

ASPECTS OF THE MIGRATION OF INVASIVE PARASITIC JUVENILE
NEMATODES IN THE TISSUES OF LABORATORY RODENTS.

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I hereby declare that this thesis was composed by myself and the work it contains is my own.

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ABSTRACT

Rigorous proof that a specific site is part of the migratory pathway of a skin penetrating nematode depends on the data satisfying the inequality $\underline{p}_o + \underline{p}_f > 1$, where \underline{p}_o is the peak proportion of the dose found in the site, and \underline{p}_f the maximum proportion of the dose reaching the destination. It is shown that third stage larvae of Strongyloides ratti (two strains) applied in exact doses in the order of 20 to the skin of the rat can be extracted from the nasal region of the head in sufficient quantities to satisfy the criteria. The timing of the peak of the dose of larvae (\underline{p}_o) in the nasal region was found to vary between the strains. The maximum proportion of the dose completing migration (\underline{p}_f) was quantified on days 5 to 8 by counting the number of egg-tracks present in the small intestine. With appropriate statistical tests, the excess of the sum of the means of these two proportions over unity is shown to be highly significant for both strains. Thus it is effectively certain that the nasal region of the rat head is part of a pathway taken by this parasite on its way from the skin to the intestine. Larvae of both strains were also recovered from the cranial region of the rat head but in insufficient numbers to satisfy the inequality. Nevertheless, the results combined with a histological study suggest events in the cranium precede those in the nasal region, with the two sites forming a single step on a specific pathway. Larvae of Nippostrongylus brasiliensis, applied to the skin of the rat in exact doses in the order of 20 partially sheathed L3's, were found in sufficient quantities in the lung ($\underline{p}_o = 35h$) to fulfil the inequality ($\underline{p}_f =$ adult worms in the small intestine on days

8/10). The result confirmed a re-analysis of the data of Twohy (1956) for subcutaneously injected larvae of N. brasiliensis.

Direct observation of the arrival of S. ratti in the small intestine is shown to be inaccurate. Compressed tissue autoradiography was attempted but abandoned after a number of failures. A new technique utilizing short pulses of anthelmintic (morantel tartrate) gave results for the arrival in the gut of N. brasiliensis that correspond with direct counts. For S. ratti (both strains), the 'drug pulse' data is 15-20h advanced of direct counts. The overall kinetics of migration suggest subtle differences between the two strains of S. ratti.

S. ratti and N. brasiliensis larvae, transferred from the nasal region and lungs respectively of donor rats to the stomach of a recipient, are shown to undergo developmental changes within the relevant site before migration can proceed to the gut. The changes can be monitored by morphological markers, namely, an oesophagus index (OI) greater than unity for S. ratti and the onset of ecdysis for N. brasiliensis. Larvae were absent from extracts of the lung (S. ratti), head (N. brasiliensis) and liver (S. ratti and N. brasiliensis). The results are discussed with reference to the rejection of the Looss-Fulleborn 'blood-lung route' as a universal model to describe the behaviour of skin-penetrating nematode juveniles inside hosts.

CHAPTER 1.

GENERAL INTRODUCTION

The birth of the subject.

The textbook account as to how the infective stage larvae of skin-penetrating nematodes reach their destination after the transition of the skin barrier is based upon the 'classic' reports of two workers in the last few years of the nineteenth century and first two decades of the twentieth century. We start therefore, with a brief review of these studies, and their influence on modern thinking.

In 1898 Looss (see Looss, 1911) published the first record of skin penetration by the infective larvae of a parasitic nematode (Ancylostoma spp.). As a direct result of that finding, Looss began a series of experiments to determine how the larvae reach the intestine after penetration of the skin. The work, based on Strongyloides sp. and the hookworms Ancylostoma duodenale and Ancylostoma caninum, was reported in a brief paper in 1905.

Subsequently a detailed account of the hookworm story appeared in a monograph in 1911. Looss carried out experiments in which he inoculated dogs with large, but otherwise unquantified, doses of the parasite, then, at subsequent times killed them and examined tissues for larvae by histological techniques. In an animal autopsied soon after infection, he found 'considerable' numbers of larvae in the lymphatics, a few in capillaries and many in the skin proper. From this he concluded that if under normal conditions larvae entered

either the circulatory system directly, or, indirectly via the lymphatics and thoracic duct, they would eventually be transported in the blood to the heart and the lungs where they would become trapped in capillary beds. From the lungs Looss postulated that the larvae would have a direct path to the oesophagus and intestine via the bronchioles, bronchi and trachea. In a second experiment where the dog was killed some 79 hours after infection, 'large numbers' of larvae were observed in the pulmonary tissue, trachea, larynx and oesophagus. He concluded that larvae did indeed migrate via what became known as the blood-lung route.

Probably the most influential work on the blood-lung route was carried out by Fulleborn & Schilling-Torgau (1911) and reported fully by Fulleborn in 1914. The experiments performed involved three dogs, each of which was given a 'heavy' dose of Strongyloides sp. larvae mixed with a small population of A. caninum. The dogs were infected on separate days, each with a different larval population. Aiming to show the exclusiveness of the pulmonary-pharyngeal route, one dog was tracheotomized, and a second had the pharyngeal-intestinal link severed at the oesophagus. The ends of the oesophagus were externalized so that the contents of the distal portion could be sampled when the dog salivated or drank. In the first, a few A. caninum and 'large numbers' of Strongyloides sp. filariform larvae were recovered in the tracheal mucus between days 3 and 6. In the second, filariform larvae of Strongyloides sp. were found in the outflow from the oesophagus from days 2 to 5. A negligible intestinal infection developed in both cases. The third dog was, on successive days, infected twice with a mixture of the two parasites. On day 19

the animal was killed, when 'large numbers' of both Strongyloides sp. and A. caninum adults were present in the small intestine. Fulleborn (1914) claimed this as a control for the involvement of the lung in the intact animal.

Despite the fact that his observations were only qualitative, the conclusions of Looss (1905, 1911) had a profound influence on future thought. Fulleborn's approach was more experimental, but it is clear from his writing that he considered proof for the circulatory system to have been obtained by Looss. Few workers at the time sided with Sambon (1908) in questioning the hypothesis, with the result that it soon achieved the status of fact for all species of skin penetrating nematode.

Criteria required for proof of part of the pathway.

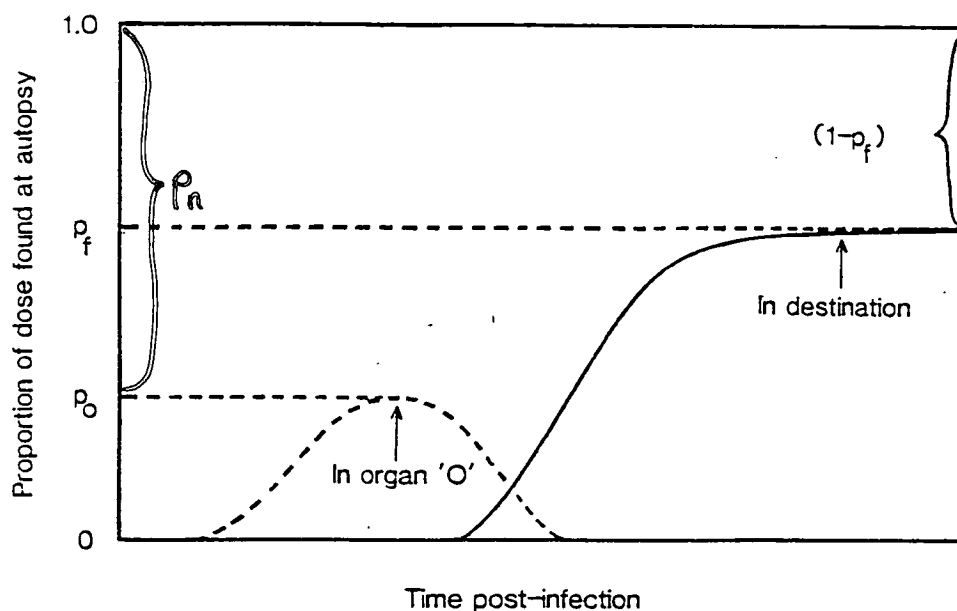
Harley & Gallicchio (1971) state with reference to Trichinella spiralis, that 'the mere presence of migratory larvae in any given body does not warrant the conclusion that this is the main pathway'. They suggest an overall picture must be obtained which takes into account (a) the complete larvipositing period of the adults, (b) the number of larvae recovered at each body site per day, (c) the total capacity of the adults, and (d) the host's anatomy and physiology. A similar argument was put forward by Wilson (1983) in his review of migratory pathways of skin penetrating roundworms, where he questions the validity of some of the conclusions of the pioneers of the subject.

Wilson (1983) describes the experiments of Looss as examples of 'sampling at autopsy'. In this type of experiment, a large group of animals is infected with the parasite, then at subsequent times, subgroups are killed and tissues examined for the presence of migrating larvae. The sites in which larvae are found, together with the chronological relationship between them, supposedly define a route of migration. Wilson (1983) has suggested that this is a fallacious argument since it depends on 'impedance' of nematode movement in host tissues. If impedance exists parasites would accumulate in a given locus and be detected by sampling at autopsy. If however, impedance is absent the parasites could still pass through yet remain undetected. Indeed, Wilson suggested that a route lacking impedance would be the biologically sensible one on first principles, and that evidence of impedance without qualification could not be taken as proof of part of a pathway. Paradoxically, it may be those tissues where none or only a few parasites are found which actually constitute a route.

Thus, in order to prove that a site of impedance is, in fact, part of a pathway, Wilson (1983) proposed the following. If a dose (d) of parasites is administered to the host at time zero, then an examination of a putative transit site Q , presenting impedance, as it does, will reveal a subsequent rise in the number of parasites therein (see Figure 1.1). The peak proportion of the dose found in Q is termed p_Q . As time progresses, the number present diminishes to zero. The relationship between p_Q and the total proportion (p_t) of the dose which passes through Q is determined by the rate of arrival and the mean time taken by individuals to pass through. If migration is totally synchronous, then all parasites

taking this route would be present in \underline{O} at the same time, in which case $\underline{p}_o = \underline{p}_t$. If the time taken to transit \underline{O} is shorter than the arrival period (the difference in time between the first and last arrivals) then $\underline{p}_o < \underline{p}_t$.

Figure 1.1 Idealised results from sampling at autopsy.



In the ideal case, all administered parasites successfully reach their destination, i.e. $\underline{p}_f = 1$, where \underline{p}_f is the proportion of the dose actually completing migration. In this case, any parasites found in \underline{O} at an earlier time can be stated with certainty to be en route to their goal. This theoretical example never occurs in the natural situation because, under operational conditions, a

proportion of the dose never reaches the end-point; i.e $p_f < 1$, and the proportion of the dose unaccounted for is therefore equivalent to $1 - p_f$. In most situations p_f is difficult to assess exactly, and, with a suitably designed experimental framework, it has to be assumed to be equal to the proportion of the dose developing to maturity.

Before Q can be proved to be part of a pathway, the total larvae passing through (p_t) must be greater than the proportion of larvae which never reach their destination ($1 - p_f$). Sampling at autopsy gives no information on the arrival period or transit time, and the relationship between p_o and p_t is uncertain; consequently p_o has to be accepted as our estimate of p_t . To summarise, proof is obtained when

$$p_o > 1 - p_f \dots \dots \dots (1)$$

which can be rewritten as

$$p_o + p_f > 1 \dots \dots \dots (2)$$

If the relationship is established, then a proportion, p_c , of the dose can be said with certainty to have passed through Q on their way to the endpoint, where,

$$p_c = (p_o + p_f) - 1 \dots \dots \dots (3)$$

Given that the inequality is satisfied, there is a high probability, if not certainty, that all parasites found in Q were on their way to their destination. If the inequality principle is to be applied to a sampling at autopsy experiment, the result relies heavily on the impedance offered by the site in question to the migrating parasite. Only if impedance is above the 'critical' level will the inequality be satisfied. As the total number of larvae passing through Q is not measured, Wilson (1983) stresses the need for an experimental framework that minimizes the proportion of the dose that never complete migration to the endpoint, ie. that maximizes p_f .

Evidence in favour of passage though the pulmonary tissue.

The findings of Looss and Fulleborn were not quantitative and therefore they cannot be subjected to the above analysis. Nevertheless most workers in the field from about 1920 have taken the Looss-Fulleborn theory to be the absolute proof, and have seen their role as one of elaborating the story (e.g Foster & Cross, 1934). Even at the discovery of new skin penetrating nematode parasites, the blood-lung route has been accepted without question as the migratory pathway. For example, Yokogawa (1922) states, with reference to Nippostrongylus brasiliensis, that 'the larvae placed on the skin of rats penetrate very easily and quickly into the tissues and travel to the lungs by means of the bloodstream.' without presenting any evidence whatever for the circulatory route, and no estimates of p_o in the lung.

Reviewing the findings of other workers in the field, Wilson (1983) concluded that, at the time of writing, the only data that met the

criteria of the inequality [(2) p6] were those obtained by Twohy (1956) after subcutaneous injection of N. brasiliensis into the rat. These results offered a prima facie proof of the involvement of the lung, though they were not presented by Twohy in that light. It is clear from his style that he believed the pulmonary migration to be a fact before undertaking his study. Twohy was only concerned with the kinetics of the migration, and the true value of his results did not emerge until they were reexamined by Wilson (1983). Together with the results of Twohy (1956), the work of Love, Kelly & Dineen (1974) constitutes proof that a significant proportion of injected larvae of N. brasiliensis take the pulmonary-oesophageal route to the intestine.

The genus Strongyloides has figured largely in studies on migration. The early work by Looss (1905, 1911) and Fulleborn (1914) has been followed by a succession of studies on different species (Griffiths, 1939; Rogers, 1939; Basir, 1950; Spindler, 1958; Abadie, 1963; Stankiewicz & Bezubik, 1967; Stone & Simpson, 1967; Supperer & Pfeiffer, 1967; Genta & Ward, 1980; Nwaorgu & Connan, 1980; Dawkins, Muir & Grove, 1981). The identification of small numbers of larvae within the lung has been taken by many of the above authors to be evidence for a migratory pathway which they already regarded as fact. Typical of this approach is that of Stone & Simpson (1967) who infected young pigs with 1×10^6 larvae of S. ransomi, a massive dose by any standards. The maximum number recovered from the lung was 255, or 0.00025 of the dose. Larvae extracted from other tissues of the body (liver, spleen, heart, kidneys and brain) reached a peak at 783. Stone & Simpson state 'it is doubtful if development occurs

anywhere else but the lung'. Other authors claim a pulmonary migration on the basis of similar findings to the above, e.g. Galliard (1967), Carlson & Goulson (1977) and Neva (1986).

Evidence supporting the involvement of the circulatory system.

Looss concluded that larvae reach the lung through the lymphatics and then the circulatory system. The role of the lymphatics has largely been ignored by the textbooks, but occasionally workers have returned to it, notably Oshio (1956) with S. ransomi, Gharib (1953, 1961a, b) and Clarke (1967) with N. brasiliensis and finally Wilson & Simpson (1982), Wilson (1983), Wilson, Simpson & Seaton (1986) and Wilson (1987) with S. ratti. However, many authors state that larvae travel to the lungs via the blood circulation (Full citation above). Wilson (1983) argues that traditional methods of 'sampling at autopsy' are unlikely to yield significant results for the role of the circulatory system since once in the blood, larvae will be transported quickly to the next capillary bed where they will become trapped, a point first noted by Looss 1911 and subsequently by Harley & Gallicchio (1971) and Croll (1977). Other authors have tried to overcome the problem of no 'impedance' in the circulatory system by the administration of massive doses of parasites to potential hosts of the smallest possible body mass. For example, Turner, Shalkop & Wilson (1960) published the results of a series of experiments on the role of the bloodstream in the migration of S. papillosus in lambs. Each lamb was inoculated with 2×10^6 larvae on the inguinal skin. Twelve hours later small numbers of larvae were recovered from the circulatory system. Larvae were not exclusively found in blood removed

from vessels leading to the lungs, but were also present in the mesenteric and hepatic systems. The authors suggestion is that recirculation of larvae can occur before finally becoming trapped in the lung. This idea was first proposed by Fulleborn (1925) and subsequently by Wilson (1977, 1980a, b).

Several authors have performed speculative experiments on the role of the blood stream by injecting larvae directly into the venous system (Fulleborn, 1914, 1925, 1929; Twohy, 1955a; Croll, 1977 and Croll & Ma, 1978). These have however been of little value to the overall understanding of migration routes. For example Twohy (1955a), working with N. brasiliensis, injected L3's into the femoral vein of rats and found that they developed to the fourth stage in the lung. On orally administering these 4th stages to recipients, he found that they developed to adults, which he claimed as evidence for the circulatory-pulmonary migration. However, when L3's were injected into the hepatic-portal vein, they developed to L4's in the liver, and subsequently to adults when given to recipients orally.

Such experiments do not address the fundamental question as to whether the circulatory system is involved in the first place so that all the evidence available for the proposition is based on the qualitative observations of the sort made by Looss (1911), Abadie (1963) and Clarke (1967).

Evidence in favour of a route other than that accepted by convention.

A lack of evidence for the blood-lung route raises the question, is there any other possible pathway from the skin to the intestine? Frickers (1953) postulated that larvae of S. ransomi may be able to bypass the lung of the pig. Nojima, Kawanabe, Noda & Sato (1986) suggested S. pavonis in chicks were randomly dispersed throughout the body after finding only small numbers of larvae in the lung. Schad, Aikens & Smith (1989) use the term 'scramble model' to indicate a random distribution of S. stercoralis larvae in the dog.

Abe (1964) described a migratory route for S. ratti through the head of the rat. Spindler (1958) recovered larvae of S. ratti from the head of the rat, but ignored these findings in favour of the pulmonary route. In a series of abstracts in Japanese (Abe, 1964; Abe, Tanaka, Kagei & Hori, 1965b; Abe, Hattori & Tanaka 1965a; Abe, Tanaka, Nagano & Izumi, 1966) it was concluded that infective larvae of this species, when injected sub-cutaneously, migrated through the muscle stroma to the head and then the lungs before reaching the digestive tract. Similar findings were described by Hattori, Tada & Nagano (1968) and Hattori (1977). Quantitative data with reference to the head were reported by Tada, Mimori and Nakai (1979); Murrell (1980, 1981) and Hattori (1981) but were not sufficient to satisfy the criteria laid down by Wilson (1983). Dawkins, Thomason & Grove (1982) and Nawa, Kiyota, Korenaga & Kotani (1985) suggested a similar migration for S. ratti in the mouse. Owor & Wamukota (1976) recovered larvae of S. stercoralis from the subarachnoidal spaces from a fatal case of human strongyloidiasis. Genta (1989) found a

'few' larvae in the cranial cavity of a dog infected experimentally with S. stercoralis.

Mimori, Korenaga, Chowdhury and Tada (1982), Nojima, Noda, Kawanabe & Sato (1987) and Tanaka, Mimori, Minematsu & Tada (1989) tried to evaluate a pathway for S. ratti through the head of the rat by observing morphological changes in the larvae. The findings are suggestive but offer no absolute proof.

In judging the emphasis to place on these reports, it must first of all be said that the evidence in favour of the head being a transit site is certainly as good as that which implicates the lung. Dawkins (1989) argues that more larvae are found in the cerebrospinal fluid of the cranium than are in the lungs, but this could be due to the ones in the lungs representing a far more dynamic worm population, with larvae spending a very short time in the lungs and airways. The larvae in the CSF, on the other hand, may represent a small population who inadvertently find their way into this site, the kind of argument put forward by Wilson (1983).

Aims of this project

The basic aim of this project was to consolidate a rational framework for the investigation of in-host migration of infective larval stages of skin penetrating roundworms, using as an illustrative system, a quantitative comparative study of Nippostrongylus brasiliensis and two strains of Strongyloides ratti in rats.

The work followed on from previous studies in this laboratory which had confirmed (a) N. brasiliensis is not milkborne whereas S. ratti can be. (Wilson et al., 1976a) (b) larvae of S. ratti which enter the milk of suckled mothers are not a separate population from those which reach the gut in unsuckled or non-reproducing rats: i.e. there is a switch in the system initiated by the suckling stimulus (Wilson et al., 1976b; Wilson, 1977; Wilson et al., 1978b; Wilson, 1979, 1980a, b; Wilson & Simpson, 1981; Wilson & Simpson, 1982; Wilson et al., 1982;. (c) Larvae of S. ratti in lactating hosts do not initially enter the venous system and the route from the skin to the mammary gland is not systemic (Wilson & Simpson, 1982; Wilson et al., 1986). (d) The two strains of S. ratti differ quantitatively in their migration in lactating hosts (Wilson & Simpson, 1981; Wilson et al., 1986). (e) Larvae labelled with radio-selenium can be tracked in rat tissue (Wilson, 1979; Wilson & Simpson, 1981). (f) The S. ratti system has been progressively refined in all aspects: in particular the exact dose (EDT) technique has brought marked statistical and practical benefits, and skin application is now a reproducible experimental procedure which mimicks the natural process (Wilson et al., 1978a; Wilson & Simpson, 1981; Wilson et al., 1986).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Parasites

2.1.1 Strongyloides ratti

Two strains of S. ratti were used in this study. The first, designated the 'homogonic' strain, was originally obtained from The Wellcome Laboratories, Langley Park, Beckenham, Kent. The 'G60 heterogonic' was isolated from a wild rat by Dr G. Graham (Philadelphia, U.S.A.) in 1960.

The two strains differ by the type of life-cycle they undertake (Figs 2.1 and 2.2). In both, parthenogenetic females (Plate 2.1a) are parasitic in the mucosa of the rat small intestine. These deposit distinct batches of eggs between the villi, resulting in the formation of 'egg-tracks' (Plate 2.1b). The eggs hatch in the rectum or faeces to release the first stage larvae. The first and second stage larvae (L1 and L2) have the characteristic free-living rhabditiform oesophagus. After the second moult, the infective filariform stage (L3) is produced in the homogonic cycle, whereas, in the heterogonic cycle, the L1's undergo 4 moults to give rise to a generation of free-living male and female worms. The free-living females produce eggs which follow a line of development similar to those deposited by the parasitic females of the homogonic cycle.

Figure 2.1 Homogonic life-cycle of *Strongyloides ratti*.

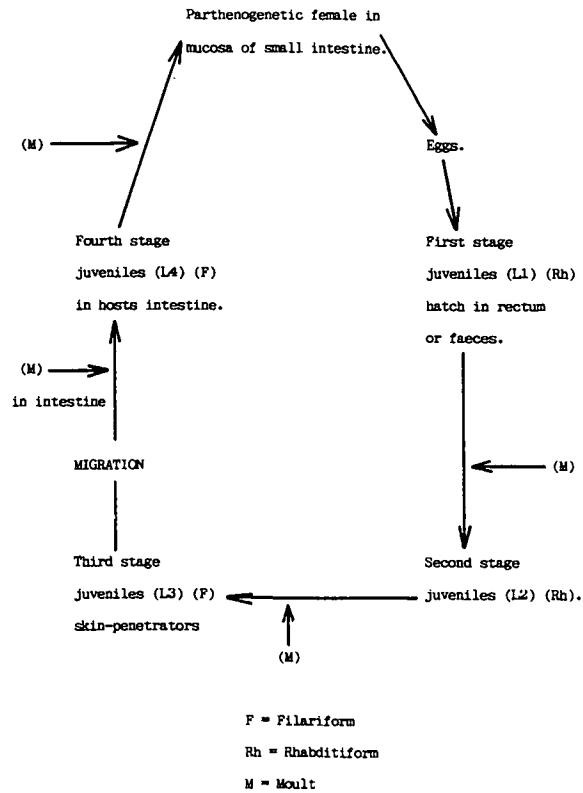
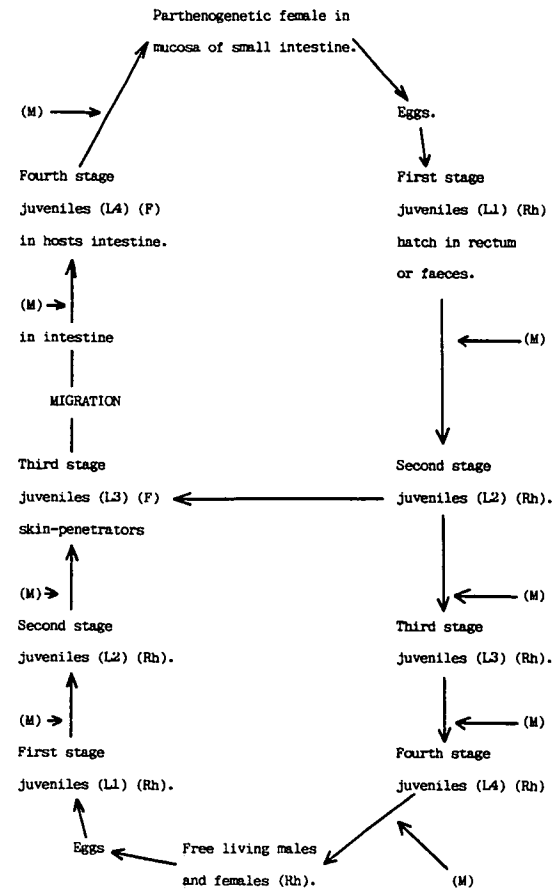


Figure 2.2 Heterogonic life-cycle of *Strongyloides ratti*.



(M) = Moult, F = Filariform, Rh = Rhabditiform.

Figure 2.3 Life-cycle of *Nippostrongylus brasiliensis*.

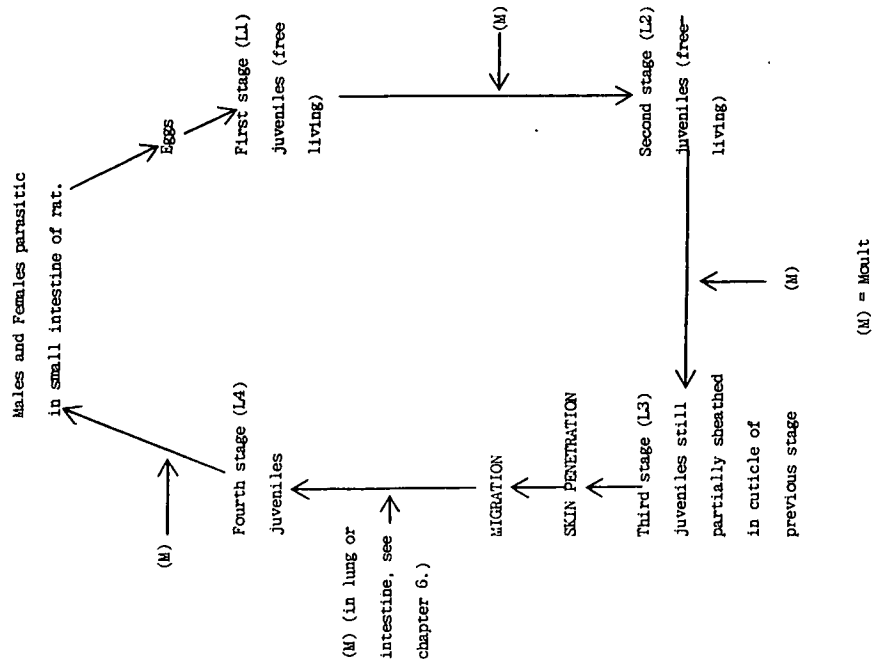


Figure 2.4 Parts of the rat head examined for larvae. nf = nasofrontal region c = cranial cavity exposed and brain removed.

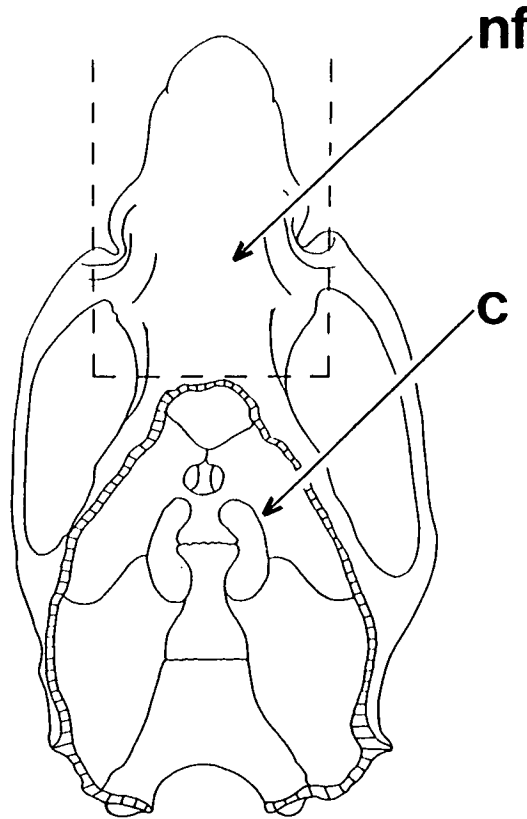
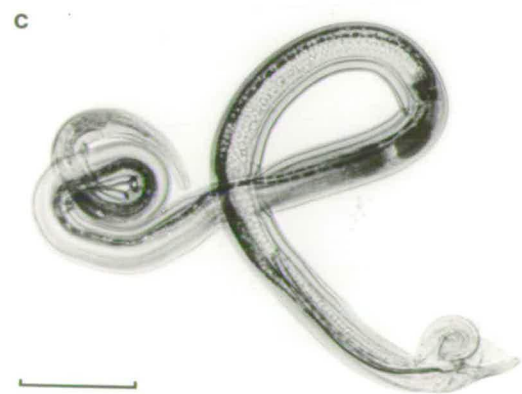
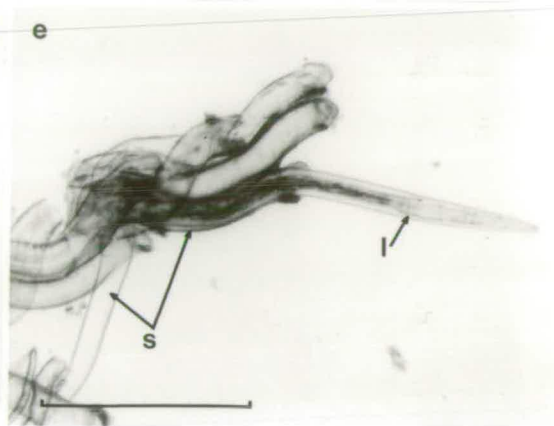
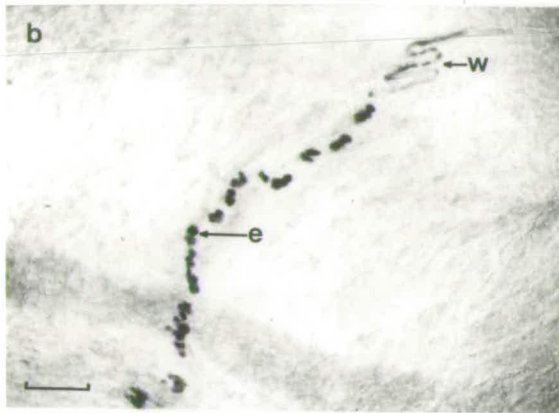


Plate 2.1

- a. Parthenogenetic female S. ratti.
- b. Parthenogenetic female S. ratti (w) and associated egg-track (e) in mucosa of small intestine.
- c. Parasitic male N. brasiliensis.
- d. Parasitic female N. brasiliensis.
- e. Third stage larvae of N. brasiliensis (l) remaining inside a sheath (s) until contact with a host. Numerous empty sheaths are also shown.

Bars represent 250 μ m.



On contact with a host, the infective larvae of both strains penetrate the skin, undergo a migratory phase, and, eventually become established in the small intestine. Here development continues to the fourth stage (L4), and finally, the parthenogenetic females.

2.1.2 Nippostrongylus brasiliensis

The life cycle of N. brasiliensis (Fig 2.3) resembles in outline that of the homogonic strain of S. ratti. However, both males and females are parasitic in the small intestine (Plate 2.1c, d). The eggs produced by the female hatch in the faeces to release the first stage larvae which subsequently undergo two moults to the infective L3. Unlike S. ratti, the infective stage remains partially sheathed in the cuticle of the L2 (Plate 2.1e). The sheath is attached to the substratum and the anterior end breaks off, allowing the larva to emerge when stimulated by a sudden rise in temperature, namely a passing host. After skin penetration, a migratory phase occurs before establishing in the gut.

2.2 Rats

All rats were derived from the outbred colony of Wistar Rattus norvegicus, originally maintained by the Institute of Occupational Medicine (I.O.M.), Bush Estate, Penicuik, Edinburgh. Experimentals were bred in The Department of Zoology, University of Edinburgh, excepting twelve week old ones which were obtained from the I.O.M. All animals initially harboured a colony of the oxyurid nematode, Syphacea muris, but this was eradicated during the study period

(Appendix 1.1). Animals were housed in one of two types of cages: stock cages, (Model RB3, North Kent Plastics Ltd, Home Garden, Dartford, Kent) with wood shavings in the bottom, or grid cages (Model R1, North Kent Plastics Ltd.). Water was provided by an automatic watering system. Food was available ad libitum (for type of food see Appendix 1.2). All rats were kept at 20-22°C in a 12:12, light:dark regime, the lights switching on at 8.00 a.m.

2.3 Culture Systems

2.3.1 S. ratti (homogonic)

(After Wilson & Simpson, 1981)


Rats with stock infections were held in grid cages suspended over trays lined with damp paper towelling, which was replaced daily. Faeces were collected overnight, rinsed rapidly with distilled water to minimize contamination with urine and then checked for patency. L1's were normally observed from 5 days post-infection. Approximately 10 faecal pellets were placed on a watch glass in the bottom of a 90mm diameter glass petri dish. A small amount of distilled water was added to the faeces if it appeared dry. The watchglass was surrounded by distilled water and the petri dish lid replaced. Cultures were incubated for 72 hours in an anhydric incubator (Vindon Scientific) at 19±1°C. After 60 hours distilled water was added to the faeces, and any infective larvae that emerged were collected from the film of water under the watchglass at 72 hours.

Material from all dishes was pooled, and the percentage of motile larvae assessed. Only if this was greater than 95% were the larvae used for experimental purposes.

Each week, two 3-week-old rats were infected for stock with 1000 infective larvae. A 0.5 ml sample of the pooled larvae was added to 9.5 ml of distilled water in a 10 ml conical centrifuge tube. A 0.2 ml sample of this diluted suspension (1:20) was placed in three watchglasses, and a mean count obtained. The number of larvae per ml of the undiluted suspension was therefore 100x the mean count from the three wells. The concentration of larvae was adjusted by either sedimentation or the addition of distilled water until the count was approximately 1000 larvae per 0.5 ml. The rats were infected by subcutaneous injection into the loose skin of the neck.

2.3.2 S. ratti (Heterogonic)

(After Wilson & Simpson, 1981)

 Cultures were set up as in 2.3.1. Water was added to the faeces on days 3, 4 and 5 so that any homogonic larvae produced would migrate out and could be discarded on day 6. Water was added again on day 7, and true heterogonic larvae harvested the following day.

2.3.3 N. brasiliensis.

(After Barakat, 1951 and Wilson & Dick, 1964)

The culture system adopted for N. brasiliensis was based on the traditional filter paper technique. On top of a small piece of absorbent cotton wool, soaked in distilled water and situated in the bottom of a 90mm petri dish, was placed a 55mm diameter piece of Whatman 54 filter paper. One piece of infected rat faeces was positioned centrally on the filter paper. After approximately 7 days of incubation at $19\pm 1^{\circ}\text{C}$, third stage larvae migrate across the paper to the edge where they become attached by the tails of their sheaths. The sheath is broken at the front end, allowing the larvae to emerge when stimulated by a sudden rise in temperature. This gives the whole paper a fringed appearance. The cultures were left for a further two days to ensure all larvae had completed development. Evaporation from the cultures was reduced by the addition of an open bowl of water to the bottom of the incubator.

To infect stock rats, the filter was removed and placed in water at 37°C for 5 minutes. This stimulated exsheathment, resulting in a suspension of larvae from which a dose of 1000 could be counted and administered to rats in a similar manner to S. ratti (page 23).

2.4 Preparation of radio-labelled larvae of S. ratti (homogonic)

(After Wilson & Simpson, 1982; Carter, 1986)

The underlying culture method was essentially that outlined on page 19. One or 2 pieces of infected rat faeces were placed in each of the 3 chambers of a cavity slide. With a 1ml tuberculin syringe, fitted with a 19G needle, either 0.3ml or 0.15ml of diluted [⁷⁵Se]-Selenomethionine (Amersham International, Code SC. 1P) was added to each well. The cavity slide was placed in a petri dish and surrounded by distilled water. The lid was replaced and the culture incubated at 19±1°C for 72 hours. A couple of drops of water were added to each well at 60 hours.

Larvae were harvested and the percentage non-motile estimated. Residual label was removed by repeated centrifugation with distilled water until the reading of a 0.5ml sample of the supernatant was approximately background. Later, centrifugation was abolished and only sedimentation used, as the former reduced the viability of the larvae (see chapter 5).

After removal of excess background label, a known number (Z) of larvae were hand counted in 0.5ml of the supernatant and the emissions produced over 100 seconds (A) recorded in a Panax NaI scintillation counter. The count produced by 0.5 ml of the supernatant was also recorded (K).

The mean number of emmissions per worm per minute was therefore:

$$\frac{\underline{A-K}}{\underline{Z}} \times \frac{60}{100}$$



2.5 Infection procedure.

2.5.1 S. ratti or exsheathed N. brasiliensis used in exact doses in the order of 20 larvae (EDT20).

(Developed from Wilson, Simpson & Seaton, 1986)

The infection procedure for both strains of S. ratti was a modified version of the 'exact dose technique' (EDT) based on 100 hand counted larvae (now termed EDT100) (Wilson et al., 1986; Wilson, 1987). The dose was lowered from one in the order of 100 to 20 so as to reduce error variances to an absolute minimum (see chapter 3). This change had the advantage, among others, that larvae could be counted more rapidly and their number confirmed more accurately before application.

Newly emerged third-stage larvae were collected from faecal culture and pooled. Samples of 20 larvae were hand counted and transferred, with a drawn out Pasteur pipette, to a solid watchglass in approximately 0.2ml of distilled water. The count n in each watchglass was then checked.

All rats were anaesthetized with sodium pentobarbitone (Sagatal, May and Baker). The amount administered was 31µg/g body weight, which is sufficient to immobilize them for 2.5 hours. For 3-week-old animals the Sagatal was diluted 1 in 10 with 0.9% sodium chloride solution, whilst for 12-week-olds, the dilution was 1 in 2. The anaesthetic was administered 1 hour prior to infection in accord with the protocol of Wilson et al. (1986).

The normal infection site was a 10mm by 10mm area of clipped skin on the left flank. Prior to infection this was moistened with a piece of absorbent cotton wool soaked in distilled water. Larvae were applied with a 1ml tuberculin syringe, and left undisturbed, but watched continuously, for 45 minutes (see page 35). The drop was then removed with a Pasteur pipette, the region washed several times with distilled water, and the washings examined for the presence of any non-penetrators, s.

Syringes and watchglasses used for the application of larvae were washed with distilled water; the washings were allowed to sediment, and were then examined for residual larvae, r.

Each rat therefore obtained a unique 'exact' dose of $\underline{n} - (\underline{r} + \underline{s})$.

2.5.2 A large dose of approximately 500 larvae of S. ratti or exsheathed N. brasiliensis.

A dose of 500 larvae in 0.2ml was estimated by a dilution count (see page 20) and applied to the infection site by methods described on

page 24. No attempt was made to estimate residual larvae and non-penetrators.

2.6 Estimation of p_f .

(After Zamirdin & Wilson, 1974; Wilson et al., 1978a)

For both S. ratti and N. brasiliensis, the method used to obtain a value for p_f was a modified gut squash. Rats were starved in grid cages for 24 hours prior to autopsy. In the case of S. ratti, killing was on day 8 post-infection, later changed to day 5 for the homogenic strain and day 6 for the heterogenic. Rats infected with N. brasiliensis were killed on days 8-10 post-infection.

The first 500mm of the small intestine was removed, cut into 5 equal pieces, slit open and squashed between two glass plates (140mm x 140mm). On viewing under 20x magnification, adult worms were counted and expressed as a proportion of the dose. For S. ratti the value of p_f was later taken as the number of egg tracks in the intestine. In preliminary studies the guts were frozen and counted at a convenient time later. As the count for S. ratti proved more accurate in fresh guts, this was subsequently adopted (see pages 33-38).

2.7 Estimation of p_o .

Sampling for p_o was done in 2 regions of the head by methods modified from Tada et al. (1979) and Murrell (1980). After

carbon dioxide asphyxiation, the head of the rat was separated from the neck, and the skin, mandible, muscles and eyes removed. The roof of the cranium was cut away with fine scissors, the brain gently removed and teased open to reveal the ventricles. The cranial cavity and brain were washed with 50ml of warm 0.9% sodium chloride solution. The resulting suspension was placed in a 120ml centrifuge tube in a water bath (Techne TE-8A) at 37°C and left to settle for 30 minutes. The supernatant fluid was aspirated to a final volume of 5ml, the sediment resuspended and divided equally between 3 solid watchglasses for counting of larvae under the medium power of a stereo microscope.

The nasofrontal region is wedge shaped and was separated from the rest of the head so that it contained the nasal cavity and the major portion of the maxilla (Fig 2.4) (Tada et al., 1979). This was chopped coarsely and mixed with 50ml of Hanks' balanced salt solution (HBSS, Gibco) in a 120ml centrifuge tube and incubated at 37°C for 4 hours. After incubation the liquid was aspirated to leave 15ml of tissue suspension which was poured through a domestic wire tea strainer. The material retained in the strainer was washed with 40ml of 0.9% sodium chloride, these washings being added to the original filtrate. The combined suspension was sedimented for 30 minutes at 37°C. After aspiration to 5ml the larvae were counted as for the cranium.

In some assays the head was examined as one entity. In this case, the brain was removed and washed with HBSS. The rest of the head was coarsely chopped and added to the washings from the brain. Incubation

and counting was as for the nasofrontal region. Livers, lungs and brains were chopped finely with a razor blade after rinsing with warm 0.9% NaCl solution to remove excess blood. They were then sampled as above.

2.8 Autoradiography for ^{75}Se labelled parasites in the intestine.

(After Mangold & Dean, 1983; Kwansa & Murrell, 1986; Wilson, Coulson & Dixon, 1986; Chandiwana, 1988)

Rats were starved for 24h prior to removal of the small intestine, so that intestinal contents likely to cause false images on the autoradiogram were eliminated as far as possible. The small intestines were placed on a 180x115mm piece of cardboard. This was covered with cling film, gently squashed under the weight of a lead brick, and placed in an incubator at 60°C for two hours. The card was then transferred to a hydraulic press and subjected to a pressure of 4.8×10^7 Pa for 2 minutes. On removal it was again placed under the lead brick and allowed to dry for 24 hours at 60°C .

A second layer of cling film was placed over the tissue to stop leakage. A piece of X-ray film (Kodak X-Omat S, 18 x 24cm) was positioned between the card and an intensifying screen (CAWO special 18 x 24cm) in an autoradiographic cassette (Harmer, 18 x 24cm) (Two cards per cassette). The cassette was closed and placed in a -60°C freezer for 4-5 weeks.

Each film was developed in Kodak LX 24 developer for 4 minutes, agitating every 30 seconds, washed and then fixed (Kodak FX 40 for 5 minutes). After washing in running tap water for 1 hour the film was allowed to dry before examination under the dissecting microscope (20x magnification) for the presence of reduced foci.

2.9 Recovery of larvae from the tissues of donor rats to infect recipients by the oral route.

The tissue in question was removed from the rat at a specific time post-infection and coarsely chopped with scissors. This was placed in a petri dish of 0.9% NaCl solution and incubated at 37°C for 15 minutes. Larvae that moved out into the saline were counted into samples of 20 with a drawn out pipette. Each sample was transferred to a separate solid watch glass in approximately 0.1ml of the saline, the count checked and then stored at 37°C until infection.

A 1ml tuberculin syringe was transformed into a stomach tube by the addition of a 19G blunt needle, onto which was inserted a 50mm piece of tubing (PP80, Portex Ltd, Hythe, Kent). 0.2ml of saline was placed in the syringe and the sample taken up in as little fluid as possible in order to reduce the number of larvae actually passing up into the barrel of the syringe. Keeping the syringe vertical, the sample was administered to the stomach of a lightly etherized rat. The syringe was washed thoroughly with distilled water, and after sedimentation, the washings were examined for the presence of any residual larvae. These along with any larvae remaining in the watchglass, were subtracted from the initial inoculum to obtain the final 'exact dose'.

2.10 Measurement of growth of worms

Worms to be measured were extracted from the tissues by methods described in 2.9. Single worms were placed on microscope slides in a drop of 0.9% NaCl solution, covered with a coverslip (Chance No 1. 22 x 22mm) and quickly heated to 60°C to immobilize them. All measurements were done with the aid of a PZO (Warsaw) microscope fitted with a camera lucida. The total body length (BL) and oesophagus length (OL) were measured. For S. ratti, the oesophagus index (OI) was calculated as described by Nojima et al. (1987), where,

$$\underline{OI} = \frac{\underline{OL}}{\underline{BL-OL}}$$

2.11 Histology.

Tissues for histological examination were processed by techniques modified from Drury & Wallington (1980) (Figure 2.5). All sections were stained by haematoxylin and eosin (Figure 2.6).

2.12 Statistical analysis of data.

Values for p_o and p_f are expressed throughout as proportions of the dose. Where means are quoted, the associated standard error is also given. All data expressed as proportions were converted to arc-sines before analysis by either Student's t-test or

analysis of variance (Sokal & Rohlf, 1981). In certain experiments, differences in the variances of the data being analysed led to the use of a modified form of the t-test (Milton & Tsokos, 1983).

Probabilities, P, were calculated from the Solid State Software for the Texas TI 59 programmable calculator. Coefficients of variation (CV) were calculated from the arc-sines of the data.

Figure 2.6 Outline Schedule for Haematoxylin and Eosin Staining.
(after Drury & Wallington, 1980).

1. Xylene I	3mins
2. Xylene II	3mins
3. Absolute alcohol	3mins
4. 96% alcohol	2mins
5. 90% alcohol	2mins
6. 70% alcohol	2mins
7. Ehrlichs Haematoxylin	5-10mins
8. Tap water	2mins
9. 1% HCl in 70% alcohol	30secs
10. 2% sodium hydrogen carbonate solution	1min
11. Tap water	5mins
12. 0.5% Eosin	30-45secs
13. Tap water	1min
14. 70% alcohol	1min
15. 90% alcohol	1min
16. 96% alcohol	1min
17. Absolute alcohol	2mins
18. 50:50, alcohol:xylene	1min
19. Xylene III	1min
20. Xylene IV	3mins
21. Mount in Canada balsam	

Figure 2.5 Outline Schedule for Tissue Processing

1. 10% formal saline	5 days
*2. Decalcification in 5% trichloroacetic acid	5 days
3. 70% alcohol	4 hours
4. 90% alcohol	4 hours
5. 96% alcohol	4 hours
6. Absolute alcohol	4 hours
7. Xylene I	3 hours
8. Xylene II	3 hours
9. Vacuum impregnation with fibrowax I	1 hour
10. Vacuum impregnation with fibrowax II	1 hour
11. Vacuum impregnation with fibrowax III	1 hour
12. Embed	

* Only if bone present in specimen.

3 - 8 done automatically in Histokinette.

CHAPTER 3

THE 'EXACT DOSE' SYSTEM

Introduction

The statistical benefits of a framework for the application of small exact doses of parasites were predicted by Wilson et al. (1978a) and confirmed by Wilson & Simpson (1981). Wilson et al. (1978a) found that, when doses were based on volume sampling of larval suspensions, the coefficient of variation, CV, of the doses themselves was 15%. In addition a CV of 10% was attributable to observer error in counting the parasites en masse in the intestine. The exact dose system eliminates both sources of variation and thus increases the overall sensitivity. Of more importance for this kind of study is the reduction of the variation between experiments such that the added component of variance between experiments (variation between experiments minus the within experiment variance) is generally eliminated, thus allowing means from different experiments to be compared directly.

The following experiments were designed to modify the exact dose system for skin application of larvae (Wilson et al., 1986) to produce the highest possible 'take' of parasites (p_f) with minimum variance, such that the probability of obtaining data of sufficient magnitude to fulfil the criteria for proof of migratory routes (Wilson, 1983) was maximized.

3.1. Strongyloides ratti (homogonic)

To reduce the errors due to the counting procedure to an absolute minimum, the original technique for the application of a known dose of larvae to the skin (now termed EDT100) (Wilson et al., 1986; Wilson, 1987) was modified so that each rat obtained a dose in the order of 20 (EDT20) (page 23). This had the advantage that larvae could be dispensed more rapidly and their number confirmed more accurately before application. Forty five minutes after infection, the drop of inoculum was removed from the rat, and the infection site washed. The washings were examined for the presence of any non-penetrators which were subtracted from the initial dose, so that the exact number of penetrators was known. To standardize the hosts, and reduce the effects of any innate immunity, 3-week-old, newly weaned rats were used throughout.

3.1.1 Test of EDT20 [Expt 1]

This experiment was designed to evaluate EDT20 and examine the relationship between worms and egg-tracks (see page 14).

Materials and Methods

Six rats were infected by EDT20 (mean exact dose = 19.0) and killed on day 8, when the small intestines were removed, frozen, and at a convenient time later, thawed and examined for worms and tracks.

Results [Expt 1]

The mean proportion of worms which became established, \bar{p}_f , was 0.766 ± 0.038 (SEM). However, when expressing the total number of egg-tracks in the gut as a proportion of the dose (irrespective of whether each track was associated with a worm or not), the mean value was 0.817 ± 0.055 (SEM). The percentage of wormless tracks was 6.9%.

3.1.2 The effect of freezing and thawing on the proportion of wormless tracks [Expt 2].

This experiment was done to see if wormless tracks were the result of freezing and thawing the guts prior to counting.

Materials & Methods

Eight rats were infected by EDT20 (mean exact dose = 18.0) and killed on day 8 when the small intestines were removed and examined fresh.

Results

The mean proportion of the dose as adult worms or egg-tracks was 0.655 ± 0.057 (SEM) and 0.689 ± 0.063 (SEM) respectively. The percentage of wormless tracks in this experiment was 5.1%.

3.1.3 Does each track represent one worm [Expts 3-6]?

The slight reduction in the proportion of wormless tracks in guts

assayed fresh led to the possibility that this was due to procedural phenomenon. Alternatively, one worm could produce more than one track over the relevant developmental period. This was examined in the following 4 experiments.

Materials and Methods

Each rat had only 1 larva applied to the skin by methods similar to EDT20. The number of rats used in each of the 4 experiments was 22, 25, 23 and 27. All rats were housed separately for the first 3 hours after infection to eliminate the possibility of cross-infection. Small intestines were removed and examined fresh on day 8 post-infection.

Results

From the total of 97 rats used in the four experiments, 10 were eliminated as the larva failed to penetrate, 31 of the remaining animals had neither tracks nor worms, 55 had a single track and a single worm and the final rat had one track but no worm. On no occasion was there more than one track present. This is strong evidence to allow the equivalence of tracks and worms.

3.1.4 Time taken to penetrate the outer layers of skin [Expt 7].

As described in the introduction to this chapter, a refinement to the exact dose technique involved recovering non-penetrators from the skin some 45 minutes post-infection. In some experiments, the anaesthetised rat occasionally moved and dislodged the drop of water containing

the larvae. This experiment was done to identify the minimum time that the drop had to be in place for \bar{p}_f to be maximal, and to verify that 45 minutes was an appropriate time to start larval recovery.

Materials and Methods

Twenty five rats infected by EDT20 were divided into 5 groups of 5. Each group had the drop of inoculum removed and the infection site washed at one of the following times post-infection; 5 minutes, 10 minutes, 20 minutes, 30 minutes and 45 minutes. All non penetrators were counted and expressed as a proportion of the original inoculum (minus the larvae remaining in the glassware). The number of tracks present in the gut on day 8 were expressed as a proportion of the inoculum.

Results

The results are shown in Figure 3.1a. The curve for the proportion of the dose recovered from the skin decreases rapidly for the first 20 minutes and reaches its minimum by 30 minutes. The curve of the proportion of the inoculum which become adult in the intestine is a mirror image of that for larvae recovered from the skin. The two curves agree in demonstrating that maximal development is achieved if doses are left undisturbed for 30 minutes. Forty five minutes was therefore a sensible time at which the process of larval recovery from the skin was initiated. The value of \bar{p}_f obtained (taking into account non penetrators) was 0.820 ± 0.035 (SEM). In further

Figure 3.1 Time taken for L3 of S. ratti to invade skin:

circles represent the mean proportion of the initial inoculum (ordinates) recovered in washings from the infection site at the times after application represented as abscissae; squares are the mean proportions of the inoculum which developed to adults at day 8 following each treatment. Bars define 95% confidence limits (data untransformed): 5 rats per treatment. The number of larvae in the inoculum was $\underline{n-r}$, where \underline{n} is the original sample count and \underline{r} the residual larvae in syringe and watchglass. (a) homogonic strain [Expt 7], (b) heterogonic strain [Expt 10].

Figure 3.2 Cross-section of the arrangement for counting exact doses of N. brasiliensis on polythene: \underline{m} , masking tape; \underline{l} , juvenile (not to scale); \underline{p} , polythene film; \underline{w} , watch glass; \underline{b} , bottle screwtop. For dimensions see text page 43.

Figure 3.1

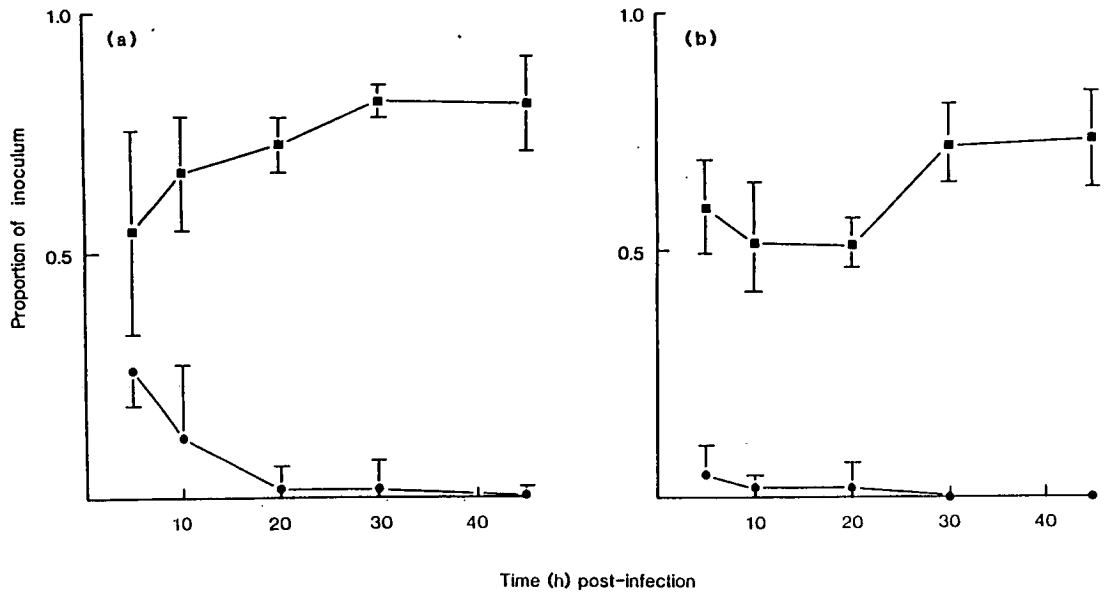
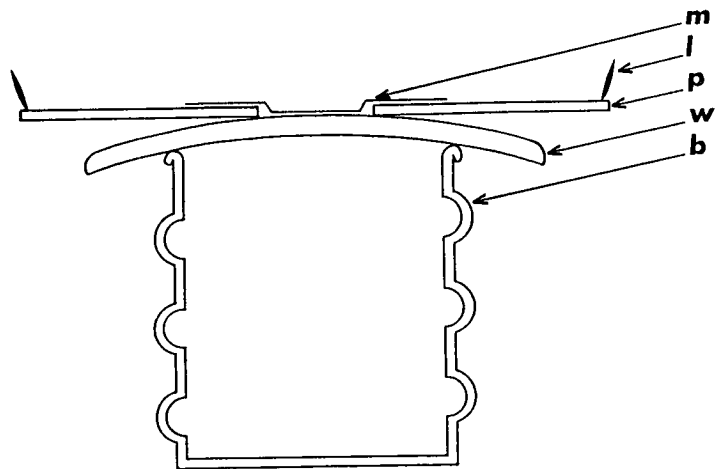


Figure 3.2



experiments, rats were discarded where the drop of inoculum rolled off prior to 30 minutes post-infection.

3.1.5. Assessment of EDT20 [Expts 8-9].

Two experiments were designed to test the amended EDT20. In the first, 8 rats were infected (mean exact dose = 19.3) and in the second, 7 (mean exact dose = 19.6)

Results [Expts 8-9]

The mean values of p_f on day 8 were 0.809 ± 0.043 (SEM) and 0.687 ± 0.088 (SEM). A one-way analysis of variance on the arc sines of these data plus data obtained for \bar{p}_f in Expts 1, 2 and 7 reveals no significant difference between the five means ($F_{[4,29]} = 1.51$, $P = 0.224$, with an overall $CV = 18.3\%$), and a total \bar{p}_f of 0.758 ± 0.028 from 34 rats.

3.2 S. ratti (heterogonic)

It was assumed that the behaviour of the parthenogenetic females in the small intestine would be similar for both strains, and that the results obtained on pages 33-35 would be equally applicable to the heterogonic strain. Therefore, p_f was taken as the proportion of egg-tracks in fresh intestines on day 8.

3.2.1 Time taken for larvae to penetrate the outer layers of skin [Expt 10].

Materials and Methods

Five groups of 6 rats were infected by EDT20 with the heterogonic strain. After 5 minutes, 10 minutes, 20 minutes, 30 minutes and 45 minutes (1 rat died) one of the groups had the drops of inocula removed, and the infection site washed. Larvae recovered from the infection site were expressed as a proportion of the original dose (minus any larvae recovered from the glassware). Egg-tracks on day 8 were expressed as a proportion of the original inoculum, minus any larvae remaining in the glassware.

Results

The results are shown in Figure 3.1b. Although virtually all larvae have penetrated by 5 minutes, the maximum take is not achieved unless the drop is left in position for 30 minutes ($\bar{p}_f = 0.733 \pm 0.036$ (SEM)). In further experiments, 45 minutes was kept as the time at which larval recovery was initiated, with rats eliminated from experiments if the drop was dislodged prior to 30 minutes.

3.2.2 Comparison of establishment on day 5 and day 8 [Expt 11].

Katz (1967) and Wilson & Simpson (1981) suggested that the maximum proportion of the applied dose of the heterogonic strain had reached maturity by 8 days post-infection. The following experiment was

designed to examine the possibility that, like the homogenic strain (see Chapter 4), this could be assessed as early as day 5.

Materials & Methods

Twelve rats were infected by EDT20, six of which were killed on day 5 (mean exact dose = 19.7), the remaining six on day 8 (mean exact dose = 20.3) when their small intestines were removed and examined.

Results

There was clearly no significant difference in the proportion of egg-tracks on day 5 ($\bar{p}_f = 0.689 \pm 0.054$ (SEM)) or day 8 ($\bar{p}_f = 0.662 \pm 0.057$ (SEM)). It was noted that on day 5, egg tracks were very short and difficult to see, and therefore to speed up the counting process and minimize errors, day 6 was adopted in further experiments.

3.2.3 Assessment of EDT20 with reference to the heterogenic strain [Expts 12 and 13].

Two similar experiments were set up, in which 11 and 14 rats respectively were infected by EDT20 (mean exact doses = 18.6 and 19.1). p_f was estimated as the proportion of the dose as egg-tracks on day 6.

Results

The mean takes of the parasite in the two experiments were

0.707±0.039 (SEM) and 0.565±0.039 (SEM). Analysis of variance of the arc sines of these data plus that obtained from Expts 10 and 11 (day 5 and day 8 data pooled) reveals a significant difference ($F_{[3,38]} = 3.034$, $P = 0.04$). The difference being attributable to the mean of 0.565±0.039 (SEM) [Expt 13]. The pooled mean of the other three experiments being 0.697±0.023 (SEM), from a total of 28 rats, with an overall CV of 16.7%.

3.3 Nippostrongylus brasiliensis

Unlike the infective stage larvae of S. ratti, those of N. brasiliensis remain partially sheathed in the second stage cuticle until contact is made with a rat. In most cases experimental infections are initiated unnaturally, in that the larvae used are artificially induced to exsheath before they are counted and applied to the skin or injected subcutaneously. Most authors have relied on subcutaneous injection as a means of initiating an infection, but this removes an important step in the natural infection process. The following experiments were designed to develop a system similar to EDT20 for the skin application of known doses in the order of 20 L3's of N. brasiliensis.

3.3.1 Establishment of exsheathed larvae [Exps 13 and 14].

Third stage larvae grown on filter paper (page 21) were artificially stimulated to exsheath in distilled water kept at 37°C for 5 minutes. Once free of their sheaths, the larvae were used to infect rats in a manner identical to the one used for S. ratti. Two

experiments with 4 (mean exact dose = 18.5) and 10 (mean exact dose = 16.8) rats respectively were conducted to evaluate the technique.

Results

The proportion of the dose that established in the first experiment was 0.628 ± 0.044 (SEM), and in the second, 0.311 ± 0.046 (SEM). A Student's t-test on the arc sines of these data reveals a highly significant difference between the two experiments ($t_{[12]} = 3.919$, $P = 0.002$). The result of the first experiment, although good by comparison to accepted norms, is well below the ideal goal of unity.

3.3.2 Establishment of larvae exsheathed in a balanced salt solution [Expt 15].

Haley (1962a) and Wilson & Dick (1964) reported that water was unsuitable as a medium for the maintenance of suspensions of N. brasiliensis larvae. Wilson & Dick (1964) found that larvae survived for prolonged periods in modified quarter strength mammal Ringer-Locke solution (1/4RLA) (Hale, 1958). This medium was tested as a vehicle in which to apply exsheathed larvae to the skin.

Materials and Methods

Sheathed larvae from the same population were stimulated to exsheath in either distilled water or 1/4RLA solution (2.3g NaCl, 0.01g KCl,

0.044g CaCl_2 and 0.04g NaHCO_3 per litre of distilled water). Seven rats were infected with an exact dose of less than 20 larvae exsheathed in distilled water (mean exact dose = 17.9) and seven with larvae from 1/4RLA (mean exact dose = 18.4). All rats were killed on day 10 and examined for the presence of adult worms.

Results

The mean \bar{p}_f for larvae exsheathed in distilled water was 0.537 ± 0.067 (SEM), whilst for the 1/4RLA treated larvae, $\bar{p}_f = 0.374 \pm 0.061$ (SEM). No significant difference between treatments is seen after the application of a Student's t -test to the arcsines of the data ($t_{[12]} = 1.775$, $\underline{p} = 0.101$).

3.3.3 The use of larvae still sheathed on the filter paper [Expt 16].

It was assumed that uncontrolled aspects of the use of exsheathed larvae arose during the artificial exsheathment process itself, or in the handling of the worm suspension subsequently. In the natural situation, the larvae probably remain sheathed until contact is made with a passing rat. In order to exploit this, a technique was devised to infect rats with larvae still sheathed on the filter.

Materials and Methods

The filter paper culture was removed from the petri dish and placed on an upturned watchglass (48mm dia) attached to a bottle top (35mm dia, 23mm depth) (Figure 3.2) and the combination manipulated under the

dissecting microscope. It was thought that at an appropriate density sheathed worms would be clearly seen and counted with 45x magnification. What was thought to be an exact number of 20 worms was counted, and the piece of paper containing the said number was cut from the whole and held in forceps while the count was checked with the microscope. The snippet of paper was then placed, worm side down, onto the dampened infection site of an anaesthetised rat (page 24). After 1 hour the paper was removed from the rat and examined for any larvae that had not exsheathed. These were subtracted from the initial sample, in the hope that the exact number of penetrators would be known. Four rats were infected by this technique (calculated exact dose = 17.0) and four with exsheathed larvae (mean exact dose = 18.0) from the same population. Worm burdens were compared on day 10.

Results

The take of exsheathed larvae was 0.667 ± 0.074 (SEM) and of sheathed ones 1.148 ± 0.074 (SEM). Clearly the counting process for the sheathed larvae was inaccurate as in 3 out of the four rats the number of adult worms present on day 10 exceeded the initial dose.

3.3.4 Juveniles sheathed on a sandwich of three filters.

It was concluded that the results obtained in 3.3.3 were due to some larvae on the top of the filter being indistinguishable from the fibres of the paper. A three layered system was therefore developed where 3 filters were sandwiched together. On removal of the upper and lower filters it was envisaged that only a thin fringe of sheathed

larvae would remain on the very edge of the middle layer. The methods for counting the larvae on the middle layer are identical to those described on page 43. Twelve exact doses (mean exact dose = 18.5) were counted out and placed in water at 37°C for 5 minutes. The number of exsheathed larvae was compared to the initial count. Only once did the figures match, on 8 occasions the number of exsheathed larvae exceeded the initial count by 1, with the remainder having an excess of 2.

3.3.5 Juveniles sheathed on a polythene disc.

Again the results of 3.3.4 were attributed to larvae being indistinguishable from the fibres of the paper. A transparent substratum was required which under realistic operational conditions would allow all larvae to be counted. Once more a three layered geometry was adopted, with the upper and lower filter discs being separated by an annulus of transparent polythene, 0.22mm thick. The polythene was cut with an outside diameter of 60mm, so as to protrude beyond the filter paper margin, and with a 20mm diameter hole in the centre. This construction permitted the flow of water from the cotton wool reservoir to the uppermost layer bearing the faecal pellet. After incubation and removal of the filters, a thin fringe of larvae was left on the polythene. The polythene was secured with a 25mm square piece of masking tape to the upturned watchglass and larvae counted into exact doses (page 43). A test of the method was performed by emersing 30 snippets of polythene, each with an exact dose in the order of 20 (mean exact dose = 18.8), into distilled water at 37°C for 5 minutes. The initial count was compared to the final

number exsheathed plus the number remaining within their sheaths. In all cases the number exsheathed added to the number still in their sheaths after the test confirmed the original count. All larvae escaped from their sheaths on 22 occasions; the number where 1 remained sheathed was 6; and in each of the remaining 2 samples, 2 larvae failed to escape. The method was therefore deemed accurate in the counting of sheathed larvae of N. brasiliensis.

3.3.6 Initiating an infection with larvae ensheathed on polythene [Expt 17].

Materials and Methods

The technique described on page 45 was used to count out 9 exact doses in the order of 20 sheathed larvae (mean exact dose = 19.4). These were administered to rats as described on pages 43-44.

Results

The mean take from the nine rats on day 10 was 0.438 ± 0.053 (SEM); i.e. lower than three of the values obtained with exsheathed larvae (see pages 41, 42 and 44).

3.3.7 Infection of sheathed larvae from polythene culture: the problem of desiccation [Expt 18].

The following experiment was designed to test the supposition that, given time, desiccation of L3's on polythene reduced their viability.

Materials and Methods

Seven rats were given doses one after the other from a single polythene culture. A final rat was infected from another fresh, damp, culture of the same sort. All rats were killed on day 10 and their small intestines examined.

Results

The p_f 's, in the order in which the rats were infected, were as follows: 0.700, 0.722, 0.571, 0.684, 0.150, 0.143, 0.182 and 0.737. Clearly, the longer the time between the removal of the filters and infection of the host, the greater the reduction in viability. In further studies, each culture supplied a maximum of 3 exact doses.

3.3.8 Comparison of p_f 's using sheathed and exsheathed larvae [Expts 19 and 20].

The expectation that sheathed individuals would meet the requirements, providing that desiccation could be circumvented, led to a comparison with exsheathed larvae.

Materials and Methods

Two experiments were set up to compare the ability of sheathed and exsheathed larvae from the same population to colonize the rat gut.

Results

The results are summarized below as Table 3.1.

Experiment	Number of rats	Sheathed		Exsheathed	
		Mean exact dose	\bar{D}_f (\pm SEM)	Mean exact dose	\bar{D}_f (\pm SEM)
(21)	6	20.2	0.820 \pm 0.040	17.2	0.319 \pm 0.052
(22)	10	18.8	0.836 \pm 0.057	16.0	0.379 \pm 0.069

Two way analysis of variance of the arcsines of the proportions that became established reveals a highly significant difference between the treatments ($F_{[1, 28]} = 1345$, $\underline{p} = 3 \times 10^{-12}$). There was no significant difference between experiments ($F_{[1, 28]} = 0.836$, $\underline{p} = 0.361$).

3.4 Conclusions

3.4.1 General 'exact dose' techniques.

(1) Exact dose systems increase the take of parasites to levels higher than normally obtained.

(2) Exact dose systems reduce variation to an extent that finer

differences between treatments can be detected.

(3) Exact dose systems improve the chances of homogeneity within treatments, between experiments.

3.4.2 S. ratti (homogonic)

(1) Samples of fresh intestines containing adult S. ratti had fewer wormless egg-tracks (5.1%) than those that had been frozen and thawed (6.9%). More comprehensive data from further experiments counting worms in fresh intestines has shown that the proportion of wormless tracks is as low as 2.9% (see Tindall & Wilson, 1988).

(2) Each female worm only produces 1 egg track, therefore egg tracks can be equated to p_f .

(3) Larvae require a maximum of 30 minutes to penetrate the outer layers of skin.

(4) Using the modified exact dose system (EDT20), a mean value for p_f of 0.758 ± 0.028 (SEM) was obtained from a total of 5 experiments. The overall coefficient of variation being only 18.6%, well below normally accepted levels.

3.4.3 S. ratti (heterogonic)

(1) Larvae require a maximum of 30 minutes to penetrate the outer layers of skin.

(2) All worms have developed to maturity by day 5, but the egg-tracks are very short and difficult to see, therefore day 6 is designated as the time to obtain values of \underline{p}_f .

(3) A mean take of 0.697 ± 0.023 (SEM) was obtained in 3 trials of the exact dose technique. As the errors are low (overall $\underline{CV} = 16.7\%$), a further experiment ($\underline{p}_f = 0.565 \pm 0.039$ (SEM)) was detected as an outlier from the general trend.

3.4.4 N. brasiliensis

(1) Larvae exsheathed in water produce highly variable takes.

(2) Exsheathment in a quarter strength mammal Ringer Locke solution produces a take similar to, if not poorer than, larvae exsheathed in water.

(3) It is possible to make an exact count of larvae sheathed on polythene, but not of ones on filter paper.

(4) If desiccation can be circumvented, the use of a known dose in the order of 20 larvae (EDT20Nb) sheathed on polythene, leads to a high take of parasites, with a low variance ($\underline{p}_f = 0.830 \pm 0.038$ (SEM) from 16 rats, with an overall coefficient of variation of 19.8%.)

CHAPTER 4

MAJOR TRANSIT SITES ON THE MIGRATORY ROUTE.

4.1 Strongyloides ratti

The first part of this chapter deals with experiments on the homogonic and heterogonic strains of S. ratti, utilizing the improved exact dose technique (EDT20) as a tool to maximize p_f , and to investigate whether the head is a major component of a pathway for either, or both, of the strains, or whether larvae found in this site are 'lost'. An attempt to estimate the time of arrival of the larvae in the small intestine by direct counting is also described.

4.2 Migration of S.ratti (homogonic).

4.2.1 Migration through the whole head.

4.2.1.1 Extraction of larvae from the head (including the brain) [Expt 1].

The aim of this experiment was to combine the refined techniques described earlier with those used by Tada et al. (1979) and Murrell (1980) (page 26) to see whether any larvae could be extracted from the head of the rat and, at the same time, determine \bar{p}_f .



Materials & Methods

Thirty three rats were infected by EDT20 (mean exact dose = 19.4). Nine were kept to obtain a value of \bar{P}_f on day 8, whilst the remainder were split into 3 groups of 8. One group was killed at 30h, 40h and 50h post-infection. The skin, mandible and eyes were removed, then, the rest of the head (including the brain) was assayed as described on page 26. The head skin was placed in 30ml of 0.9% sodium chloride solution in a petri dish and incubated at 37°C for 2 hours, after which the saline was examined for larvae. The washings from the head tissues contained a lot of debris attributed to the brain. This made detection of the larvae difficult, with the result that the material had to be fixed in 10% formal saline, and counted at a later date.

Results

Larvae were extracted from the head tissues, but the proportions recovered were thought to be an inaccurate assessment of the true population in the head (0.042±0.023 at 30h, 0.006±0.006 at 40h and zero at 50h). Non-motile worms in the fixed tissue suspension proved difficult to detect, resulting in the introduction of errors associated with the counting process. No larvae were recovered from the skin.

4.2.1.2 Extraction of larvae from the head (brain removed) [Expts 2-7].

The following 6 experiments were modified from Expt 1 by the

removal of the brain from the head prior to incubation. This was to reduce the debris present at the time of counting, enabling motile worms to be seen.

Materials and Methods

All rats were infected by EDT20. At specific times post-infection, groups were killed and the head examined after removal of the brain (see page 26). In each experiment, a further group was kept until day 8 when a value of \bar{p}_f was obtained. Details of the protocols of Expts 2-7 are given in Table 4.1. The experiments were done primarily to locate p_o , consequently the sampling times became more restricted as the sequence evolved.

Results

The results are given in Table 4.1. All the values for \bar{p}_o were obtained at 30h, excepting experiment 2 when the peak appeared at 40h. In all 6 experiments, the first criterion of proof is satisfied arithmetically, that is $\bar{p}_o + \bar{p}_f > 1$ (for statistical analysis see below). The skin of the head was also examined in Expt 2, but no larvae were recovered, a similar finding to an examination of the brain in Expt 3.

Table 4.1 Protocols and results of Expts 2-7 on the migration of S. ratti (homogonic) through the head of the rat (brain removed).

Table 4.2 Testing the inequality $\bar{p}_o + \bar{p}_f > 1$ for the S. ratti (homogonic) in the head of the rat. \bar{p}_o taken at 30h post-infection, except * (40h).

$$p_c = \bar{p}_f - \bar{p}_n$$

Table 4.1

Exp	Mean exact dose	Mean proportion of dose (\pm SEM) recovered from the head at the following times (h) post-infection (n).					\bar{P}_f
		25	30	35	40	50	
2	18.9	—	0.153 ± 0.053 (4)	—	0.172 ± 0.035 (4)	0.042 ± 0.027 (4)	0.843 ± 0.030 (7)
3	19.5	—	0.284 ± 0.051 (4)	—	0.088 ± 0.033 (4)	0.013 ± 0.013 (4)	0.815 ± 0.019 (7)
4	18.8	0.133 ± 0.030 (6)	0.218 ± 0.041 (5)	0.174 ± 0.047 (6)	0.092 ± 0.011 (6)	—	0.843 ± 0.038 (6)
5	18.8	—	0.248 ± 0.034 (6)	—	—	—	0.821 ± 0.015 (5)
6	19.3	—	0.246 ± 0.011 (6)	—	—	—	0.818 ± 0.036 (5)
7	19.0	—	0.250 ± 0.037 (6)	—	—	—	0.858 ± 0.037 (5)

Table 4.2

Exp	\bar{P}_n	\bar{P}_f	\underline{p}_c	Probability that \underline{p}_c is due to chance (df)	
2*	0.828 ± 0.035	0.843 ± 0.030	0.015	0.283	(9) N.S
3	0.716 ± 0.051	0.815 ± 0.019	0.099	0.060	(9) N.S
4	0.782 ± 0.041	0.843 ± 0.036	0.061	0.296	(9) N.S
5	0.752 ± 0.034	0.821 ± 0.015	0.069	0.119	(9) N.S
6	0.754 ± 0.011	0.818 ± 0.036	0.064	0.091	(9) N.S
7	0.750 ± 0.037	0.858 ± 0.037	0.108	0.092	(9) N.S
Pooled data	0.762 ± 0.014	0.841 ± 0.012	0.079	0.00019	(62)

4.2.1.3. Statistical proof of migration through the head

The proof requires that $\bar{p}_o + \bar{p}_f > 1$(1)

which can be rewritten as $\bar{p}_f > 1 - \bar{p}_o$(2)

or $\bar{p}_f > \bar{p}_n$(3) where

$(1 - \bar{p}_o) = \bar{p}_n$(4)

If the excess of $\bar{p}_o + \bar{p}_f$ over unity (the proportion that can be said with certainty to have passed through a particular organ) is equivalent to p_c , then,

$$p_c = (\bar{p}_o + \bar{p}_f) - 1.....(5)$$

$$p_c = \bar{p}_f - \bar{p}_n.....(6)$$

As the values for \bar{p}_o and \bar{p}_f are from separate animals, a statistical analysis of the data is required, such that not only is the relationship defined by the inequality (2) satisfied numerically, but also that the mean values have a sufficiently small variance to render it statistically significant.

Table 4.2 summarizes the results of Expts 2-7, such that only the values of \bar{p}_n (head) and their associated \bar{p}_f 's are given.

For each experiment a t-test was performed on the arc-sines of the data for p_f against the arcsines of p_n , in order to determine whether $\bar{p}_f > \bar{p}_n$ could have occurred by chance.

Column 5 shows that, taken individually, each result was non-significant. However analysis of variance on all the data for p_n

(arc sine transformed) reveals homogeneity ($F_{[5,25]} = 1.035$, $P = 0.419$), with an overall mean of 0.762 ± 0.014 (SEM) (untransformed). A

similar analysis of the data for \underline{p}_f gives the same result ($\underline{F}_{[5,29]} = 0.853$, $\underline{P} = 0.524$), with an overall mean of 0.841 ± 0.012 (SEM) (untransformed). It is therefore permissible to pool the data within treatments from these experiments and test the significance of the difference between the grand means of the relevant arcsines with a single comprehensive \underline{t} -value. Examination of the variance within the \underline{p}_f (variance = 66.47 with 29 df, transformed data) and \underline{p}_n data (variance = 28.36 with 25 df, transformed data) showed them to be significantly different ($\underline{F}_{[29,25]} = 2.343$, $\underline{P} = 0.017$). Thus the method for comparison of means drawn from populations with different variances recommended by Milton & Tsokos (1983) was used. The resultant \underline{t} -value was $\underline{t}_{[60]} = 3.97$, $\underline{P} = 0.00019$. It can therefore be stated with confidence that a proportion (\underline{p}_c) of 0.079 of the dose passed through the head of the rat on their way to the intestine. There is a high probability, if not certainty, that all those larvae found in the same site at the same time were similarly on course. Therefore it is highly likely that a proportion of at least 0.238 (\underline{p}_o) of the dose of larvae reached their destination after passage through the head in this series of experiments.

4.2.2. The specific site within the head

The head as a whole is a heterogeneous system made up of many sites. In an attempt to refine the application of the inequality, and taking note of the results of Tada et al. (1979), two specific locations were examined, namely, the nasal region and the cranium.

4.2.2.1. Examination of the cranium and nasofrontal regions of the head [Expts 8-12]

Experiments 8-12 were a series conducted chronologically in numerical order essentially to locate p_o in time in the two regions, and to obtain a precise estimate of \bar{p}_f . Each successive protocol was designed to answer questions raised by the evidence accumulated from its predecessors. In consequence, the assays in the head became more restricted in time as the sequence evolved.

Materials and Methods

All rats were infected by EDT20. In each experiment one group of rats was kept until day 8, when they were autopsied for egg-tracks in the intestine. The remaining animals were divided into subgroups, and each subgroup killed at a specific time post-infection, when the nasofrontal and cranial regions were examined (see page 26). The general protocols for Expts 8-12 are given in Table 4.3.

Results

The results for the larvae recovered from the nasofrontal region and the cranium are shown in Tables 4.4 and 4.5 respectively. \bar{p}_f for each experiment is also given in Table 4.4. A general survey of the nasofrontal data places p_o in this part of the head somewhere in the range 20-25h, with the most probable time of those sampled of 23h (Expts 10 and 12). This is considerably earlier than the estimate of the peak in the head as a whole, probably due^{to} the restricted timings

Table 4.3 The protocols for Experiments 8-20. (S. ratti homogonic) a. Nasal region and cranium examined. b. Nasal region, cranium and small intestine examined. c. Cranium and small intestine examined. d. Small intestine only examined. * See Table 4.6.

Table 4.4 Larvae (S. ratti homogonic) in the nasal region and egg tracks in the small intestine (day 8) for Experiments 8-20. * = larvae died during incubation. # = data from both treatments combined. \$ = mean p_f lower than general trend, therefore data from these experiments ignored in any analysis. This decision is defended later (see page 65). \underline{p} = probability that all means could have been drawn from the same population.

Table 4.3

Expt	Mean exact dose	The number of rats in which the head and intestine were sampled at each of the following times (h) post-infection:												The number of rats used to obtain a value of \bar{D}_f on day 8.			
		15	16	18	20	23	25	26	30	35	40	48	60		72	96	120
8	19.2	--	--	--	5a	--	4a	--	4a	5a	5a	--	--	--	--	--	5
9	18.1	10a	--	--	9a	--	10a	--	--	--	--	--	--	--	--	--	5
10	18.6	--	8a	8a	8a	8a	--	--	--	--	8a	--	--	--	--	--	6
11	18.3	--	--	7a	7a	--	6a	--	--	--	--	--	--	--	--	--	6
12	19.4	--	--	--	8a	8a	--	--	--	--	--	--	--	--	--	--	6
13*																	
14	17.2	5a	--	--	5a	--	--	--	--	--	--	--	--	--	--	--	4
15	18.8	--	--	4a	--	--	--	4a	--	--	--	--	--	--	--	--	4
16	19.3	--	--	--	--	6b	--	--	--	--	6b	--	6b	--	--	--	6
17	19.3	--	--	--	--	6b	--	--	--	--	6b	--	6c	--	6d	6d	6
18	19.8	--	--	--	--	6b	--	--	--	--	6b	6b	--	--	6d	--	6
19	19.1	--	--	--	--	5b	--	--	--	--	6b	6b	6d	--	6d	--	6
20	18.7	--	--	--	6a	--	5a	--	6a	6a	6a	--	--	--	--	--	6

Table 4.4

Expt	Mean proportion of the dose (\pm SEM) recovered from the nasofrontal region at the following times (h) post-infection:												Egg-tracks as mean proportion of dose (\pm SEM)	
	15	16	18	20	23	25	26	30	35	40	48	60		
8	--	--	--	0.395 ± 0.076	--	0.164 ± 0.016	--	--	0.246 ± 0.047	0.233 ± 0.038	--	--	--	0.818 ± 0.027
9	N11	--	--	0.236 ± 0.030	--	0.184 ± 0.049	--	--	--	--	--	--	--	0.811 ± 0.017
10	--	0.050 ± 0.013	*	*	0.359 ± 0.048	--	--	--	--	--	0.203 ± 0.045	--	--	0.785 ± 0.041
11	--	--	0.052 ± 0.016	0.148 ± 0.029	--	0.308 ± 0.109	--	--	--	--	--	--	--	0.800 ± 0.056
12	--	--	--	0.141 ± 0.026	0.395 ± 0.040	--	--	--	--	--	--	--	--	0.902 ± 0.023
13#	--	--	--	0.191 ± 0.016	0.266 ± 0.028	--	--	--	--	--	--	--	--	0.871 ± 0.014
14	N11	--	--	0.023 ± 0.014	--	--	--	--	--	--	--	--	--	0.464 ± 0.046
15	--	--	0.177 ± 0.058	--	--	--	0.038 ± 0.013	--	--	--	--	--	--	0.819 ± 0.022
16	--	--	--	--	*	--	--	--	--	0.145 ± 0.044	--	N11	--	0.857 ± 0.030
17	--	--	--	--	0.307 ± 0.047	--	--	--	--	0.139 ± 0.025	--	--	--	0.860 ± 0.032
18	--	--	--	--	0.187 ± 0.026	--	--	--	--	--	0.033 ± 0.017	N11	--	0.783 ± 0.017
19	--	--	--	--	0.119 ± 0.025	--	--	--	--	--	0.025 ± 0.025	N11	--	--
20	--	--	--	0.095 ± 0.037	--	0.094 ± 0.033	--	0.035 ± 0.011	0.037 ± 0.019	0.028 ± 0.013	--	--	--	0.599 ± 0.045
P (D.F.)	N.A.	N.A.	0.028 (9)	0.0007 (4,36)	0.001 (5,39)	0.379 (2,17)	N.A.	N.A.	N.A.	0.463 (2,17)	0.507 (10)	N.A.	N.A.	0.153 (9,52)
Pooled Mean	N11	0.050 ± 0.013	Means treated separate	0.183 ± 0.013 except exp 8	0.156 ± 0.020 (exps 18 & 19) rest = 0.325 ± 0.021	0.217 ± 0.041	0.038 ± 0.013	0.246 ± 0.047	0.233 ± 0.038	0.167 ± 0.023	0.029 ± 0.014	N11	--	0.835 ± 0.010

Table 4.5 Larvae recovered from the cranium in Experiments 8-20 (S. ratti homogonic). * = larvae died during incubation # = data from both treatments combined. \$ = \bar{p}_f very low, all data from these experiments ignored in all analysis (see text). \underline{p} = probability that all means could have been drawn from the same population. a = Expts 10, 13, 17 & 18. b = Expt 12. c = Expts 16 & 19. d = Expt 8. e = Expts 6, 16 & 17.

Table 4.6 The effect of bandaging the site of infection [Expt 13] (S. ratti homogonic).

Table 4.5

Mean proportion of the dose (\pm SEM) recovered from the cranium
at the following times (h) post-infection:

Expt	15	16	18	20	23	25	26	30	35	40	48	60
8	—	—	—	0.135 \pm 0.031	—	0.038 \pm 0.023	—	0.038 \pm 0.013	N11	0.042 \pm 0.019	—	—
9	0.031 \pm 0.018	—	—	0.150 \pm 0.038	—	0.013 \pm 0.013	—	—	—	—	—	—
10	—	0.019 \pm 0.009	0.086 \pm 0.023	0.085 \pm 0.018	0.057 \pm 0.017	—	—	—	—	—	—	—
11	—	—	*	0.176 \pm 0.114	—	0.052 \pm 0.011	—	—	—	N11	—	—
12	—	—	—	0.066 \pm 0.015	0.085 \pm 0.020	—	—	—	—	—	—	—
13#	—	—	—	0.057 \pm 0.022	0.063 \pm 0.021	—	—	—	—	—	—	—
14\$	N11	—	—	0.108 \pm 0.012	—	—	—	—	—	—	—	—
15	—	—	0.074 \pm 0.046	—	—	—	0.051 \pm 0.020	—	—	—	—	—
16	—	—	—	—	0.009 \pm 0.009	—	—	—	—	N11	—	N11
17	—	—	—	—	0.027 \pm 0.012	—	—	—	—	0.008 \pm 0.008	—	N11
18	—	—	—	—	0.016 \pm 0.010	—	—	—	—	—	0.008 \pm 0.008	N11
19	—	—	—	—	0.010 \pm 0.010	—	—	—	—	—	N11	N11
20\$	—	—	—	0.076 \pm 0.018	—	0.043 \pm 0.034	—	N11	N11	N11	—	—
p (D.F.)	N.A.	N.A.	0.494 (10)	0.893 (5,42)	0.015 (6,45)	0.038 (2,17)	N.A.	N.A.	N.A.	0.069 (3,21)	0.341 (10)	N.A.
Pooled data	0.031 \pm 0.018	0.019 \pm 0.009	0.082 \pm 0.020	0.122 \pm 0.019	0.046a \pm 0.010 0.085b \pm 0.020 0.009c \pm 0.006	Means treated separate	0.051 \pm 0.020	0.038 \pm 0.013	NIL	0.042d \pm 0.019 0.002 \pm 0.003	0.004 \pm 0.004	N11

Table 4.6

Mean proportion of the dose (\pm SEM) recovered as larvae or egg
tracks in the following sites (6 rats per group; mean exact
dose = 19.1)

Sampling time	CRANIUM		NASO-FRONTAL		INTESTINE	
	with bandage	without bandage	with bandage	without bandage	with bandage	without bandage
20h	0.127 \pm 0.038	0.067 \pm 0.017	0.175 \pm 0.031	0.206 \pm 0.004	—	—
23h	0.093 \pm 0.035	0.034 \pm 0.022	0.299 \pm 0.028	0.234 \pm 0.048	—	—
8 days	—	—	—	—	0.876 \pm 0.018	0.804 \pm 0.023

in the earlier study. As not all experiments included every sampling time, it is difficult to pinpoint the exact time of p_o , but, for the purpose of the proof, the maximum value irrespective of timing is taken. Clearly in five experiments, the first criterion of proof is satisfied arithmetically, in that $\bar{p}_o + \bar{p}_f > 1$ in all cases (for statistical analysis see page 62).

The proportions of the dose recovered from the cranium appear to peak at approximately 20h. The inequality $\bar{p}_o + \bar{p}_f > 1$ is not met in respect of the cranium in any of the experiments (\bar{p}_f 's in Table 4.4).

4.2.2.2. Internal migration or direct transfer of larvae from the skin to the head? [Expt 13]

When the rats regained consciousness from the EDT20 procedure, they immediately licked the site of infection. It was therefore important to decide whether larvae reached the head by superficial transfer during grooming, or by internal migration.

Materials and Methods

Thirty six rats were infected by EDT20. After removal of the drops of inoculum, some 45 minutes post-infection, the infection site of 18 of the rats was covered with a bandage. Immediately overlying the site was a 10mm x 10mm piece of Whatman No.1 filter paper, which was held in place by a 25mm x 25mm square of adhesive waterproof strapping (T. J. Smith and Nephew Ltd). This in turn was secured by a 250mm strip of

surgical tape (Micropore, 3M Co) cut from a roll 25mm wide. The strip was shaped like a watch and watch strap, with the 25mm square 'watch' placed over the waterproof layer and the 'strap', 10mm wide, wound round the body of the rat approximately two-and-a-half times. For security, a furrow was clipped in the fur to receive the surgical tape, except in the immediate vicinity of the infection site, where a 5mm fringe of fur was retained. The bandages were left in place until the animals were killed or for 23 hours, whichever was the shorter. The bandaging prevented the rat from licking the site of infection, and directly transferring larvae to the head tissues. The remaining 18 rats had their infection sites left uncovered. Six rats from both groups were killed at 20h and 23h when the cranium and the naso-frontal region were examined for larvae. The remaining six rats were killed on day 8 and the egg-tracks within the intestine expressed as a proportion of the dose.

Results

The results are shown in Table 4.6. From the hypothesis under test, one would expect fewer parasites, if any at all, in the head of bandaged animals if larvae reach the head by direct transfer. No detailed analysis is needed to see that the results falsify the hypothesis, for the mean parasite content of the head in bandaged animals is arithmetically greater in 3 of the 4 comparisons depicted in Table 4.6. Moreover, \bar{p}_f for bandaged animals is also greater arithmetically. Thus larvae must reach the head by internal migration and not external transfer.

The results of Expt 13 suggest that the peak in the nasal region is at 23 hours. A sample t -value calculated for the difference between the mean arcsines of p_o for the bandaged and non-bandaged animals confirms that these data are homogeneous ($t_{[10]} = 1.133$, $p = 0.284$), with an overall mean of 0.266 ± 0.028 (SEM). The same applies to the accompanying p_f 's ($t_{[10]} = 0.378$, $p = 0.713$), mean value = 0.870 ± 0.014 (SEM).

4.2.2.3 Statistical proof of migration through the nasal region (see also Appendix 2).

Table 4.7 summarizes the values for \bar{p}_n and \bar{p}_f obtained in Expts 8-12, along with the pooled values for Expt 13 (bandaged and unbandaged). In each case the inequality $\bar{p}_o + \bar{p}_f > 1$ is satisfied arithmetically, but as described on page 55, a statistical analysis is required to show that p_c (column 5) did not occur by chance. Column 6 shows the results of a t -test on p_n and p_f for each experiment. The results are significant in 3 experiments [Expts 8, 12 and 13], marginally so in 1 [Expt 10] and non-significant in the remaining 2 [Expts 9 and 11]. Therefore in at least 3 out of 6 experiments the case is proven. A one-way analysis of variance on the arcsines of p_n for all experiments shows that the means could have been drawn from the same population ($F_{[5,42]} = 1.62$, $p = 0.176$). A similar analysis leads to the same conclusion with reference to p_f ($F_{[5,34]} = 1.66$, $p = 0.171$). An examination of the error variances for p_n and p_f (variance = 98.79 with 42 df and 46.94 with 34 df respectively, calculated from the analysis of variance on the arcsines of all the

Table 4.7 Testing the inequality $\bar{p}_o + \bar{p}_f > 1$ for
Experiments 8-13 (S. ratti homogonic). * = data from
bandaged and unbandaged animals combined.

Table 4.8 Larvae recovered from the small intestine in Expts
16-19 (S. ratti homogonic). \underline{p} = probability that all means
could have been drawn from the same population.

Table 4.7

Expt	Time of estimate of p_0 in naso frontal region (hours)	\bar{p}_n	\bar{p}_f	P_c	Probability that p_c is due to chance (D.F.)
8	20	0.605 ± 0.076	0.818 ± 0.027	0.213	0.026 (8)
9	20	0.764 ± 0.030	0.811 ± 0.017	0.047	0.358 N.S. (12)
10	23	0.641 ± 0.048	0.785 ± 0.041	0.144	0.051 N.S. (12)
11	25	0.692 ± 0.109	0.800 ± 0.056	0.108	0.367 N.S. (10)
12	23	0.605 ± 0.040	0.902 ± 0.023	0.297	0.00004 (12)
13*	23	0.734 ± 0.028	0.870 ± 0.014	0.136	0.0085 (22)
All data pooled		0.684 ± 0.021	0.837 ± 0.013	0.153	0.0000003 (83)

Table 4.8

Expt	Mean proportion of the dose (\pm SEM) observed in the small intestine at the following times (h) post-infection:							\bar{p}_f day 8 (\pm SEM)
	23	40	48	60	72	96	120	
16	N11	N11	—	0.233 ± 0.040	—	—	—	0.857 ± 0.030
17	N11	N11	—	0.354 ± 0.041	—	0.892 ± 0.030	0.884 ± 0.039	0.860 ± 0.032
18	N11	—	0.073 ± 0.011	0.369 ± 0.064	—	—	0.810 ± 0.039	0.783 ± 0.017
19	N11	—	0.173 ± 0.038	0.304 ± 0.042	0.618 ± 0.054	—	0.752 ± 0.031	—
P (D.F.)	N.A.	N.A.	0.028 (10)	0.191 (3, 20)	N.A.	N.A.	0.067 (2, 15)	See Table 4.12
pooled means	N11	N11	Means treated separate	0.315 ± 0.025	0.618 ± 0.054	0.892 ± 0.030	0.815 ± 0.024	0.835 ± 0.010 (total from all exps 8- 20)

data for each quantity), reveals a significant difference in the sampling processes. This emerged from a variance ratio test ($F_{[42,34]} = 2.10$, $P = 0.014$). Therefore the modified t -test (Milton & Tsokos, 1983) was applied to the pooled data. It gave a $t_{[83]}$ -value of 5.59 with a probability of 2.83×10^{-7} . From the last line of Table 4.7 it can be stated that 0.153 (p_c) of the dose actually pass through the nasal region en route to the intestine, and without much doubt, a proportion of 0.316 (\bar{p}_o) transit this site before reaching their goal.

4.2.3 Kinetics of migration through the head tissues and subsequent arrival in the small intestine determined by direct observation [Expts 14-20].

(It should be noted at this point that arrival of S. ratti in the intestine is better determined by methods other than direct observation - see Chapter 5)

Materials and Methods

Seven similar experiments were set up. In each, a large group of rats were all infected by EDT20. Sub-groups were killed at specific times post-infection (1 group being kept until day 8 for p_f), when the cranium and the nasal regions were examined (page 26). At autopsy, the rats involved in Expts 16-19 had their small intestines examined by direct observation (page 25). The detailed protocols of each experiment are given in Table 4.3 (page 58).

Results

The results of the proportions of the dose recovered from the three sites (naso-frontal, cranium and small intestine respectively) are given in Tables 4.4, 4.5 and 4.8. The data obtained in Expts 8-13 are also shown in the tables. Analysis of variance on the arcsines of all the \underline{p}_f data reveals two definite outliers from the general trend, Experiments 14 and 20 ($\underline{F}_{[11,60]} = 8.79$, $\underline{P} = 4.9 \times 10^{-9}$). Omitting the two outliers an analysis on the remaining data shows homogeneity ($\underline{F}_{[9,52]} = 1.556$, $\underline{P} = 0.153$, with an overall \underline{CV} of 9.6%). In further calculations the data from Experiments 14 and 20 have been ignored as they clearly do not belong to the same population as those from the remaining experiments. Possible causes of such failure are dealt with in Appendix 2.

All data obtained for the nasal region, cranium and small intestine were analysed for each site at a specific time by either a Student's \underline{t} -test or one way analysis of variance (see Tables 4.4, 4.5 and 4.8) of the arcsines. Where homogeneity ($\underline{P} > 0.05$) exists, the mean value of the pooled data has been calculated. Where the data are heterogeneous, a 'least significant difference' test was applied to obtain a pool of data and its outliers (e.g. nasal data at 23 hours). Where only two sets of data were present, and proved heterogeneous, the two means are treated separately (see small intestine data at 48h).

Figure 4.1 shows the relationship of the larvae recovered from the nasal region with those from the cranium. The curves are drawn through

Figure 4.1 Migration of S. ratti (homogonic) through the cranium (squares) and naso-frontal region (triangles). The curves are drawn through the most comprehensive data. Bars represent 95% confidence limits.

Figure 4.2 Migration of S. ratti (homogonic) through the nasal region (triangles) and subsequent arrival in the small intestine (squares) judged by direct observation. Bars represent 95% confidence limits.

Figure 4.1

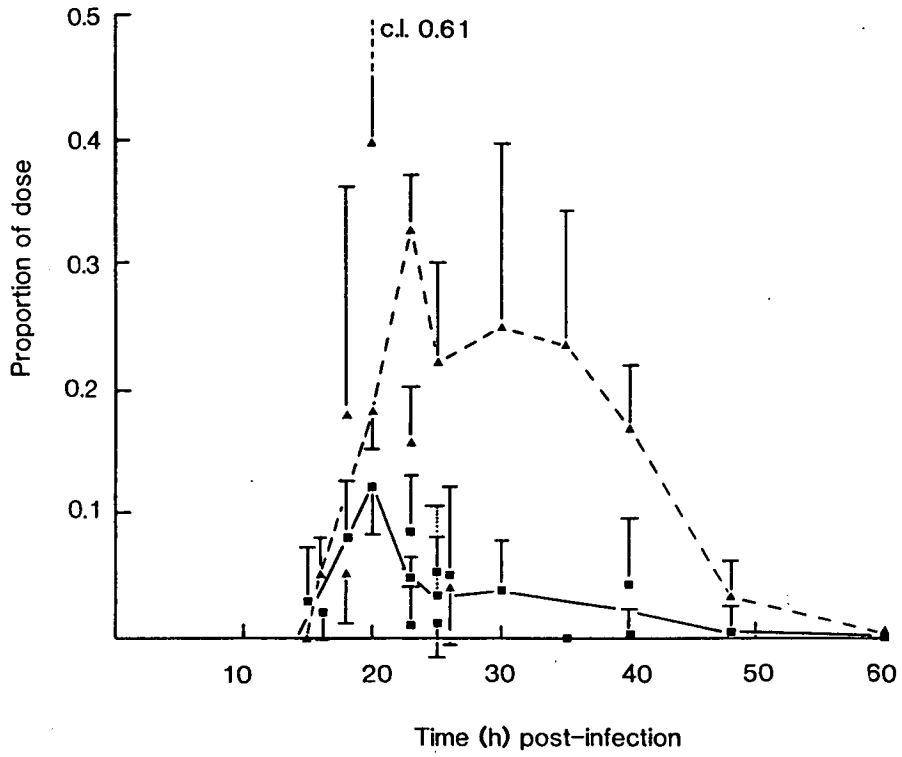
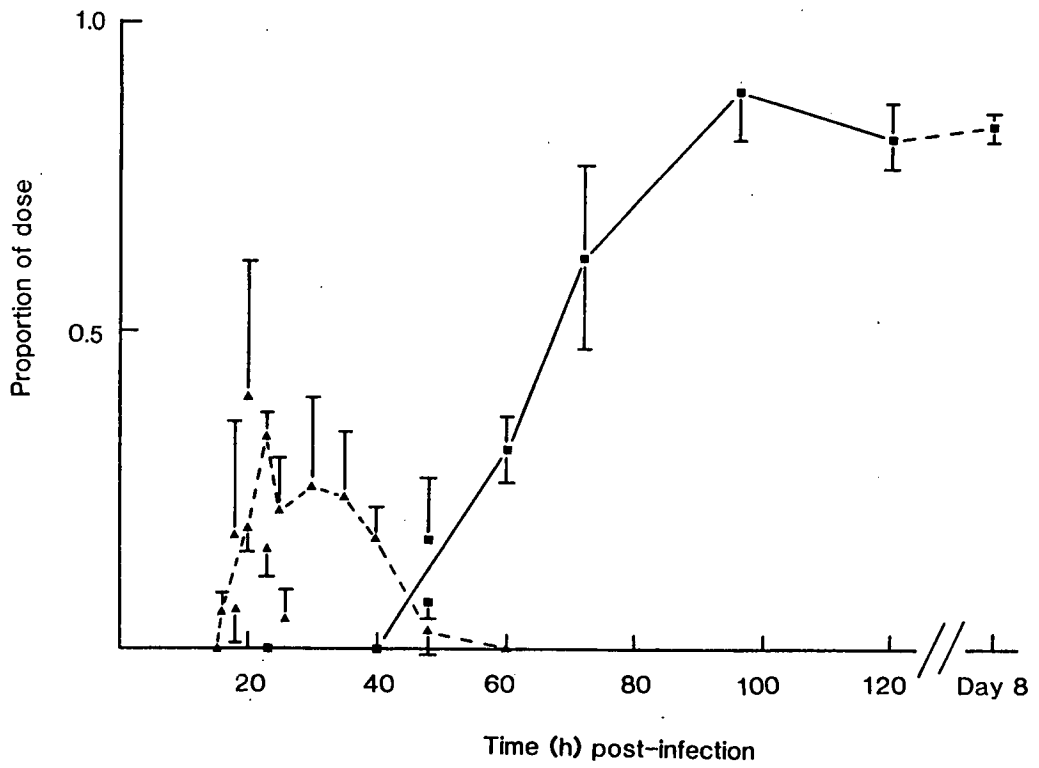


Figure 4.2



the most comprehensive data, outliers from the general trend being plotted separately. Larvae first appear in the cranium at 15h, rise to a peak by 20h, and the majority vacating the head by 50 hours. In the nasal region larvae were first detected after 16h, with the peak at approximately 23 hours. No larvae were recovered at 60h. This again suggests that the cranium-nasal region is a specific step on a single pathway, as the events in the cranium precede those in the nasal region. Factors, uncontrolled in these experiments, that cause heterogeneity between assays make interpretation of the exact timing of peaks difficult.

Figure 4.2 shows the migration through the nasal region and the subsequent arrival in the small intestine judged by direct observation. The first larvae were detected in the small intestine at 48h, with maximal arrival apparently occurring by 96 hours, some 36 hours after all have vacated the head. The technique of direct observation of larvae in the small intestine is flawed because the worms are extremely small ($<0.7\text{mm}$) and virtually transparent. The result is that larvae arriving in the intestine go unnoticed, with one's ability to detect them increasing as they grow (Wilson et al., 1986). The 'drug pulse' data recorded in Chapter 5 offer an alternative picture in this context.

All data obtained from the small intestine at 120h are expressed as egg-tracks, not adult worms. No statistical analysis is required to show that the proportion recovered at this time is not significantly different from the data obtained on day 8. In further studies 120h (Day 5) was adopted to evaluate p_f , as this increased the turn-

over of experiments.

4.3 S.ratti (heterogonic)

Wilson & Simpson (1981) suggested subtle differences in the migratory behaviour of the homogonic and the G60 heterogonic strains of S. ratti. Tada et al. (1979), Murrell (1980) (G60 strain) and Hattori (1981) calculated that the strains of S. ratti used in their experiments peaked in the head at 30-40h, 48h and 48h respectively, some 7-25h later than the data obtained in this study for the homogonic strain (pages 57-67). A series of experiments was therefore conducted to ascertain whether the G60 strain followed a similar pathway to the homogonic strain, and, whether any differences in the kinetics of such a migration could be observed in this system.

4.3.1 Migration through the head and subsequent arrival in the small intestine determined by direct observation [Expts 20-29].

A series of experiments similar to those performed on the homogonic strain was conducted to get an exact timing of p_0 in the nasal region, and to examine the relationship between larvae recovered from both sites within the head. The final experiments in the series were attempts to define the kinetics of migration from the nasal region to the small intestine using direct observation. The 'drug pulse' data recorded in Chapter 5 offer an alternative picture in this context. The protocols are given in Table 4.9.

Table 4.9 Protocols for Experiments 20-29 using S.

ratti (heterogonic). a. Cranium and naso-frontal region sampled. b. Cranium, naso-frontal region and small intestine sampled. c. Small intestine only sampled.

Table 4.10 Larvae of S. ratti (heterogonic) recovered from the nasal region and egg-tracks in the small intestine (day 6) for Experiments 20-29. \bar{p}_f = mean \bar{p}_f lower than general trend, therefore data from this experiment ignored in any analysis. P = probability that all means could have been drawn from the same population.

Table 4.9

Expt	Mean exact dose	The number of rats in which the head and intestine were sampled at each of the following times (h) post-infection												The number of rats used to obtain a value of \bar{p}_t on day 6.		
		15	20	25	30	35	40	45	50	60	70	80	90		120	
20	18.7	6a	6a	5a	6a	6a	4a	—	5a	—	—	—	—	—	—	5
21	18.8	—	—	6a	6a	6a	6a	—	—	—	—	—	—	—	—	6
22	18.1	—	—	—	—	—	8a	—	—	—	—	—	—	—	—	8
23	19.4	—	—	—	—	6a	6a	6a	6a	6a	—	—	—	—	—	6
24	19.2	6a	6a	6a	—	—	—	—	—	—	—	—	—	—	—	6
25	18.7	—	—	—	—	—	5b	—	6b	6b	6b	—	—	—	—	5
26	19.3	5a	6a	5a	4a	—	6a	—	—	—	—	—	—	—	—	5
27	18.7	—	—	—	—	—	6b	5b	6b	6b	6b	—	—	—	—	6
28	19.9	—	—	—	—	—	—	—	—	—	6c	6c	6c	—	—	6
29	19.0	—	—	—	—	—	—	—	—	6c	6c	6c	6c	6c	—	6

Table 4.10

Expt	Mean proportion of the dose (\pm SEM) recovered from the nasofrontal region at the following times (h) post-infection:											Egg-tracks as mean proportion of dose (\pm SEM)
	15	20	25	30	35	40	45	50	60	70		
20	N11	0.043 ± 0.021	0.227 ± 0.022	0.333 ± 0.030	0.430 ± 0.049	0.458 ± 0.046	—	0.133 ± 0.035	—	—	—	0.678 ± 0.021
21	—	—	0.155 ± 0.023	0.289 ± 0.043	*	0.370 ± 0.044	—	—	—	—	—	0.748 ± 0.039
22	—	—	—	—	—	0.262 ± 0.024	—	—	—	—	—	0.4385 ± 0.041
23	—	—	—	—	0.400 ± 0.072	0.428 ± 0.048	0.381 ± 0.026	0.179 ± 0.043	—	—	—	0.761 ± 0.045
24	N11	0.091 ± 0.023	0.198 ± 0.034	—	—	—	—	—	—	—	—	0.675 ± 0.021
25	—	—	—	—	—	0.503 ± 0.054	—	0.094 ± 0.054	N11	N11	—	0.648 ± 0.051
26	N11	0.038 ± 0.019	0.133 ± 0.037	0.165 ± 0.056	—	0.314 ± 0.073	—	—	—	—	—	0.697 ± 0.051
27	—	—	—	—	—	0.344 ± 0.047	0.247 ± 0.052	0.073 ± 0.018	N11	N11	—	0.713 ± 0.027
28	—	—	—	—	—	—	—	—	—	—	—	0.795 ± 0.045
29	—	—	—	—	—	—	—	—	—	—	—	0.771 ± 0.016
P (D.F.)	N.A.	0.253 (2, 15)	0.139 (3, 18)	0.065 (2, 13)	0.589 (10)	0.166 (5, 27)	0.090 (9)	0.195 (3, 19)	N.A.	N.A.	—	0.130 (8, 41)
Pooled mean	N11	0.057 ± 0.013	0.178 ± 0.016	0.274 ± 0.028	0.410 ± 0.046	0.396 ± 0.024	0.309 ± 0.033	0.119 ± 0.022	N11	N11	—	0.722 ± 0.013

Table 4.11 Larvae of S. ratti (heterogonic) recovered from the cranium in Experiments 20-29. \$ = mean p_f lower than general trend, therefore data from this experiment ignored in any analysis (see text). \underline{P} = probability that all means could have been drawn from the same population. a = Expts 20 and 23. b = Expt 21. c = Expts 20, 23 and 26. d = Expts 21, 25 and 27.

Table 4.12 Larvae of S. ratti (heterogonic) observed in the small intestine in Experiments 20-29. \underline{P} = probability that all means could have been drawn from the same population.

Table 4.11

Mean proportion of the dose (\pm SEM) recovered from the cranium at the following times (h) post-infection:

Expt	15	20	25	30	35	40	45	50	60	70
20	N11	0.038 \pm 0.029	0.052 \pm 0.029	0.036 \pm 0.023	0.017 \pm 0.011	N11	—	0.014 \pm 0.014	—	—
21	—	—	0.138 \pm 0.020	0.118 \pm 0.030	0.119 \pm 0.033	0.064 \pm 0.028	—	—	—	—
22	—	—	—	—	—	0.021 \pm 0.015	—	—	—	—
23	—	—	—	—	0.033 \pm 0.025	0.008 \pm 0.008	N11	N11	N11	—
24	N11	0.027 \pm 0.012	0.118 \pm 0.035	—	—	—	—	—	—	—
25	—	—	—	—	—	0.009 \pm 0.037	—	0.010 \pm 0.010	N11	N11
26	N11	0.009 \pm 0.009	0.069 \pm 0.029	0.070 \pm 0.018	—	N11	—	—	—	—
27	—	—	—	—	—	0.110 \pm 0.037	0.033 \pm 0.014	0.036 \pm 0.027	N11	N11
28	—	—	—	—	—	—	—	—	—	—
29	—	—	—	—	—	—	—	—	—	—
P (D.F.)	N.A.	0.574 (2,15)	0.169 (3,18)	0.036 (2,13)	0.037 (2,15)	0.0007 (5,27)	0.024 (9)	0.31 (2,15)	N.A.	N.A.
Pooled data	N11	0.025 \pm 0.011	0.090 \pm 0.014	means treated separate	0.025a \pm 0.013 0.119b \pm 0.033	0.003c \pm 0.003 0.082d \pm 0.019	means treated separate	0.015 \pm 0.010	N11	N11

Table 4.12

Mean proportion of the dose (\pm SEM) recovered from the small intestine at the following times (h) post-infection:

Expt	40	45	50	60	70	80	90	120
25	N11	—	N11	0.044 \pm 0.016	0.221 \pm 0.028	—	—	—
27	N11	N11	N11	0.085 \pm 0.022	0.140 \pm 0.021	—	—	—
28	—	—	—	—	0.170 \pm 0.026	0.188 \pm 0.025	0.425 \pm 0.061	—
29	—	—	—	—	0.214 \pm 0.044	0.338 \pm 0.026	0.429 \pm 0.066	0.770 \pm 0.029
P (D.F.)	N.A.	N.A.	N.A.	0.109 (10)	0.240 (3,20)	0.002 (10)	0.980 (10)	N.A.
Pooled data	N11	N11	N11	0.065 \pm 0.011	0.186 \pm 0.016	Means treated separate	0.427 \pm 0.043	0.770 \pm 0.029

Results

The proportions of the dose recovered from the three sites are shown in Tables 4.10, 4.11 and 4.12. Analysis of variance of all the data for \underline{p}_f shows one definite outlier from the general trend, Experiment 22 ($\underline{F}_{[9,48]} = 5.61, \underline{P} = 2.8 \times 10^{-5}$). The larvae in this experiment were impaired as the mean \underline{p}_f is depressed below the level of the others in the series. The data obtained in Experiment 22 have therefore been ignored in further calculations.

The last row in each table represents the overall mean for each site at each specific sampling time. Where homogeneity exists ($\underline{P} > 0.05$) between the data at a given sampling time the pooled mean is calculated. Where heterogeneity exists, a 'least significant difference' test was applied to obtain a pooled mean and its outliers.

The pooled data from Tables 4.10 and 4.11 have been plotted as Figure 4.3. As all the data for each sampling time within the nasal region are homogeneous, the curve for these data goes through every point. The data obtained for the cranium are highly heterogenous, with two or three distinct groups of data at each of the following sampling times: 30h, 35h, 40h and 50h. Where this occurs the curve has been drawn through the mean of the means of the groups. Larvae first appear in both regions at 20h, and have apparently vacated the head completely by 60h. The peak in the nasal region appears at 35-40h. An examination of the data for each experiment reveals that, in both cases where a comparison is possible, the peak is at 40h rather than 35h (Expts 20

Figure 4.3 Migration of S. ratti (heterogonic) through the cranium (squares) and naso-frontal region (triangles). The curves are drawn through the most comprehensive data. Bars represent 95% confidence limits.

Figure 4.4 Migration of S. ratti (heterogonic) through the nasal region (triangles) and subsequent arrival in the small intestine (squares) judged by direct observation. Bars represent 95% confidence limits.

Figure 4.3

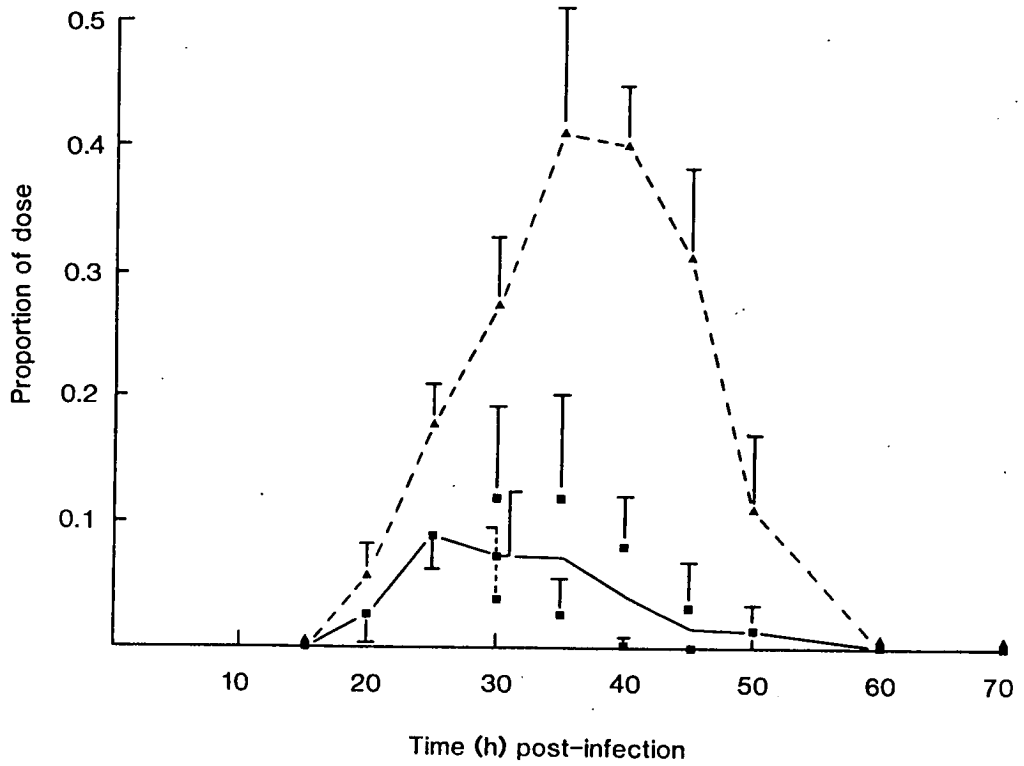
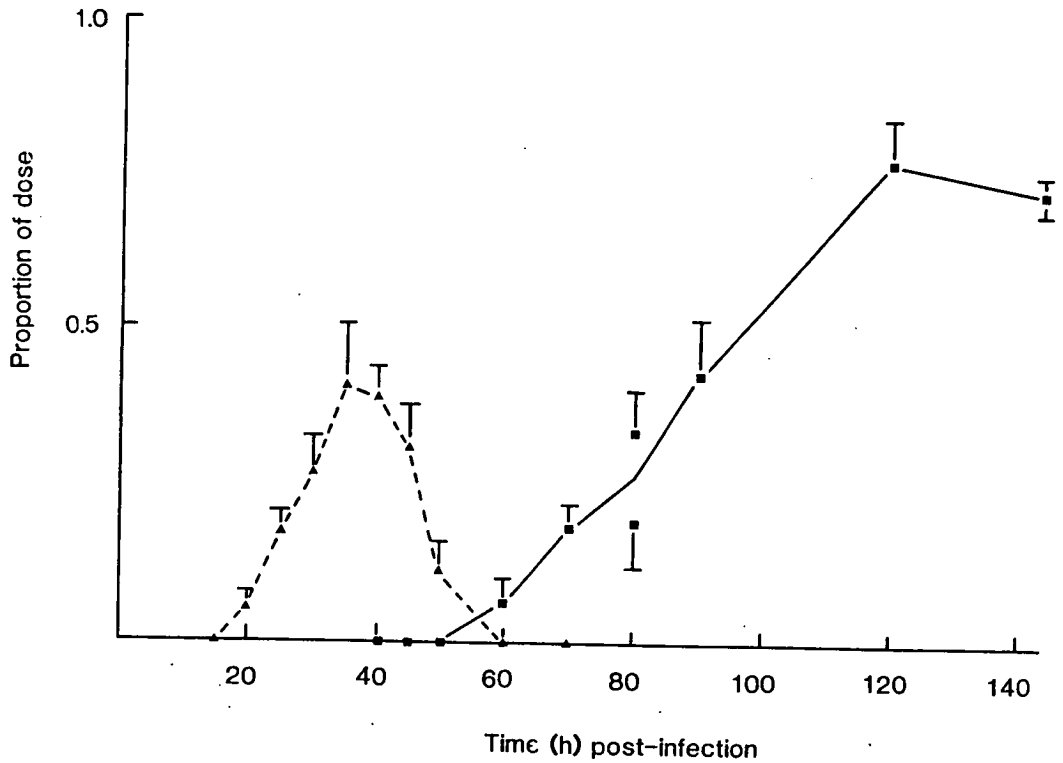


Figure 4.4



and 23). In all six experiments where the nasal region was sampled at 40h, the data obtained were high enough arithmetically to fulfil the inequality (for statistical analysis see below). The peak in the cranium is more difficult to pin-point because of the nature of the data. An examination of the results of each experiment (Tables 4.10 and 4.11) suggests that the peak in the cranium precedes that in the nasal region by 5-15h (Expts 20, 21, 23 and 26).

The pooled data for the arrival in the small intestine (Table 4.12) have been plotted with the nasal data as Figure 4.4. Direct observation suggested that larvae arrived in the small intestine by 60h with migration complete by 120h, some 60h after all have disappeared from the head. The results presented in Chapter 5 show that direct counts are flawed and that migration to the intestine is further advanced than the results presented here suggest.

4.3.2 Statistical proof for the involvement of the nasal region in the pathway.

Table 4.13 shows the values for \bar{p}_n , \bar{p}_f and p_c for experiments involving a sampling time of 40h in the nasal region. Column 6 shows the results of a t-test on the values of \bar{p}_n against \bar{p}_f for each experiment. The results are significant in 2 experiments, and not so in the remaining 4 (Expts 21, 23, 24 and 25). A one way analysis of variance of the arcsines of all the data for \bar{p}_n reveals homogeneity ($F_{[5,27]} = 1.71$, $P = 0.166$), with a similar result for p_f ($F_{[5,27]} = 1.07$, $P = 0.401$), justifying pooling of all the data. Examination of the error variances

Table 4.13

Expt	\bar{E}_n	\bar{E}_f	P_c	Probability that P_c is due to chance (D.F.)
20	0.542 ± 0.046	0.678 ± 0.021	0.136	0.023 (7)
21	0.630 ± 0.044	0.748 ± 0.039	0.118	0.075 (10) N.S.
23	0.576 ± 0.048	0.761 ± 0.045	0.183	0.029 (10)
25	0.497 ± 0.054	0.648 ± 0.051	0.151	0.078 (8) N.S.
26	0.686 ± 0.073	0.697 ± 0.051	0.011	0.944 (9) N.S.
27	0.656 ± 0.017	0.711 ± 0.027	0.057	0.576 (10) N.S.
Pooled data	0.603 ± 0.024	0.711 ± 0.017	0.108	0.0006 (64)

Table 4.14

Lung				Intestine		
Source (mean dose)	Time (h)	P_o	P_n	Source (mean dose)	Time	P_f
Table 1	15	0.50	0.50	Table 2	59h	0.60
	15	0.33	0.67		68h	0.55
(1040)	15	0.45	0.55	(1170)	75h	0.83
	16	0.24	0.76		95h	0.93
	18	0.44	0.56			
	19	0.34	0.66			
	19.5	0.40	0.60			
Table 2	26	0.69	0.31	Table 4	8 days	0.79
	32	0.42	0.58		8 days	0.90
(1070)	37	0.72	0.28	(270)	8 days	0.86
Mean		0.453	0.547			0.780
S.D.		0.152	0.152			0.148

of the p_n and p_f data (variance = 60.845 with 27 df and 42.141 with 27 df respectively; transformed) suggests no significant difference with a variance ratio test ($F_{[27,27]} = 1.44$, $P = 0.173$). A parametric t -test applied to the pooled data gave a $t_{[64]}$ -value of 3.61, with an overall probability of 6×10^{-4} . From the last line of Table 4.13 it can be said with certainty that 0.108 of the dose actually pass through the nasal region on their way to the gut, and without much doubt, a proportion of at least 0.397 (\bar{p}_0) arrive at their destination after migration through the nasal region. The data for the cranium does not satisfy the criteria in any experiment.

4.4 N. brasiliensis.

The aim of this section is to show the analysis of the data of Twohy (1956) in its full form to prove that subcutaneously injected larvae of N. brasiliensis transit the lung, then, to follow this up with a study on the migration after skin application.

4.4.1 Analysis of the data of Twohy (1956).

Twohy (1956) wrote his report without concern for a proof of the type Wilson (1983) proposes. Consequently, his tabulated values require interpretation and recalculation to reveal their relevance to the current debate. The results of the reappraisal are presented in Table 4.14. The major statistical problem is that Twohy's assays were not replicated sufficiently to give a precise measure of the associated variances. As a conservative approximation his numbers (recalculated

to give a proportion of the dose) reported at successive times have been treated as replicates. A t -value of 3.28 (df = 15) emerges for the difference between the means of the arcsines of \bar{p}_f and \bar{p}_n , having a probability of occurring by chance of 1 in 198 times. From this it can be deduced that p_c , the mean proportion of the dose that certainly passed through the lungs of the rats on their way to the intestine in Twohy's experiments was 0.23.

4.4.2 Migration of N. brasiliensis following skin application of larvae.

The bulk of Twohy's (1956) assays (and those from which the conclusions obtained in 4.4.1 were drawn) were obtained following subcutaneous injection of larvae. It is therefore still necessary to prove that the unnatural means of administration of larvae produced no abnormalities in migration. This is more imperative since Twohy's limited assays with skin application were accompanied by a large variance and a lower recovery of parasites from his rats compared with injection (14% vs 61%).

Materials and Methods [Expts 30-39].

Ten experiments were set up to investigate migration through the lung and subsequent arrival in the small intestine after skin application of larvae. In each, a large group of rats was infected by EDT20Nb. Subsequently, sub-groups were killed when their lungs and small intestines were examined (see pages 25 & 26). A final group was kept until day 8/10 to obtain a value for \bar{p}_f . The protocols for the

Table 4.15 Protocols for Experiments 30-39. a, lung sampled only. b, lung and small intestine sampled. c, small intestine only sampled.

Table 4.16 Larvae of N. brasiliensis recovered from the lung and adults in the intestine on days 8-10 post-infection for Experiments 30-39. P is the probability that all the means could have been drawn from the same population.

Table 4.15

Expt	Mean exact dose	The number of rats in which the lung and intestine were sampled at each of the following times (h) post-infection:													The number of rats used to obtain a value for \bar{P}_r on day 8/10.
		12	15	16	18	20	23	25	30	35	40	45	50	60	
30	18.8	—	—	—	—	6a	—	—	6a	6a	6a	—	—	—	6
31	19.0	—	—	—	—	—	—	—	—	6b	6b	6b	6b	—	4
32	19.7	—	—	—	—	—	—	—	—	5b	5b	—	5b	5b	5
33	19.9	—	—	—	—	—	—	—	—	6b	—	—	—	—	6
34	18.6	—	—	—	—	5a	6a	—	—	—	—	—	—	—	5
35	17.7	—	—	—	—	8a	—	—	—	—	—	—	—	—	8
36	20.0	5a	—	5a	5a	—	—	—	—	—	—	—	—	—	5
37	19.7	—	—	—	—	—	—	—	6a	—	6a	—	6a	—	6
38	20.5	—	6a	—	6a	6a	—	6a	6a	—	—	—	—	—	6
39	19.9	—	—	—	—	—	—	—	—	—	—	5c	—	—	—

Table 4.16

Expt	Mean proportion of the dose (\pm SEM) recovered from the lung at the following times (h) post-infection:													Adults as mean proportion of dose (\pm SEM)
	12	15	16	18	20	23	25	30	35	40	45	50	60	
30	—	—	—	—	0.328 ± 0.062	—	—	0.333 ± 0.051	0.484 ± 0.054	0.320 ± 0.066	—	—	—	0.782 ± 0.030
31	—	—	—	—	—	—	—	—	0.516 ± 0.049	0.280 ± 0.065	0.045 ± 0.016	N11	—	0.731 ± 0.037
32	—	—	—	—	—	—	—	—	0.508 ± 0.034	0.492 ± 0.037	—	0.009 ± 0.009	N11	0.802 ± 0.042
33	—	—	—	—	—	—	—	—	0.442 ± 0.063	—	—	—	—	0.788 ± 0.026
34	—	—	—	—	0.358 ± 0.083	0.319 ± 0.021	—	—	—	—	—	—	—	0.683 ± 0.052
35	—	—	—	—	—	0.357 ± 0.053	—	—	—	—	—	—	—	0.762 ± 0.025
36	0.033 ± 0.022	—	0.253 ± 0.036	0.333 ± 0.010	—	—	—	—	—	—	—	—	—	0.681 ± 0.082
37	—	—	—	—	—	—	—	0.411 ± 0.034	—	0.286 ± 0.033	—	0.011 ± 0.011	—	0.771 ± 0.057
38	—	0.113 ± 0.030	—	0.217 ± 0.049	0.388 ± 0.065	—	0.366 ± 0.054	0.382 ± 0.054	—	—	—	—	—	0.846 ± 0.061
39	—	—	—	—	—	—	—	—	—	—	—	—	—	—
P (D.F.)	N.A.	N.A.	N.A.	0.058 (9)	0.811 (2,14)	0.607 (12)	N.A.	0.487 (2,15)	0.743 (3,19)	0.060 (3,19)	N.A.	0.593 (2,14)	N.A.	0.291 (8,42)
Pooled data	0.033 ± 0.022	0.113 ± 0.030	0.253 ± 0.036	0.269 ± 0.032	0.358 ± 0.038	0.341 ± 0.031	0.366 ± 0.054	0.370 ± 0.027	0.487 ± 0.025	0.338 ± 0.031	0.045 ± 0.016	0.007 ± 0.005	N11	0.764 ± 0.016

10 experiments are given in Table 4.15.

Results

The proportions recovered from the lungs, along with their respective \bar{p}_f 's are recorded in Table 4.16. Table 4.17 shows the proportion of the dose recorded in the small intestine by direct observation (see also Chapter 5). Larvae first appeared in the lungs at 12h, peaked by 35h and finally disappeared at 60h. They were first detected in the small intestine at 40h with maximal arrival apparently occurring by 60h (Expt 9). A t -test or one way analysis of variance on the arcsines of all the data from each site (lung or intestine) at each specific sampling time demonstrated homogeneity in every case ($P > 0.05$), with the resultant mean value being calculated and plotted as Figure 4.5. The disappearance from the lung is a virtual mirror image of the estimated arrival (see Chapter 5) in the small intestine, suggesting that the larvae migrate directly from the lung to the gut.

4.4.3. Statistical proof of migration through the lungs.

The peak in the lungs appears at 35 hours in the four experiments in which this timing was sampled. Table 4.18 shows the outcome of the statistical analysis of the data obtained for \bar{p}_n against \bar{p}_f for these four experiments (30-33). A t -test (column 5) on the arcsines of p_n against p_f for each experiment shows that the results are significant in each case ($P = 0.0015, 0.0062, 0.00094$ and 0.0059 for Expts 30-33 respectively). A one way analysis of variance on the arc sines of all the data for \bar{p}_n and \bar{p}_f

Table 4.17 Larvae (N. brasiliensis) recovered from the small intestine of the rat for Experiments 30-39.

Table 4.18 Testing the inequality $\bar{p}_o + \bar{p}_f > 1$ for N. brasiliensis in the lung of the rat.

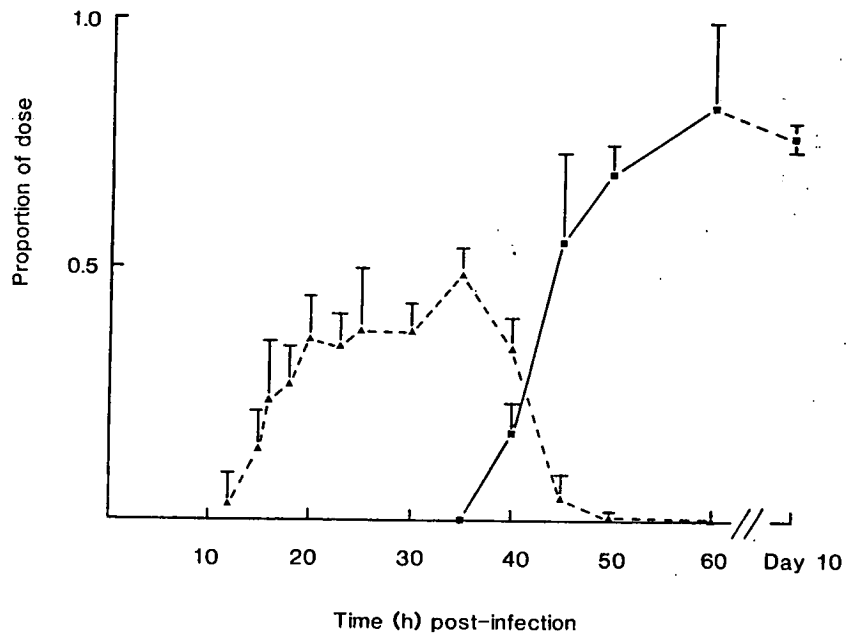
Table 4.17

Expt	Mean proportion of the dose (\pm SEM) recovered from the small intestine at the following times (h) post-infection:				
	35	40	45	50	60
31	Nil	0.198 ± 0.047	0.547 ± 0.073	0.621 ± 0.047	—
32	Nil	0.141 ± 0.020	—	0.770 ± 0.093	0.822 ± 0.063
33	Nil	—	—	—	—
39	—	—	—	0.667 ± 0.040	—
$\frac{P}{(D.F.)}$	N.A.	0.371 (9)	N.A.	0.159 (2,16)	N.A.
Pooled data	Nil	0.172 ± 0.027	0.547 ± 0.073	0.680 ± 0.034	0.822 ± 0.063

Table 4.18

Expt	\bar{P}_n	\bar{P}_f	P_c	Probability that P_c is due to chance (D.F.)
30	0.516 ± 0.054	0.782 ± 0.030	0.266	0.0015 (10)
31	0.484 ± 0.049	0.713 ± 0.037	0.229	0.0062 (8)
32	0.492 ± 0.034	0.802 ± 0.042	0.310	0.00094 (8)
33	0.558 ± 0.062	0.788 ± 0.026	0.230	0.0059 (10)
All data pooled	0.513 ± 0.025	0.779 ± 0.016	0.266	1×10^{-10} (42)

Figure 4.5



reveals homogeneity in both cases ($F_{[3,19]} = 0.417$, $P = 0.743$ and $F_{[3,17]} = 0.709$, $P = 0.56$ respectively), justifying the pooling of the data. A variance ratio test between the errors associated with counting larvae in the lung and those in the gut (variance from lung data = 53.93 with 19 df, variance from gut data = 31.67 with 17 df) reveals no significant difference ($F_{[19,17]} = 1.703$, $P = 0.138$). Thus a parametric t -test applied to the pooled data results in a $t_{[42]}$ -value of 8.606 with a probability of occurring by chance of 1 in 10^{10} . Therefore it can be said with certainty (from last row of Table 4.18) that at least 0.266 of the dose of skin applied N. brasiliensis pass through the lung en route to the gut, and without much doubt, all larvae within that site (mean $p_o = 0.487$) will reach their destination. ✓

4.5 Conclusions

4.5.1. S. ratti (homogonic)

(1) Preliminary experiments revealed that larvae of S. ratti (homogonic) migrate through the head of the rat on their way to the intestine. The peak in the head was 0.238 ± 0.014 (SEM) at 30h post-infection. The corresponding value for \bar{p}_f was 0.841 ± 0.012 (SEM). No larvae were recovered from the skin of the head. The brain was discarded as early assays were fouled with a debris attributable to this tissue.

(2) The specific site of migration was found to be the nasal region ($\bar{p}_o = 0.306$, $\bar{p}_f = 0.837$ in six experiments), where it

could be said with certainty that 0.153 of the dose actually passed through on their way from the skin to the small intestine. The timing of p_o within this site varied between experiments, but was always in the range 20–25h, with the most likely time of 23h.

(3) Larvae were also found in the cranium, but not in sufficient quantities to fulfil the inequality $\bar{p}_o + \bar{p}_f > 1$.

(4) Larvae reach the head through internal migration not superficial transfer.

(5) Although the cranium is unproven as part of a pathway, evidence is in favour of a hypothesis that this region is traversed before the nasal region, and that the cranium–nasal region forms a single step in a specific pathway. Larvae peak in the cranium at 20h and have virtually all disappeared by 50h, whilst those in the nasal region reach a peak at 23h and have disappeared from this region by 60h.

(6) Direct observation suggests larvae first appear in the small intestine at 48h, with migration complete by 96h, some 36h after all larvae have apparently vacated the head. The technique of direct observation is shown in Chapter 5 to be flawed with migration further advanced than these results suggest.

(7) The proportion of egg-tracks present on day 5 is not significantly different from day 8.

(8) Factors uncontrolled in these experiments lead to heterogeneity in the data at given times, but overall estimates of \bar{p}_O and \bar{p}_f are generally more consistent. (Note: Important questions in regard to the analysis of these data are addressed in the Discussion and Appendix 2.)

4.5.2 S. ratti (heterogonic)

(1) Larvae of the G60 heterogonic strain migrate via the nasal region of the rat head ($\bar{p}_O = 0.397 \pm 0.024$ (SEM), $\bar{p}_f = 0.711 \pm 0.017$ (SEM)), where it can be said with certainty that 0.108 of the dose actually pass through en route to the intestine.

(2) Larvae were also recovered from the cranium, but not in sufficient quantities to fulfil the inequality $\bar{p}_O + \bar{p}_f > 1$.

(3) Evidence from the kinetics of the migration suggest that events in the cranium possibly precede those in the nasal region. Larvae appear in the cranium and nasal region at 15h and peak at 25-35h and 40h respectively. Both regions yield no parasites by 60h.

(4) Factors uncontrolled in these experiments lead to heterogeneity in the data for the cranium.

(5) Direct observation suggests that larvae first appear in the small intestine at 60h and complete migration by 120h (see Chapter 5 where the technique of direct observation is shown to be flawed).

4.5.3 N. brasiliensis.

(1) Larvae of N. brasiliensis migrate via the lung of the rat, where it can be said with certainty that at least 0.266 of the dose actually pass through on their way to the intestine. It is highly likely that at least 0.487 (\bar{p}_0) of the dose actually utilize the lung as a transit site.

(2) Larvae first appear in the lung at 12h, peak at 35h and cannot be recovered from the region by 60h post-infection. The first larvae arrive in the small intestine at 40 hours, with migration completed by 60h (see Chapter 5 for assessment of direct observation of N. brasiliensis larvae in the gut).

MIGRATION TO AND FROM THE PROVEN TRANSIT SITES

5.1 Introduction.

Tada et al. (1979) proposed that larvae of S. ratti migrate to the head of the rat through the subcutaneous tissues. This was concluded from experiments in which the skin of the neck was circumsized, resulting in a delay in the larvae reaching the head. From the head they suggest that larvae migrate down the oesophagus with nasal excreta, bypassing the lung completely. This contradicted the conclusions of Abe (1964), Abe et al. (1965a, b), Hattori et al. (1968) and Hattori (1977) who suggested that the migration to the head was through the muscle stroma, with larvae passing through the lungs before reaching the gut. The views of Tada et al. (1979) are shared by Hattori (1981), Mimori et al. (1987) and Nojima et al. (1987).

5.2 Migration of S. ratti (homogonic) to the head in three-week-old rats.

It was envisaged that larvae applied to the normal infection site on the left flank of the rat would take longer to migrate to the head than those placed on the back of the neck if migration was through the muscle stroma or subcutaneous tissues rather than the circulatory system. Also, the proportion of the dose reaching the gut would be inversely proportional to the distance between the infection site and

the head, since the greater the distance from the transit site, the weaker the stimulus to migrate towards it, resulting in more larvae becoming 'lost'. Conversely, if the migration involved the circulatory system then, once in the blood stream movement would be a passive process, and the distance between the infection site and the head have no effect on the proportion arriving therein.

5.2.1 Distance travelled from the infection site to the head [Expt 1] in 3-week-old rats.

The distance from the infection site to a line drawn across the top of the head from eye to eye was measured in 10 rats and found to be 67.4mm (mean) from the flank, or 26.8mm from the neck.

5.2.2 Migration to the head and 'take' in the small intestine after infection in the two sites [Expts 2-5].

A series of 4 experiments was designed to try and ascertain whether the time for the larvae to peak in the head (23h for flank application, see Chapter 4) differed with the site of application. 20h and 25h were also examined in Expt 5.

Materials and Methods

The protocol for each experiment was essentially the same. Two groups of rats were infected on the neck and flank respectively by EDT20. At 23h, half of each set were killed and their nasal regions and craniums examined (page 26) for the presence of larvae. The remaining rats were killed on day 8 to obtain a value for \bar{p}_f . The numbers of rats

used in each experiment are given along with the results in Table 5.1.

Results

A t-test applied to the arcsines of the data for neck- and flank-applied larvae recovered from the cranium and nasal regions is non significant for each experiment. The same applies to the values for \bar{p}_f . On close examination, there is some evidence to suggest that larvae applied to the neck peak in the head earlier than those applied to the flank. In all four experiments, the proportion recovered from the nasal region at 23h is arithmetically (though not significantly) greater for neck administered larvae, whilst in Expt 5, by 25h the reverse is true. If the two sites in the head form a single step in a specific pathway, then the peak in the cranium would be immediately prior to the one in the nasal region (see Chapter 4). From the data of the first 3 experiments, the proportion recovered at 23h from the cranium is always greater for the flank applied larvae, the reverse of the numbers recovered from the nasal region at the same time. This could be tentatively presented as evidence that neck applied larvae are slightly more advanced in their migration through the head at 23h post-infection.

The proportion reaching the end point in these experiments was depressed below the mean value for the 11 experiments described in 4.2 ($\bar{p}_f = 0.835 \pm 0.010$ (+SEM)). Moreover, \bar{p}_o 's are also dramatically lower. Factors related to unavoidable changes in the diet of the rats were incriminated as the cause (see Appendix 1.2). The validity of any conclusions from this part of the study is therefore

questionable.

5.2.3 Migration of S. ratti (homogonic) after application to the neck or flank of 12-week-old rats.

The results obtained in 5.2.2 hinted that migration from the neck to the head was slightly quicker than from the flank to the head in 3-week-old newly weaned rats. It was possible that an increase in the difference in the distance between the infection sites would amplify this effect if it was a real one. To this end, a similar study utilizing 12-week-old mature rats as the host was performed. A prerequisite for the main study was to confirm that the peak in the head in mature rats occurred at the same time as in weanlings.

5.2.3.1 Migration of larvae through the head after flank application [Exp 6].

Materials and Methods

Fifteen mature female rats were infected by EDT20 (mean exact dose = 18.9) on the left flank, with larvae of S. ratti (homogonic). Three were kept until day 5 to obtain a value for \bar{p}_f , with the remaining 12 being split into 6 groups of 2. A pair of rats was killed at the following times post-infection: 20h, 25h, 30h, 35h, 40h and 50h when the mean proportion of the dose recovered from the nasal region was 0.027±0.027, 0.200±0.100, 0.077±0.024, 0.027±0.027, 0.027±0.027, 0.056±0.003 (SEM) respectively. Only a single larva was recovered from the cranium of one of the rats killed at 20h. \bar{p}_f was 0.572±0.049.

Table 5.1 The results of Experiments 2-5 for the proportion of the dose of S. ratti (homogonic) recovered from the cranium and/or nasal region after infection on either the left flank or the neck of 3-week-old rats. The proportion of the dose as egg-tracks in the intestine on day 5 is also given (\bar{p}_f). Sample size (n) is 6 except # where n = 5, * where n = 10 and, \$ where n = 8. \underline{P} is the probability that the proportions of larvae recovered after flank or neck application are the same.

Table 5.2 The results of Experiments 7-10 for the proportion of the dose of S. ratti (homogonic) recovered from the nasal and/or cranial regions after infection on either the flank or neck of 12-week old mature rats. The proportion of the dose as egg-tracks in the intestine on days 5 or 8 is also given. Sample size (n) is 6 except * where n = 5, and, ** where n = 4. \underline{P} is the probability that the proportions of larvae recovered after flank or neck application are the same. # Not significant by a simple \underline{t} -test, but an analysis of variance on all the data for the nasal region in this experiment, to get a better estimate of the error variance, shows a significant difference (see text). ? Tubes dropped during the experiment.

Table 5.1

Expt	Infection site	Mean exact dose	Mean proportion of the dose recovered from the cranium and nasal regions at the following times (h) post-infection:				\bar{E}_F
			Cranium		Nasal region		
			23	18	23	25	
2	Neck	18.9	0.042 ± 0.020	—	0.060 ± 0.025	—	0.764 ± 0.037
	Flank	18.3	0.043 ± 0.016	—	0.052 ± 0.012	—	0.766# ± 0.054
<u>P</u>			0.785		0.899		0.913
3	Neck	18.3	0.075* ± 0.022	—	0.121* ± 0.038	—	0.725 ± 0.074
	Flank	18.2	0.099* ± 0.012	—	0.073* ± 0.017	—	0.681\$ ± 0.051
<u>P</u>			0.132		0.536		0.560
4	Neck	19.1	0.028 ± 0.012	—	0.154 ± 0.059	—	0.661# ± 0.065
	Flank	18.2	0.053 ± 0.033	—	0.088 ± 0.022	—	0.624 ± 0.040
<u>P</u>			0.686		0.492		0.584
5	Neck	18.9	—	0.090# ± 0.031	0.234 ± 0.039	0.127# ± 0.044	0.751 ± 0.059
	Flank	19.0	—	0.083 ± 0.024	0.218 ± 0.026	0.184 ± 0.074	0.761 ± 0.083
<u>P</u>				0.956	0.777	0.462	0.485

Table 5.2

Expt	Infection site	Mean exact dose	Mean proportion of the dose (\pm SEM) recovered from the nasal-frontal region at the following times (h) post-infection:								\bar{E}_F
			Cranium				Nasal region				
			20	23	25	30	20	23	25	30	
7	Neck	45.9	—	—	—	—	0.190 ± 0.024	—	0.141 ± 0.021	—	0.510* ± 0.042
	Flank	45.1	—	—	—	—	0.089 ± 0.016	—	0.078 ± 0.014	—	0.565** ± 0.031
<u>P</u>							0.0078		0.045		0.167
8	Neck	46.2	—	0.064 ± 0.018	0.071 ± 0.021	—	—	0.190 ± 0.048	?	—	0.667 ± 0.035
	Flank	39.2	—	0.033 ± 0.009	0.056 ± 0.011	—	—	0.143 ± 0.030	0.118 ± 0.015	—	0.423* ± 0.075
<u>P</u>				0.164	0.686			0.431			0.010
9	Neck	50.3	0.044* ± 0.018	—	—	0.004 ± 0.004	0.287* ± 0.031	—	—	0.296 ± 0.042	0.833 ± 0.022
	Flank	48.5	0.035* ± 0.016	—	—	0.004 ± 0.004	0.233* ± 0.016	—	—	0.428 ± 0.055	0.873* ± 0.034
<u>P</u>				0.789		0.999	0.191			0.094#	0.285
10	Neck	48.4	—	—	0.013 ± 0.008	0.052* ± 0.017	—	—	0.111 ± 0.035	0.216* ± 0.033	0.608 ± 0.032
	Flank	47.3	—	—	0.016 ± 0.001	0.012 ± 0.009	—	—	0.150 ± 0.048	0.111 ± 0.029	0.580 ± 0.031
<u>P</u>					0.956	0.068			0.726	0.041	0.572

5.2.3.2 Migration to the head from infection sites on the flank and neck [Expts 7-10]. in 12-week old rats.

Four experiments were conducted to compare the migration from both infection sites. The protocols and results are summarized in Table 5.2. Each experiment was designed to supplement the results obtained in the previous one. To aid in detection, the dose size was increased to 50, as the amount of tissue debris present at the time of counting [Expt 6] was considerably greater than with 3-week-old rats. \bar{p}_f was estimated on day 5.

Experiment 7 (sampling times 20h and 25h, nasal regions only).

At both sampling times a significantly higher proportion of the neck applied larvae are found in the nasal region. No significant difference is seen in the \bar{p}_f data. The mean distance from the centre of the infection site to the head was measured for 12 rats in each group and found to be 43mm and 123mm for the neck and flank sites respectively.

Experiment 8 (sampling times 23h and 25h).

Experiment 8 was designed to see whether larvae applied to the flank, peaked in the nasal region in the period of time between the samples in Expt 7. Five weanling rats were infected on the flank by EDT20 as controls. A significantly greater proportion of the dose was recovered from the flank than the neck on washing the skin of mature rats some 45 minutes post-infection (0.178 ± 0.036 (SEM) versus 0.020 ± 0.007 (SEM),

$t_{[33]} = 5.16$, $P = 0.000012$). No larvae were recovered from the skin of the weanlings. No significant difference was seen in the proportions of neck or flank larvae (assumed to have penetrated) recovered from the nasal region or the cranium, at any of the sampling times, but, the proportions of neck larvae are arithmetically higher in all cases. A t -test on the arcsines of the p_f data reveals a highly significant difference ($t_{[9]} = 3.26$, $P = 0.0098$), with the larvae entering the neck establishing to a greater degree. This result could be due to the larvae on the flank having a greater difficulty in penetrating the skin than those on the neck, as seen from the proportion of the dose recovered from the infection site after 45 minutes. Even though non penetrators are recovered by the EDT procedure, 'partial penetrators' cannot be quantified. A high proportion of the former probably implies a significant number of the latter.

Experiment 9 (sampling times 20h and 30h).

In order to get a view of migration over a longer period of time, the sampling times involved in experiment 9 were 20h and 30h. The take on day 5 (6 rats with neck application and 5 with flank) was compared to that on day 8 (5 rats infected at each site). The aim of this was to decide whether migration was complete in adult rats by day 5 as it had proved to be in weanlings. The proportion of the dose recovered from the cranium at either time was not significantly different. Larvae recovered from the nasal region at 20h were higher from neck administration, and the reverse by 30h. However, the result at 30h became significant after applying an analysis of variance to the

arcsines of all the data for the nasal region ($F_{[3,18]} = 3.76$, $P = 0.025$).

The proportion of the dose applied to the neck developing to maturity by day 5 (0.833 ± 0.022 (SEM)) is clearly not different from those on day 8 (0.838 ± 0.037 (SEM)). The same applies to the data from flank applied larvae (0.873 ± 0.034 (SEM) and 0.820 ± 0.035 (SEM) for day 5 and day 8 respectively).

In this experiment the data obtained from the nasal region at 30h was sufficient, for both flank and neck applied larvae, to fulfil the inequality $\bar{p}_o + \bar{p}_f > 1$. For the flank, $\bar{p}_o = 0.426 \pm 0.055$ (SEM), $\bar{p}_f = 0.846 \pm 0.025$ (SEM) (day 5 and 8 combined) resulting in a value for p_c of 0.272. A t -test on the arcsines of p_n against p_f reveals a significant difference ($t_{[14]} = 4.956$, $P = 0.00021$). For the neck, $\bar{p}_o = 0.296 \pm 0.042$ (SEM), $\bar{p}_f = 0.836 \pm 0.021$ (SEM) (day 5 and 8 combined) resulting in a value for p_c of 0.132. A t -test of p_n against p_f gives a significant difference ($t_{[16]} = 2.99$, $P = 0.0086$). Therefore it can be said with certainty that larvae of this strain actually pass through the nasal region en route to the gut in adult rats.

Experiment 10. (sampling times 25h and 30h).

The proportion of the dose recovered from the nasal region at 30h was significantly higher for the neck applied larvae ($t_{[9]} = 2.384$, $P = 0.041$), the reverse of the results in Expt 9. The data here

are suggestive that larvae applied to the flank peak in the head first. No significant difference was seen in the day 5 \bar{p}_f 's.

Conclusions from Expts 7-10

The results of larvae administered to different sites in 3-week old rats suggested that larvae applied to the neck reached the head earlier than ones applied to the flank. Expts 9 and 10 using 12-week old rats as the host, produced contradictory results. It is therefore difficult to make any conclusions from these experiments, especially after examining the data from Expt 8 where there is a suggestion that larvae experience a greater difficulty in penetrating the skin if placed on the flank rather than the neck of mature rats. The differences in rates of migration may be due to this, rather than the hypothesis in question. Moreover, the adverse effects on the parasite of dietary disturbances place the majority of these results in question (see Appendix 1.2).

5.3 Migration from the head to the gut re-examined (see Chapter 4).

Tada et al. (1979) suggested that larvae migrated directly from the head of the rat down the oesophagus to the stomach and small intestine. If this were the case, disappearance from the head should correlate with arrival in the gut, i.e. the two curves would be mirror images of each other, unless the larvae are subjected to an impedance in the oesophagus or the stomach. The results on pages 66 & 72 confirmed that both the homogonic and heterogonic strains of S. ratti had disappeared from the head by 60h, but appeared not to peak

in the gut until 96h and 120h respectively. Wilson et al. (1986) concluded that the shape of the curve for arrival in the small intestine determined by direct observation was a resultant of (a) the actual arrival of L3 at their destination, and (b) the improvement in one's ability to see an individual worm after it has grown larger; or, if methods of larval extraction from the tissue are used, the change in 'extractability' of worms as time progresses. Attempts to eliminate the bias suggested to exist by Wilson et al. (1986) are described in the following section and ascertain whether the delay in the peak in the gut after leaving the head is due to a procedural phenomenon or to the fact that larvae are 'impeded' in their migration between the two sites.

5.3.1 Arrival of S. ratti (homogonic) in the small intestine assessed by compressed tissue autoradiography.

The technique of compressed tissue autoradiography has been widely accepted as a tool to elucidate the migratory pathway of Schistosoma mansoni in an unnatural host, the mouse (Georgi, 1982; Georgi, Dean & Chandiwanna, 1982; Georgi, Dean & Mangold, 1983; Mangold & Dean, 1983; Dean & Mangold, 1984; Dean, Mangold, Georgi & Jacobson, 1984; Mastin, Wilson & Bickle, 1985; Georgi, Wade & Dean, 1986; Wilson & Coulson, 1986; Wilson, R. A., Coulson & Dixon, 1986; Georgi, Wade & Dean, 1987). Indeed, assuming the equivalence of radiographic foci to worms, the data obtained by Mangold & Dean (1983) are sufficient to fulfil the inequality principle and prove the involvement of the lung in the migratory pathway of S. mansoni in the mouse (see General Discussion). It has also been used to investigate the migration of

Toxocara canis in the mouse (Wade & Georgi, 1987). Kwansa & Murrell (1986) showed that labelled S. ratti larvae produced images on compressed tissue autoradiograms. If the signal:noise ratio is high, autoradiography is more sensitive than instrumental radioassay because nuclear emissions can be collected by a photographic emulsion, with each reduced focus being equivalent to one labelled organism. Thus the loss of label from the worm during migration is not as important (Georgi, 1982).

Experiment 11

Wilson (1979) and Wilson & Simpson (1982) devised a system for labelling L3's by the addition of 0.3ml of an aqueous solution of selenomethionine containing approximately 22kBq of ^{75}Se to a faecal pellet added to each well of a counting slide (page 22). Using this system they successfully obtained a label on the third stage larvae of 1.49 counts per minute (c.p.m/L3). In the present study the above concentration of label was compared to a five fold increase, i.e. 110kBq per well. Three petri dishes were set up with counting slides containing label at a concentration of 22kBq per well, 3 with the label at 110kBq per well and a final 3 dishes with just 0.3ml of water added to each well to obtain unlabelled larvae for control purposes. All were incubated at 19°C for 72h, with a drop of distilled water being added at 60h. The cultures appeared very wet at 72h, with only a few larvae being able to migrate out into the surrounding water. This meant that no estimate of the label associated with each worm was possible. The supernatant fluid had an initial count near to background, consequently no centrifugation or washing

was required.

Ten rats were infected by EDT20 with the larvae from the lower isotope concentration (mean exact dose = 17.1), 9 rats with larvae from the higher concentration (mean exact dose = 18.7) and 4 rats with the unlabelled larvae (mean exact dose = 18.0). Direct counting of larvae in the gut (page 64) suggested that at least 0.5 of the dose had completed migration by 72h. With this in mind, 5 of the rats infected with worms from the lower isotope level were killed at 72h, along with 4 from the group infected with worms presumed to have the higher label. Rats were starved for 24h prior to removal of the guts, so that the intestinal contents were reduced to a minimum. The small intestines of these animals were placed on a piece of straw board, covered in cling film then squashed and dried (page 27). The dried guts were placed in autoradiographic cassettes (2 per cassette) with a piece of x-ray film. The cassettes were left for 4 weeks, then developed (page 27). No reduced foci were present on any of the autoradiograms.

The control animals were killed on day 5 and their small intestines examined for the presence of egg tracks. The proportion of the dose as egg tracks for the low intensity larvae was 0.795 ± 0.067 (SEM), for the high intensity larvae 0.785 ± 0.041 (SEM), and for the controls 0.700 ± 0.049 (SEM).

Experiment 12

An experiment virtually identical to experiment 11 was conducted. The

concentrations of label used were the same. In this experiment, enough larvae migrated out of the culture to enable an assessment of the activity on each to be made. Many larvae still failed to escape as the cultures appeared very wet. After centrifugation to remove free label in the supernatant, 600 hand counted larvae were measured for activity (page 27). The c.p.m for low intensity larvae was 4.04 and for high labelled larvae 21.5. Ten rats were infected with high intensity larvae (mean exact dose = 18.3), 10 with low labelled larvae (mean exact dose = 17.8) and 4 with unlabelled controls (mean exact dose = 19.8), all by EDT20. Five rats from both groups infected with labelled larvae were killed at 72h and guts subjected to autoradiography. The remaining animals were killed on day 5 for \bar{p}_f . After exposure for 4 weeks the autoradiograms were developed. In all cases the amount of background noise was so high that detection of any foci caused by larvae proved impossible.

A one way analysis of variance on the arcsines of the data for \bar{p}_f (0.345±0.060, 0.537±0.062 and 0.822±0.060 (+SEM) for low, high and unlabelled larvae respectively), reveals a significant difference between the migratory success of the labelled larvae compared to the controls ($F_{[2,10]} = 10.99$, $\underline{p} = 0.003$).

Experiment 13.

To maximize the possibility of detection of any foci and reduce background noise to a minimum, Wilson R. A. et al. (1986) placed an intensifying screen in each cassette and let exposure take place at -80°C. Both these techniques were employed in Experiment 13. The

problem of the cultures being too wet was circumvented by the addition of the label to each well in only 0.15ml of water as compared to the 0.3ml used previously.

To each of three cultures was added either 22kBq ^{75}Se , 110kBq ^{75}Se or distilled water in a total volume of 0.15 ml. At 72h the activity was 4.73 c.p.m and 17.07 c.p.m for low and high labelled larvae respectively. To assess the proportion of the larvae actually labelled, a dose in the order of 20 L3's was placed on each of 4 coverslips (64mm x 22mm) and instantly heat fixed. The number of larvae on the coverslip was then recorded, the coverslip covered in sellotape and finally placed in an autoradiographic cassette with an intensifying screen. Nine rats were infected by EDT20 with low intensity larvae (mean exact dose = 19.2), nine with the high intensity larvae (mean exact dose = 18.6) and 5 rats with unlabelled larvae as controls (mean exact dose = 19.0). Four rats from both groups receiving labelled larvae were killed at 72h, and their small intestines removed and used for autoradiography. The remaining rats were killed on day 5 for \bar{p}_f .

Two further rats were infected with 500 L3's (one with the low intensity and one with high intensity larvae) on the flank. These were killed at 72h and larvae extracted from the gut (page 28) were spotted onto coverslips as above to ascertain whether the label remained on the worms throughout migration. All the high intensity larvae produced a distinct image prior to infection, whereas, 100 out of 105 of the low intensity ones were recognisable as foci (Plate 5.1). However, when extracted from the small intestine at 72h, all the high intensity

Plate 5.1 Contact prints of autoradiograms of labelled larvae of S. ratti (homogonic) spotted onto coverslips.

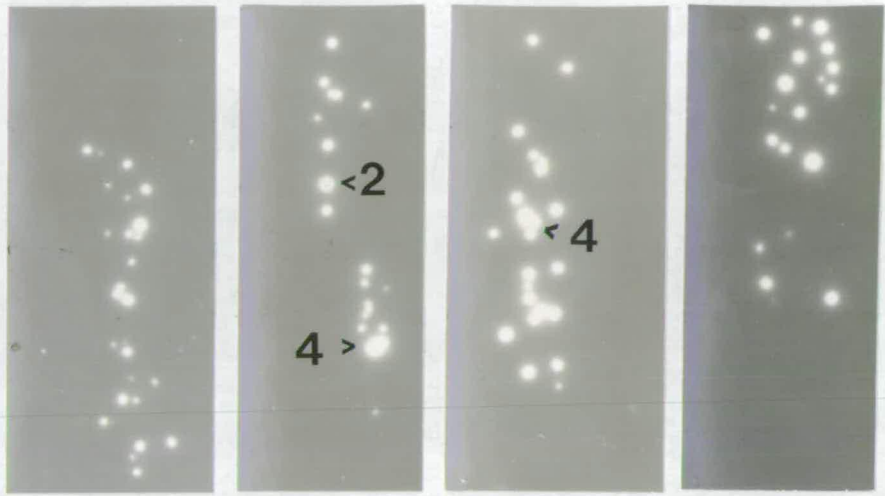
(a) Larvae from cultures labelled with 110kBq ^{75}Se -methionine. Left to right, the number of larvae spotted were 27, 23, 24 and 18. The number of foci are 27, 23, 24 and 18. The numbers on the autoradiogram represent foci that are the result of more than one larva.

(b) Larvae from cultures labelled with 22kBq ^{75}Se -methionine. Left to right, the number of larvae spotted were 23, 31, 25 and 26. The numbers of foci are 22, 29, 23 and 26.

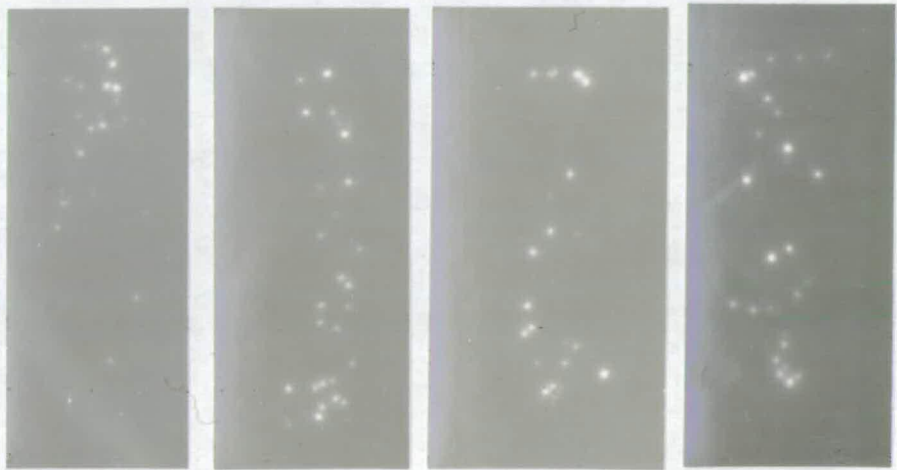
(c) Larvae extracted from the gut of a rat at 72h. The initial larvae had been labelled with 110kBq of ^{75}Se -methionine. The numbers of larvae spotted left to right were 14, 13, 13 and 14. In each case, every larvae can be seen as a focus on the autoradiogram.

(d) Larvae from gut of rat at 72h. The initial larvae came from cultures labelled with 22kBq ^{75}Se -methionine. The number of larvae spotted were 12, 10, 9 and 12. The number of foci present is 11, 5, 7, & 4. These foci are very faint and difficult to detect.

(a)



(b)



(c)



(d)

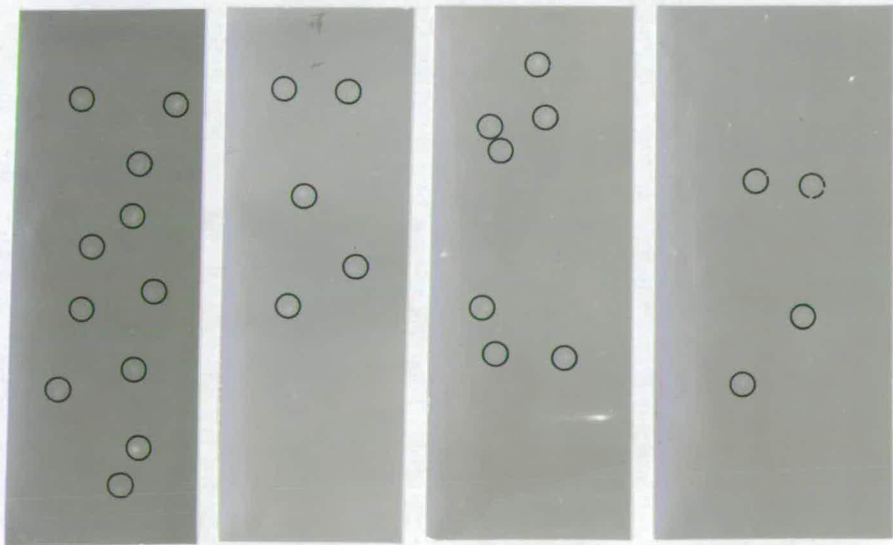
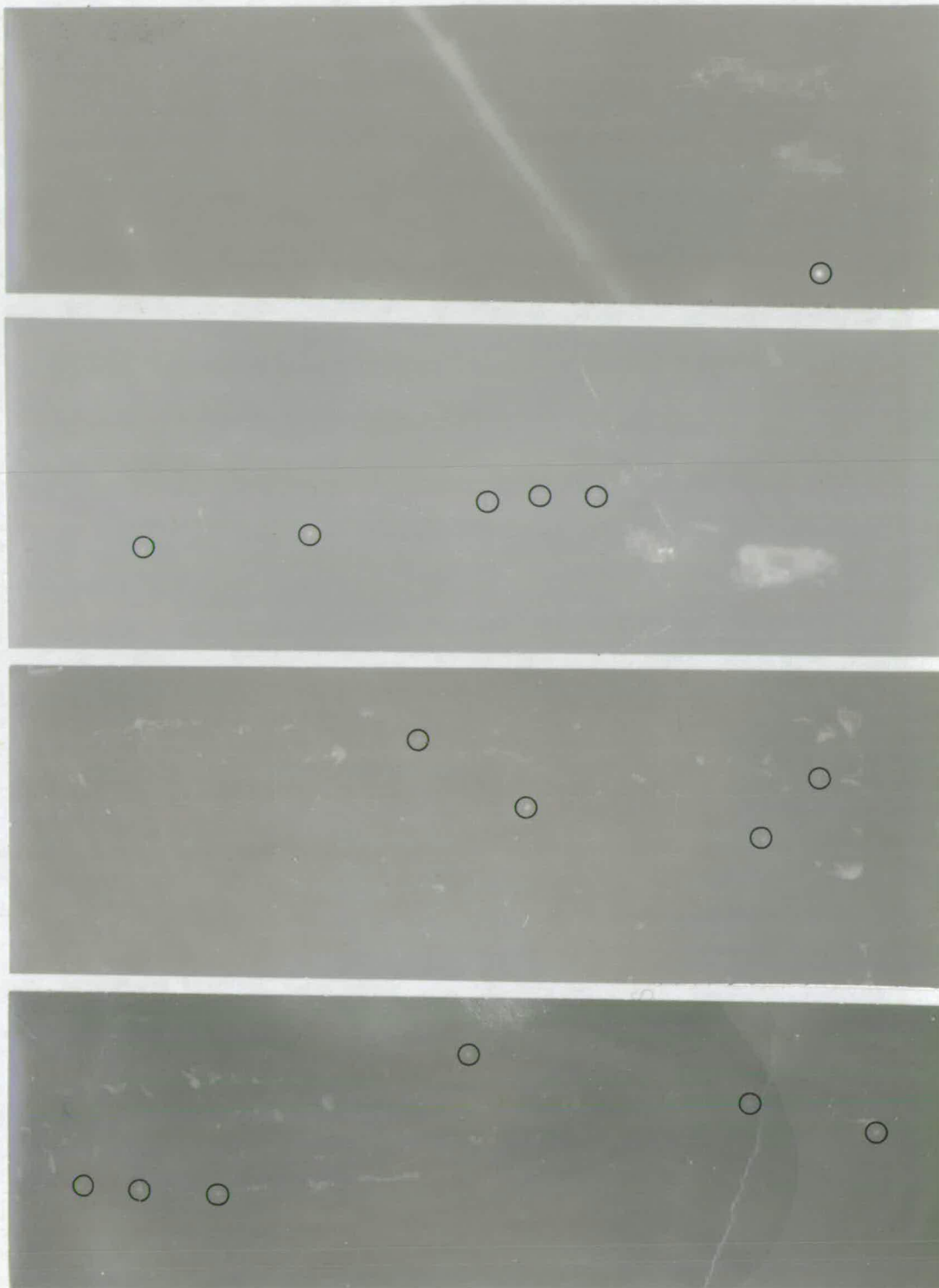


Plate 5.2 Contact prints of autoradiograms of the small intestines of rats infected 72h previously with labelled larvae of S. ratti (homogonic).

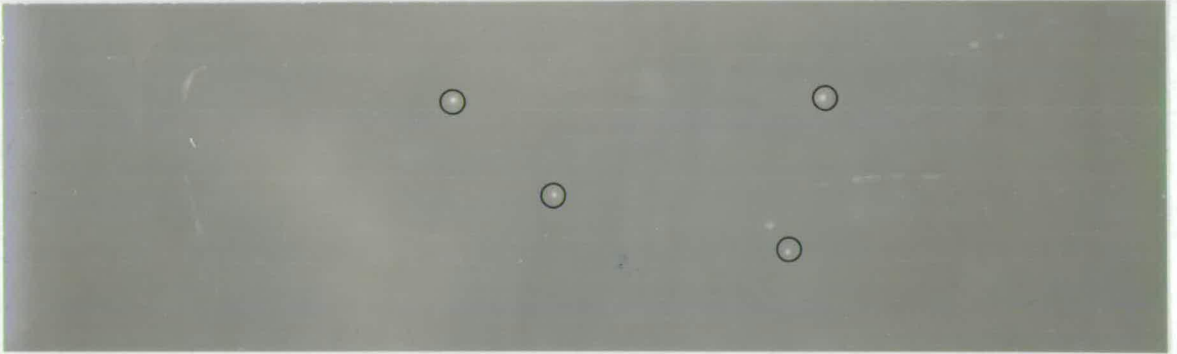
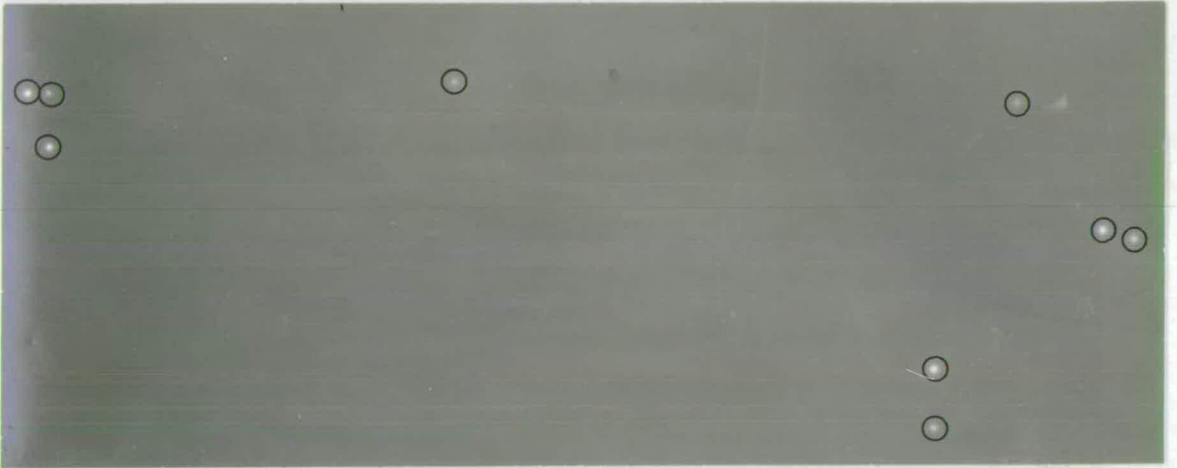
(a) Larvae from cultures labelled with 22kBq. From top to bottom, the dose/foci are as follows, 21/1, 19/5, 19/4, 18/6.

(b) Larvae from cultures labelled with 110kBq. From top to bottom the dose/foci are as follows, 20/9, 19/9, 19/4, 19/4.

(a)



(b)



larvae produce a distinct image, whereas the low intensity ones proved difficult to detect, with only 27 out of 43 distinguishable.

Plate 5.2 shows the autoradiograms of the rat small intestines removed at 72h post-infection. The images produced by the low labelled larvae are faint and difficult to detect. From this evidence, only 63% of larvae extracted from the gut at 72h produced an image, therefore the 0.214 ± 0.086 (SEM) present in the small intestine in these animals may be well below the actual numbers present. An examination of the data for the high labelled larvae shows the proportion of the dose as foci after 72h (0.336 ± 0.073) to be arithmetically higher than the \bar{p}_f value determined as egg-tracks (0.253 ± 0.041). This suggests that migration is complete by 72h, much earlier than estimated by direct counts (96h). However, the data obtained for the high labelled larvae have to be treated with care, as an analysis of variance on the arcsines of all the values of \bar{p}_f shows these to be significantly lower than the controls (0.692 ± 0.042) and low labelled larvae (0.624 ± 0.074) ($F_{[2,12]} = 16.81, p = 0.00033$).

Experiment 14. Does the label inhibit the migratory success of the larvae?

The results of Expt 13 suggested that larvae labelled with 110kBq per well were inhibited in their migration. This experiment was designed to investigate the effect of the label on migrating larvae. Three cultures were set up with 180kBq of [^{75}Se]-selenomethionine in 0.15ml of water. Three further cultures had just 0.15ml of water

added to each well. The larvae were harvested after 72h and the labelled ones centrifuged several times to eliminate free 75 selenium in the supernatant. The mean c.p.m./L3 of 800 hand counted L3's was 24.01. Eight rats were infected by EDT20 with labelled larvae (mean exact dose = 17.9), and 7 with control larvae (mean exact dose = 19.0). On day 5 the guts were examined and the worm burdens assessed. A significant difference is seen between the arcsines of p_f for control larvae ($\bar{p}_f = 0.858 \pm 0.026$ (SEM)) and labelled larvae ($\bar{p}_f = 0.439 \pm 0.048$ (SEM)) ($t_{[13]} = 7.397$, $p = 5 \times 10^{-5}$.)

Experiment 15. The effect of centrifugation on the 'take'.

The results of Expts 13-14 clearly show that labelled larvae are inhibited in their migration. This could be the effect of the label, or, the fact that labelled larvae undergo centrifugation to reduce the activity of the supernatant. In Experiment 11 where no centrifugation took place, migration appeared normal. Experiment 15 was designed to investigate the effects of centrifugation on both labelled and unlabelled larvae. Three cultures were set up with each well receiving 110kBq of [75 Se]-selenomethionine in 0.15ml of water. A further 3 cultures were set up for control larvae. At 72h, each group of larvae was split into 2 sub-groups. One sub-group was subjected to centrifugation until the activity of the supernatant was approaching background (9 times at 1500 rpm for 2 minutes), whilst the count of the supernatant in the other group was reduced by a series of dilution and sedimentation. The unlabelled control larvae were split into two subgroups and each treated as the labelled ones. The c.p.m of the

centrifuged larvae was 15.6. No estimate was made of the activity on larvae cleaned by sedimentation.

Groups of five rats were infected by EDT20 with either labelled and centrifuged larvae, labelled and sedimented larvae, unlabelled and centrifuged larvae or unlabelled and sedimented larvae (mean exact doses = 16.6, 18.2, 17.2 and 18.2 respectively). Values for \bar{p}_f on day 5 were as follows,

Labelled and centrifuged larvae,	$\bar{p}_f = 0.118 \pm 0.104$ (SEM)
Labelled and sedimented larvae,	$\bar{p}_f = 0.797 \pm 0.056$ (SEM)
Control larvae after centrifugation,	$\bar{p}_f = 0.272 \pm 0.036$ (SEM)
Control larvae after sedimentation,	$\bar{p}_f = 0.778 \pm 0.053$ (SEM)

No detailed analysis is required to show that the effect of the label on migratory success is negligible since the \bar{p}_f for labelled larvae is arithmetically higher than the unlabelled controls. However after centrifugation it is clear that the larvae are impaired since the \bar{p}_f of both labelled and unlabelled larvae is appreciably depressed below that of the sedimented larvae. In further experiments, all larvae were cleaned by sedimentation.

Experiment 16. Arrival of larvae in the small intestine of the rat.

The results of Expt 13 led to the possibility that migration may be complete by 72h. Experiment 16 was planned to explore arrival in the intestine up to 70h. Three cultures were set up with 110kBq of [⁷⁵Se]-selenomethionine in 0.15ml of distilled water as described

previously. Control larvae were produced by a similar culture method. All larvae were cleaned by sedimentation at 72h after which, the activity present on the labelled worms was 3.4 c.p.m/L3. As this was considerably lower than previous experiments, the autoradiograms were exposed for 5 weeks. Twenty-two rats were infected by EDT20 with labelled larvae (mean exact dose = 19.1) and at subsequent times, their small intestines removed for autoradiography. Four rats were infected (EDT20) with unlabelled larvae (mean exact dose = 18.5) as controls. The results of the experiment are shown below as Table 5.3.

Time of killing (h) post- infection	Number of animals	Proportion of the dose (\pm SEM) as reduced foci on the autoradiogram
40	4	0.277 \pm 0.084
50	5	0.470 \pm 0.097
60	4	0.141 \pm 0.068
70	4	0.406 \pm 0.049
\bar{p}_f labelled	5	0.737 \pm 0.036
\bar{p}_f control	4	0.617 \pm 0.099

Clearly the labelled larvae were not impaired since the mean \bar{p}_f is greater than the unlabelled controls. The autoradiograms were difficult to interpret as the foci were very faint, due to the low activity on each worm.

Experiments 17-21

Unfortunately at this point in the project the normal rat diet (Oxoid 41B, Styles Ltd) ceased to be manufactured, and the rats were initially transferred onto PCD food (Special Diets Services), which proved inadequate for the culturing of S. ratti (see Appendix 1.2). As a result, very few infective larvae were obtained from the cultures in Expts 17-21, and consequently the technique of autoradiography was abandoned.

5.3.2 The 'drug pulse' technique.

Previous work in this laboratory (Wilson unpublished) suggested that a mirror image of the arrival of larvae in the stomach and small intestine could be obtained by the oral administration of a short 'pulse' of anthelmintic to rats in a series. If the drug has a short persistence it will remove larvae already in the gut but have little or no effect on subsequent arrivals. A comparison between the takes in such hosts (p_m) to a mean from untreated controls (\bar{p}_f) gives the proportion of \bar{p}_f present at the time of administration as

$$\frac{\bar{p}_f - p_m}{\bar{p}_f}$$

Wilson (unpublished) suggested that, with suitable controls, morantel tartrate was a candidate for this purpose at a dose rate of 0.02mg/g

body weight. The drug was applied to a lightly etherized rat by stomach tube. As a control measure, the technique was first applied to N. brasiliensis, since it was assumed that as the larvae arriving at the gut in this case are large and easily quantifiable, the direct counts in the gut were accurate. A comparison between the results obtained by direct counting and the drug pulse technique would evaluate the efficiency of the system.

5.3.2.1 Arrival of N. brasiliensis detected by the drug pulse technique [Expt 22].

Materials and Methods

Forty six rats were infected with N. brasiliensis by EDT20Nb (mean exact dose = 22.6), and then, in groups of 5 or 6, given an oral dose of morantel tartrate (0.02mg/g body weight) at the following times post-infection (after 24h starvation), 10h, 30h, 40h (5 rats), 50h, 60h (5 rats), 70h and 80h. All rats were fed immediately after treatment to aid the expulsion of the drug from the gut. The final 6 rats were left as untreated controls. All rats were killed on day 8 and the number of adult worms in the small intestine counted and expressed as a proportion of the dose (p_m for treated animals, p_f for controls).

Results

The worms recovered on day 8 were 0.788 ± 0.030 , 0.735 ± 0.039 and 0.104 ± 0.031 of the exact dose from rats treated 10h, 30h, and 40h

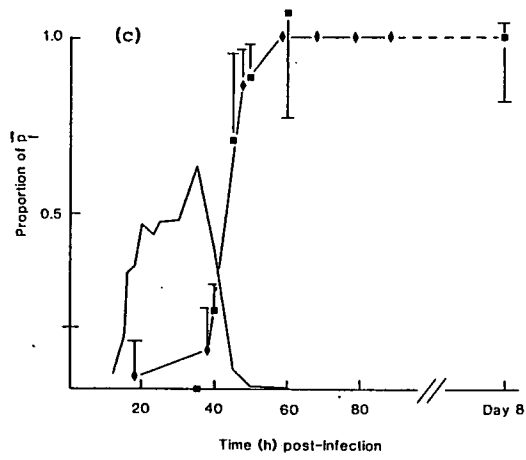
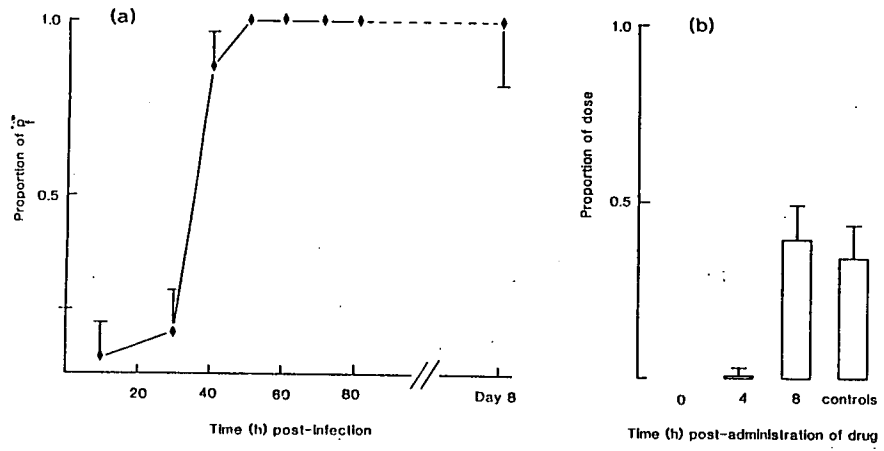
Figure 5.1

(a) The results of the drug pulse for N. brasiliensis translated to the proportion of \bar{p}_f present at the time of application of the drug. Day 8 results are the untreated controls (\bar{p}_f).

(b) The persistence of a dose (0.02mg/g body weight) of morantel tartrate with respect to N. brasiliensis (Expt 23). The histogram shows the development of larvae orally administered at different times after the drug. The controls received no drug.

(c) The migration of N. brasiliensis through the lung and subsequent arrival in the gut assessed by direct counts (squares) (pages 75-78) and the drug pulse (diamonds). The data for the drug pulse has been moved 8h to the right to take into account the maximum persistence of the anthelmintic. All the data are expressed as a proportion of the control larvae that complete migration (\bar{p}_f).

Figure 5.1



post-infection. No adult worms were recovered from rats treated after 40h. The data, translated to give the proportion of \bar{p}_f (0.827±0.070) present in the gut at the time of the treatment, are shown as Figure 5.1a. The first time the drug has any effect is at 40h, as the means of the arcsines of the raw data obtained prior to this time are not significantly different from the controls (at 10h, $t_{[10]} = 0.905$, $p = 0.387$, and at 30h, $t_{[10]} = 1.438$, $p = 0.181$).

5.3.2.2 Persistence of the drug with reference to N. brasiliensis [Expt 23].

Although all measures available to eliminate the drug from the gut of the host were employed, a period of persistence is inevitable, resulting in an effect on arrivals during this time. A system was developed whereby larvae extracted from the lungs of donors (see Chapter 6) were administered by stomach tube to groups of recipient rats at different times after the drug. A further group of controls received similar larvae without anthelmintic. A comparison between the takes in drug treated and untreated animals reveals the length of time the anthelmintic effects persist.

Materials and Methods

Six rats, intended as donors, were infected with 500L3's of N. brasiliensis on the left flank. At 45h post-infection, the rats were killed and the lungs removed, and larvae therein extracted (page 28). The larvae were prepared for exact doses in the order of 20.

Eighteen rats were given a standard dose of morantel tartrate (0.02mg/g body weight) after 24 hours starvation. At 0h, 4h and 8h later, groups of six were given an oral infection of 20 N. brasiliensis extracted from the lungs of the donors (overall mean exact dose = 20.7). A further six control rats were given larvae without anthelmintic (mean exact dose = 22.7). All the rats were killed on day 8 and the adult worm burdens expressed as a proportion of the dose.

Results

The results are shown in Figure 5.1b. When the drug and larvae are administered simultaneously, no larvae develop, but 8h after administration, the effect of the drug has worn off completely, with the take comparable to that in the controls. The maximum persistence of the drug is therefore 8h. To correct the curve of arrival in the gut for this persistence, it must be displaced by 8h to the right. The corrected curve along with the data obtained by direct counting (see Chapter 4) are shown as Figure 5.1c. Both sets of data lie on the same curve. This agreement validates the method using anthelmintic, and is prima facie evidence that morantel tartrate used in this way has no effect on migrating larvae. ✓

5.3.2.3 The anthelmintic has no parenteric effects [Expt 24].

As mentioned in 5.3.2.2 agreement between the methods was preliminary evidence that the effect of morantel tartrate was confined to the gut. Added to this, the development of larvae after a drug pulse is

administered at 10h or 30h was not significantly different from the untreated controls. Nevertheless, further evidence was gathered to confirm that the anthelmintic has no parenteric effects.

Materials and Methods

Four rats were infected with 500L3's on the skin of the left flank. At 40h, two of the rats were given an oral dose of morantel tartrate (0.02mg/g body weight). All the animals were killed at 45h, when the lungs were removed, and larvae extracted from them were used to infect 12 recipients by oral administration of exact doses of the order of 20. Six were given larvae from the treated donors (mean exact dose = 19.7) whilst the others were given larvae from the controls (mean exact dose 22.7). All were killed on day 8 and their worm burdens compared.

Results

The mean take of control larvae was 0.359 ± 0.028 (SEM) while larvae from the treated animals gave a take of 0.416 ± 0.045 (SEM) in the recipients. A t -value calculated for the arcsines of these data confirms homogeneity ($t_{[10]} = 1.069$, $p = 0.31$). Thus larvae extracted from the lung 5h after the oral administration of morantel tartrate are not impaired in their capacity to reach maturity.

5.3.2.4 S. ratti (homogonic) arrival in the gut [Expts 25-28].

Materials and Methods

Four experiments similar to that performed on N. brasiliensis (page 108) were completed. The protocols and results for each experiment are given in Table 5.4.

Results

The data for each experiment translated to the proportion of \bar{p}_f actually present in the intestine at the time of administration of the drug are shown in Figure 5.2. The results are highly variable and therefore each experiment has been treated separately. An examination of the Figure reveals that the earliest time that the drug has any effect is when it is administered at 30h. In all the experiments, the means of the raw data obtained prior to 30h are not significantly different from the control \bar{p}_f 's. The earliest time by which maximal arrival can be said to have occurred is when the drug is administered at 60h (Expts 25 & 26), and not until 90h in Expts 27 and 28. The curve of arrival plateaus at 40h (Expts 26-28) before an increase occurs again at 90h. This is most apparent in Expt 27. Two theories could account for this feature, (a) larvae arrive from two different sites, one before the other, or, (b) once in the small intestine the larvae burrow deep into the mucosa to undergo two moults, effectively shielding themselves from the action of the drug, before re-emerging at a later stage as the more susceptible adult.

5.3.2.5 Persistence of the drug with reference to S. ratti
(homogonic) [Expt 29].

A similar experiment to the one carried out for N. brasiliensis was done for S. ratti. Larvae extracted from the nasal region of the head at 30h were found to be orally infective to recipients (see Chapter 6). These were used to measure the length of time that the drug remained active in the gut of the rat.

Materials and Methods.

Fourteen rats were infected with 500L3's on the left flank. Larvae were extracted from these and used in exact doses of the order of 20 (overall mean exact dose = 19.7) to infect recipients orally which had previously been treated with the anthelmintic (0.02mg/g body weight). Groups of 4, 5 or 6 rats were given the anthelmintic (after 24h starvation) at the following times prior to the larvae; 0h, 4h, 5h (4 rats), 6h (5 rats), 8h (5 rats) and 15h (5 rats). All rats were fed immediately after medication. A further group of rats received only larvae as controls. All rats were killed on day 5 and small intestines examined for the presence of egg-tracks.

Results

The results are shown as Figure 5.3a. When larvae and drug were administered simultaneously, no larvae survived but, the proportion developing when infection was 4h after the drug was not significantly different from the controls. This suggests a maximum persistence of 4h.

Table 5.4 The results of Experiments ~~25-28~~ showing the proportion of the applied dose of S. ratti (homogonic) in the small intestine of the rat on day 5 after the application of a pulse of morantel tartrate (0.02mg/g body weight) at different times post-infection.

Figure 5.2 The results shown in Table 5.4 (S. ratti homogonic) translated to the proportion of \bar{p}_f present at the time of administration of the drug. (a) = Expt ~~25~~, (b) = Expt ~~26~~, (c) = Expt ~~27~~ and (d) = Expt ~~28~~.

Table 5.4

Mean proportion of the dose (\pm SEM) as egg-tracks in the small intestine on day 5 after oral administration of the drug at the following times (h) post-infection: (n)

Expt	Mean exact dose										\bar{E}_T
		10	20	30	40	50	60	70	80	90	
25	20.8	0.775 ± 0.019 (3)	0.771 ± 0.029 (3)	0.602 ± 0.053 (6)	0.278 ± 0.055 (5)	0.404 ± 0.063 (6)	0.192 ± 0.074 (6)	0.153 ± 0.053 (5)	—	—	0.708 ± 0.023 (5)
26	20.5	—	0.574 ± 0.050 (6)	0.463 ± 0.030 (6)	0.318 ± 0.033 (6)	0.312 ± 0.055 (6)	0.090 ± 0.042 (6)	0.033 ± 0.016 (5)	0.380 ± 0.048 (5)	0.019 ± 0.012 (6)	0.686 ± 0.036 (6)
27	22.5	—	0.814 ± 0.039 (6)	0.598 ± 0.051 (5)	0.712 ± 0.099 (6)	0.481 ± 0.055 (5)	0.435 ± 0.094 (5)	0.579 ± 0.109 (6)	0.505 ± 0.071 (6)	0.142 ± 0.019 (6)	0.760 ± 0.037 (6)
28	20.8	0.818 ± 0.023 (6)	0.788 ± 0.053 (5)	0.690 ± 0.052 (6)	0.368 ± 0.053 (6)	0.308 ± 0.059 (6)	0.351 ± 0.040 (6)	0.256 ± 0.071 (5)	0.241 ± 0.036 (6)	0.078 ± 0.022 (6)	0.874 ± 0.022 (6)

Figure 5.2

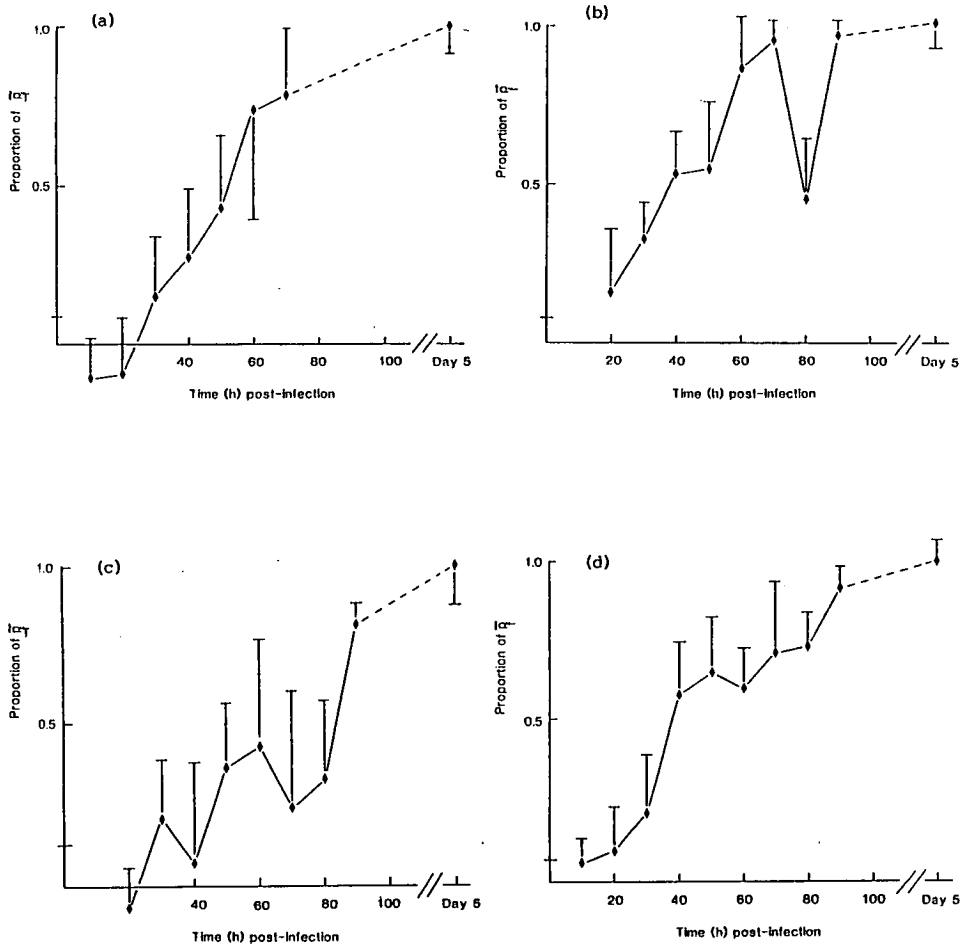


Figure 5.3 The persistence of morantel tartrate with respect to S. ratti (homogonic). The histograms show the proportion of the dose of larvae as egg-tracks on day 5 after oral administration at different times after the drug. The controls received no drug. (a) The results of Experiment 29, where the dose rate was 0.02mg/g body weight. (b) The results of Experiment 33, where the dose rate was 0.06mg/g body weight.

Figure 5.4 The effect of the drug on different stages of S. ratti (homogonic) in the gut of the host. The histogram shows the development of larvae orally administered to recipient rats in exact doses after extraction from the nasal tissues of donor rats at 30h. A pulse of the anthelmintic was administered at different times after the larvae. The infection time for recipients is taken as 0h, even though the initial infection is 30h old. The controls received no drug.

(a) Experiment 30 where the dose rate was 0.02mg/g body weight.

(b) Experiment 32 where the dose rate was 0.06mg/g body weight

Figure 5.3

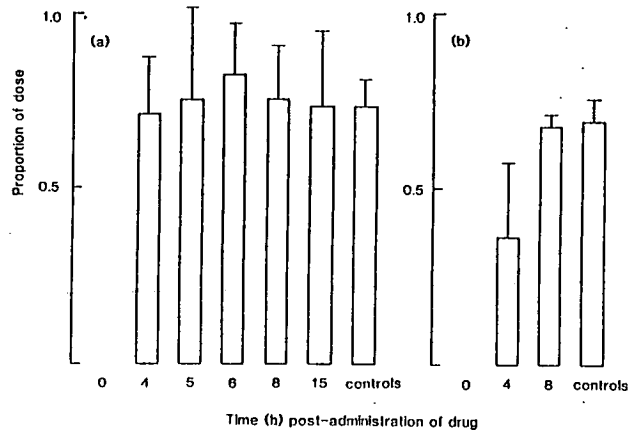
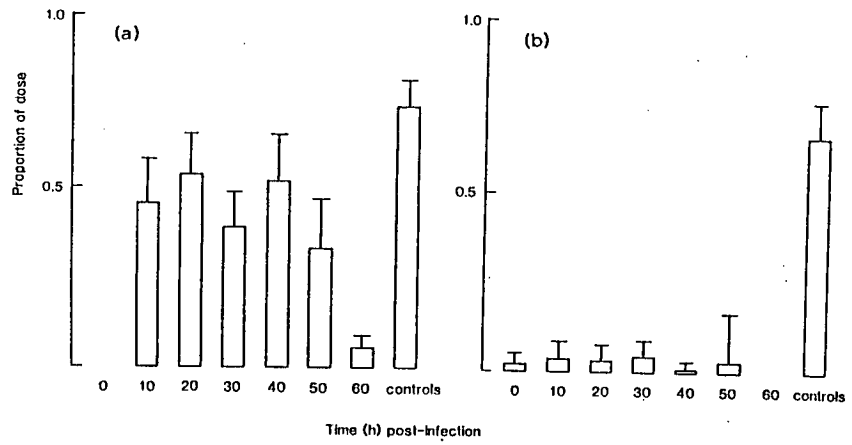


Figure 5.4



5.3.2.6 Effect of the drug on the intestinal worms [Expt 30].

The following experiment was designed to investigate the effect of the drug on larvae in the intestine, with a view to obtaining an answer as to why the arrival curve in the gut reached a plateau between 40h and 80h post-infection.

Materials and Methods

Sixteen rats were infected with 500L3's on the left flank. At thirty hours larvae were extracted from the nasal region and used to infect 46 recipients orally with exact doses of the order of 20. The recipient rats were divided into groups of six, and given a standard dose (0.02mg/g body weight) of morantel tartrate at the following times post-infection; 0h, 10h (5 rats), 20h (5 rats), 30h, 40h, 50h and 60h. The remaining 6 control rats were given larvae without anthelmintic as controls. All were killed on day 5 and their worm burdens assessed.

Results

The results are shown as Figure 5.4a. When the larvae were administered at the same time as the drug, no adults developed. Between 10h and 50h a large proportion of the larvae appear to be able to protect themselves from the action of the drug. By 60h (90h if the 30h in the donor is taken into account) the protection is lost, with the drug exerting a greater effect, although still only 95% efficient. These results appear to back up the hypothesis that once within the

gut the larvae burrow into the mucosa, where they are partially protected from the action of the drug.

5.3.2.7 Increasing the concentration of the drug [Expt 31].

This experiment and the next were designed to test the possibility that all ^{intestinal phases} could be eliminated from the gut if the drug concentration was high enough.

Materials and Methods

Thirty rats were divided into five groups of six and all infected by EDT20 (mean exact dose = 20.3). At ninety hours post-infection, one group was given a standard dose of morantel tartrate (0.02mg/g body weight) diluted so that a 50g rat received 0.1ml. Group 2 received 0.2ml of the drug, and group 3, 0.3ml. Group 4 was killed at 90h and the guts examined by direct counts so that the mean number of larvae present at the time of administration of the drug was known. The final group was kept to obtain a value for \bar{p}_f . All the rats (except group 4) were killed on day 5 and the egg-tracks present in the gut expressed as a proportion of the dose.

Results

The proportion observed in the gut at 90h was arithmetically higher than the egg-tracks present on day 5 ($\bar{p}_f = 0.869 \pm 0.036$ (SEM) and 0.812 ± 0.042 (SEM) respectively). The takes were 0.322 ± 0.071 , 0.272 ± 0.096 and zero after administration of 0.1ml, 0.2ml and 0.3ml of

the drug solution respectively.

5.3.2.8 Effect of 0.06mg/g body weight of morantel tartrate on larvae in the intestine [Expt 32].

Although a three fold increase in the concentration of the drug administered to rats completely eliminated all worms present at 90h, the effect on the most resistant stage of the development in the gut (40h to 80h) was not known.

Materials & Methods

Ten rats were infected with 500L3's on the left flank. At thirty hours larvae were extracted from the nasal region and used to infect 40 recipients orally with exact doses of the order of 20. The recipient rats were divided into groups of five, and given a dose (0.06mg/g body weight in 0.3ml distilled water) of morantel tartrate at the following times post-infection; 0h, 10h, 20h, 30h, 40h, 50h and 60h. All rats were starved for 24h prior to treatment and fed 30 minutes later. The delay between administration of the drug and feeding was to allow the drug to exert its full effect before being eliminated from the body. The remaining 5 controls received larvae only. All were killed on day 5 and their worm burdens assessed.

Results

The results are shown in Figure 5.4b. The increased concentration of drug is effective against 95% of the dose of larvae at any given time.

At 60h (90h if the 30h in the donor is taken into account), the drug is 100% effective against adult parasites.

5.3.2.9 Persistence of the increased concentration of drug in the rat gut [Expt 33].

The experiment on the persistence of the drug within the rat gut had to be repeated with the increased dose of morantel tartrate to ascertain whether this level would be accompanied by an unacceptably prolonged anthelmintic effect in the intestine.

Materials and Methods

Eighteen rats, which had been starved for 24h, were given a dose (0.06mg/g body weight) of morantel tartrate at time zero, and fed thirty minutes later. At 0h, 4h and 8h later, groups of 6 were given an oral infection of 20 larvae (mean exact dose = 22.0) extracted from the nasal region of 8 donor rats infected some 30 hours earlier. A further six control rats were given larvae without the anthelmintic. All animals were killed on day 5 and egg-tracks expressed as a proportion of the dose.

Results

The results are shown in Figure 5.3b. No larvae developed after feeding simultaneously with the drug. The development in the rats given the drug some 8h before the larvae is indistinguishable from

that in the controls.

5.3.2.10 Arrival in the small intestine assessed by the increased drug pulse technique.

As the efficiency of the drug at the higher concentration (0.06mg/g body weight) is in excess of 95% at all times, and the persistence is only 8h, the experiment to obtain a curve of arrival in the gut of the rat was repeated with this concentration.

Materials and Methods

Fifty three rats were infected by EDT20 (mean exact dose = 20.2), split into 8 groups of 5 or 6, and each group given the drug (0.06mg/g body weight) at one of the following times post-infection (after 24h starvation); 20h (5 rats), 30h, 40h (5 rats), 50h, 60h, 70h, 80h and 90h. The rats were fed 30 minutes after treatment. The remaining 6 rats were left as untreated controls. All rats were killed on day 5 and the egg tracks present in their small intestines expressed as a proportion of the dose.

Results

The results are summarized as Table 5.5.

Mean proportion of the dose (\pm SEM) as egg-tracks in the small intestine of the rat on day 5 after oral administration of the drug at the following times (h) post-infection; (n)

20	30	40	50	60	70	80	90	\bar{p}_f
0.750 ± 0.038 (5)	0.629 ± 0.064 (6)	0.392 ± 0.058 (5)	0.183 ± 0.068 (6)	0.080 ± 0.030 (6)	0.137 ± 0.029 (6)	0.031 ± 0.015 (6)	0.028 ± 0.019 (6)	0.821 ± 0.048 (6)

A t -test of the arcsines of the raw data for drug treatment at 20h against the controls reveals no statistical difference ($t_{[9]} = 1.217$, $P = 0.254$), but at 30h, the difference is significant ($t_{[10]} = 2.457$, $P = 0.0034$). Thus, the first time the drug has any effect is at 30h post-infection, the same as the lower concentration (page 114). Figure 5.5a shows the results translated to the proportion of \bar{p}_f present at the time of administration of the drug. Figure 5.5b is corrected to allow for a persistence of 8h, along with the results for the direct counts obtained on page 65. The curve derived by the drug pulse precedes that of the direct counts by some 15-17h, but is parallel to it. This difference could be due to larvae being held up in the stomach and/or being affected by the drug during migration. However, since for *N. brasiliensis* both the data obtained by the drug pulse and that from direct counting lie on the same line, the delay in the stomach in this case must be negligible.

Figure 5.5

(a) The arrival of S. ratti (homogonic) in the gut assessed by the drug pulse technique. The results are translated to show the proportion of \bar{p}_f present at the time of administration of the drug.

(b) The migration of S. ratti (homogonic) through the nasal region and subsequent arrival in the small intestine assessed by direct counts (squares) (pages 64-68) and the drug pulse (diamonds) (0.06mg/g body weight). The drug pulse curve has been displaced by 8h to the right to allow for the maximum persistence of the drug. The results are plotted as the proportion of the control larvae (\bar{p}_f) that actually complete migration.

Figure 5.6

(a) The arrival of S. ratti (heterogonic) in the gut of the rat assessed by the drug pulse technique. The data are translated to the proportion of \bar{p}_f present at the time of administration of the drug. Day 6 animals are the controls.

(b) The migration of S. ratti (heterogonic) through the nasal region and the subsequent arrival in the small intestine assessed by direct counts (squares) and the drug pulse (diamonds) (0.06mg/ g body weight). The drug pulse curve has been displaced by 8h to the right to allow for the maximum persistence of the drug. The results are plotted as the proportion of the control larvae (\bar{p}_f) that actually complete migration.

• Figure 5.5

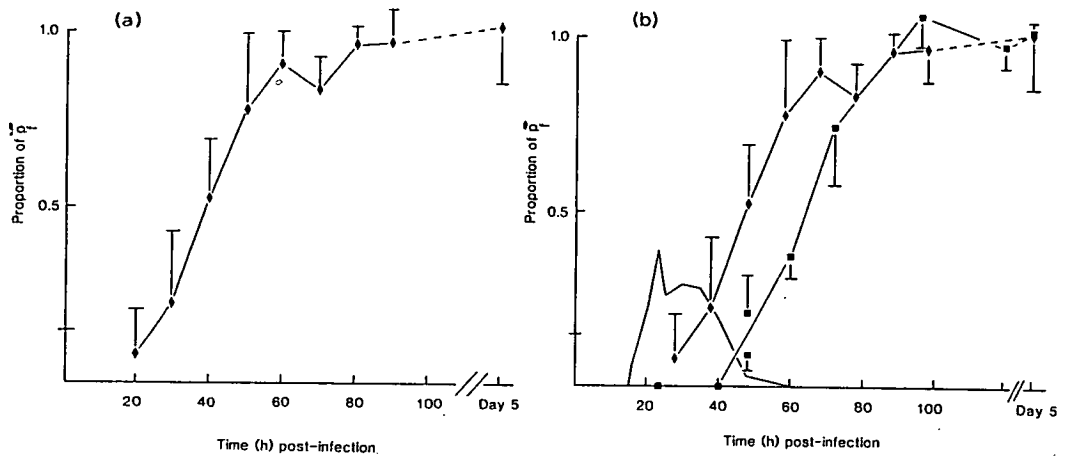
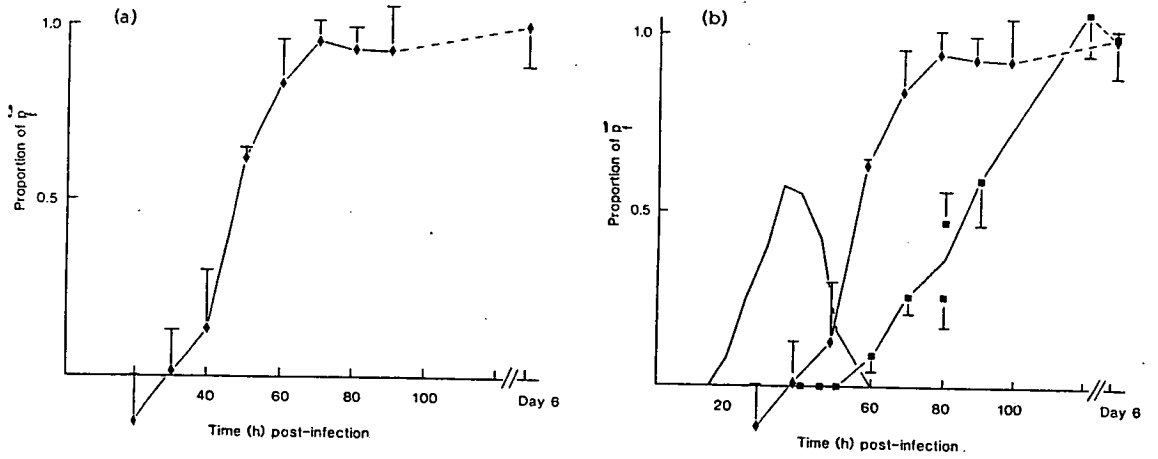


Figure 5.6



As transit of the stomach for both species is governed by the same forces, it is reasonable to conclude that S. ratti also passes quickly into the small intestine, with the curve obtained by the drug pulse being a true picture of the arrival in the small intestine. The arrival in the gut is a virtual mirror image of the apparent vacation of the head, suggesting that larvae do migrate directly from the head to the stomach without the involvement of any other transit site. This conclusion is secure if it can be demonstrated that larvae in transit are unaffected by the drug.

5.3.2.11 Effect of the drug on migrating larvae [Expt 34].

It can already be said that the early stages of migration are unaffected by the drug, since the drug pulse data at 20h are not significantly different from the untreated controls. The following 2 experiments were designed to investigate the possibility that the significant difference that occurs after 30h could be due to an effect on migration.

Experiment 34

Materials and Methods

Four donor rats were infected with 500L3 on the flank at time zero. At thirty hours, two of the rats were given a dose (0.06mg/g body weight) of the drug. At thirty five hours, all the rats were killed, and larvae extracted from the nasal regions used to infect recipients with exact doses in the order of 20. Six rats received larvae from treated

animals (mean exact dose = 20.3) and six with larvae from the controls (mean exact dose = 20.8).

Results

The proportion of egg tracks present on day 5 was 0.624 ± 0.044 (SEM) and 0.597 ± 0.050 (SEM) for larvae from treated and untreated donors respectively. A t -test on the arcsines of these data gives

$$t_{[10]} = 0.409, \underline{p} = 0.691.$$

Experiment 35

The results of Expt 34 clearly show that larvae taken from the head 5h after oral administration of morantel tartrate are not impaired in their capacity to reach maturity. However, they give no indication as to whether the drug affects the numbers of larvae alive in the head.

Materials and Methods

Twenty-two rats were infected by EDT20 (mean exact dose = 20.9). At 30h, 11 rats were given a dose of morantel tartrate (0.06mg/g body weight) after 24h starvation. The rats were fed 30 minutes later. At 35 hours, 5 of the treated animals and 6 of the untreated controls were killed and their nasal regions and craniums examined for larvae. The remaining animals were killed on day 5.

Results

The proportions recovered from the craniums were 0.052 ± 0.029 (SEM) and 0.008 ± 0.008 (SEM) ($t_{[9]} = 1.66$, $P = 0.13$) for the treated and untreated animals. 0.186 ± 0.036 (SEM) and 0.146 ± 0.012 (SEM) ($t_{[9]} = 0.82$, $P = 0.43$) were recovered from the nasal regions respectively. On day 5, the proportion developing to maturity was 0.536 ± 0.048 (SEM) for treated hosts and 0.739 ± 0.036 (SEM) for the untreated. A t -test on the arcsines of these data reveals a significant difference ($t_{[9]} = 3.292$, $P = 0.0093$). This difference must be attributable to larvae in the gut at the time of administration of the drug, since it had no effect on the larvae in the head. A proportion of \bar{p}_f equivalent to 0.274 ± 0.065 must therefore have been in the intestine by 38h, allowing for a persistence of 8h. This is a similar finding to that suggested by the arrival curve estimated by the drug pulse where a proportion of \bar{p}_f equivalent to 0.233 ± 0.078 was present at this time (page 122).

5.3.2.12 Arrival of S. ratti (heterogonic) in the gut of the rat.

A similar experiment to the one performed on S. ratti (homogonic) for the arrival in the stomach/small intestine was performed utilizing the drug pulse technique. It was thought unnecessary to repeat the experiments on the persistence of the drug, and the effect of the drug on larvae in the gut at different times post-infection, since these had already been carried out on the homogonic strain, and it is reasonable to assume that the results would be similar for both strains.

Materials and Methods

Fifty two rats were infected with S. ratti (heterogonic) by EDT20 (mean exact dose = 21.2), and then, in groups of 5 or 6, given an oral dose of morantel tartrate (0.06mg/g body weight) at the following times (h) post-infection, 20h, 30h, 40h, 50h (5 rats), 60h, 70h, 80h and 90h (5 rats). The final 6 rats were left as untreated controls. All rats were killed on day 6 and the egg-tracks present expressed as a proportion of the dose.

Results

The results of the proportions of egg-tracks in the small intestine are shown below as Table 5.6.

Mean proportion of the dose (\pm SEM) as egg-tracks in the small intestine of the rat on day 6 after oral administration of the drug at the following times (h) post-infection; (n)

20	30	40	50	60	70	80	90	\bar{p}_f
0.795 ± 0.029 (6)	0.717 ± 0.034 (6)	0.622 ± 0.045 (6)	0.269 ± 0.006 (5)	0.117 ± 0.034 (6)	0.034 ± 0.017 (6)	0.050 ± 0.019 (6)	0.052 ± 0.035 (5)	0.721 ± 0.032 (6)

Clearly the drug has no effect when administered at 20h or 30h, and, when applied at 40h the proportion of larvae developing to maturity is not significantly different from the controls ($t_{[10]} = 1.793$, $P = 0.103$). By 50h, the drug has a significant effect ($t_{[9]} =$

12.027, $\underline{p} = 7.5 \times 10^{-7}$). Migration is essentially complete when the drug is administered at 60h.

Figure 5.6a shows the data translated to the proportion of \bar{p}_f present at the time of administration of the drug. Taking the same persistence as that for the homogonic strain, Figure 5.6b shows the curve transposed some 8h to the right. Also shown are the data for the migration through the nasal region and arrival in the gut assessed by direct counts. The drug pulse data therefore suggests that migration to the gut starts at around 50h and is essentially complete by 68h, some 50h earlier than assessed by direct counts.

5.4 Conclusions

5.4.1 Migration of S. ratti (homogonic) from the infection site to the head.

(1) Experiments suggested (though not significantly) that larvae applied to the neck region of weanling rats reached the head earlier than those applied to the flank.

(2) Similar experiments on mature rats gave contradictory results.

(3) The skin of the flank appears to be more difficult for larvae to penetrate than the skin of the neck in mature rats.

(4) Conclusive proof was gained that larvae migrate via the nasal region in adult rats as well as weanlings.

5.4.2 Autoradiographic analysis of arrival of S. ratti (homogonic) in the gut of the host.

(1) Cultures set up with 22kBq per well of [⁷⁵Se]-selenomethionine produced larvae with an activity of 4.03–4.73 c.p.m/L3. 95% of these larvae produced an image on an autoradiogram as L3's, but when extracted from the gut at 72h, only 63% could be detected as foci.

(2) Larvae labelled with 110kBq per well (17.07 c.p.m/L3) all produced a distinct image as infective L3's and when extracted from the intestine at 72h.

(3) Larvae labelled with 110kBq per well of [⁷⁵Se]-selenomethionine first appeared as foci on autoradiograms taken of the small intestine at 40h, with migration essentially complete by 72h.

(4) Autoradiograms suffered from background noise.

(5) Reduced viability was confirmed to be due to centrifugation of the larvae.

(6) A forced change in the diet of the host rats adversely affected the experimental results in a number of contexts dealt with in Chapter 5, and led to the abandonment of autoradiography.

5.4.3 Drug pulse technique

(1) A new technique was devised using short pulses of a non-systemic, non-persistent anthelmintic (morantel tartrate) to obtain a mirror image of the arrival of larvae in the gut of the host.

5.4.3.1 N. brasiliensis

(1) Using 0.02mg/g body weight of the drug (dissolved in 0.1ml of water), a curve for the arrival in the gut was obtained.

(2) The persistence of the drug was found to be less than 8h.

(3) Taking into account the persistence of 8h, the arrival curve obtained by the drug pulse lies directly on the one obtained by direct counts.

(4) The drug has no effects on migrating larvae.

5.4.3.2 S. ratti (homogonic).

(1) Using 0.02mg/g body weight of the drug a curve of the arrival in the gut was obtained which had a plateau between 40h and 80h post-infection.

(2) The plateau was thought to be due to larvae becoming shielded from the action of the drug by burrowing deep into the intestinal mucosa. By 90h the larvae have undergone two moults to the adults, when they

once more become susceptible to the drug.

(3) The persistence of 0.02mg/g body weight of the drug with reference to S. ratti was found to be 4h.

(4) Increasing the drug to 0.06mg/g body weight resulted in 95%+ efficiency at eliminating worms from the gut at all times.

(5) 0.06mg/g body weight of the drug had a persistence of 8h.

(6) A curve of arrival in the gut was obtained using 0.06mg/g body weight of the drug. Allowing an 8h persistence, this was found to mirror the disappearance from the head. The first larvae appeared in the gut between 30h and 38h with arrival complete by 60-68h.

5.4.3.3 S. ratti (heterogonic)

(1) Allowing for a persistence of the drug of 8h (0.06mg/g body weight), a curve of the arrival in the gut was obtained which suggested that they first appeared there at 50-58h, with migration complete by 68h.

CHAPTER 6

THE LUNGS AND HEAD AS EXCLUSIVE SITES FOR N. BRASILIENSIS AND S. RATTI RESPECTIVELY.

6.1 Introduction

In chapter 4 it was rigorously demonstrated that both strains of S. ratti transit the nasofrontal region of the head in their migration from the skin to the intestine of the rat, and also N. brasiliensis uses the lung in reaching the same goal. The aim of the work described in this chapter was to try and decide whether the routes of the two parasites are mutually exclusive and whether a number of pathways exist for each. Twohy (1955a, 1956) presented good evidence to suggest that N. brasiliensis needs to undergo a moult in the lung before migration can proceed. Mimori et al. (1982) and Nojima et al. (1987) have shown differences in the form of S. ratti L3's recovered from the skin and the head. Kawanabe et al. (1988) state without supporting evidence that S. ratti larvae extracted from the skin were unable to infect by oral administration, whereas those from the nasal region could do so after a period of development. Tanaka et al. (1989) conclude, after injecting larvae directly into the cranium, that a mandatory developmental period is required in this site. Therefore for both S. ratti and N. brasiliensis there was tentative evidence available that each uses the specific site proven to be part of its route to undergo developmental changes before migration proceeds. In other words, that these locations act as 'nurseries' to the parasite

concerned. The results obtained from the drug pulse technique (Chapter 5), for the arrival of larvae in the gut, are a virtual mirror image of the disappearance from the head (S. ratti, both strains) or the lung (N. brasiliensis). This is consistent with the idea that larvae migrate directly from the relevant transit site to the intestine by the most direct route, in both cases the oesophagus.

However, when one examines the data for the peak in each site (\bar{p}_0 = 0.316 for the homogonic strain, 0.397 for the heterogonic strain and 0.487 for N. brasiliensis) a considerable proportion of the larvae actually completing migration is still unaccounted for. As described in the General Introduction (Chapter 1), the probability that p_0 would equal p_t is low, and therefore the peak proportion recovered is likely to be much less than p_t . Nevertheless, the possibility that the migration is sufficiently synchronous for p_0 to equal p_t cannot be excluded on the evidence so far.

6.2 S. ratti (homogonic)

6.2.1 Other possible transit sites [Expts 1-4]

In this section the possibility of S. ratti (homogonic) larvae appearing in other transit sites in the period from infection (time zero) to maximal arrival in the gut (68h) is examined.

Materials and Methods

Experiments 1-4 were designed to look for larvae in the lung and liver

after infection with exact doses of about 20 infective stages. The sites examined were ones in which larvae would have direct access to the alimentary tract. The experiments in Chapter 4 showed that larvae of N. brasiliensis could be extracted from the lung of the rat and therefore the probability is high that the same method would detect larvae of S. ratti in the same location. No data are available on the extractability of larvae from the liver, but the architecture of the said tissue would not appear to present a barrier to the extraction technique, bearing in mind the diversity of other tissues in which the method succeeds.

In each experiment a large number of animals was infected with S. ratti (homogonic) by EDT20. At subsequent times subgroups were killed and their lungs and livers examined for migrating larvae (page 26). A further group of rats was kept until day 5/8 for \bar{p}_f to confirm the ability of the larvae to reach their goal. The protocols and results are shown in Table 6.1.

Results

Only once was a single larva recovered from the lung in a total of 49 rats. No larvae were ever recovered from the liver tissue from the 12 rats examined. The values of \bar{p}_f in all four experiments were normal, and therefore, the viability of the larvae was not impaired.

Table 6.1 Experimental protocols and results for migration of S. ratti (homogonic) through the liver and lungs (Expts 1-4).

Table 6.2 Establishment of S. ratti (homogonic) transferred from a donor to a recipient (Expts 5-9). Controls were infected at the same time as donors. Each group of 6 recipients was infected from the pooled worms from 2 donors. \bar{d} is the mean exact dose.

Table 6.3 Total body length of S. ratti (homogonic) recovered from the nasal region of rats infected with 500L3's on the flank (Expt 13). * = significant growth over the infective L3 stage (Time 0). 25 larvae measured at each time.

Table 6.1

Expt	\bar{d}	Tissue	Numbers of rats sampled at the following times (h) after infection with exact doses (\bar{d}) (a single larva was recovered from a single rat in the group marked *):						$\bar{P}_f \pm \text{SEM}$ (n)
			18	23	26	40	48	60	
1	18.8	lung	4	—	4*	—	—	—	0.819 ± 0.022 (4)
2	19.4	lung/liver	—	6	—	6	—	—	0.860 ± 0.032 (6)
3	19.8	lung	—	6	—	—	6	—	0.783 ± 0.017 (6)
4	19.4	lung	—	5	—	—	6	6	0.752 ± 0.031 (6)

Table 6.2

Expt	Mean proportion ($\pm \text{SEM}$) of larvae which established in the intestines of recipients after oral transfer from donors at the following times (h) post-infection of the donor (6 rats at each sampling time):							$\bar{P}_f \pm \text{SEM}$ controls ($n = 6$)
	20	23	25	30	35	40	48	
5	—	0.160 ± 0.038 21.0	—	—	—	—	0.743 ± 0.047 19.2	—
6	nil	—	—	0.648 ± 0.059 19.8	—	0.536 ± 0.041 21.8	—	—
7	nil	—	—	0.754 ± 0.048 22.2	—	—	—	—
8	nil	—	0.386 ± 0.045 21.7	0.820 ± 0.044 21.7	0.779 ± 0.020 21.2	—	—	0.809 ± 0.047 21.7
9	nil	—	0.124 ± 0.023 21.5	0.784 ± 0.037 22.3	0.790 ± 0.045 22.2	0.523 ± 0.056 20.7	—	0.778 ± 0.025 22.0

Table 6.3

Time (h) post-infection	Total body length (mm) mean \pm (SEM)
0	0.625 \pm 0.003
20	0.619 \pm 0.003
25	0.632 \pm 0.003
30	0.627 \pm 0.003
35	0.638 \pm 0.005*

6.2.2 Development in the nasal region [Expts 5-9].

The drug pulse technique confirmed that the first arrivals in the small intestine appear at 38h post-infection (Chapter 5). If an obligatory process of development is a precondition for the colonisation of the intestine, then that process has been completed by some parasites before 38h. The aim of the work described in this section was to extract larvae at different times from the nasal region, to discover when they could successfully infect recipient rats orally, and thus to decide the time at which development in the nasal region is complete.

Materials and Methods

Five comparable experiments were set up to examine whether development takes place in the nasal region prior to colonisation of the gut. In each, exact doses of larvae extracted from the nasal region of donor rats were administered to experimental subjects by stomach tube and the ability to mature in the intestine of larvae extracted at different times compared. Experiment 5 is given as an example of the general protocol applicable in this context. Four rats were infected with 500 L3 on the flank and killed at 23h (2 rats) and 48h (2 rats), at each of which times larvae were extracted from the nasal region and used to infect 6 recipients orally with exact doses of about 20 (page 28). All recipients were killed on day 5 and their worm burdens assessed. In Expts 8 and 9, at the same time as the donors received their dose of 500 larvae, six control animals were infected by EDT20 to be sure that the p_f 's attained by the particular larval

populations were of a sufficiently high standard. The outline protocols and results are given in Table 6.2.

Results

The results clearly show that a period of development is required in the nasal region before larvae can inhabit the small intestine of the host. Larvae extracted at 20h are unable to withstand the rigours of the alimentary tract, whilst by 23h, 0.16 of the larvae have undergone the physiological changes required. Maximum potential for development is achieved by 30h. As established in Chapter 4, the earliest arrivals in the nasal region appear at 16h post-infection. Therefore from these results it can be said that a period of development of at least 7 hours is required before migration can proceed. Any larvae migrating through other sites could require a similar period of development and be delayed in their transit of such tissues. Sampling at autopsy (page 3) should reveal such other sites. The negative results for the liver and lungs (page 134) can therefore be taken to mean that these do not act as nurseries for the developing larvae.

6.2.3 Morphological markers for development

The results of 6.2.2 prove that S. ratti (homogonic) must undergo developmental changes in the nasal region before migration can be completed. The following describes an attempt to define morphological markers to identify the completion of this mandatory development.

6.2.3.1. Body length as a marker [Expt 13].

Mimori et al. (1982) noted a significant growth in larvae from infection to being present in the nasal region at 48h. The overall body length of the worms extracted from the head was measured in the following experiment to see if a change could be detected by 30h, the time at which development is complete.

Materials and Methods

Four rats were infected with 500 L3's on the flank. At 20h, 25h, 30h and 35h, a rat was killed, and larvae present in the nasal region extracted, with 25 randomly chosen to be measured (page 29).

Results

The results are shown in Table 6.3. Analysis of variance of the data for the infective L3's and the parasitic phases demonstrates significant growth ($F_{[4,120]} = 4.53$, $P = 0.0019$). This growth was not apparent until 35h post-infection.

6.2.3.2. The oesophagus index as a morphological marker [Expt 14].

Nojima et al. (1987) and Tanaka et al. (1989) have shown that changes in the size of the oesophagus relative to the overall body length occur whilst the larvae are present in the head of the rat. Kawanabe et al. (1988) state, without supporting data, that larvae recovered from the skin were unable to infect orally, whereas those

Table 6.4 Growth of S. ratti (homogonic) in the nasal region of the rat head (Expt 14). * = no significant change from infective L3 prior to infection (time 0). 25 worms measured at each sampling time.

Table 6.5 Establishment of S. ratti (heterogonic) transferred from a donor to a recipient (Expt 15). The controls were infected at the same time as the donors. Each group of five recipients was infected with larvae from the pooled worms from 2 donors. \bar{d} is the mean exact dose.

Table 6.6 Growth of S. ratti (heterogonic) in the nasal region of the rat head (Expt 16). # = significant reduction over infective stage. * = significant growth over infective L3 stage (time 0). Twenty five worms measured at each sampling time except \$ where $n = 24$.

Table 6.4

Time (h) post-infection	Total body length (mm) mean \pm SEM	Oesophagus length (mm) mean \pm SEM	Oesophagus index (OI)	Proportion of larvae where OI > 1
0	0.621 ± 0.0036	0.272 ± 0.0015	0.781 ± 0.0075	nil
20	0.616* ± 0.0022	0.274* ± 0.0020	0.804* ± 0.0085	nil
25	0.632* ± 0.0028	0.309 ± 0.0020	0.955 ± 0.0095	0.24
30	0.630* ± 0.0043	0.324 ± 0.0035	1.066 ± 0.0170	0.96
35	0.659 ± 0.0065	0.351 ± 0.0046	1.143 ± 0.0018	0.92

Table 6.5

Mean proportion (\pm SEM) of larvae which established in the intestines of recipients after oral transfer from donors at the following times (h) post-infection of the donor (5 rats at each sampling time):

Expt	Time (h) post-infection						\bar{P}_f \pm SEM controls
	20	25	30	35	40	45	
15	nil	nil	0.250 ± 0.100	0.603 ± 0.017	0.678 ± 0.028	0.329 ± 0.023	0.691 ± 0.049
\bar{d}	17.8	22.8	22.6	20.2	21.2	22.0	24.2

Table 6.6

Time (h) post-infection	Total body length (mm) mean \pm SEM	Oesophagus length (mm) mean \pm SEM	Oesophagus index (OI)	Proportion of larvae where OI > 1
0	0.626 ± 0.006	0.280 ± 0.003	0.812 ± 0.010	nil
20	0.580# ± 0.004	0.261# ± 0.0020	0.818 ± 0.009	nil
25	0.598# ± 0.006	0.274 ± 0.003	0.848 ± 0.009	nil
30	0.604 ± 0.005	0.294 ± 0.003	0.929* ± 0.021	0.12
35§	0.631 ± 0.011	0.314* ± 0.006	0.997* ± 0.013	0.63
40	0.655* ± 0.008	0.333* ± 0.004	1.034* ± 0.019	0.72
45	0.650* ± 0.006	0.330* ± 0.003	1.037* ± 0.016	0.76

from the nasal region could do so, if the oesophagus index (OI) was greater than 1.

Materials and Methods

The experiment was identical to Expt 13, excepting that the oesophagus length was measured as well as the body length and the oesophagus index, OI, calculated from the two (page 29).

Results

The results are shown in Table 6.4. Analysis of variance of the data for body length prior to infection and the four sampling times reveals a significant growth ($F_{[4,120]} = 16.17$, $P = 1 \times 10^{-10}$).

Again this does not occur until 35h post-infection. Two similar analyses of oesophagus length and oesophagus index indicate a change ($F_{[4,120]} = 129$, $P = 1.56 \times 10^{-42}$ and $F_{[4,120]} = 151$, $P = 6.5 \times 10^{-46}$ respectively). The increase this time is apparent by 25h. Column 5 shows the proportion of the larvae in which the oesophagus index is greater than 1. By 25h, 0.24 of the larvae have developed to this degree, whilst by 30h virtually all have done so (0.92). Comparing the OI to oral infectivity (Table 6.2) shows a correlation between the proportion of the larvae in which OI > 1 and the establishment of the parasite in a donor rat. It therefore appears that OI is a good indicator of oral infectivity of S. ratti (homogonic).

6.3 S. ratti (heterogonic)

The results on pages 62, 134 and 137 prove that S. ratti (homogonic) migrates through the nasal region of the rat head and not the lung. Proof that the heterogonic strain of S. ratti utilizes the nasal region was presented on page 73. It was thought unnecessary to repeat all the experiments for the heterogonic strain, but differences in kinetics already detected were worth amplifying.

6.3.1 Development of S. ratti (heterogonic) in the nasal region of the rat [Expt 15].

An experiment similar in design to Expts 5-9 was completed for S. ratti (heterogonic).

Materials and Methods

Twelve rats were infected with 500 L3's of S. ratti (heterogonic) on the left flank. These were killed in pairs at the following times post infection, 20h, 25h, 30h, 35h, 40h and 45h, at each of which times larvae were extracted from the nasal region and used to infect 5 recipients orally (page 28). At the same time as the donors received their doses, a further group of 5 animals was infected by EDT20 as controls, to be sure that the p_f 's attained by the larval population were sufficiently high. Recipients and controls were killed on day 6 and the worm burdens expressed as a proportion of the dose.

Results

The results are shown in Table 6.5. Larvae extracted at 20h or 25h were incapable of developing in the gut. By 30h, a quarter of the dose had undergone sufficient development to withstand the intestinal environment. Larvae first appeared in the nasal region at 20h (Table 4.18), therefore a period of development of at least 10h is required before migration can proceed. Maximal development had been achieved by 40h.

6.3.2 The oesophagus index as a morphological marker for the completion of development in the nasal tissues [Expt 16].

Materials and Methods

Six rats were infected with 500L3's on the left flank. At 20h, 25h, 30h, 35h, 40h and 45h, one of the rats was killed and 25 randomly chosen larvae, extracted from the nasal region, were measured (page 29). The measurements were compared to those of L3's prior to infection.

Results

The results are given in Table 6.6. Analysis of variance of all the data for body length shows significant changes over the time period ($F_{[6,167]} = 16.88$, $P = 3.45 \times 10^{-15}$), with larvae in the nasal region at 20h shorter than infective L3's. A significant growth over the L3 stage is not seen until 40h. An analysis of oesophagus

length reveals a similar result ($F_{[6,167]} = 65.03$, $P = 3.62 \times 10^{-42}$). The oesophagus index shows no reduction in early arrivals in the nasal region, but a significant increase is not seen until 30h, with the maximum level being attained by 40h ($F_{[6,167]} = 47.25$, $P = 1.5 \times 10^{-33}$). Again a correlation is seen between oral infectivity (Table 6.5) and the proportion of the larvae in which OI is greater than 1 (Table 6.6 column 5). Larvae first became orally infective at 30h (0.250±0.100 of the dose) when 0.12 had an OI greater than 1. By 40h, the larvae extracted from the head had reached a peak in their ability to inhabit the gut of a recipient after oral administration (0.678±0.028 (SEM)), whilst 0.72 had an OI greater than 1.

6.4 N. brasiliensis

6.4.1 Possible transit sites other than the lung [Expts 17-18].

An examination of all the published material that can be discovered on the subject gives no evidence for a pathway other than the one through the lung. The possibility that this could however occur was investigated since the value for \bar{p}_o obtained in these studies was significantly lower than that of \bar{p}_f (see Chapter 4).

Materials and Methods

The possibility that the liver and/or the head is part of the pathway of N. brasiliensis was investigated in two experiments. The protocols were essentially the same as those for S. ratti

Table 6.7 Protocols and results of the migration of N. brasiliensis through the head and liver (Expts 17 & 18). \bar{d} is the mean exact dose, n = the sample size, # = heads only, * = heads and livers.

Table 6.8 Establishment of N. brasiliensis after oral transfer from a donor to a recipient (Expts 19-22). Controls infected at the same time as donors. \bar{d} is the mean exact dose.

Table 6.9 Growth of N. brasiliensis in the lung of the rat (Expt 23). * = no significant growth over previous sampling time.

Table 6.7

Expt.	\bar{d}	Number of rats in which larvae were absent from extracts of the heads and livers sampled at the following times (h) post-infection:					\bar{P}_f +SEM (n)
		20	23	30	40	50	
17#	18.6	5	6	—	—	—	0.683±0.052 (6)
18*	21.8	6	—	6	6	6	0.742±0.049 (6)

Table 6.8

Expt	Mean proportion of <i>N. brasiliensis</i> that established in the guts of recipients after oral transfer from the lungs of donors at the following times postinfection of the donor (6 rats at each time):						\bar{P}_f +SEM controls n = 6
	20	25	30	35	40	45	
19	nil	nil	nil	nil	0.328 ±0.039	—	—
\bar{d}	21.3	19.5	20.3	22.0	20.3		
20	—	—	nil	nil	0.092 ±0.015	0.179 ±0.018	—
\bar{d}			20.2	20.8	19.8	22.5	
21	—	—	—	0.073 ±0.030	0.700 ±0.020	—	—
\bar{d}				21.3	21.2		
22	nil	—	nil	0.041 ±0.015	0.545 ±0.020	—	0.738 ±0.083
\bar{d}	20.0		19.7	20.3	21.2		20.8

Table 6.9

Time (h) post-infection	Total body length (mm) mean±SEM	Sample size
0	0.649±0.005	30
15	0.645±0.008*	20
20	0.757±0.010	20
25	0.918±0.011	21
30	0.962±0.007	21
35	1.029±0.011	20
40	1.021±0.014*	16
45	0.989±0.011*	10

(homogonic) (page 133) in the lungs and liver. A large group of rats was infected by EDT20Nb, then at subsequent times, sub-groups were killed, and the liver and head examined for migrating larvae (page 26). A final sub-group was kept until day 8 to obtain a value for \bar{p}_f to confirm the ability of the larvae to reach their destination.

Results

The outline protocols and results are shown in Table 6.7. On no occasion were any larvae recovered from the liver (24 rats) or the head (35 rats), though the migratory success of the larvae was normal as judged by \bar{p}_f (column 8).

6.4.2 Development of N. brasiliensis in the lung [Expts 19-22].

Good evidence has been presented by Twohy (1955a, 1956) that N. brasiliensis L3's in the lung of the rat must moult before they continue their migration to the gut. The following experiments were designed to quantify the time course of this change. On page 108, it was demonstrated that larvae first appear in the small intestine 40h post-infection, therefore some individuals in the lungs have completed development in advance of this time.

Materials and Methods.

Four comparable experiments were designed to answer the above question. In each, donor rats were infected with 500L3's on the left

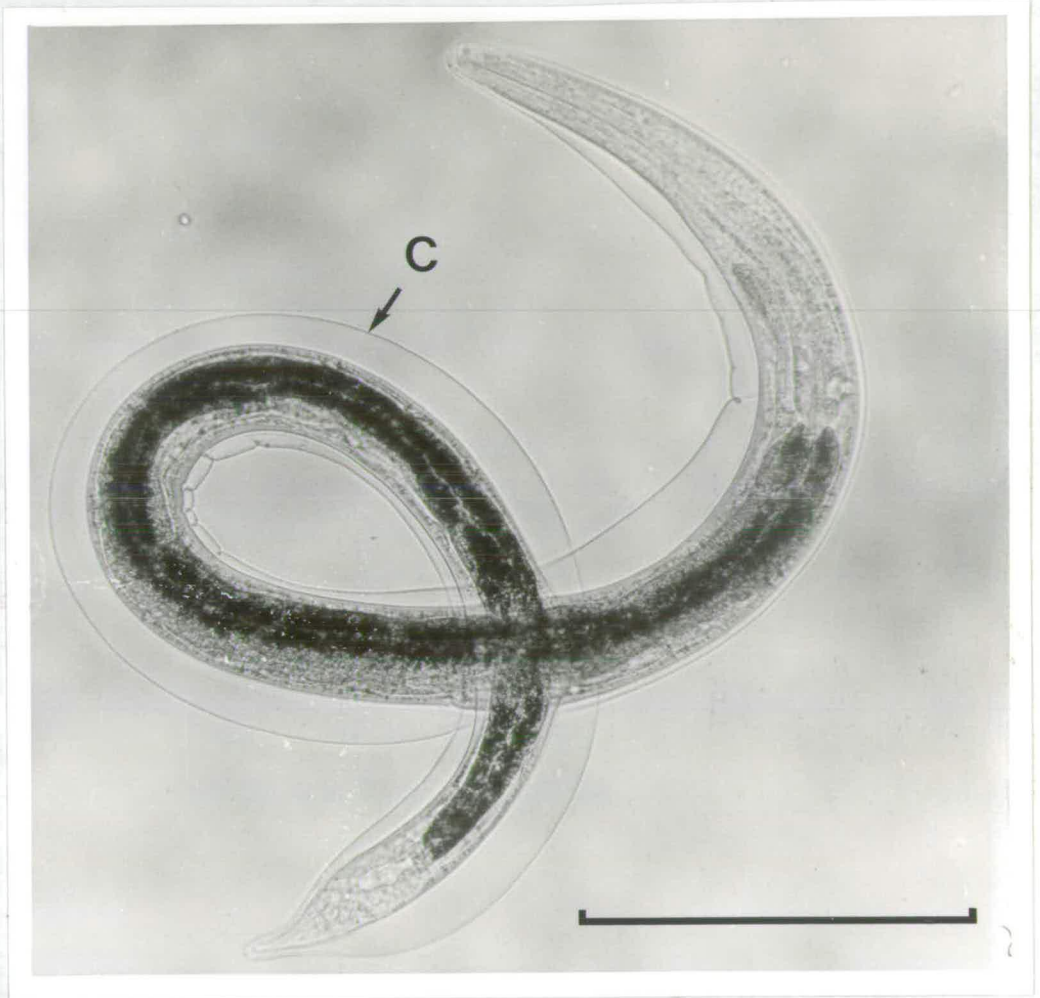
flank (2 donors for every six recipients), and then at subsequent times killed, and the larvae within the lung used in exact doses in the order of 20 to infect recipients orally. In the final experiment (Expt 22) a group of six rats were infected by EDT20Nb at the same time as the donors, to quantify the migratory success of the particular larval population. The detailed protocols and results are shown in Table 6.8.

Results

It is clear from the data of Table 6.8 (page 139), that larvae in the lungs were unable to withstand the digestive tract of the host if transferred earlier than 35h. Indeed, in 2 of the four experiments, this was not possible until 40h post-infection. The developmental period in this case is much longer than that for S. ratti, where 10h was required by the heterogonic strain and 7h for the homogonic strain. N. brasiliensis was first recovered from the lungs at 15h (page 78), consequently, a developmental period of at least 20h is required. Any larvae present in other sites would need a similar developmental period, therefore the negative results for the liver and head can be taken to mean these tissues do not act as nurseries.

6.4.3 The identification of a morphological marker for completion of development in the lungs [Expt 23].

This experiment was designed to confirm and amplify the observations of Twohy (1955a, 1956) to the effect that N. brasiliensis had to initiate moulting before continuing migration to the endpoint.



Materials and Methods

Twenty-one rats were infected with approximately 100L3's (still ensheathed) on the left flank. At the following times post-infection, groups of 3 were killed, 15h, 20h, 25h, 30h, 35h, 40h and 45h. The lungs were removed and any larvae therein extracted (page 29). After heat fixation, the larvae were measured and the stage of development noted.

Results

The results are shown in Table 6.9. Analysis of variance identifies significant growth between the infective L3 and larvae in the lung at 45 hours post-infection ($F_{[7,150]} = 320$, $P = 1 \times 10^{-11}$), but those arriving in the lungs at 15 hours are not significantly longer than infective L3's. Maximum growth has been achieved by 35h, the earliest time at which some larvae are infective by adoptive transfer. All larvae extracted at 35h were undergoing a moult (Plate 6.1). Therefore it appears that the onset of moulting is the point at which the larvae can continue their migration to the gut.

6.5 Conclusions

6.5.1 S. ratti (homogonic)

(1) As not all larvae were accounted for in the nasal region, other organs and tissues, from which there was a direct pathway to the gut, were examined over the period from infection to maximal arrival in the

digestive tract. Only one larvae was ever recovered from the lungs of the 49 rats examined, with none from the livers (12 rats). Larvae of N. brasiliensis can be extracted from the lungs, so there would appear to be no reason why S. ratti cannot be. The architecture of the liver should present no problems for the extraction procedure.

(2) Larvae need a period of development of at least 7 hours in the nasal region of the rat before migration can proceed to the gut. With this in mind, larvae cannot migrate through the lungs or liver, assuming they need a similar period of development, since they are not detected by sampling at autopsy in these tissues. Therefore the head migration is probably exclusive, with the occasional larvae recovered from other tissues being part of the dose which never complete migration ($1 - p_f$).

(3) Development within the nasal region is complete when the oesophagus index (OI) is greater than 1. Virtually all larvae have reached this stage of development by 30h (0.92 of the dose).

6.5.2 S. ratti (heterogonic)

(1) A period of development of up to 10 hours is required before migration can proceed from the nasal region to the gut.

(2) Development is complete when the oesophagus index is greater than 1. The maximum proportion of larvae (0.72 of the dose) have developed to this extent by 40h.

6.5.3 N. brasiliensis

(1) An examination of the head (35 rats) and liver (24 rats) over the relevant developmental period revealed no migrating larvae. The results for S. ratti from the head acts as a control for the extractability of larvae from such tissues.

(2) Larvae require a period of 20h development in the lungs. Any larvae present in other tissues might require a similar period of development, and therefore be detected by sampling at autopsy. The negative results for the liver and head can accordingly be taken to mean that these tissues are not on the pathway of migrating N. brasiliensis .

(3) The first larvae complete development in the lung by 35h, with the onset of the first parasitic moult.

HISTOLOGICAL EXAMINATION OF THE MIGRATION OF
S. RATTI (HOMOGONIC) THROUGH THE HEAD OF THE RAT.

Introduction

The results of Chapter 4 categorically prove the involvement of the nasal region in the migratory pathway from the skin to the gut. Tada et al. (1979) suggested that the larvae migrated through the cranium as well as the nasal region. Hattori (1981) postulated that the pathway involved a stepwise move through the sub-arachnoidal spaces to the nasal cavities. Mimori et al. (1987) and Nojima et al. (1988) using studies on the change in the morphology of the migrating larvae came to the same conclusion. Dawkins et al. (1982) and Nawa et al. (1985) found larvae in the cerebrospinal fluid of infected mice. Proof for the involvement of the cranium in the pathway has not been obtained in the present study (Chapter 4) and there is no detailed knowledge of exactly which tissues in the head contain the larvae.

Non quantitative histological techniques could not offer proofs of a pathway in any sense comparable to those forming the core of the present study. Description of a histological investigation of the heads of parasitised rats at different times post-infection is nevertheless presented in the following Chapter. It was carried out with the expectation that enough information would emerge to form the basis of a testable theory as to the detailed sequence of events in

this region.

Materials and Methods

Four rats were infected with 500 L3's of S. ratti (homogonic) on the left flank. At 20h, 30h (2 rats) and 40h, the rats were killed and the head removed. The skin of the head, the underlying muscles, the mandible and the eyes were removed. The posterior halves of the cranium and brain were discarded. The remaining head tissue was then fixed in 10% formal-saline, and processed (page 29). Serial sections, 10 μ m thick, were cut and stained with haematoxylin and eosin and examined for the presence of larvae.

Results

Plate 7.1 is a parasagittal section through the rat head showing the cranium and nasal regions as examined in chapter 4. Anterior to the cerebral hemispheres can be seen the large olfactory bulb (OB) in which terminate the olfactory nerves. The olfactory nerves arise from the olfactory receptor cells located in the epithelium that covers the ethmoid turbinals (E) in the nasal cavity. Axons from the olfactory receptors pass through the foramina of the cribriform plate and engulf the whole surface of the olfactory bulb.

The surface of the brain is enveloped by three layers of tissue, the meninges. The most outer, know as the dura mater, is a tough elastic layer closely applied to the inner surface of the cranium. Beneath this is the arachnoid and, finally the pia-mater, both of which

consist of delicate connective tissue lined by flattened mesothelium (Wheater, Burkitt & Daniels, 1981). The pia-mater is intimately attached to the surface of the brain. The arachnoid and pia mater are connected by thin web like strands, with the space between the two known as the sub-arachnoid space and filled with cerebrospinal fluid. The arteries and veins of the CNS pass in the sub-arachnoid space and are loosely attached to the pia-mater. A space between the arachnoid and dura mater, termed the sub dural space is also fluid filled. This is not continuous with the sub-arachnoid space.

Examination of sections taken from rats infected 20h and 30h earlier disclosed similar results. At both times relatively large numbers of larvae were seen in the sub-arachnoidal spaces, but they were not restricted to this area (Plate 7.2, 7.3 & 7.4). Larvae were also detected in the sub-dural space, and tissues of the brain directly beneath the pia-mater. Deep penetration of the brain was rarely observed, with most larvae remaining in close proximity to the meninges. No larvae were ever seen in the cerebral cortex, although a number were identified in the sub-arachnoidal space (Plate 7.5) around this area of the brain, and especially in the meningeal tissue separating it from the olfactory bulb (Plates 7.2, 7.3 & 7.4). The most noticeable concentration of larvae was in the dense plexus of the olfactory nerve layer on the surface of the olfactory bulb (Plate 7.6). Larvae were seen closely applied to the meninges, and deep within the nerve bundles in this region. Large numbers lay parallel to nerves running through the cribriform plate to the ethmoid turbinals (Plates 7.7 and 7.8). The occasional larvae appeared to be breaking

through the epithelium of the turbinals into the nasal cavities (Plate 7.9a, b, c & d). Very few larvae were seen in the cavities between the turbinals.

At 40h, relatively small numbers of larvae were detected in the tissues of the brain or meninges, except the nerve plexus at the anterior region of the olfactory bulb. Larger numbers were observed in the nerves and tissues associated with the ethmoid turbinals.

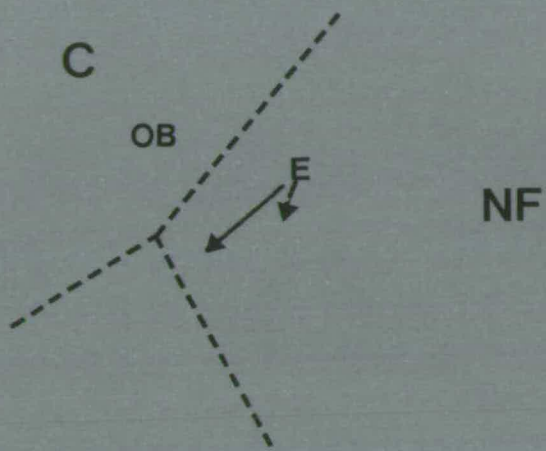
Conclusions

The results from the sections cut at 20h and 30h revealed larvae associated with the meningeal tissue at the anterior region of the cerebral cortex and the olfactory bulb. The relatively highest concentration of larvae was in the nerve plexus enveloping the olfactory bulb and in the nerve bundles of the ethmoid turbinals. By 40h virtually all larvae are in the anterior of the olfactory bulb in close proximity to the cribriform plate, or in the tissues of the nasal region. This is consistent with the possibility that larvae arrive in the cranium first, and become associated with the meningeal tissue of the olfactory bulb and cerebral cortex. As time progresses the larvae could pass forward to the nerve plexus surrounding the olfactory bulb in either the sub-arachnoidal space, the sub dural space or nervous tissue beneath the meninges. From this region the larvae appear to follow the direction of the olfactory nerves through the cribriform plate into the tissues of the ethmoid turbinals. Plate 7.9 a-d would suggest that larvae break through the sensory epithelium covering the turbinals into the nasal cavities. From here the

larvae have a direct path to the intestine down the oesophagus. The impence to movement in the nasal cavities would be negligible, and thus, migration through this region would be rapid and therefore difficult to detect.

Plate 7.1 A parasagittal section of the rat head showing the naso-frontal region (NF), the cranium (C), the olfactory bulb (OB) and the ethmoid turbinals (E).

Plate 7.2 is a parasagittal section through the region separating the olfactory bulb and the cerebral cortex (C). The section shows larvae closely associated with the pia-arachnoid (PA) around the sheath of olfactory nerves on the surface of the olfactory bulb. Larvae are indicated by arrows (x100).



OB

PA

C



Plate 7.1

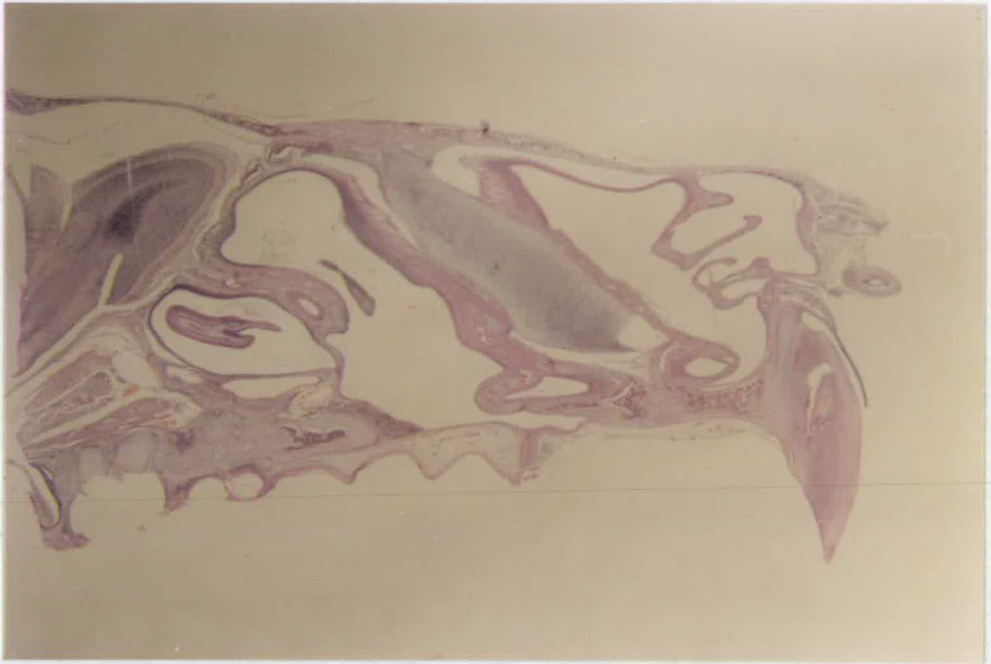


Plate 7.2

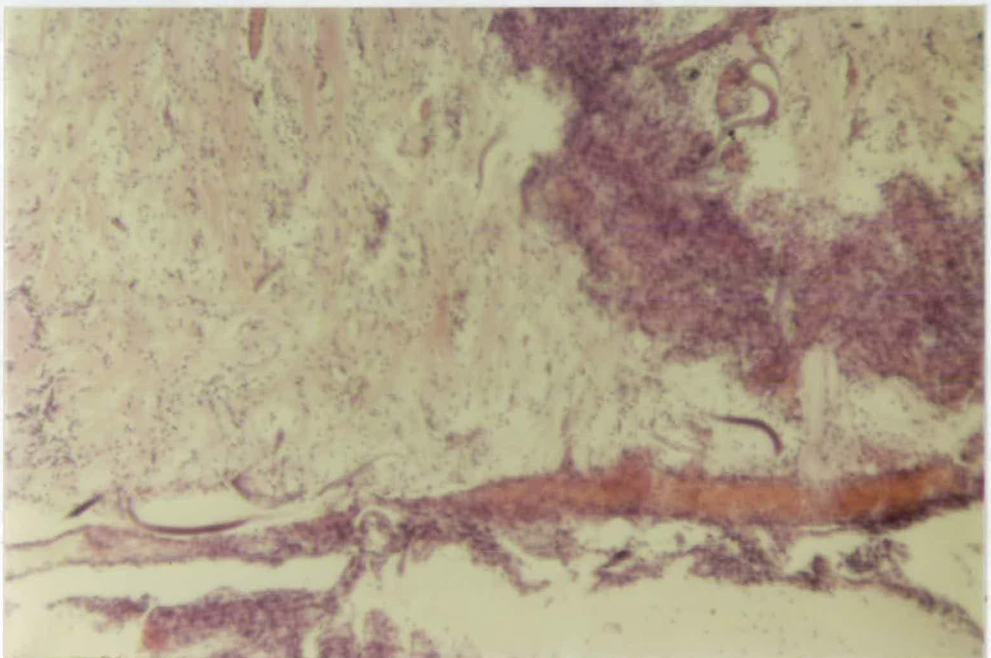


Plate 7.3 is a high power (x200) of a section taken from the same region as Plate 7.2. It demonstrates larvae in association with the meningeal tissue. The pia-arachnoid is seen as the area of dense purple nuclei. (C) is the cerebral cortex and (ON) the olfactory nerves engulfing the olfactory bulb. (OB) is the base of the cranium.

Plate 7.4 is a transverse section through the region shown in Plate 7.2. The section shows the relationship between the arachnoid (A) and the pia-mater (P). Sections of larvae can be seen in the sub-arachnoidal space (SS), the sub-dural space, and between the nerve bundles around the olfactory bulb (OB). (x200)

C

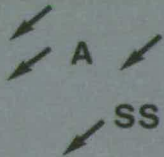


ON

OB



P



A

SS

Plate 7.3



Plate 7.4

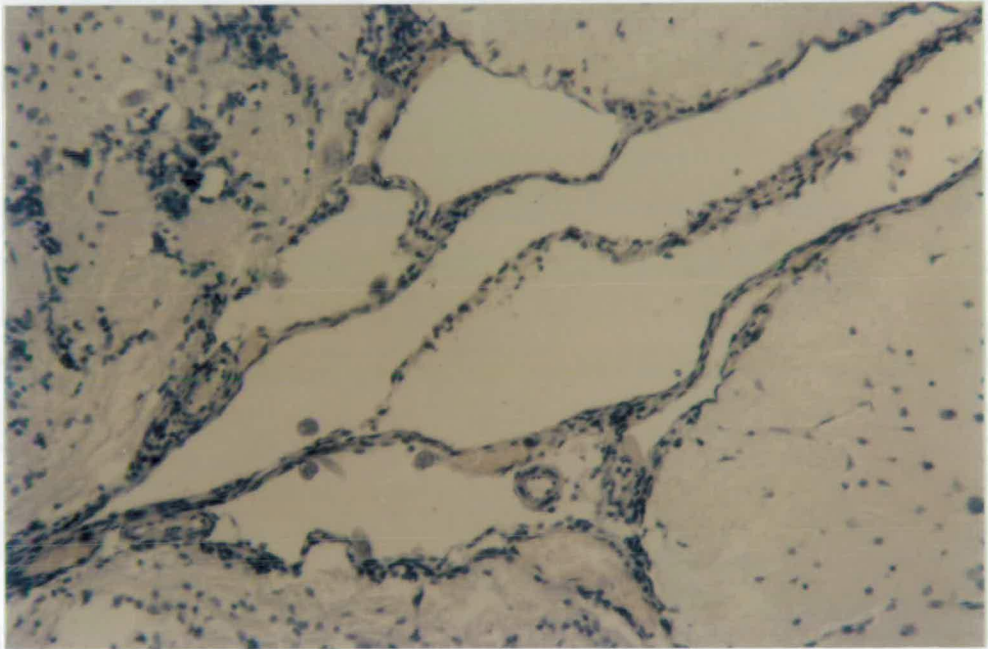


Plate 7.5 is a parasagittal section showing a single larva in the sub-arachnoid space around the cerebral cortex.
(x400)

Plate 7.6. is a transverse section through the anterior of the olfactory bulb, showing an accumulation of larvae associated with the nerve fibres. (M) is the meningeal tissue between the two lobes. (x200)

c

M

M

Plate 7.5

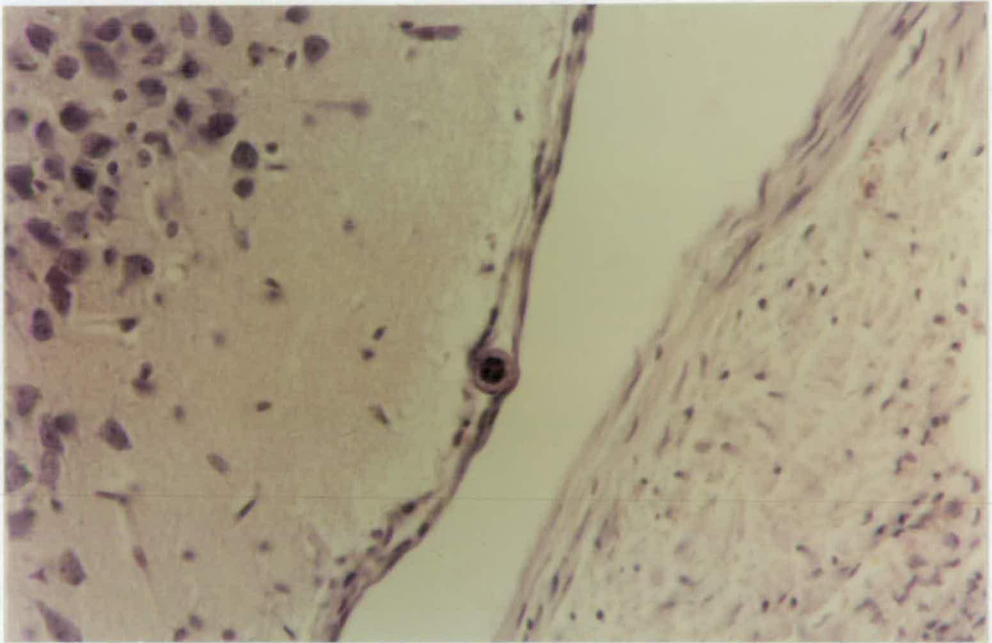
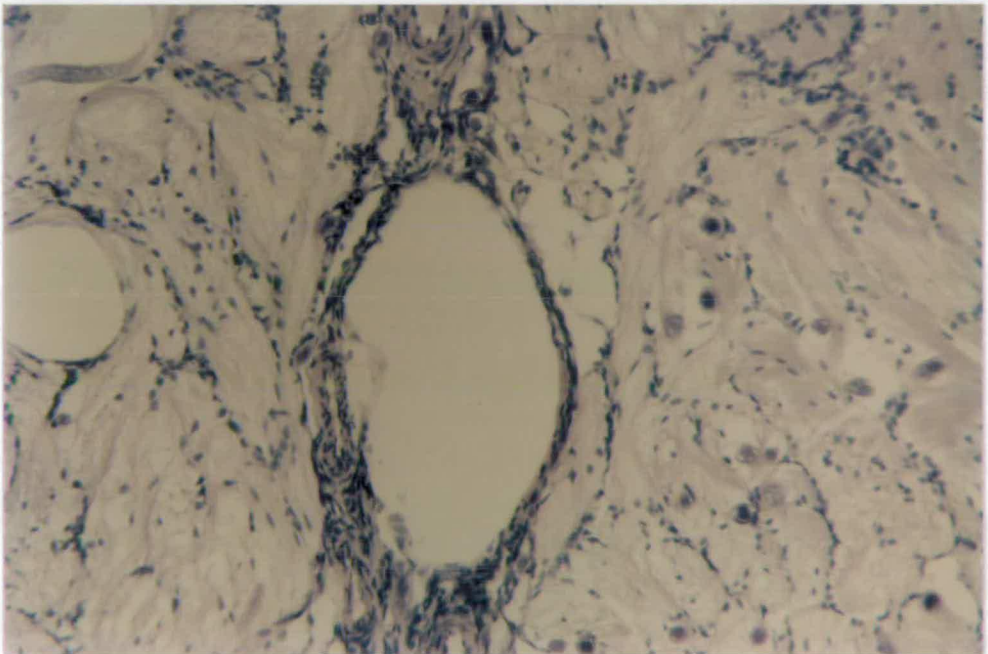


Plate 7.6



Plates 7.7 and 7.8 are low and high powers respectively of the nerve bundles passing through the foramina of the cribriform plate (CP). Larvae closely associated with the nervous tissue are indicated with arrows. (x100 and x200)

CP



CP



CP



Plate 7.7

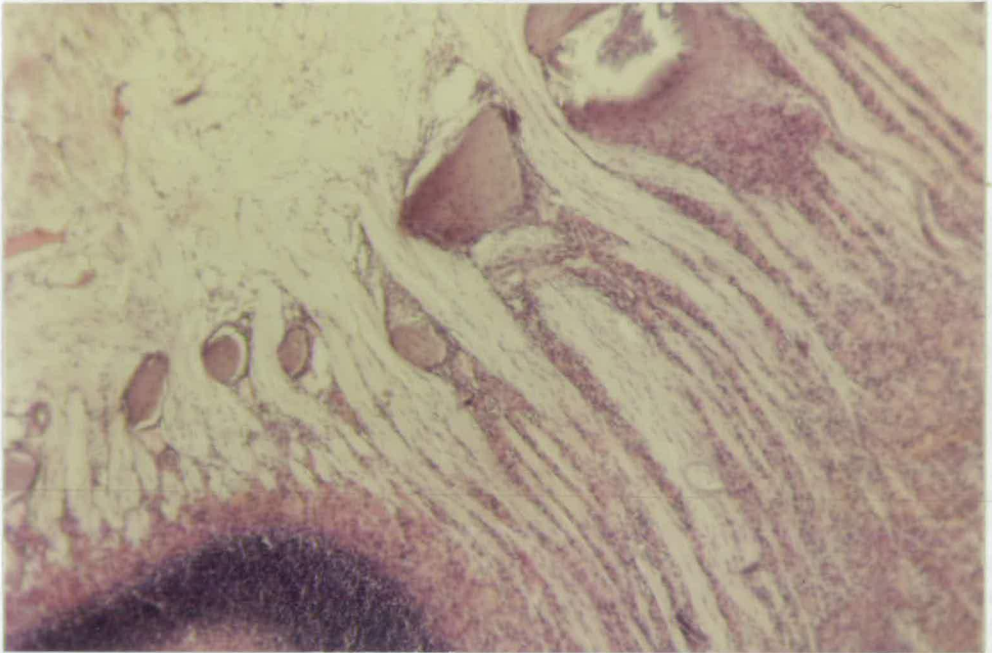


Plate 7.8

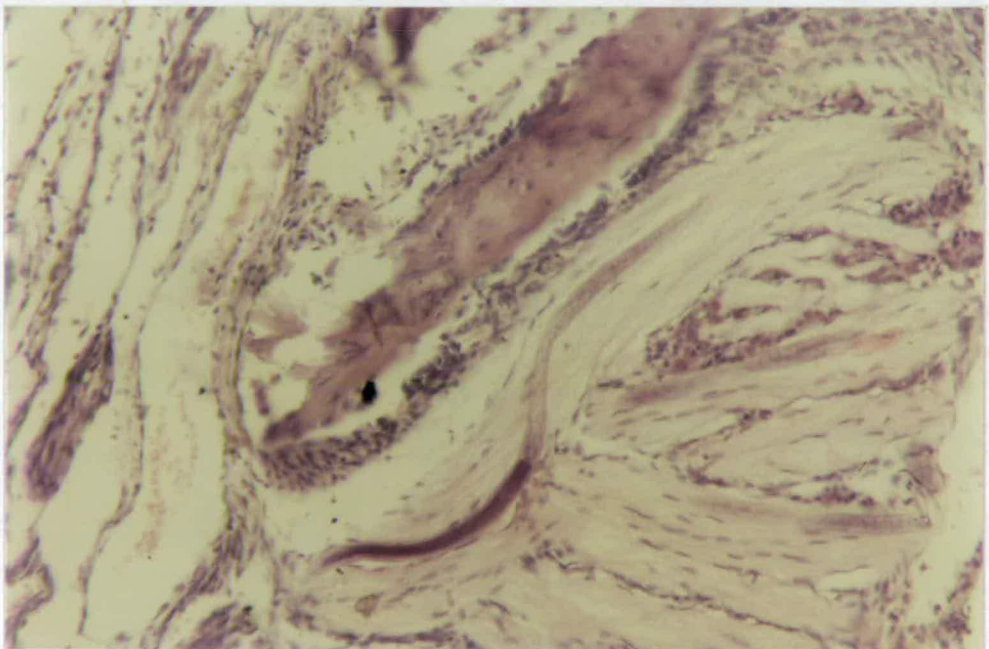


Plate 7.9 a, b, c & d are sequential sections showing a single larva apparently breaking through the olfactory epithelium (OE) into the nasal cavity. (x200)

NC

OE



Plate 7.9a

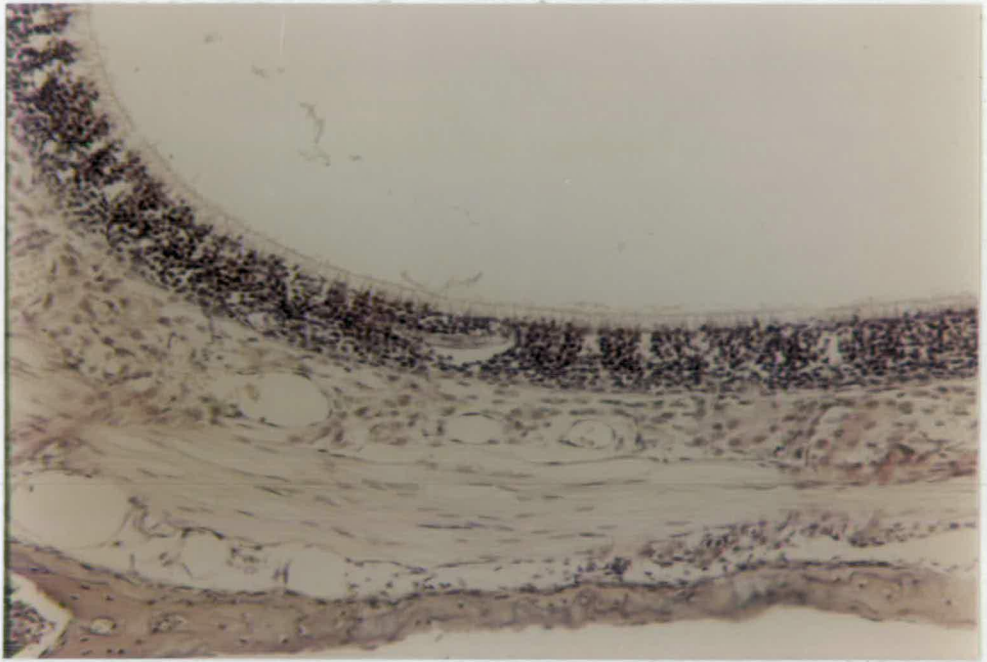


Plate 7.9b

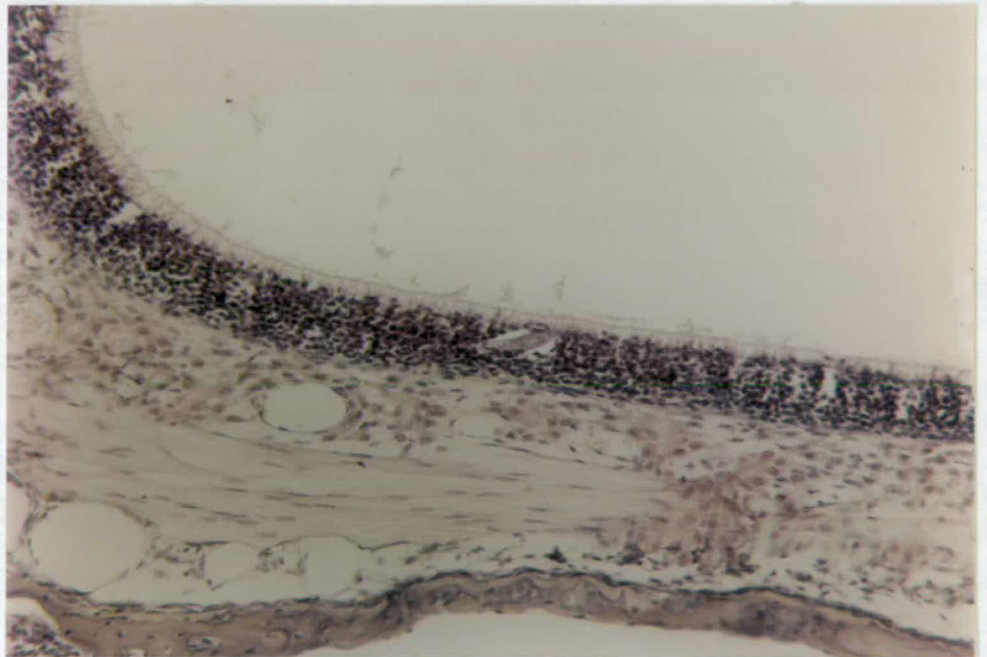




Plate 7.9c

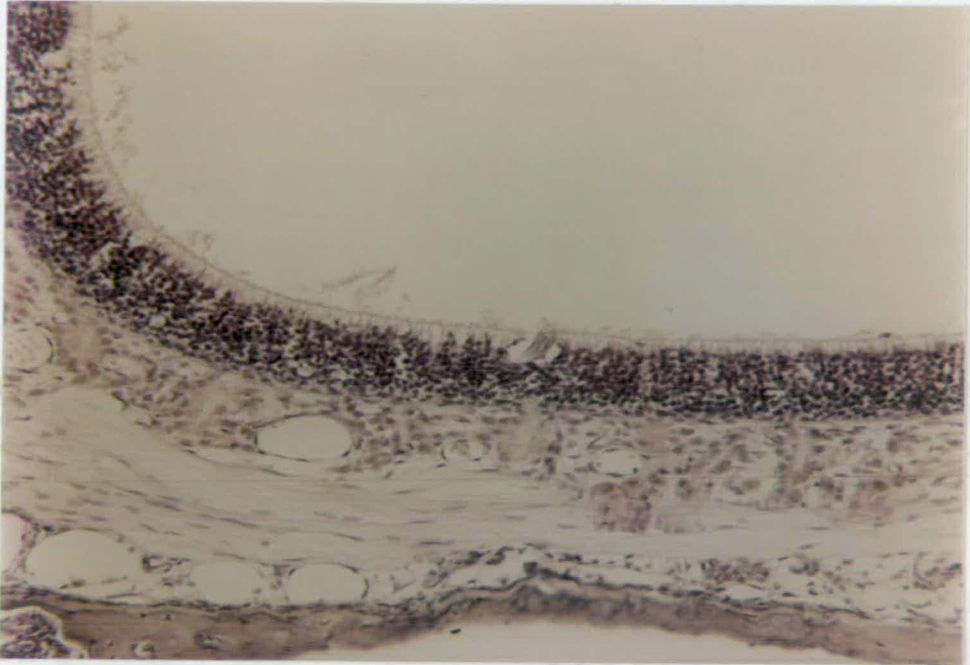
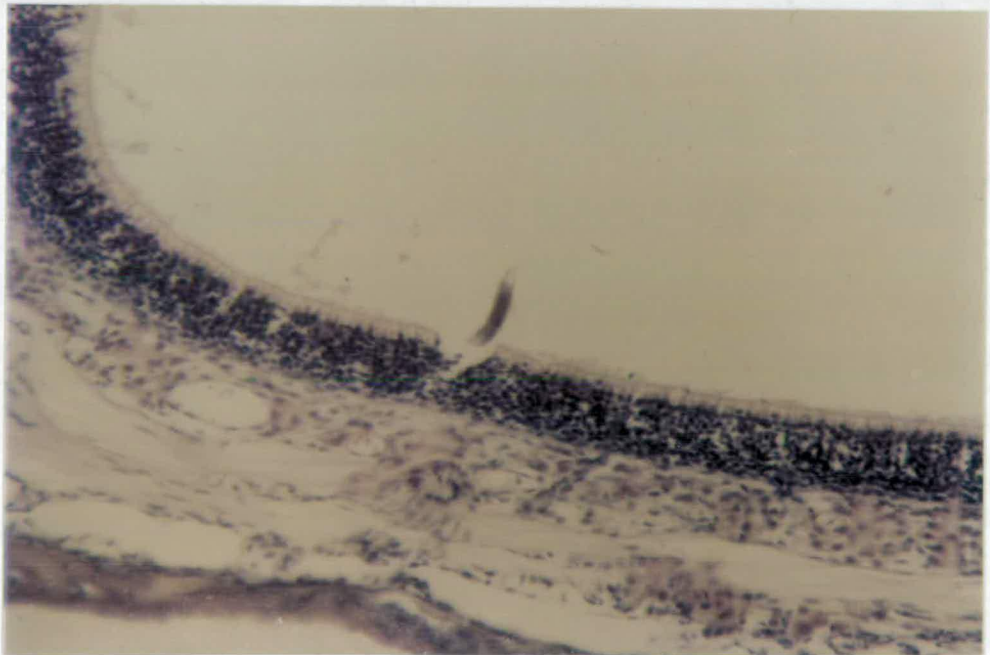


Plate 7.9d



CHAPTER 8

DISCUSSION

The site finding behaviour of many juvenile stages of macro-parasites involves extensive migration in the body of the host. The pathways they take are supposedly well known, and are baldly stated as fact in textbooks of parasitology, but the truth is that in most cases the type of evidence used to support these assertions is at best inconclusive and, at worst, completely misleading.

Wilson (1983) in his review of roundworm migration suggested that for a putative transit site (Q) to be proved to be part of a migratory pathway, the inequality $p_o + p_f > 1$ must hold in respect of it, where p_o is the peak proportion of the dose found within the site, and p_f the maximum proportion of the dose reaching the endpoint. Irrespective of the exact method of estimation it is crucial for this form of proof that the experimental system is designed to yield the highest possible value for p_f . If for example all parasites complete migration, i.e. $p_f = 1$, then any parasites found in the body other than the destination must be on course for the destination. If however, $p_o = 0.4$ and $p_f \leq 0.6$, it is impossible to decide whether parasites in Q are on or off their proper course; but with the same p_o and a p_f of 0.65 we can say for certain that 0.05 of the dose passed through Q on their way to the destination. That being so, it is highly probable, even if not certain, that all parasites in Q were en route to the endpoint. Wilson (1983) could find only one set of data for a

skin penetrating roundworm that satisfied the criteria at the time of writing, viz the report of Twohy (1956) for N. brasiliensis in the rat lung. A further review of the literature encompassing all invasive helminth parasites revealed only one further instance where the inequality can be satisfied. The data of Mangold & Dean (1983) proves categorically that Schistosoma mansoni migrates through the lungs of the mouse after skin application, before finally establishing in the mesenteric circulatory system (\bar{p}_O on day 7 = 0.775 ± 0.133 , \bar{p}_f on day 45 = 0.411 ± 0.085 , giving a p_c of 0.186). Each of the above has its limitations. The former was obtained after subcutaneous injection, whilst the latter is based on an unnatural host-parasite relationship and the assumption that autoradiography does not err in any way.

At first sight, the data of Behnke, Paul & Rajasekariah (1986) fulfil the criteria for the lung as a transit site in the migration of Necator americanus in neonatal hamsters. But, as noted by the authors, the upper limit of the range of larval recovery from tissues is in excess of the dose. The authors state that the inaccuracy in the counting of the dose only occurred in 1 of the 5 experiments performed. Nevertheless, the presentation of the data does not permit a separate assessment of the remaining four experiments and therefore no accurate conclusions can be drawn.

More recently, Schad et al (1989) have adopted a system of compartmental analysis to study migration routes. As the authors point out, the technique has advantages over the inequality approach in that it can be used to study the sequence of events and therefore

possibly identify sites where impedance to the movement of the nematode is below the 'critical' level. However, the model used by the authors of S. stercoralis in the dog is flawed in many ways, primarily: (a) The proportion of the dose reaching the endpoint has a mean value in the region of only 0.06 of the dose. (b) The variance associated with larval recovery is extremely large. (c) The results were obtained by compressed tissue autoradiography, where, as the authors note, only 21% and 92% of the larvae from two different labelling techniques were successfully labelled (see also Aikens & Schad, 1989). From their results, Schad et al. conclude that the migration of S. stercoralis in the dog is not necessarily confined to the lung, but, more probably, that a number of pathways exist. This they term the 'scramble model'.

The low establishment obtained by Schad et al. (1989) raises doubts as to the role of the dog as a true host for S. stercoralis. The sheep parasite S. papillosus is also quoted as a natural parasite of rabbits (e.g. Brumpt, 1921; Sandground, 1926), but unpublished work in this laboratory (Wilson, personal communication) as well as that of Nwaorgu & Connan (1980) produced takes in rabbits of similar magnitude to those obtained by Schad et al. (1989) with S. stercoralis in dogs. A possibility that arises is that these so-called hosts (dogs) are accidentally infected in areas with a high incidence of the parasite in the true human host. In this case the scramble model proposed by Schad et al. may bear no relation to the events in the true host, with the random distribution of larvae a result of disorientation in an unnatural environment. Moreover, although in this study a ⁷⁵Se label was shown not to be

damaging to L3 of S. ratti, the very high activities obtained by Schad et al. may, in fact, have affected the migration and viability of S. stercoralis.

Migration of 3rd-stage larvae of Strongyloides ratti

Since Abe (1964) first reported the occurrence of larvae of S. ratti in the head of the rat, there have been numerous other accounts from various Japanese workers (see General Introduction) and latterly by Murrell (1980, 1981), Bell Adams & Gerb (1981) and Murrell & Graham (1983) in the USA. Similar results were obtained by Dawkins et al. (1982) and Nawa et al. (1985) in the mouse. These observations have largely been ignored in the wider literature (e.g. Schmidt & Roberts, 1985) despite the lack of evidence for the commonly held belief that the route of migration involves the lung (Abadie, 1963; Wertheim & Lengy, 1965; Moqbel, 1980; Genta & Ward, 1980). However until now, none of the sets of data for either route satisfies the criteria laid down by Wilson (1983), i.e the inequality has always been in the wrong direction ($p_f < p_n$, where $p_n = 1 - p_o$). On past evidence, therefore, larvae in the head or the lung could just as well have been off, as on, their proper course.

Wilson (1983) argues that much of the data obtained by other workers falls short of the requirements because of their failure to understand the relevance of p_f . In two experiments, Tada, et al. (1979) inoculated rats with 500 third stage larvae into the skin of the hind leg, with a resulting \bar{p}_o in the head = 0.38 at 40 hours and

0.42, at 30 hours respectively. Unfortunately, no value for \bar{p}_f was obtained in the first experiment, and therefore the result is meaningless in the context of a proof. In the second, a peak value of $\bar{p}_f = 0.44$ was obtained at 60 hours, clearly not of a magnitude great enough to satisfy the necessary requirements. Moreover, these experiments were conducted in such a way that associated variances were essentially unknown.

Murrell (1980) confirmed that larvae of the G60 heterogonic strain could be found in the head of the rat. In the most optimistic experiment, \bar{p}_O head = 0.20 and $\bar{p}_f = 0.37$ (rats infected with 1000 L3's subcutaneously in the nuchal region). In a further experiment, where no value of \bar{p}_f was obtained, \bar{p}_O head = 0.16. All estimates of p_O were obtained at 48 hours post-infection.

Hattori (1981) described 2 experiments. In the first he inoculated rats with 1000 L3's into the skin of the femur and, in the second, a dose of 1000 was applied to the skin of the tail. The resulting \bar{p}_O 's were 0.34 at 36h and 0.19 at 48 hours for the two experiments respectively. In both experiments the sampling time ran to 120h in the gut, by which time migration would be expected to be complete (see Chapters 4 & 5) and the maximum value for p_f obtained. However, the proportions recovered were only 0.33 and 0.16 for femur and tail applied larvae respectively.

The data obtained in this study prove categorically that two strains of S. ratti migrate through the nasal region of the rat head on

their way to the small intestine. The experimental framework, when it could be implemented to its operational limit (see later), resulted in both cases in a high proportion of the dose reaching the endpoint, with homogeneity between experiments such that results from many experiments could be pooled. The key to this was the development of the exact dose technique (EDT₂₀) for the skin application of small numbers of larvae. Wilson et al. (1978a) and Wilson & Simpson (1981) predicted the benefits of such a system, and indeed other workers have explained the need to keep the dose to a minimum in order to prevent random dispersion of larvae throughout the body (Wertheim & Lengy, 1965; Tada et al., 1979). Dawkins (1989) states that the mean establishment of S. ratti is in the range 0.21-0.27. Past results utilizing exact doses of less than 100 larvae have in this laboratory resulted in a substantially greater proportion completing migration. Wilson et al. (1986) and Carter & Wilson (1989) obtained mean takes with the homogonic strain ranging from 0.451-0.662 and 0.538-0.726 respectively and 0.681 with the heterogonic strain (Carter & Wilson, 1989). Further refinements to the technique, by the reduction of the initial dose to 20 larvae and the recovery of non-penetrators some 45 minutes post-infection have enabled the number of larvae entering the rat to be estimated with unprecedented precision. The small dose meant that errors due to counting the larvae prior to infection, and in the gut as adults, were removed from the system. The result was the production of a high quality sytem where the proportion of the dose actually completing migration to the end-point was substantially greater than that in studies in the past. The associated variance was well below normally accepted levels. In six experiments to locate p₀ in the nasal region of the rat head, the mean

proportion of the dose reaching the endpoint (\bar{p}_f) was 0.837 ± 0.013 with an overall coefficient of variation derived from the within sample error of only 10.2%. A pooled value for \bar{p}_o (nasal region) was calculated as 0.316 ± 0.021 . Therefore it can be stated as fact that at least a proportion of $0.153 [(\bar{p}_o + \bar{p}_f) - 1]$ actually passed through the nasal region en route to the small intestine in these experiments. It is highly likely, if not certain, that all the parasites in the nasal region would have completed migration to the endpoint. The exact timing of the peak in the nasal region varies between experiments, but is always in the range 20h-25h with the most likely of the times sampled being 23h. The fact that times are slightly different in different experiments in no way diminishes the main import of the results since, for the purpose of the proof, the timing is irrelevant.

When testing the inequality as it applied to the homogenic strain it will have been noticed that some of the data that might have been relevant in Tables 4.4 (page 58), 5.1 and 5.2 (page 89) were ignored. In most cases \bar{p}_o and \bar{p}_f added together are less than unity and, therefore, they must be relegated to the 'unproven' category. Such inferior results by Edinburghs standards were generally obtained at a time when the experimental framework was vitiated by the cessation in supply of the rat food known to be essential for the exceptionally high levels of parasite success required. They are disregarded on these grounds. However, the general question of how data of this sort should be interpreted is addressed explicitly in Appendix 2.

For the heterogonic strain the results of six experiments gave a pooled value for \bar{p}_f of 0.711 ± 0.017 with a CV of 11.2% and mean \bar{p}_o of 0.397 ± 0.024 (nasal region). The timing of the peak in the nasal region in this case was consistent at 40h.

Tada et al. (1979) reported that S. ratti could also be located in the cranium. Hattori (1981), Mimori et al. (1987), Nojima et al. (1987) and Tanaka et al. (1989) advocate a two step migration through the sub-arachnoidal space to the nasal region. In the present study, larvae of both strains were recovered from the cranial region of the rat head, but in each case $p_f < p_n$. Nevertheless, an examination of the data for the cranium and the nasal regions together shows the events in the cranium to precede those of the nasal region by some 5h to 15h for both strains, which is consistent with a stepwise move between the two sites. The histological study of the migration of the homogonic strain confirmed that larvae could be found in the cranial tissues as well as the nasal region. Sections from rats infected 20h and 30h previously with 500L3's of S. ratti (homogonic) revealed relatively large numbers of larvae associated with the meningeal tissue of the olfactory bulb and anterior cerebral cortex, the nerve bundles enveloping the olfactory bulb, the olfactory nerves passing through the cribriform plate, and the tissues of the nasal region. Sections from rats infected 40h previously supported the idea that larvae had migrated forward through the cranium to the cribriform plate and tissues of the nasal region, confirming the results of the extraction (Chapter 4), that, by 40h, virtually all larvae have disappeared from this region. On this evidence it can be suggested that the larvae first pass

through the cranium before reaching the nasal region by following the path of the olfactory nerves through the cribriform plate. Larvae were not exclusive to the sub-arachnoidal space in the cranium, as they were also seen at 20h and 30h in the sub-dural space and the nervous tissue of the brain directly beneath the meninges. Larvae were rarely observed deep in the tissues of the brain.

Two experiments performed on the extraction of larvae from the brain tissues were hampered by the vast amounts of fine tissue debris present in the final suspension. This effectively obscured any larvae that might have been present. Further investigations of the role of the brain and meninges were planned utilizing the technique of compressed tissue autoradiography. It was envisaged that data obtained would supplement that from cranial washings. Unfortunately the technique was abandoned after a number of failures trying to estimate the time of arrival in the small intestine (see later). Uncontrollable factors relating to the diet led to failures in the culturing system. The technique has been widely used in tracking S. mansoni in the mouse where authors claim no ambiguity between foci and actual parasites (Georgi, 1982; Georgi, Dean & Chandiwanna, 1982; Georgi, Dean & Mangold, 1983; Mangold & Dean, 1983; Dean & Mangold, 1984; Dean, Mangold, Georgi & Jacobson, 1984; Mastin, Wilson & Bickle, 1985; Georgi, Wade & Dean, 1986; Wilson & Coulson, 1986; Wilson, R. A., Coulson & Dixon, 1986; Georgi, Wade & Dean, 1987). The low specific activity associated with S. ratti larvae in this study (max c.p.m./L3 = 21.5) led to problems in assessing the number of true foci present on the autoradiogram. Recently, Aikens & Schad (1989) obtained a specific activity of 75-330 c.p.m./L3 for S. stercoralis by

feeding the developing larvae on ^{75}Se labelled Escherichia coli. If such a system could be developed for S. ratti, the increased activity would result in foci easily distinguishable from noise. This assumes that the label does not impair the larvae in any way. For the moment, no absolute judgement on the role of the cranium can be made. All theories are speculative, and must be viewed with caution until hard evidence is available.

Migration of N. brasiliensis 3rd stage larvae.

In the natural situation, larvae of N. brasiliensis remain partially sheathed in the cuticle of the second stage until contact is made with a passing rat. In the laboratory larvae have traditionally been stimulated to exsheath prior to subcutaneous injection into a rat. Indeed this can lead to a reasonable establishment of the parasite. Haley (1962b) stated that an average of 72% of the inocula were recovered as adults on the 10th day of infection in one of his studies. Ogilvie (1965) obtained a take of 61% on day 11. Twohy (1956) found that 78% of the dose became established from subcutaneous injection whilst 73% of those applied to the skin reached the gut if they were allowed a penetration time of 4 hours. However when examining the tissues for the presence of migrating stages, Twohy (1956) found many fewer following skin application (14 versus 61%). In the most comprehensive collation of data from a single laboratory, Haley (1962a) recorded a mean of 55% in 219 rats over a dose range of 50-3000 third stage juveniles.

Subcutaneous injection removes an important step in the life-cycle of these parasites, and thus for migratory studies it is imperative to use skin application. The results obtained in Chapter 3 suggest that skin applied exsheathed larvae are relatively ineffective as opposed to those remaining sheathed. Answers as to why this might occur include: (a) Artificial exsheathment leads to the disruption of a sequential process initiated naturally by contact with a host. The effect of temperature change on the oxygen uptake of L3's reported by Wilson (1965b) was consistent with this idea. (b) Larvae in suspension are damaged physiologically. Overcrowding has been identified as a cause of damage to S. ratti L3's (see effect of centrifugation, page 104). (c) Artificially exsheathed larvae are possibly robbed of solutes present in the aqueous film inside their sheaths. Wilson & Dick (1964) reported that water was an unsuitable medium for the maintenance of suspensions of N. brasiliensis exsheathed larvae. The comparative success of a balanced salt solution (Wilson 1965a) to maintain exsheathed L3 in vitro does not prove that it mimics the fluid in the sheaths of the third stage worms. As reported in Chapter 3, skin application using 1/4RLA failed to reverse the damage that somehow results from artificial exsheathment.

The development of a system for the application of known doses of sheathed larvae maximized p_f and reduced the variance to a minimum, such that a proof was obtained for the migration of N. brasiliensis through the lungs of the rat. These results correspond well with those obtained by Twohy (1956) (see Figure 8.1) after subcutaneous injection of larvae. Larvae first appear in the lung 12h, rise to a plateau by 20h, with a sudden increase to a peak at 35h

($\bar{p}_o = 0.487$). Very few larvae remain in the lung after 50h. A value for \bar{p}_f was calculated from 4 experiments on day 8/10 as 0.779 ± 0.016 with a CV of 9%.

Arrival of N. brasiliensis and S. ratti (homogonic) in the small intestine.

An accurate picture of the overall kinetics of migration is the starting point for the development of further proofs of the pathways involved. The traditional methods are direct observation of larvae within the intestine (e.g. Twohy, 1956), methods of extracting larvae from the intestine (e.g. Murrell, 1980) or radioactive tracking (Wilson, 1979). Wilson et al. (1986) claim that the shape of a curve determined by direct observation for arrival in the small intestine is not only dependent on the actual arrival of larvae, but also on the improvement in one's ability to see an individual worm after it has been in place for some time and grown larger; or, if methods of extraction are used, the change in extractability as time progresses.

According to the conventional blood-lung hypothesis of migration, larvae of N. brasiliensis migrate directly from the lungs via the trachea, larynx and oesophagus to the small intestine. The theory first proposed by Tada et al. (1979) suggested that S. ratti pass directly with nasal excreta into the oesophagus and subsequently the intestine. If both theories are correct, then vacation of the respective transit site should mirror the arrival in the gut. The results obtained by the direct counting of such larvae suggest this is

so for N. brasiliensis. The larvae in this case are large (>1mm) and easily quantifiable on arrival in the small intestine.

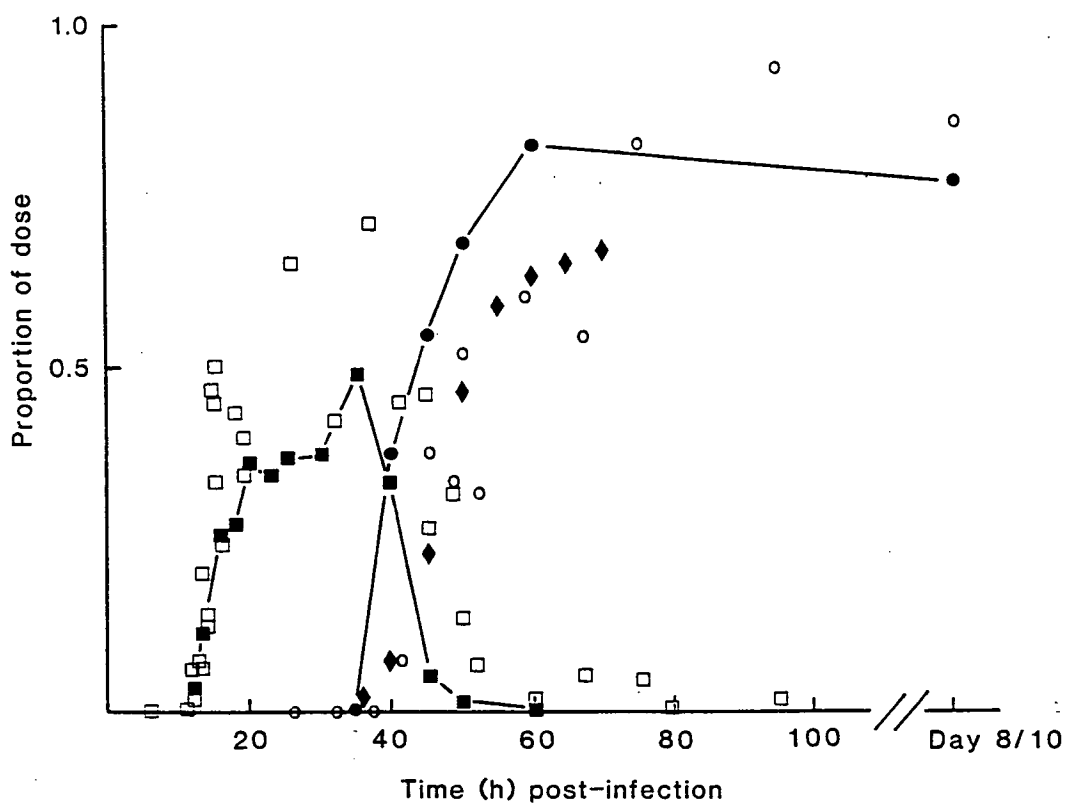
The larvae of S. ratti are much smaller (<0.7mm) and very delicate, consequently detection in the small intestine proved extremely difficult. The disappearance from the head (60h) was followed by a large delay before all larvae were detected in the small intestine (96h). Compressed tissue autoradiography was used to try and ascertain whether this difference was due to either (a) a large observer error in counting the larvae in the intestine or (b) larval arrival to the gut was actually delayed. The results suggest that a significant proportion of the dose of S. ratti (homogonic) has arrived in the gut by as early as 40h, with migration complete by 72h in one experiment. If a large proportion of the label is attached to the cuticle of the larvae, problems could arise after the initiation of a moult, in that either (a) moulting would result in the worms no longer being detectable (Kwansa & Murrell (1987) claim an 82% reduction in the label between infection and adults in the gut on day 8), (b) shed cuticles as well as true worms may produce foci resulting in a count greater than the true numbers present. Wertheim & Lengy (1963) suggest that the first parasitic moult takes place at 60h, therefore any results obtained after this time have to be viewed with caution.

The fully developed drug pulse technique lacks the problems associated with compressed tissue autoradiography when used in the gut and also eliminates the points noted by Wilson et al (1986) as the worms that are counted are all at the same late stage of development, when

Figure 8.1 The migration of N. brasiliensis through the lung and subsequent arrival in the small intestine.

- Migration through lung (present data).
- Arrival in small intestine (present data).
- Migration through lung (Twohy, 1956). Each point represents a single assay.
- Arrival in small intestine (Twohy, 1956). Each point represents a single assay.
- ◆ Data of Love et al. (1974) for larvae collected in oesophageal fistula. The results of experiment 1 replotted as the total proportion of the dose of 3000 L3's recovered at the end of each 5 h period.

Figure 8.1



individuals (or egg-tracks) are unambiguously visible in an intestinal squash preparation. It also eliminates any larvae present in the stomach at the time of administration and consequently the exact time of arrival in the stomach as distinct from the small intestine is known. Combined with an exact dose technique, and suitable controls to ensure that parenteric phases are unaffected, this procedure offers the possibility of a high degree of precision. The susceptibility of the two species to the drug was found to differ. All stages of N. brasiliensis were eliminated from the digestive tract of the rat when morantel tartrate was administered at a dose rate of 0.02mg/g body weight, whereas a three fold increase was required to obtain a 95% efficiency against S. ratti (homogonic). The drug was 100% efficient against adult S. ratti at the lower concentration, but stages up to 80h post-infection showed a marked resistance. This phenomenon was attributed to L3's of S. ratti arriving in the digestive tract and burrowing into the mucosa to undergo two moults. It was envisaged that the larvae in the mucosa were effectively shielding themselves from the action of the drug, whereas the adults on the surface were more susceptible. Wertheim & Lengy (1963) reported the first adults at 70h, although Mimori et al. (1987) placed this event at 78h, with 50% of the intestinal dwellers being adult by 90h. This correlates with the increase in susceptibility of S. ratti to the drug. N. brasiliensis on the other hand attach to the mucosa with the major part of the body hanging free in the lumen of the intestine, consequently all stages are equally affected by the drug.

The results for N. brasiliensis from the direct counts and the corrected data for the drug pulses for this species fall on the same curve. In this case, therefore, there is no observer bias in direct counts, nor is there a significant delay in traversing the stomach. The results of the present study suggest that migration to the gut is approximately 7 hours earlier than that outlined by Twohy (1956) and Love et al. (1974) who used chronic catheterization of the oesophagus to collect hourly samples of N. brasiliensis larvae as they approached the stomach in non-immune rats (see Figure 8.1). If the rate of development is inversely proportional to the density of larvae within the lung, due to competition for nutritional factors for example, the higher doses used by Twohy and Love et al. (dose = 270-1170 and 3000 respectively) would result in larvae remaining in the lung for a longer period. Alternatively, both the studies of Twohy and Love et al. relied on subcutaneous injection, which could have disrupted the early part of migration. This would seem unlikely since the curve of the arrival in the lung from the data of Twohy corresponds well with the present data. In four experiments, Love et al. (1974) recovered 67%, 63%, 48% and 30% of the inocula, with most of these appearing in samples between 40h and 60h post-infection. The peak rate of movement down the oesophagus was recorded at 48h.

The curve for S. ratti (homogonic) obtained by direct observations lags 15-17h behind, and parallels that for the corrected drug related counts. The discrepancy between the two curves is unlikely to be due to the larvae being delayed in traversing the stomach as those of N. brasiliensis were not. For both species it can be imagined that the same forces govern the rate of movement

through the stomach, in which case the passage of S. ratti is likely to be similar in its kinetics to that of N. brasiliensis. Accepting the curve for the drug pulse as the more correct picture for S. ratti (homogonic), and therefore, comparing it with both types of evidence for N. brasiliensis, a distinct difference in kinetics emerges. At 40h a proportion of 0.20 of the total which will ultimately reach the gut has arrived in both cases. Following this, the increase to a proportion of 0.90 is achieved in 8h by N. brasiliensis and in 28h by S. ratti (homogonic). Though the fastest migrators of both species take the same length of time to reach their destination, S. ratti (homogonic) is much less synchronised in its movements, with a time interval between early and late arrivals more than three times longer than that of for N. brasiliensis.

Migration to the respective transit site

Looss (1905) described the initial part of the migratory pathway of A. caninum through the lymphatics before reaching the circulatory system via the thoracic duct. Most textbooks, ignoring this suggestion, have stated that larvae enter venules directly. Only occasionally have workers returned to the idea of the involvement of the lymphatics. Gharib (1961a) and Clarke (1967) found numbers of N. brasiliensis larvae in lymph nodes soon after infection. Both took this to mean that the normal migration route was via the lymphatic system. Tada et al. (1979) performed surgery on rats to remove a strip of skin from around the neck. A delay was thus seen in the

peak of larval recovery from the head tissues. The authors proposed that the gap held up larvae migrating in the subcutaneous tissues. Wilson & Simpson (1982) argued that larvae migrating in lymphatics would be hindered by such major surgery. Evidence obtained from the lactating rat system would suggest that at least the early part of the migration of S. ratti is likely to involve the lymphatics (Wilson & Simpson, 1982; Wilson et al., 1982; Wilson, 1983; Wilson et al., 1986; Wilson, 1987). Wilson & Simpson (1982) have shown that lymph nodes that drain the infection site on the flank of the rat are in an intimate association with the mammary gland. Secondly, in 14 out of 26 lactating rats which were injected with heat killed radio-isotope labelled larvae, the distribution of the label within the mammary gland was similar to when live larvae were injected. The local lymphatic system is an obvious candidate to concentrate dead material like this. The possible role of the lymphatic system in transporting larvae to the head tissues is difficult to assess since very little is known of it's anatomy and physiology in the rat. Tilney's (1971) maps of lymphatic drainage suggest that the inguinal nodes draining the site of infection have efferent vessels leading to the axillary nodes whose efferents then empty into the circulatory system via the subclavian duct. Direct connection to the head through the lymphatic system is unknown but is not impossible.

Larvae entering the circulatory system by the subclavian duct would be transported via the heart to the lungs. To reach the head in the blood stream, larvae of S. ratti would have to be able to recirculate in the blood. Many authors claim that Toxocara spp. reach the brains

of mice through the circulatory system (e.g. Sprent, 1955; Bisseru, 1969; Dunsmore, Thompson & Bates, 1983). Sprent (1955) suggests a recirculation through venous arteriolar anastomoses in the lung before reaching the brain, a similar hypothesis to that of Wilson (1977, 1980a, b, but now discarded) for the switch in the migration route of S. ratti in the lactating host. The movement of the larvae would be passive once in the blood stream, and the direction they take would be highly dependent on the channels open to them. Wilson (1977) suggested that the diameter of arteriolar-venous shunts in the lung (15-25 μ m) would permit passage of larvae of S. ratti (diameter = 17 μ m) but not N. brasiliensis (diameter = 27 μ m). Fulleborn (1925) proposed that larvae could break out of capillaries in the lung, and then re-enter the venous system for re-circulation to occur. With regard to S. ratti, these ideas have been falsified (Wilson & Simpson, 1982).

Sprent (1954) suggests that the cerebral arteries on the surface of the mouse brain have a lumen between 15 μ m-20 μ m. He concludes that Toxocara spp. larvae (diameter 15-20 μ m) will thus leave the circulatory system at this point, with smaller larvae such as microfilaria able to pass through the fine capillaries and remain in the blood. S. ratti larvae would fall into the former category. It was noted in the histological study that large numbers of S. ratti larvae were in close association with the blood vessels of the meningeal tissue, but no haemorrhaging was observed.

A study of two different strains of S. ratti showed subtle differences in the behaviour in the lactating rat system (Wilson &

Simpson, 1981). The two strains (homogonic and G60 heterogonic) differed in that the time for most larvae to reach the milk exceeded 30h for the homogonic strain and 36h for the heterogonic strain, and the split in the proportion of the dose recovered as adults in the intestine of the mother or pups was significantly more in favour of the mother in the heterogonic strain. It is clear from the results of the present study that the outline migration of the two strains is similar but the timing of specific events differs substantially. The homogonic strain first appears in the head tissues at 15h (cranium) reaches a peak in the nasal region by 20-25h and vacates the head completely by 60h. Migration to the small intestine starts by 38h and is complete some 30h later. The first heterogonic larvae appear in the head by 20h, peak in the nasal region by 35-40h, and seem to have vacated the head completely by 60h. In this case the migration to the gut starts by 48h and is complete by 68h. Present findings with the heterogonic strain are comparable to those of Murrell (1980) who was probably working with the same strain. The least dynamic larvae of both types require a similar time to complete migration, but the vast majority of the population of the homogonic strain are 5-15h more advanced in their migration than those of the heterogonic strain. These results thus reinforce the findings of Wilson & Simpson (1981) that the two strains show subtle differences in migration, and therefore the theory of recirculation is inadequate, for implicit in it is a somewhat passive role for the larvae as far as direction finding is concerned, and differences between the two strains cannot be accounted for in this way. Wilson (1983) refers to unpublished experiments in which labelled larvae were injected directly into the femoral veins of rats and virtually 100% of the label quickly became

trapped in the lung, apparently refuting the idea of re-circulation and, in particular, blocking the passage of larvae from the subclavian vein to the head by way of the lung.

No attempt was made to recover either N. brasiliensis or S. ratti larvae from the circulatory system as the 'impedance' would be negligible, and larvae would be quickly transported to the next capillary bed, assuming no recirculation. Negative results in this context would have no real meaning, as the detection of the larvae in sufficient quantities to warrant a proof would not be possible. Harley & Gallichio (1971) recovered over 70% of the tissue larvae of T. spiralis by cannulation of the thoracic duct of the rat. These larvae are assumed to enter the blood flow in unoperated animals, but that assumption may be false. Chronic thoracic duct cannulation may radically alter the interrelations of the two systems and create forces which compel larvae into an outflow which does not normally occur. When Harley and Gallichio ⁽¹⁹⁷¹⁾ examined the blood by conventional heart puncture very few larvae were recovered. The time for a complete circuit through the circulatory system in the rat is approximately 5 seconds and the cardiac output is approximately 120ml/minute (Prosser & Brown, 1962). Accordingly, the larvae in 1ml of blood have many opportunities every minute to leave the circulatory system by lodging in capillaries and breaking through the endothelium. Harley & Gallichio calculated, from the highest number of T. spiralis larvae recovered from the thoracic duct (53,261) over a 24h period, that only 37 larvae were entering the bloodstream every minute, or one larvae in every 3ml of blood. They conclude 'with these physiological facts in mind, it seems incredible that one is able to find any larvae

in the bloodstream'.

As seen from the curves of arrival in the transit sites and the digestive tract, the larvae of both N. brasiliensis and S. ratti are unsynchronised in their movements. For S. ratti (homogonic), there is at least 8h between the first arrivals in the head and the peak. Assuming blood borne migration, larvae from the initial dose of 20 would be entering the circulatory system throughout this 8 hour period. Bearing in mind the facts stated by Harley & Gallichio, (1971) an attempt to investigate the role of the blood by sampling at autopsy, would not have justified the use of the rats. Moreover, the events in lactating rats provide strong circumstantial evidence that the blood system is not the immediate target of skin penetrating S. ratti (Wilson & Simpson, 1982).

The results obtained from the application of S. ratti (homogonic) larvae to the different sites on the rat were suggestive but not conclusive that larvae applied to the neck of the 3-week-old rats peaked in the head earlier than those applied to the flank, supporting the idea that migration is not through the blood flow, as such a migration would be passive, and the site of infection bear no relevance to the time of arrival in the transit site. Results obtained in 12-week-old mature rats were contradictory. This was attributed to the fact that a greater proportion of the dose of the larvae was recovered from the skin of the rat if applied to the flank rather than the neck. The conclusion that has to be drawn is that larvae applied to the flank in these rats faced obstacles irrelevant to the internal pathway and that any difference in the rate of transit to the head

could be attributed to this fact.

It seems reasonable to conclude that the hypothesis of Wilson & Simpson (1981) is correct in that the migration of S. ratti is unlikely to be passive as the differences between the strains cannot be accounted for in this way. Added to this is the marked asynchrony exhibited by both strains of S. ratti compared with that of N. brasiliensis, suggesting that, if the latter is blood borne, the former is unlikely to be so.

The head and lungs as exclusive routes for S. ratti (homogonic) and N. brasiliensis respectively.

It has been shown in this study that larvae of both S. ratti and N. brasiliensis accumulate within their given sites for a considerable period of time. Twohy, (1955a and 1956) argued that N. brasiliensis needs to initiate a moult in the lung before continuing migration to the gut. Mimori et al. (1982) and Nojima et al. (1987) have shown that small changes occur in the form of S. ratti between the skin and the head. Kawanabe et al. (1988) state, without supporting data, that larvae recovered from the skin were unable to infect by oral administration, whereas those from the nasal region could do so if the oesophagus index (OI) was greater than 1. Tanaka et al. (1989) showed that L3's achieved an ability to survive and grow in the small intestine of the rat during their migration through the head, and they conclude, after injecting larvae directly into the cranium, that this development takes place over a number of hours. It therefore appeared that both parasites used the

respective transit sites as a 'nursery' to undergo developmental changes before completing migration, thus the term 'impedance' (Wilson, 1983) can be extended to cover the delay required for this process.

In chapter 6 it was shown that larvae adoptively transferred from the lung or nasal region when they first arrive in those tissues cannot develop in the gut, whereas larvae transferred later have acquired this capability. The relevant processes take over 20h for N. brasiliensis and 7-14h for the homogonic strain of S. ratti, a similar result to that of Tanaka et al. (1989) after injecting larvae directly into the cranium. In each case a morphological marker identified the larvae competent to survive the intestine. They were an OI greater than unity for S. ratti and the onset of ecdysis for N. brasiliensis. The sudden peak in N. brasiliensis extracted from the lung at 35h (see Chapter 4) is probably attributed to the fact that some of the larvae have completed development by this time (chapter 6) and become active in their quest to reach the small intestine. Thus they are more likely to migrate out of the minced tissue into the surrounding fluid.

The requirements of a substantial developmental period have a major significance for testing the general hypothesis that either parasite reaches its goal by more than one route. Larvae migrating by a fundamentally different pathway would require an alternative nursery in which the relevant development could take place. The possibilities are few, assuming the nursery has an open path to the alimentary tract, they are the nasal region, the lungs and the liver. The time

taken to prepare for the enteric environment is substantial for both S. ratti (homogonic) and N. brasiliensis; so impedance in its wider connotation would be sufficient to identify alternative nurseries by sampling at autopsy if the associated pathways were quantitatively significant.

The negative results for the head for N. brasiliensis and the lung for S. ratti (homogonic) can therefore be taken to mean that these sites do not function as nurseries for the species concerned. Without much doubt the pathways of the two species are therefore fundamentally different. The fact that identical methods were used for S. ratti from the head and N. brasiliensis from the lung in numbers sufficient for a rigorous proof in both cases reinforces the conclusion that negative results have real meaning.

It is difficult to assess the negative results of the assays of the liver, because there is no comparable controls for either species. However, the architecture of the liver would not appear to present a barrier to the extraction procedure bearing in mind the diversity of the tissues where the technique is successful. It therefore seems reasonable to conclude that neither species utilizes the liver as a nursery in which mandatory development takes place.

The significance of negative results depends entirely on the time of sampling. These were defined by the overall kinetics determined by the drug pulse technique. The conclusion that no part of the head is involved in the pathway of N. brasiliensis is uncontroversial because, as far as can be discovered, no other workers have

investigated the possibility as the traditional pulmonary route has always been taken as fact (Schwartz & Benjamin, 1934; Taliaferro & Sarles, 1939; Twohy, 1955a, b, 1956; Weinstein & Jones, 1956; Gharib, 1961a,b; Haley, 1961; Solomon, 1966; Clarke, 1967; Love et al., 1974; Croll, 1977; Croll & Ma, 1978). That the lung is not a significant point of transit for S. ratti runs counter to the consensus view, past and present (Dawkins, 1989). A substantial number of reports have recorded larvae of this parasite in rat's lungs (Spindler, 1958; Abadie, 1963; Wertheim & Lengy, 1963; Moqbel & Denham, 1977; Tada et al., 1979; Moqbel, 1980; Murrell, 1980; Bell et al., 1981; Nojima et al., 1987) and the majority of those authors interpreted their findings as denoting a route. Few workers outside this laboratory have questioned these findings, the exceptions being the Japanese workers (full citation on page 10), Murrell (1980, 1981), Bell et al. (1981), Schad (1989) and Schad et al. (1989). Most studies implicating the lung were reported in qualitative terms and none gives a comprehensive quantitative picture of events there. Using a system that differed in many respects from the one used in this study, Murrell (1980) found proportions of 0.011 and 0.017 of a subcutaneously injected dose of 1200 larvae per rat in the lungs of two groups of 6 sampled at 22 and 48h postinfection. In my own case, only one larva out of a total of 767 that were applied to the skin of 39 rats was found in the lungs over four sampling times (23-48h inclusive). The period chosen was that most likely to reveal sites of mandatory development judged by events in the head and gut. Nojima et al. (1987) concluded that the pulmonary route was accidental for their strain of S. ratti because the few larvae they found in the lung had a mean oesophagus index which was less than unity and

unchanged with time. This feature could be an important diagnostic tool for the elucidation of the migratory pathways of other Strongyloides species, if an obligatory developmental period is required before establishing in the gut of the host.

The efficiency of the extraction procedure is an important factor. S. ratti (homogonic) is three fold less synchronised in its migration than N. brasiliensis. The magnitude of asynchrony in the former case is such as to make it certain that, assuming extraction of all individuals present, p_o 's would still not represent the total proportion which passes through a transit site. It would appear that a conservative estimate of the efficiency would be in excess of 50%. Even if it were as low as 50%, the data are categorical in demonstrating that the lung is effectively not a nursery for migrating S. ratti of the homogonic strain in Wistar rats of the Edinburgh colony.

Conclusions

This thesis describes the first two rigorous proofs applicable to the migration pathway of infective juveniles of macroparasites inside their hosts. The proof refers to two strains of Strongyloides ratti and Nippostrongylus brasiliensis in an experimental framework which mimicked the natural situation in all respects but one, namely, the rats were anaesthetised at the time of infection. As far as can be judged from previous work (Wilson et al., 1986), the procedure used to induce anaesthesia introduces no artefacts with regard to migration. Thus it can be claimed with reasonable certainty

that the only site to act as a nursery for the required development of S. ratti migrating from the skin to the intestine is the nasal region of the rat head. Conversely, N. brasiliensis develops only in the lung. S. ratti was also found in small numbers in the cranial region of the rat head. Although the data obtained were not of sufficient magnitude to warrant a proof of involvement in the migration, the overall kinetics and a histological study of the rat head suggests that the cranium-nasal region forms a specific step on a single migratory pathway.

The exact mode of migration from the skin to the respective transit site is uncertain, but it would seem that if N. brasiliensis is blood borne, then S. ratti is unlikely to be, as there is a three fold difference in the asynchrony of the kinetics. Added to this is the fact that subtle differences in the rate of migration of two differing strains of S. ratti cannot be accounted for in a passive migration such as one involving blood circulation.

Therefore it is effectively certain that two skin penetrating nematodes, related in that they are both of rhabditid ancestral stock and having a host, point of entry, and destination inside the host all in common, nevertheless achieve their goal by fundamentally different routes. Within a species, different strains show considerable variation in the rates of the migration, even though the specific pathway appears to be the same. These conclusions uphold the criticism by Wilson (1983) of the inductive ideas that have dominated the subject of migration in hosts since its inception, and which, to a

large extent, have led to the erroneous acceptance of a uniform model to describe all such migrations.

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APPENDIX 1.1

ERADICATION OF SYPHACIA MURIS WITH PIPERAZINE CITRATE UNDER NORMAL ANIMAL ROOM CONDITIONS.

Introduction

The Oxyurid nematodes of the genus Syphacia are common parasites of the caecum and large intestine of laboratory rodents (Hoag, 1961; Weber, 1976; Taffs, 1976; Burgu, Doganay & Yilmaz, 1986; Kang, Kim & Kim, 1987). They are even found in so-called specified pathogen free (SPF) colonies (Owen and Turton, 1979; Matsuzawa, 1986). Although the parasites appear to cause little pathology, Taffs (1975) stresses the need to eliminate them, as in critical experiments the results could be influenced by their presence, especially in nutrition or blood studies, because normal physiological reactions and blood counts are not obtained when such animals are used (Habermann & Williams, 1958). In helminth investigations established natural infections may also interfere with or inhibit an artificial infection, and make a specific immunological study difficult or impossible.

Attempts have been made in the past to rid a colony of rodents of Syphacia sp. by chemotherapy, (Hoag, 1961; Taffs, 1975, Weber, 1977; Weiss & Ernst, 1981; Matsuzawa, 1986) and have been successful in the short term. The simple direct life-cycle, and the persistence of the eggs in the environment, makes eradication difficult. Hoag (1961) found infective pinworm eggs in dust, on equipment and in ventilation units associated with animal rooms. Soulsby (1982) claims that the

only way to be certain of achieving a Syphacia free colony of rodents is to obtain the young by caesarian section, and barrier sustain them. Owen & Turton (1979) successfully eradicated S. obvelata from a SPF colony of mice after caesarian section followed by a treatment period of 3 months with thiabendazole. Matsuzawa (1986) suggests that S. muris can be eliminated from a rat colony by a four week anthelmintic treatment of pyrvinium pamoate in the diet, but the rats again have to be barrier sustained. Weiss & Ernst (1981) claimed that a colony of rats remained clear of S. muris for 18 months after treatment with pyrvinium pamoate in the drinking water.

The classic anthelmintic for the treatment of oxyurids is piperazine and its derivatives. Hoag (1961) suggests a three week regime whereby mice infected with S. obvelata receive piperazine citrate (3g/litre drinking water) for 2 separate periods of one week, with a break of one week between treatments. Soulsby (1982) recommends a single treatment period of 7-10 days with piperazine citrate/adipate (4- 7g/litre of drinking water) substantially to reduce the population of pinworms. The efficiency of piperazine compounds at eliminating immature stages of Syphacia sp. has been questioned by Lynch & Hoegl (1959). Owen and Turton (1979) claim an 8 week treatment period with piperazine citrate (2g/litre of drinking water) failed to eradicate S. obvelata from a mouse colony, even though strict hygiene measures were employed.

Approximately 18 months after commencement of the present project, the cultures of Strongyloides ratti began to fail. This was thought to be associated with either a change in the diet of the rats (see

Appendix 1.2), or the presence of S. muris in the faeces.

Faecal pellets were coated in copious amount of mucus which was attributed to what appeared to be a sudden escalation in the numbers of adults and eggs of S. muris. It was thought that the presence of the mucus was having a detrimental effect on the cultures such that the overall development of S. ratti was impaired. The aim of this study was to develop a system which could be used under normal animal room conditions to eliminate S. muris endemic in the colony of Wistar rats.

Materials and Methods

A 'quarantine' room was thoroughly scrubbed with 1% Tego solution (T. H. Goldschmidt Ltd, Ruislip, Middx) before the admission of rats. Strict hygiene measures were implemented such that personnel and equipment could not move from infected areas into the quarantine room. All cages and tops were soaked in 0.0025% Tego solution at 70°C for 30 minutes before being scrubbed and finally rinsed in cold tap water. Clean sawdust was added to each cage. All cages, tops and food were changed every 7 days. The choice of anthelmintic was dependent on the ease of administration, with the most obvious way being in the drinking water. Weiss & Ernst (1981) had shown that pyrvinium pamoate was suitable, but as the drug is non soluble, it has to be administered in the form of a suspension, which makes administration in the drinking water difficult. Piperazine citrate is very soluble and therefore chosen. It was administered at a rate of 7g/litre of drinking water for one week, then removed for 5 days before re-administration for a further week. The break between treatments was

reduced from 7 days (Hoag, 1961) to 5 days to minimize the possibility of the development of gravid females in this period as the life-cycle of S. muris can be completed in six days (Stahl, 1961).

An assessment of the population of the parasite within the caecum was made by washing the contents into a 90mm diameter bowl with 150ml of 0.9% sodium chloride solution (saline). After vigorous agitation, the suspension was left to sediment for 5 minutes, after which the supernatant was discarded and the process repeated two more times. The final sediment was diluted to 50ml, from which a 10ml sample was taken and examined under the dissecting microscope. The number of worms therein was multiplied by a factor of 5 to get the total worms in the caecum. The diagnosis of an infection without the need for necropsy was based on positive microscopic examination of an adhesive cellophane tape (Sellotape) which was applied to the perianal skin of the rat (van der Gulden, 1967). All examinations of the peri-anal skin were completed at 14.00h to catch the peak in circadian deposition of eggs (Van der Gulden, 1967; Lewis & D'Silva, 1980).

Experiments and Results

(a) A failed attempt.

Twenty male and twenty female 12-week-old rats were paired for mating and placed in clean cages in the quarantine room. One pair was killed immediately and their caeca examined. The numbers of S. muris present were 176 and 103 for the female and male rat respectively. The remaining 19 pairs all proved positive by the

sellotape test. Six weanlings from breeding animals carrying the parasite were killed at this time and their caecae examined. The mean worm burden per rat was 495 ± 106 .

After the second treatment (see Materials and Methods), all 19 pairs of rats provided negative sellotape smears. The first generation pups were weaned 7 weeks after commencement of the treatment. Five randomly chosen pups were killed and their caeca examined. The mean worm burden was 242 ± 33.7 . A t -test on the results of worm burdens of the weanlings from adults carrying the parasite and those from treated parents reveals no significant difference ($t_{[9]} = 2.08$, $p = 0.067$). An examination of a further 20 pups, by the sellotape method, proved positive in each case, as did the 19 breeding pairs.

(b) A successful attempt.

It was assumed that although eggs were not present on the peri-anal skin after the second treatment with the drug some may have been adhering to the fur of the rats and could conceivably initiate a second infection. Alternatively piperazine compounds have been questioned as to their efficiency at eliminating larval stages of Syphacia sp. from the gut of the host (Lynch & Hoegl, 1959; Owen & Turton, 1979). In a second attempt to eradicate the parasite, the treatment period was lengthened on the assumption that the high standard of husbandry in the animal rooms together with the administration of the drug over a longer period would eliminate the possibility of ova remaining in the room, and subject all worms to the drug in their most vulnerable state.

Twenty female and twenty five male 3-week old rats (third generation pups from the treated animals in (a)) were placed in groups of five in breeding cages cleaned as above. The sexes were kept separate. All gave positive sellotape tests. A further 8 rats were killed and their caeca examined. The mean worm burden was 448.1 ± 67.4 . The experimentals received the drug for 7 periods of 7 days, with a 5 day break between each treatment. After 1.5 and 2.5 treatments, 2 of the rats were killed and their caeca examined. All proved negative. After 5 treatments, 18 breeding pairs were set up, with the remaining 5 rats killed and an examination of the caecum of each performed. All gave negative results for S. muris. At the end of the treatment period the adult rats were examined fortnightly by the sellotape method. All breeders were culled after producing 7 litters, at which time eggs were still absent from perianal smears. Twenty randomly chosen pups from a pool of each of the first seven litters also provided negative smear tests. Ten male and ten female rats from the fifth litter were kept to maintain the breeding colony. The first two successive litters from these showed no signs of eggs in peri-anal smears.

Discussion

The production of a colony of rats free from the parasite S. muris was of prime importance because it was thought that the presence of the worms and eggs disrupted the faecal environment in which S. ratti develop. The short term use of piperazine citrate on the breeding colony failed to reduce the population of S. muris. This was probably due to an inefficiency of the drug against larval

stages and the presence of eggs in the environment, or fur of the rat, after treatment was complete.

Owen & Turton (1979), suggest that the treatment period must be of sufficient time to allow all ova in the environment to either pass through a host or lose their viability otherwise, on ceasing with the drug, an infection will reappear. Owen & Turton (1979) applied an 8 week continuous treatment period of ^Piperazine citrate to mice infected with S. obvelata, but failed to eradicate the problem. In this study S. muris was successfully eradicated from a rat colony after 7 treatment periods of 7 days, with a 5 day break between each. The success of this study could possibly be attributed to a 3.5 fold increase in the concentration of the drug over that used by Owen & Turton (1979). These authors were concerned about the toxicity of high levels of the drug over a long period of time. Consequently they reduced the concentrations in their experiments to a minimum. No apparent suffering was observed in the animals in this study. The reproductive capacity of the rats was not impaired as the mean size (+SEM) of the first seven litters after treatment was 9.8 ± 0.31 compared to 9.4 ± 0.39 in rats before the treatment programme. The colony has now been free of the parasite for 12 months and therefore with careful management, it should be possible to maintain this state.

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APPENDIX 1.2

ASSESSMENT OF THE ABILITY OF DIFFERENT RAT DIETS TO SUPPORT THE DEVELOPING STAGES OF S. RATTI (HOMOGENIC).

At a point midway in the study, failure of the experimental system occurred sporadically. This was traced to variation in the Oxoid 41B rat food manufactured by Styles Ltd, Bewdley. Ultimately, supply from this source ceased altogether. The possibility of this sort of failure had been documented early in this laboratory (Zamiridin & Wilson, 1974). It was essential to maintain the high proportion of S. ratti completing migration to the end point, so a series of trials was performed to try and find a suitable replacement diet.

Trial 1.

The first trial was a comparison between the normal Styles 41B and PCD (Special Diet Services, Witham, Essex.). Two rats were fed with each of the diets for one week, before infection by subcutaneous injection with 1000 L3's of S. ratti (homogenic). The infection was found to be patent in both cases by 5 days. Faeces from the infected rats were collected overnight from day 5-6 and cultures set up. After incubation at 19°C for 60h, each culture was watered, and emerging larvae were harvested at 72h, 96h and 120h. Cultures from rats fed PCD food were covered in a mass of fungal growth. The yield of 3rd stage larvae from Styles 41B and PCD was 237662 and 0 at 72h, 80000 and 63000 at 96h and finally 0 and 143000 at 120h. The results indicate that the development of larvae from rats fed the PCD diet is much slower than in the Styles 41B, and that the total number of

larvae recovered was reduced even though the hosts were infected with the same initial dose. The first larvae to emerge were used to infect 2 groups of 10 rats by EDT20 (72h for Styles food, and 96h for PCD food). Experimental animals were kept on the food corresponding to the food of the stock animals from which the infective material was derived. \bar{p}_f 's on day 5 were 0.813 ± 0.022 and 0.718 ± 0.029 for Styles 41B and PCD food respectively. A t -test on the \bar{p}_f 's shows a significant difference between the two populations of larvae ($t_{[18]} = 2.23$, $p = 0.038$).

The slower rate of development in the culture, the reduction in numbers of larvae recovered from the culture, and the reduced capacity of larvae to inhabit the small intestine were indications that the PCD diet was far from ideal as a replacement for Styles 41B.

Trial 2

The second trial was a comparison between Styles 41B and RMI-C (Special Diet Services, Witham, Essex). Two rats were fed on either of the diets for 1 week prior to subcutaneous injection of 1000 L3's of S. ratti (homogonic). Faeces were collected overnight between days 5 and 6 post-infection. Cultures were set up and incubated at 19°C , with water being added at 60h. Faecal cultures from rats fed on RMI-C supported a heavy growth of fungi. Larvae were harvested at 72h from cultures from rats fed on Styles 41B and 96h from RMI-C. Two groups of 6 rats were infected by EDT20 with one of the larval populations. The experimentals were fed with the diet corresponding to the stock from which the infective material was derived. All the

rats were killed on day 5 and the egg-tracks present in the guts counted. The values for \bar{p}_f were 0.865 ± 0.027 (SEM) and 0.884 ± 0.031 (SEM) for Styles 41B and RMI-C respectively. No statistical analysis is required to show that the means are of a similar magnitude.

Fifteen infective L3's from both sources were heat killed and measured with the aid of a camera lucida. The mean size (mm) was 0.608 ± 0.007 and 0.639 ± 0.006 for larvae derived from cultures from rats fed on RMI-C and Styles 41B respectively. A t -test on the results reveals a significant difference, $t_{[28]} = 3.26$, $p = 0.003$.

The significant difference in the length of the infective larvae might lead to the conclusion that the conditions for development were sub-optimal in the faeces from rats fed on RMI-C, but the migratory capacity of the larvae from this medium was not impaired. Wilson (1954) suggested that a reduction in the length of Trichostongylus retortaeformis larvae was observed if they were starved of essential food requirements present in the faecal environment. Competition between nematodes and fungi could lead to a lack of nutritional requirements. Alternatively factors may be lacking in faeces from rats fed on RMI-C or PCD foods, suppressing the growth of the larvae and thus allowing the fungi to become established, whereas in cultures from Styles 41B, the rapid development of larvae suppresses the growth of the fungi.

Trial 3

A second trial was completed on RMI-C. Again 2 rats were fed on Styles 41B for a week, and 2 on RMI-C. Both groups were infected with 1000 L3's subcutaneously. Cultures were set up on day 6 and larvae harvested 72h later from the Styles 41B cultures and at 96h from RMI-C cultures. Fungal growth was intense on faecal cultures from rats fed on RMI-C. Fifteen infective L3's from both populations were heat fixed and measured. The mean body length for the larvae was 0.580 ± 0.004 and 0.581 ± 0.004 for the larvae derived from rats fed on Styles 41B and RMI-C food respectively. No statistical analysis is required to show that the body lengths are equal. Two groups of five rats were infected by EDT20 with one of the populations of larvae. All experimentals were fed the same food as that of the stock from which the infective larvae were derived. The proportion of egg-tracks present in the small intestines was measured on day 5. The \bar{p}_f 's were 0.784 ± 0.025 and 0.540 ± 0.112 for Styles 41B and RMI-C respectively. A t -test on the arcsines shows the migratory success of both groups of larvae not to be significantly different ($t_{[8]} = 2.11$, $p = 0.067$).

Trial 4

The aim of this trial was to make a comparison between PCD and RMI-C as the results obtained in the first three trials are difficult to compare. As controls, two separate batches of Styles 41B (designated A and B) were used. Batch A. was the same food used in Trials 2 and 3. Batch B had not been used. Stock animals were fed the appropriate food for 1 week prior to infection with 1000 L3's. Faecal cultures were

set up on day 5 post-infection, and larvae harvested 72h (Styles 41B batch A and B) or 96h (PCD and RMI-C) later. A significant growth of fungi was observed on the RMI-C and PCD cultures. Each population of larvae was used to infect 6 rats by EDT20, with experimentals being fed the same food as the stock animals from which the infective larvae were derived. All rats were killed on day 5 and the \bar{p}_f 's obtained were 0.809 ± 0.040 (Styles batch A), 0.888 ± 0.032 (Styles batch B), 0.427 ± 0.082 (RMI-C) and 0.907 ± 0.0234 (PCD). Analysis of variance on the arc sines shows the RMI-C data to be an outlier ($F_{[3,20]} = 12.62$, $P = 0.00007$).

These results quite clearly show that PCD was the superior of the two new foods in its ability to support the growth and development of S. ratti (homogonic). It was by no means ideal since development to the infective stage took 1 day longer than in Styles 41B, suggesting that the conditions were sub-optimal for larval stages of S. ratti.

With these results, and the cessation in production of Styles 41B, PCD was adopted as the food for all experimental rats for a period of approximately two months. Unfortunately, experimental results of larvae of the homogonic strain completing migration were depressed below the levels attained with Styles 41B food (see page 87). It was at this point that increased numbers of S. muris in the faeces were noted (see Appendix 1.1). Successful elimination of this particular parasite failed to significantly improve the culture system for S. ratti. In the long term it became increasingly difficult to obtain infective L3's of both strains of S. ratti from culture. Development proceeded to the moulting L2, but a large proportion of

the larvae were unable to cast the old cuticle of this stage and therefore unable to penetrate skin (see page 107). The cultures supported a vast growth of fungus throughout, and development took 96h at 19°C to reach the infective stage.

Trial 5

Three new foods were tested against a small quantity of Styles 41B (batch B). Two of the diets were based on the original Oxiod 41B formula, Pilsbury 41B (Pilsbury Ltd, Birmingham) and Quest 41B (Quest Nutrition, Canterbury, Kent). The final food was a high protein Pilsbury breeding diet. Stock animals and the culturing of larvae was as in Trials 1-4. Fungal growth on all cultures was at a minimum, with infective L3's present at 72h in each case. As a comparison to see the effect of the food on worms establishing in the gut of the host, a batch of 8 rats was infected by EDT20 and fed on the food corresponding to that of the stock rats from which the infective material was derived. A further group were all fed on PCD. All rats were killed on day 5, when the results below were obtained.

Source of larvae	Food of experimentals	\bar{p}_f (+SEM)
Pilsbury 41B	Pilsbury 41B	0.835±0.017
Pilsbury 41B	PCD	0.838±0.014
Styles 41B	Styles 41B	0.779±0.029
Styles 41B	PCD	0.656±0.048
Pilsbury breeder	Pilsbury breeder	0.717±0.093
Pilsbury breeder	PCD	0.813±0.029
Quest 41B	Quest 41B	0.729±0.019
Quest 41B	PCD	0.573±0.068

Analysis of variance of the arc sines of the results shows a significant reduction in the establishment of larvae from cultures from stock animals fed either Styles 41B or Quest 41B when used to infect experimentals feeding on PCD.

With these results in mind Pilsbury 41B was adopted as the food on which all stock and experimentals were fed. It appears from the results using PCD as the food for the experimentals, that the establishment within the small intestine may be impaired although the results are not conclusive. Both stock and experimentals were therefore fed on Pilsburys 41B. Results obtained were indistinguishable from those using 'good' batches of Styles 41B.

The specific effect of the food on the nematodes is not known. The three different 41B type diets along with the Pilsbury breeder failed to support the growth of fungi on the surface of the culture. There appears to be an inverse correlation between the growth of fungus and the rate of development of larvae. The reason for this may be competition between the larvae and the fungi for certain growth factors, resulting in a slower rate of development of the larvae. This however does not explain the reason for vast fungal growth on cultures of RMI-C and PCD food, and not on any of the others tested.

Alternatively it is possible that, under ideal conditions, fungal growth is suppressed by the larvae in the cultures either through the larvae feeding or movement within the faecal pellets. Whatever is missing from cultures of RMI-C and PCD slows the rate of development of the larvae down to such an extent that the fungus is allowed to

become established.

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APPENDIX 2

EVALUATION OF DATA RELEVANT TO THE INEQUALITY, $\bar{p}_o + \bar{p}_f > 1$

(Supervisor's comment)

At its initiation the project described in this thesis was dedicated exclusively to the question of proof by the 'inequality principle' that the head of the rat is part of a functional pathway of the homogonic strain of Strongyloides ratti. The relevant experiments were nos. 2-13 (Tables 4.1, page 54, and 4.4, page 58) inclusive, the results of which constitute a successful demonstration of the method.

Because of the importance of these data to the subject of in-host migration, experiments 8-13 (Table 4.4) were published (Tindall & Wilson, 1988).

Subsequently, experiments with the homogonic S. ratti designed for other purposes were conducted and, in some of them, data were collected which were potentially relevant to the inequality, $\bar{p}_o + \bar{p}_f > 1$ (expts 14-20, Table 4.4, page 58; expts 2-5, Table 5.1, page 89). In the majority of these assays the inequality was in the 'wrong' direction, i.e. $\bar{p}_o + \bar{p}_f < 1$. As recorded elsewhere in this thesis, the relative failures occupied a period in the middle of the project during which unforeseen problems related to the host's diet arose and were, eventually, solved. In presenting his arguments Mr Tindall has (correctly, in my view) followed the history of the investigation and summarised the data for the proof of the case

as it applied to homogonic S. ratti when Tindall & Wilson (1988) was in preparation. The question addressed here is whether a series of results in which $\bar{p}_O + \bar{p}_f < 1$ emerges should modify one's view of those cases where it had already been proven that $\bar{p}_O + \bar{p}_f > 1$. It is argued on the following grounds that no change of view is justified, even if there is no identifiable explanation of the 'failures'.

The application of standard statistical analysis to these data could be misleading as to the purpose in hand and the nature of the null hypothesis involved. Some of the data apparently satisfy the inequality and these are tested by analysis of variance to decide whether to discard the null hypothesis that the excess of the sum of \bar{p}_O and \bar{p}_f over unity is due to chance. When $\bar{p}_O + \bar{p}_f < 1$, no analysis is required: it is obvious by inspection that the null hypothesis holds. But it is important to observe that the null hypothesis is simply a statement about the properties of the data. When the null hypothesis is accepted it leads to no conclusions about the nature of the pathway other than the implication that, in such cases, proof for the particular variant under investigation is lacking. The relevant animals, although they do not provide proof, still supply evidence in support of that variant if p_O 's in an appropriate site are measurable at all. The exercise at this stage is not concerned with the question of the existence of other variants but, simply, with irrefutable identification of one of them. As long as $\bar{p}_O + \bar{p}_f > 1$ can be proved in a significant number of cases, the existence of any number in which the inequality is not satisfied is immaterial. The question resolves into what should be

regarded as a significant number of proven cases. For the present investigation, the evaluation process is exemplified as follows. The pooled data of Table 4.1 (page 54) provide a \bar{p}_O in the heads of 31 rats which was such as to predict that a proportion of the larvae in this site would certainly have migrated on to the intestine if the rats had not been killed. In the same set of experiments, 35 rats had a \bar{p}_f which proved that some, if not all, of the worms in the intestine could only have reached the that destination after passage through the head. Thus these experiments constitute proof that, in 66 animals, the head was part of a functional pathway. By similar argument, a total of 176 young rats used in this project (Tables 4.1 and 4.4) gave irrefutable proof of the involvement of the head in one pathway to the destination taken by homogonic S. ratti: a significant number by any standards.

In mature rats the operational problems inherent in achieving high enough values of p_O and p_f are greater. Consequently, proof is available from only 22 animals in this age group (expt 9, Table 5.2, page 89).

These comments refer to the homogonic S. ratti. Data in this thesis gathered for heterogonic S. ratti and Nippostrongylus brasiliensis were uniform in validating the specific inequality under test.

P.A.G. Wilson, January, 1990.

Criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of *Strongyloides ratti* in the rat

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SUMMARY

The first rigorous proof applicable to the migration pathway of an infective juvenile macroparasite inside its host is presented. Third-stage larvae of a homogonic strain of *Strongyloides ratti* applied in exact doses of less than 20 to the skin of the flank of young rats were recovered 16-40 h later in the naso-frontal part of the head. The peak proportion of the dose (\bar{p}_o) recovered between 20 and 25 h in this site had a mean value of 0.316 ± 0.021 in 48 animals. In 40 other rats infected simultaneously the mean proportion of the dose (\bar{p}_f) that reached the small intestine was at least 0.837 ± 0.013 . Proof resides in verification of the inequality $\bar{p}_o + \bar{p}_f > 1$. With appropriate statistical tests the excess of the sum of the means of these two proportions over unity is shown to have a probability of occurring by chance of 1 in 3.5×10^6 . Thus it is effectively certain that the naso-frontal portion of the head is part of at least one pathway taken by this parasite on its way from the skin to the intestine of its host. By suitable protection of the infection site it was confirmed that migration to the head was achieved by an internal route and not as a result of grooming. Larvae were recovered from the cranium in the same rats over the period 15-40 h, but the peak proportion of the dose occurred at 20 h, and $\bar{p}_o + \bar{p}_f < 1$ in this location. Whether the cranium is also part of the pathway is therefore still undecided. The significance of this novel analysis in the general context of in-host migration of infective stages is discussed and it is concluded, following its application to data sets from other authors, that the only cases in which proof can be demonstrated are the anterior skull of the rat for *S. ratti* (present data) and the lung of the same host for *Nippostrongylus brasiliensis* (Twohy, 1956).

INTRODUCTION

Since its inception, the study of migration pathways of the juvenile stages of macroparasites inside hosts has lacked a rigorous framework of proof. The experiments of Fülleborn (1914) and Fülleborn & Schilling-Torgau (1911) with skin-penetrating roundworms were appropriate in conception in one context, but their results fell short of a rigorous proof on procedural grounds (Wilson, 1983). Apart from these particular experiments, attempts to define such pathways have been heavily dependent on the procedure termed 'sampling at autopsy' by Wilson (1983). This procedure, in its most precise execution, conforms to the following general protocol: (1) infect N hosts with a known dose of parasites; (2) kill samples of n animals at times $(0+t)$, $(0+2t)$, $(0+3t)$, ... ($n = a$ factor of N); (3) estimate the parasite content of selected tissues thought to be involved in the pathway; (4) conclude from the sites of parasite occupancy and the sequence of appearance and disappearance in those sites that the pathway is such-and-such.

The major assumption underlying this procedure is that parasites are subjected to no impedence in the natural course of their migration. While impedence is possible, of course, it is not what one would expect to evolve from the likely selection pressures on

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parasites of this type. In fact, unless there is good evidence to the contrary, it would seem logical to conclude that parasites which accumulate in a given locus are individuals which are 'lost' or sequestered following host-inflicted damage. In other words, the true pathway may be precisely through tissues where few parasites, or none are found by sampling at autopsy. A supposed corollary to the main assumption, i.e. that sites containing many parasites are quantitatively more important in terms of a pathway than those with few, is also fallacious since it implies not only that impedence is likely in the natural pathway but also that it has a constant value in different tissues.

In order to prove the case, therefore, criteria in addition to the mere occurrence of parasites in a given tissue must be applied. In this paper we describe experiments in which the proposals of Wilson (1983) are given expression. He argued that data from sampling at autopsy would have meaning in terms of a pathway only if they satisfied the inequality $p_o + p_f > 1$, where p_o = the peak proportion of a dose of parasites found in an organ ('O') which is a putative transit site, and p_f = the peak proportion of the dose reaching the destination. This relationship is examined experimentally in the context of the role of the anterior region of the skull as a putative transit site in the migration of juvenile *Strongyloides ratti* from the skin to the small intestine of the rat (Tada, Mimori & Nakai, 1979).

MATERIALS AND METHODS

The homogonic strain of *S. ratti* used in the experiments reported here is the one that has figured in previous publications from this laboratory. It was cultured in the manner already described by Wilson & Simpson (1981). The exact dose technique ('EDT') for experimental infections by skin application (Wilson, Simpson & Seaton, 1986) was used with refinements dictated by the goals in view. Paramount among these was the need to obtain as high a 'take' (i.e. p_f) as possible to test the inequality, $p_o + p_f > 1$. An additional goal of importance was to reduce the error variances to the absolute minimum. To this end the original technique (now termed 'EDT100'), in which the starting point was 100 hand-counted larvae/rat, was changed to a dose of 20 ('EDT20'). This had the advantage that larvae could be dispensed more rapidly and their number confirmed more accurately before application. Following application to the clipped and dampened skin of the anaesthetized rat, residual larvae in syringes and glassware were counted as in the earlier method. In our revised procedure larvae that had not penetrated the skin after 45 min were recovered by removing the 0.2 ml of water which had contained the dose, followed by several washes of the infection site with water. Larvae in the washings were allowed to sediment and were then counted. The timing of this last part of the method was based on evidence given under 'Experiments and Results'. The unique 'exact dose' given to a rat was determined as $20 - (R + S)$, where R is the number of larvae recovered from the tuberculin syringe and solid watch-glass which originally contained the dose, and S represents those in washings from the infection site.

The experimental animals were 3-week-old male and female Wistars from an outbred colony maintained in this laboratory, the sexes being evenly distributed among treatments. Young rats were chosen to minimize the effects of the host's innate immunity. They were anaesthetized by intraperitoneal injection of pentobarbitone sodium ('Sagatal', May & Baker) diluted 1 in 10 in 0.9% sodium chloride. The amount administered was 31 $\mu\text{g/g}$ body weight, which is sufficient to immobilize them for 2.5 h. Larvae were applied with a 1 ml tuberculin syringe to the clipped and dampened patch

on the left flank of each rat 1 h after the initiation of anaesthesia. Animals were undisturbed after application, but were watched continuously for 45 min, i.e. until loose larvae were washed from the skin. Individuals whose doses 'rolled' off within 30 min of application were removed from the experiment (see results of Exp. 1).

The infection site was left uncovered in most cases but in Exp. 7, after the removal of non-penetrant larvae, a bandage was placed around the hindquarters of some of the animals in order to prevent outside interference. Immediately overlying the site was a 10 × 10 mm piece of Whatman No. 1 filter paper which was held in place by a 25 × 25 mm square of adhesive waterproof strapping (T. J. Smith & Nephew Ltd). This in turn was secured by a 250 mm strip of surgical tape (Micropore, 3M Co.) cut from a roll 25 mm wide. The strip was shaped like a watch and watch strap, with the 25 mm square 'watch' placed over the waterproof layer and the 'strap', 10 mm wide, wound approximately two-and-a-half times round the body of the rat. For security, a furrow was clipped in the fur to receive the surgical tape, except in the immediate vicinity of the infection site, where a 5 mm fringe of fur was retained. The bandages were left in place until the animals were killed or for 23 h, whichever was the shorter.

A modified form of the intestinal squash technique for adult worms (Zamirdin & Wilson, 1974) was used to obtain p_f at day 8. Parasitic *S. ratti*, as in other *Strongyloides* spp., are parthenogenetic females lacking male equivalents. They deposit packets of eggs in distinct 'tracks' which, when few in number, are easily seen and counted in intestinal squashes under the low power of a stereo-microscope. Moreover, in some cases more tracks are seen than worms. Using EDT20 we have found that 6.9% of tracks are wormless in guts which were frozen and then thawed in the manner adopted by Zamirdin & Wilson (1974). In contrast, if intestines are assayed fresh, the wormless tracks are reduced to 2.4%. This led us to the possibility that such tracks are related to preceudural phenomena and not to the laying of more than one track per worm over the relevant developmental period. The supposition was tested by infecting 87 rats by skin application with a single larva. Of these, 31 animals had neither tracks nor worms on day 8, 55 had a single track and a single worm and the remaining rat had one track and apparently no worm. We considered this to be sufficient evidence to allow the equivalence of tracks and worms. Consequently, all p_f 's reported here are based on egg tracks counted in fresh intestines and expressed as a proportion of the EDT20 dose. In 152 such estimates 7 p_f 's of 1.00 have been observed, but the number of tracks has never exceeded the exact dose applied. We see this as confirmation of the validity of our assumption.

Sampling for p_o was done in 2 regions of the head by methods modified from Tada *et al.* (1979) and Murrell (1980). After killing, the head was separated from the neck, and the skin, mandible, muscles and eyes removed. The roof of the cranium was cut away with fine scissors, the brain gently removed and teased open to reveal the ventricles. The cranial cavity and brain were washed with 50 ml of warm 0.9% sodium chloride solution. The resulting suspension was placed in a 120 ml centrifuge tube in a water bath at 37 °C and left to settle for 30 min. The supernatant fluid was aspirated to a final volume of 5 ml, the sediment resuspended and divided equally among 3 solid watch-glasses for counting of larvae under the medium power of a stereo-microscope. The naso-frontal region is wedge shaped (Fig. 1) and was separated from the rest of the head so that it contained the nasal cavity and the major portion of the maxilla. This was chopped coarsely and mixed with 50 ml of Hanks balanced salt solution (HBSS, Gibco) in a 120 ml tube which was incubated at 37 °C for 4 h. After incubation the liquid was aspirated to leave 15 ml of tissue suspension which was poured through a domestic wire

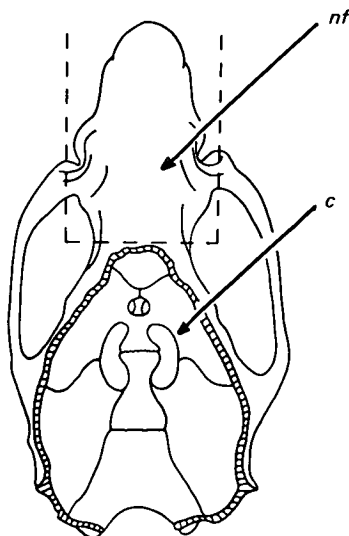


Fig. 1. Parts of the head sampled for larvae: *nf*, naso-frontal region; *c*, cranial cavity exposed and the brain removed.

tea-strainer. The material retained in the strainer was washed with 40 ml of 0.9% sodium chloride, these washings being added to the original filtrate. The combined suspension was sedimented for 30 min at 37 °C. After aspiration to 5 ml the larvae were counted as for the cranium.

In preliminary assays the tissue of the brain was macerated and incubated in Hanks solution in the same way as was applied to the naso-frontal region. Larvae were consistently absent from these preparations, so the procedure was abandoned for the definitive study.

The raw data obtained in these experiments were proportions. They were analysed by parametric statistical methods after arcsine transformation. In most cases the techniques were standard, but assays of p_o in the head had a marginally greater variance than those of p_f in the intestine. This difference became significant when large numbers of observations were pooled. In this event an appropriately modified *t* test was employed (Milton & Tsokos, 1983).

EXPERIMENTS AND RESULTS

Time taken for 3rd-stage larvae to penetrate the skin

A planned refinement of the EDT procedure to maximize p_f values was to remove larvae that were incapable of penetration from the infection site, and count them. In addition, anaesthetized rats which had received doses occasionally moved enough to dislodge the drop of water containing the larvae. Experiment 1 was done to identify the best time to wash skin for the recovery of non-penetrators and the minimum time the dose had to be in place for p_f 's to be maximal.

Twenty-five rats were infected by skin application. In groups of 5 their doses were washed from the site of infection after 5, 10, 20, 30 or 45 min, and larvae from the washings were recovered after sedimentation. All rats were killed and autopsied 8 days later.

Results are recorded in Fig. 2. They show that the proportion of the dose recovered

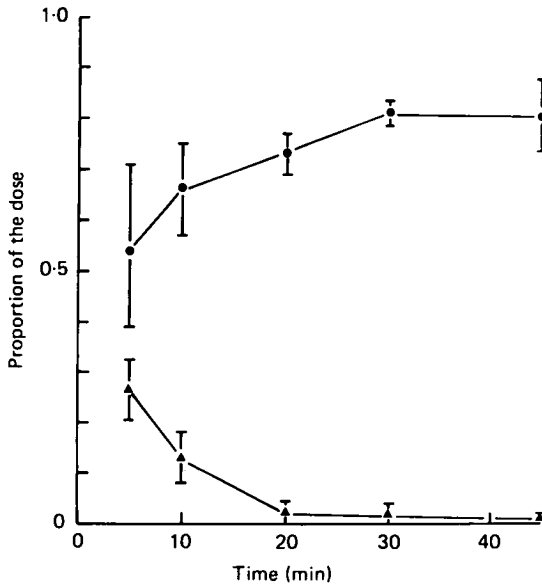


Fig. 2. Time taken for L3 of *Strongyloides ratti* to invade the skin. (▲) Mean proportions of the inoculum (ordinates) recovered in washings from the infection site at the times after application represented as abscissae. (●) Mean proportions of the inoculum which developed to adults at day 8 following each treatment. Bars define approximate 95% confidence limits (data untransformed): 5 rats/treatment. The number of larvae in the inoculum for each rat was $20 - R$, where R = residual larvae in syringe and watch-glass.

from the skin decreased rapidly in the first 20 min and reached its minimum by 30 min. The curve for the proportion of the dose (in this case, $20 - R$, not $20 - (R + S)$ - see Materials and Methods) which became adult in the intestine was a mirror image of that for non-penetrators. The two curves agree in demonstrating that maximal development is achieved if doses are left undisturbed for 30 min. In our definitive experiments, therefore, we discarded animals whose doses 'rolled off' within this period; and we started the process of larval recovery from the skin at 45 min. In all the data which follow the exact dose is given by $20 - (R + S)$, where 20 is the initial handcounted inoculum, R = residual larvae in the watch-glass and syringe, and S = larvae recovered from the skin 45 min after application.

Sampling for p_o and p_f

Experiments 2-6 were a series conducted chronologically in numerical order essentially to locate p_o in time in the two regions of the head, and to obtain a precise estimate of p_f . Each successive protocol was designed to answer questions raised by the evidence accumulated from its predecessors. In consequence, the assays in the head became more restricted in time as the sequence evolved. The general pattern of the experiments was uniform and is exemplified in the following description of Exp. 2.

Twenty-eight rats were infected by EDT20 (mean exact dose = 19.2) and 23 of them were used to sample the cranial and naso-frontal regions of the head (Fig. 1) at 20 h (3 rats), 25 h (4 rats), 30 h (4 rats), 35 h (5 rats) and 40 h (5 rats) by the technique described in the Materials and Methods section. The 5 remaining animals were killed and autopsied for egg-tracks in the intestine on day 8. This protocol, and those for Exps 3-6, are recorded in Table 1.

In the interests of clarity, the results are presented in two tables. In Table 2 are the

Table 1. *Experimental protocols*

Exp.	Mean exact dose (EDT20)	No. of rats in which the head was sampled at each of the following times (h) post-infection									The no. of rats in which egg-tracks were counted in the intestine on day 8 post-infection
		15	16	18	20	23	25	30	35	40	
2	19.2	—	—	—	5	—	4	4	5	5	5
3	18.1	10	—	—	9	—	10	—	—	—	5
4	18.6	—	8	8	8	8	—	—	—	8	6
5	18.3	—	—	7	7	—	6	—	—	—	6
6	19.4	—	—	—	8	8	—	—	—	—	6

Table 2. *Larvae in the naso-frontal region and egg-tracks in the intestine*

Exp.	Mean proportion of the dose (\pm s.e.m.) recovered from the naso-frontal region at the following times (h) post-infection									Egg-track as mean proportion of the dose (\pm s.e.m.)
	15	16	18	20	23	25	30	35	40	
2	—	—	—	0.395 \pm 0.076	—	0.164 \pm 0.016	0.256 \pm 0.053	0.233 \pm 0.038	0.207 \pm 0.029	0.818 \pm 0.027
3	Nil	—	—	0.236 \pm 0.030	—	0.184 \pm 0.049	—	—	—	0.811 \pm 0.017
4	—	0.050 \pm 0.013	* *	* *	0.359 \pm 0.048	—	—	—	0.203 \pm 0.045	0.785 \pm 0.041
5	—	—	0.052 \pm 0.016	0.148 \pm 0.029	—	0.308 \pm 0.109	—	—	—	0.800 \pm 0.056
6	—	—	—	0.142 \pm 0.025	0.395 \pm 0.040	—	—	—	—	0.902 \pm 0.023

* Larvae died during incubation.

mean proportions of the dose found as larvae in the naso-frontal region in the experiments, together with their respective \bar{p}_f 's. A general survey of these data places p_o in this part of the head somewhere within the range 20–25 h, with the most probable time of those sampled of 23 h. In the 5 experiments, irrespective of differences in time of sampling, the first criterion of proof is satisfied arithmetically in that $\bar{p}_o + \bar{p}_f > 1$ in all cases (but see the analysis which follows and the Discussion).

Proportions of the dose recovered as larvae in the cranium in Exps 2–6 are recorded in Table 3. Overall, p_o appears to occur at approximately 20 h in this location. The inequality $\bar{p}_o + \bar{p}_f > 1$ is not met in respect of the cranium in any of the experiments (compare with \bar{p}_f 's in Table 2). Judgement must be reserved therefore as to whether this region of the head is a component of a pathway to the intestine (see Discussion).

Internal migration or direct transfer of larvae to the head?

When rats regained consciousness from the EDT20 procedure they immediately licked the area of infection. It was important, therefore, to decide whether larvae reached the head by superficial transfer during grooming, or by internal migration. In Exp. 7 infections initiated in the same way as before in 18 control rats were compared

Table 3. *Larvae in the cranium*

Mean proportion of the dose (\pm S.E.M.) recovered from the cranium at the following times (h) post-infection

Exp.	15	16	18	20	23	25	30	35	40
2	—	—	—	0.135 ± 0.031	—	0.036 ± 0.023	0.038 ± 0.013	Nil	0.042 ± 0.019
3	0.031 ± 0.018	—	—	0.150 ± 0.038	—	0.013 ± 0.013	—	—	—
4	—	0.019 ± 0.009	0.086 ± 0.023	0.095 ± 0.018	0.057 ± 0.017	—	—	—	Nil
5	—	—	*	0.176 ± 0.114	—	0.052 ± 0.011	—	—	—
6	—	—	—	0.096 ± 0.015	0.085 ± 0.020	—	—	—	—

* Larvae died during incubation.

Table 4. *Effect of bandaging the site of infection (Exp. 7)*

Mean proportion of the dose (\pm S.E.M.) recovered as larvae or egg tracks in the following sites (6 rats/group; mean exact dose = 19.1)

Sampling time	Cranium		Naso-frontal region		Intestine	
	With bandage	Without bandage	With bandage	Without bandage	With bandage	Without bandage
20 h	0.127 ± 0.038	0.067 ± 0.017	0.175 ± 0.031	0.206 ± 0.004	—	—
23 h	0.093 ± 0.035	0.034 ± 0.022	0.299 ± 0.028	0.234 ± 0.048	—	—
8 days	—	—	—	—	0.876 ± 0.018	0.864 ± 0.023

directly with those in another 18 which were bandaged after infection and before they were awake (see Materials and Methods). Among each group, 6 animals were killed at 20 h and 6 at 23 h to estimate p_o in the compartments of the head. The remaining 6 animals of each type were killed on day 8 for measurement of p_f . Bandages were removed from this last group at 23 h.

From the hypothesis under test in Exp. 7, one would expect fewer parasites, or none at all, in the head of bandaged animals if significant numbers of larvae reached that position in Exps 2–6 as a result of grooming. No detailed analysis is needed to see that the results falsify that hypothesis, for the mean parasite content of the head in bandaged subjects is arithmetically greater in 3 of the 4 comparisons depicted in Table 4. Moreover, \bar{p}_f for bandaged animals is also greater, arithmetically. Thus larvae must reach the head by internal migration and not by external transfer.

Leaving the short-term purpose of Exp. 7 on one side, results from it can be assembled in the general case we are attempting to prove. The peak parasite content of the naso-frontal region is identified at 23 h, as in Exps 4 and 6. A sample t value

Table 5. *Testing the inequality $\bar{p}_o + \bar{p}_f > 1$*

Exp.	Time of estimate of p_o in naso-frontal region (h)	$\bar{p}_n^* = 1 - \bar{p}_o$	\bar{p}_f^*	$\bar{p}_f^* - \bar{p}_n = p_c$	Probability that p_c is due to chance (D.F.)
2	20	0.605 ±0.076	0.818 ±0.027	0.213	0.0002 (8)
3	20	0.764 ±0.030	0.811 ±0.017	0.047	0.358 N.S. (12)
4	23	0.641 ±0.048	0.785 ±0.041	0.144	0.0511 (12)
5	25	0.692 ±0.109	0.800 ±0.056	0.108	0.367 N.S. (10)
6	23	0.605 ±0.040	0.902 ±0.023	0.297	0.00004 (12)
7†	23	0.734 ±0.028	0.870 ±0.014	0.136	0.0085 (22)
All data pooled	—	0.684 ±0.021	0.837 ±0.013	0.153	0.0000003 (83)

* These relationships are derived and summarized in expressions (1)–(5) in the Discussion.

† Data from both treatments combined (see Table 4).

calculated for the difference between the mean arcsines of p_o for bandaged and non-bandaged animals confirms that these data are homogeneous ($P = 0.284$). The same applies to the accompanying \bar{p}_f 's ($P = 0.713$). Thus they have been combined as a single sample for each parameter in the comprehensive analysis presented below.

Testing the inequality $\bar{p}_o + \bar{p}_f > 1$ with reference to the anterior skull

If $p_n = 1 - p_o$, the relationship we have to prove can be rewritten as $p_f > p_n$ and, in terms of statistical tests of experimental data, $\bar{p}_f > \bar{p}_n$ (see Discussion). In Table 5, p_o 's for the naso-frontal region from Exps 2–7 have been coded in terms of \bar{p}_n and listed irrespective of their time of sampling. Column 5 of this table records the quantity, p_c , i.e. the excess of \bar{p}_f over \bar{p}_n . The significance of the difference between the means of the arcsines of p_n and p_f was tested within each experiment by calculating the probability of the associated t value. These probabilities appear in column 6. They show that the inequality was clearly significant in Exps 2, 6 and 7 ($P = 0.0002$, 0.00004 and 0.0085 respectively) and marginally so in Exp. 4 ($P = 0.0511$). It was not significant in Exps 3 and 5.

That the significance of the inequality was such as to prove the case in 4 experiments out of the 6 might be considered an adequate outcome for our purpose. The properties of the data, however, allow us to make a more emphatic statement than that. One-way analysis of variance of the arcsines of p_n for all experiments shows that the means could have been drawn from the same population ($F_{[5,42]} = 1.62$, $P = 0.176$). A similar analysis leads to the same conclusion with reference to p_f ($F_{[5,34]} = 1.66$, $P = 0.171$). It is quite in order, therefore, to pool the data within treatments from these experiments

and to test the significance of the difference between the grand means of the relevant arcsines with a single comprehensive t value. One qualification was needed in this connection. The standard computation of t would not have been appropriate because, with such large values of n , the difference between the variances of the sampling processes for p_o and p_f became significant. This emerged from a variance ratio test carried out on the error variances calculated in the one-way analyses referred to above ($F_{[42,34]} = 2.10$, $P = 0.014$). Thus the method for comparison of means drawn from populations with different variances recommended by Milton & Tsokos (1983) was used. It gave a $t_{[83]}$ -value of 5.59, with a probability of 2.83×10^{-7} .

DISCUSSION

Derivation of the inequality: $p_o + p_f > 1$

Assuming for the moment that organ 'O' (see Introduction) is truly a component of the natural pathway, p_o is unlikely to represent the total proportion (p_T) of the input which passes through 'O' even if our experimental procedure detects every parasite present at the peak time; but sampling at autopsy provides no direct measure of p_T and, therefore, we have to accept that, *for the purposes of proof*, p_o must be taken as our estimate of p_T .

With regard to p_f , different parasites have different features which present problems in its experimental determination. In the case of *Strongyloides ratti*, direct detection and/or extraction from a rat's intestine of newly arrived larvae is subject to errors which give seriously biased estimates. When the input is less than 100 worms, a better measure of p_f in non-immune hosts is obtained by counting adult *S. ratti* at day 8 (Wilson *et al.* 1982) (now translated into egg tracks - see Materials and Methods section). Irrespective of the exact method of estimation it is crucial for this form of proof that the experimental system is designed to yield the highest possible value for p_f . Taking concrete examples, if $p_f = 1$ (i.e. all parasites introduced reach their destination), then any larvae found in sites in the body other than the destination must be on course *for* the destination. When $p_o = 0.4$ and $p_f \leq 0.6$, it is impossible to decide whether parasites in 'O' are on or off their proper course; but with the same p_o and a p_f of 0.65 we can say that, *for certain*, 0.05 of the dose passed through 'O' on their way to the destination. That being so, it is highly probable, even if not certain, that all parasites in 'O' were *en route* to the endpoint.

Thus the proof requires that

$$p_o + p_f > 1, \quad (1)$$

which can be rewritten as:

$$p_f > 1 - p_o. \quad (2)$$

If the relationship is established, then a proportion, p_c , of the dose can be said with certainty to have passed through organ O on their way to the endpoint where

$$p_c = (p_o + p_f) - 1. \quad (3)$$

The quantity $(1 - p_o)$ is important in the handling of real data and is represented by the symbol p_n . The case is proved when,

$$p_f > p_n. \quad (4)$$

In addition, from equation (3);

$$p_c = p_f - p_n. \quad (5)$$

It is obviously impossible by sampling at autopsy to obtain p_o and p_f in one and the

same host. Consequently, experimental protocols must have allocated within them groups of animals relevant either to p_o or to p_f . In this sort of design it is necessary to show not only that the numerical relationship is as defined by inequality (4), but also that mean values have a sufficiently small variance to render it statistically significant.

Migration of 3rd-stage juveniles of Strongyloides ratti

Since Abe (1964) first reported the occurrence of larvae of *S. ratti* in the head of the rat there have been corroborating records from associated Japanese workers over the years and, latterly, by Murrell (1980) and Bell, Adams & Gerb (1981) in the USA (full documentation in Mimori, Korenaga, Chowdhury & Tada, 1982; Wilson, 1983 and Wilson *et al.* 1986). These observations have been largely ignored in the wider parasitological literature (see, for example, Schmidt & Roberts, 1985) despite the fact that the evidence presented has been at least as good as that available for the commonly held belief that the route of migration involves the lung (Abadie, 1963; Wertheim & Lengy, 1965; Moqbel, 1980; Genta & Ward, 1980). Notwithstanding the last statement, it has to be said that none of the sets of data offered till now meets the standard of proof we require. In all cases where quantitative estimates have been attempted the inequality has been in the 'wrong' direction, i.e. $p_f < p_n$. On past evidence, therefore, larvae in the head or the lung could just as well have been off as on their proper course.

Our data in Table 5 are arithmetically in agreement with a proof that the naso-frontal region of the head is truly a component of the pathway since $\bar{p}_f > \bar{p}_n$ in every experiment, despite the fact that we were simultaneously trying to locate p_o in time as well as prove the inequality. In 3 of the 6 experiments the inequality is statistically significant, and in one of the remainder the result only just falls short of the standard criterion (i.e. a probability of 1 in 19.6 versus 1 in 20). The dominant feature of the data, however, is their homogeneity within treatments. This permits the synthesis set down in the bottom row of Table 5, which confirms the case without any doubt whatever (i.e. the probability that the overall excess of \bar{p}_f over \bar{p}_n is due to chance is 1 in 3.5×10^6). We can say with certainty, therefore, that a proportion of the dose, p_o , with a mean value of 0.153, passed through the naso-frontal region of the skull on their way to the intestine. There is a high probability, if not certainty, that all those larvae in the same site at the same time were similarly on course. Thus, without much doubt, a proportion of at least 0.316 (\bar{p}_o) of the larvae reached their destination after passage through the naso-frontal region of the head of the rat in these experiments.

Inspection of the contents of Tables 2 and 3 reveals that we cannot be sure of the exact timing of p_o either in the cranium or in the anterior skull. Analysis of variance of the data for 20 h in the naso-frontal region (Table 2) highlights this point, for, in this case, the mean values for Exps 2 and 3 are significantly higher than those for 5 and 6. The precise time of the peaks within 3 h or so is thus sensitive to variables which we failed to control in our experimental design. This in no way diminishes the main import of our results; it merely affects the detail of the relationship, if any, between parasites in the two parts of the head. Since $\bar{p}_f < \bar{p}_n$ in the cranium we have to reserve judgement and admit, strictly, that larvae there could be among those lost *en route*. Nevertheless, there are hints in the results taken as a whole (Tables 2, 3 and 4) that the peak in the cranium is earlier than that in the anterior region. This is consistent with the theory that the two sites form successive steps in a true pathway.

With regard to other aspects of the problem, we have no further comment as yet on how larvae travel from the skin to the head, except to confirm from Exp. 7 (Table 4)

Table 6. *Twohy's (1956) data for Nippostrongylus brasiliensis recalculated*

(For interpretation of symbols see Table 5 and text.)

Lung				Intestine			
Source (mean dose)	Time (h)	p_o	p_n	Source (mean dose)	Time	p_f	
Table 1 (1040)	15	0.50	0.50	Table 2 (1170)	59 h	0.60	
	15	0.33	0.67		68 h	0.55	
	15	0.45	0.55		75 h	0.83	
	16	0.24	0.76		95 h	0.93	
	18	0.44	0.56				
	19	0.34	0.66				
	19.5	0.40	0.60				
Table 2 (1070)	26	0.69	0.31	Table 4	8 days	0.79	
	32	0.42	0.58	-[adults]-	8 days	0.90	
	37	0.72	0.28	(270)	8 days	0.86	
Mean		0.453	0.547			0.780	
S.D.		0.152	0.152			0.148	

that the route is internal and not superficial (see Tada *et al.* 1979; Wilson *et al.* 1986; Wilson, 1987). Nor can one say whether the pathway involving the head is exclusive or merely one among a number of competing alternatives. Research into these aspects continues in this laboratory.

Application of the analysis to data reported by others

In this paper we have developed a form of analysis first suggested in outline by Wilson (1983). He noted that Twohy's data (1956) referring to *Nippostrongylus brasiliensis* in the rat offered a *prima facie* and hitherto undetected proof of part of a migration pathway, (in this case, the lung of the host). Wilson could find no other unequivocal data in support of the supposedly factual descriptions of pathfinding by skin-penetrating roundworms in the expert literature. In a more comprehensive search, we have been unable to find evidence apart from that already cited that satisfies the criteria for proof relevant to any invasive helminth in its natural host. Among unnatural host/parasite combinations, the data of Mangold & Dean (1982) seem to stand alone since, when analysed by our methods, they can be shown to prove that the lung is part of the pathway of schistosomula of *Schistosoma mansoni* from the skin to the mesenteric veins of the mouse.

Twohy (1956) wrote his report without concern for a proof of the type we are considering. Consequently, his tabulated numbers require interpretation and recalculation to reveal their relevance to the current debate. The results of our reappraisal are presented in Table 6. The major statistical problem is that Twohy's assays were not replicated sufficiently to give a precise measure of the associated variances. As a conservative approximation we have treated his numbers (recalculated to give proportion of the dose) reported at successive times as replicates (see Table 6). A *t* value of 3.28 (D.F. = 15) emerges for the difference between the means of the arcsines of p_f and p_n , having a probability of occurring by chance of less than 1 in 100 times. From this it can

be deduced that p_c , the mean proportion of the dose that certainly passed through the lungs of the rats on their way to the intestine in Twohy's experiments, was 0.23.

One proviso must be entered with reference to Twohy's results, in that the bulk of his assays (and those from which our conclusions are drawn) were obtained following subcutaneous injection of the larvae. It is therefore still necessary to prove that the unnatural means of administration produced no abnormalities in migration. This is the more imperative since Twohy's limited assays with skin application were accompanied by a large variance and a lower recovery of parasites from his rats compared with injection (14 versus 61%).

Conclusions

In this paper we describe the first rigorous proof applicable to the migration pathway of infective juveniles of a macroparasite inside the host. The proof refers to a homogenous strain of *S. ratti* in an experimental framework which mimicked the natural situation in all respects except one, namely, that the rats were anaesthetized at the time of infection. As far as can be judged from previous work in this laboratory, the procedure we used to induce anaesthesia introduces no artefacts with regard to migration (Wilson *et al.* 1986). Thus we claim to have demonstrated with reasonable certainty that the naso-frontal region of the skull is one of the stations this parasite passes through on its way to the intestine.

In addition, from our own analysis of Twohy's (1956) data, we offer proof that larvae of *N. brasiliensis* injected subcutaneously into rats travel to the intestine by way of the lung.

As far as we know, these are the only proven cases of their kind. They establish the existence of impedance in two pathways, but that does not mean that related hypotheses can be given the status of fact without further evidence (see Introduction).

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A technique for skin application of
exact doses of Nippostrongylus
brasiliensis to rats.

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SUMMARY

Conventional methods for percutaneous infection of rats using third stage juveniles of Nippostrongylus brasiliensis which have been artificially stimulated to exsheath lead to highly variable, and relatively poor, establishment in the intestine. A new system has been developed in which larvae applied to the skin still remain partially sheathed, as they would be in nature. Cultures of the freeliving stages of the parasite contain an annulus of clear, colourless polythene film to which some of the third stage juveniles attach. Rats are infected with an individually counted, exact dose applied to the skin on polythene. Using this technique ('EDT20Nb'), consistently high values for the mean proportion of the dose that becomes established (\bar{p}_f) have been obtained, along with a variance well below the normally accepted level ($\bar{p}_f = 0.784 \pm 0.015$ from a total of 73 rats in 12 separate assays). In particular, the added component of variance between assays in this study was insignificant, so that the probability of quantitative agreement in replicate experiments based on the method is high. It is recommended for an experimental design in which small numbers of parasites can be used.

INTRODUCTION

Work in this laboratory is primarily concerned with obtaining proofs for the migratory pathways of skin penetrating nematodes. In order to fulfil the necessary criteria, a system has to be developed in which almost all the parasites administered complete their migration inside the host (Wilson, 1983). The nature of the proof we have invented also requires minimal levels of experimental error. The method for Nippostrongylus brasiliensis described in this paper was developed from long experience in this laboratory with Strongyloides ratti, also a skin penetrating parasite of the rat. In the latter case the use of small, hand-counted, exact doses of third stage juveniles in the 'EDT' procedure has produced significant benefits (Wilson, Cameron & Scott, 1978; Wilson, Simpson & Seaton, 1986). In its most refined form to date ('EDT20') it has allowed us to establish for the first time a rigorous proof of part of a pathway, namely, the involvement of the nasofrontal region of the head in the migration of third stage S. ratti from the skin to the intestine of the host (Tindall & Wilson, 1988). Unlike the infective third-stage juveniles of S. ratti, those of N. brasiliensis in the natural state remain partially sheathed in the second stage cuticle until they make contact with a passing rat. In most cases experimental infections of N. brasiliensis are initiated unnaturally, in that the juveniles used are artificially stimulated to exsheath before they are counted and applied to the skin or injected subcutaneously. Until the present study, most authors have concentrated on subcutaneous injection. Indeed, in some cases this can result in a large proportion of the dose becoming established. Haley (1962b) stated that an average of 72% of the inocula were recovered as adults in rats on the 10th day of infection in one of his studies. Ogilvie (1965) obtained a take of 61% on day 11. Twohy (1956) found that 78% of the dose became established from subcutaneous injection, whilst 73% of those applied to the skin reached the gut if they were allowed a penetration time of 4 hours. However, when examining tissues for the presence

of migrating stages, Twohy (1956) found many fewer following skin application (14 versus 61%). In general, subcutaneous injection produces lower takes than those quoted above. In the most comprehensive collation of data from a single laboratory, Haley (1962a) recorded a mean of 55% in 219 rats over a dose range of 50 - 3000 third stage juveniles. From these data Haley concluded that the proportion that established was not a function of dose size within this range.

The following experiments were designed to produce a system, similar to EDT20, that would give a consistently high establishment of N. brasiliensis, with minimum variance, after the application of an exactly known dose of either sheathed or exsheathed third stage juveniles (L3) to the skin of a rat.

MATERIALS AND METHODS

The animals used were 3 week old male and female Wistars from an outbred colony maintained in this department. Stock rats were each infected with a mean of 1000 exsheathed larvae of N. brasiliensis by subcutaneous injection. Faeces for experimental culture were collected overnight from day 6-12. Experimental rats were anaesthetized one hour before infection by an intraperitoneal injection of pentobarbitone sodium ('Sagatal', May and Baker) diluted 1 in 10 with 0.9% sodium chloride solution. The amount administered was 31µg/g body weight, which immobilized the animals for 2.5 hours. The infection site was a 15mm long by 10mm deep area of clipped skin on the left flank.

Experimental cultures to produce infective third stage juveniles were developed from the traditional filter paper technique (Barakat, 1951; Wilson & Dick, 1964). On top of a small piece of absorbent cotton wool, soaked in distilled water and situated in the bottom of a 90mm glass petri dish, is placed a 55mm diameter circle of Whatman 54 filter paper. One pellet of infected rat's faeces is positioned centrally on the filter paper and the lid of the dish put in place. After approximately seven days of incubation at 19°C third stage larvae migrate across the paper to the edge where they

become attached by the tails of their sheaths. The sheath is broken at the front end, allowing the juveniles to emerge when stimulated by a sudden rise in temperature. A rough naked eye assessment of the level of development can be made at this point, since the nematodes form a distinct fringe around the periphery of the filter paper disc. The cultures are left for a further two days to ensure that all larvae have completed development. Evaporation from them can be reduced by placing an open bowl of water in the bottom of the incubator.

To obtain exsheathed juveniles, filters from the petri dishes are placed in distilled water at 37°C for 5 minutes. The sheaths are removed with the filters, leaving worms in a suspension which can be used to infect by a method of skin application identical to EDT20 for S. ratti. Samples of 20 are handcounted by pipette into solid watch glasses and the amount of water in each reduced to approximately 0.3ml. This is taken up in the plastic barrel of a 1ml hypodermic syringe and applied, without the addition of a needle, to the lightly wetted infection site already prepared (see above). Each syringe is washed ten times in distilled water, the washings pooled, and juveniles that sediment therefrom counted. Those that remain in the solid watch glasses are also counted. The actual number applied to the skin of any given rat is determined by subtracting the residual individuals from 20.

Distilled water was the medium for containment and administration of exsheathed larvae in all experiments involving them except one. In the exceptional case half the rats were infected by larvae applied in quarter strength Ringer Locke balanced salt solution containing the following per litre: 2.3g NaCl, 0.01g KCl, 0.044g CaCl₂ and 0.04g NaHCO₃ (Wilson & Dick, 1964: '1/4PLA' minus the antibiotics).

First attempts to obtain a known dose of sheathed juveniles were made using variations of the traditional filter paper culture. The plan had been to determine numbers while juveniles were still attached to the filter paper, then to apply an appropriate snippet of the paper to the skin of a rat.

Residual worms still in their sheaths would be counted when the paper was removed at the end of the period allocated to skin penetration. All modifications of the technique using filter paper as the counting substratum failed. The number of juveniles stimulated to exsheath was almost always in excess of the number deemed to be present in the fringe. It was concluded that some worms were indistinguishable from filter paper fibres under realistic operational conditions, and that a transparent substratum was needed to circumvent this problem. The filter paper itself was necessary to support the water film for nematode movement. A 3-layered geometry was adopted, therefore, with upper and lower filter paper discs, but with a middle layer provided by an annulus of heavy duty transparent polythene, 0.22mm thick. The polythene was cut with an outside diameter of 60mm, so as to protrude beyond the filter paper margin, and with a 20mm diameter hole in the centre. This construction permitted the flow of water from the cotton wool reservoir to the uppermost layer bearing the faecal pellet. After incubation and removal of the filters, a thin fringe of juveniles was left on the polythene. The polythene was secured by means of a 25mm square piece of masking tape to an upturned watchglass (48mm dia) attached to a bottle top (35mm dia, 23mm depth) (Figure 1) and the combination manipulated under the dissecting microscope. At an appropriate density, sheathed worms could be clearly seen and counted with 45X magnification. In a test of the method, 30 snippets, each bearing a number of juveniles in the order of 20, were immersed in water at 37° for 5 minutes. In all cases the number exsheathed added to the number still in their sheaths after the test confirmed the original count. All juveniles escaped from their sheaths on 22 occasions; the number where 1 remained sheathed was 6; and in each of the remaining 2 samples, 2 juveniles failed to escape. The above technique of culturing and counting N. brasiliensis L3's on polythene being validated, it was adopted as the basis for experimental infection of rats with an exactly known dose in the order of 20 sheathed juveniles ('EDT20Nb'). Instead of immersion in water at 37°, as in the

trial, each snippet was placed with its larvae innermost on the infection site of an anaesthetized rat. One hour after application the snippets were removed and the number of larvae still in their sheaths was recorded for each animal. Each host therefore acquired a dose of $\underline{n} - \underline{w}$, where \underline{n} represents the initial count and \underline{w} the number still sheathed after 1h. No more than 3 rats were infected from any one culture as polythene does not retain moisture and the worms quickly dry out on removal of the filters (see Experiments and Results). The number of cultures required for an experiment was approximately 1.5 per infection, as in some cases the concentration of juveniles was too high or too low to facilitate an accurate count. All rats were killed 10 days post-infection, after being starved for the previous 24 hours. The 'take' was assessed by counting worms in a modified gut squash technique (Tindall & Wilson, 1988). The first 500mm of the small intestine was cut into 100mm pieces, each piece being slit open, squashed between two glass plates and viewed under the low power of a stereo microscope. The number of adult worms present was expressed as a proportion of the applied dose (\underline{p}_f). Parametric statistical methods were used to analyse the data after arcsine transformation.

EXPERIMENTS AND RESULTS

A. Establishment of N. brasiliensis using exsheathed juveniles.

In 4 similar experiments, 4, 10, 7 and 4 rats were infected with exact doses of the order of 20 exsheathed third stage juveniles (L3) of N.

brasiliensis in water by a method identical to EDT20 for S. ratti. The mean proportions recovered as adults from the small intestine 10 days post infection ($\bar{\underline{p}}_f$) are summarized in Table 1.

Analysis of variance of the arcsines of the \underline{p}_f 's shows the variance between groups to be significantly greater than that attributable to error ($\underline{F}_{[3,21]} = 8.128$, $\underline{p} = 8.8 \times 10^{-4}$). The main generator of the high \underline{F} value is the single mean, 0.311 (Table 1); but, since all tests were judged to be replicated as rigorously as possible, this highly significant outlier is

sufficient evidence that the technique suffers from severe systematic variables of an uncontrolled nature. Moreover, the highest take (0.667), although good by comparison with accepted norms, is well below our ideal goal of unity.

The possibility that water was not an appropriate medium to contain the doses was tested in an experiment in which 7 rats were infected in the same way as before, but with the juveniles in a balanced salt solution (see Materials & Methods). The \bar{p}_f was 0.374 ± 0.061 compared with 0.536 ± 0.067 in 7 controls infected with worms in water.

B. Infection with sheathed juveniles from polythene cultures: the problem of desiccation.

It was assumed that the uncontrolled aspects of the use of exsheathed L3's arose during the artificial exsheathment process itself, or in the handling of the worm suspension subsequently. However, the first experiment using combined filter paper and polythene film cultures, omitting the exsheathment step, was unsatisfactory. Nine rats, each infected with an exact dose in the order of 20 juveniles applied to the skin on polythene snippets (Materials & Methods), had a mean \bar{p}_f on day 10 of 0.438 ± 0.053 (SEM); i.e. lower than three of the values obtained with exsheathed worms (Table 1).

The following experiment was designed to test the supposition that, given time, desiccation of L3's on polythene reduced their viability. Seven rats were given doses one after the other from a single polythene culture. A final rat was infected from another fresh, damp, culture of the same sort. The \bar{p}_f 's, in the order in which the rats were infected, were as follows: 0.700, 0.722, 0.571, 0.684, 0.150, 0.143, 0.182 and 0.737. Clearly, the longer the time between the removal of the filters and infection of the host, the greater the reduction in viability. In further studies, each culture supplied a maximum of 3 exact doses.

C. Comparison of \bar{p}_f 's using sheathed and exsheathed juveniles.

From the foregoing, we had the expectation that sheathed L3's applied to the

skin on polythene would meet our requirements providing the problem of desiccation could be circumvented. To verify that this was so, and that such juveniles were better than exsheathed individuals, two replicate experiments were set up to make the comparison. The results are summarized in table 2. Two way analysis of variance of the arcsines of the proportions that became established reveals a highly significant difference between the treatments ($F_{[1, 28]} = 1345, p = 3 \times 10^{-12}$). There was no significant difference between experiments ($F_{[1, 28]} = 0.836, P = 0.361$).

D. Routine establishment using sheathed juveniles.

The comparison above clearly indicated that sheathed L3's were superior to exsheathed ones. The polythene film method has now been adopted in this laboratory as a standard means of infecting rats with an exactly known dose of the order of 20 N. brasiliensis. The results of 10 experiments using such juveniles (along with the 2 results from section C.) are shown in Table

3. Analysis of variance reveals that all 12 means could have been drawn from the same population ($F_{[11, 61]} = 1.30, P = 0.247$). The overall mean proportion of the population which becomes established is therefore 0.784 ± 0.015 (S.E.M.) from a total of 73 rats.

DISCUSSION

In the past, most workers have relied on subcutaneous injection of skin penetrating nematode juveniles to initiate infections. Although the method can sometimes lead to reasonable establishment of N. brasiliensis, the procedure appears to introduce an undesirable element of variation, and, in general, the level of development is moderate (see Introduction). Moreover, it serves to bypass a major step in the life cycle of such parasites.

In most studies it is obviously desirable to mimic natural events as far as practicable; consequently the process of skin penetration should become part of the experimental design applied to N. brasiliensis and comparable nematodes. This requirement is of paramount importance when dealing with the question of migration itself. When artificially exsheathed juveniles were

used for skin application, however, present results showed that they were even less effective than when they were injected. We see the polythene film culture combined with the 'EDT20Nb' skin application of sheathed L3 as a solution to some of the problems inherent in experiments with N.

brasiliensis. It has proved to be a powerful method in our own framework, in which the use of small numbers of parasites has become routine (Tindall & Wilson, 1990a, 1990b).

Questions arise as to why artificially exsheathed juveniles were comparatively ineffective when applied to the skin. Answers to these might involve (a) perturbation of a stepwise physiological process by the exsheathment technique, utilising, as it does, the 'thermotactic' response of these stages; (b) physiological damage resulting from overcrowding in the handling of exsheathed worms in suspension; or (c) the possibility that the juveniles, after artificial exsheathment, are robbed of solutes important for their well-being in the aqueous film inside their sheaths. Indications exist that all of these might be involved: (a) The effect of temperature change on the oxygen uptake of L3's reported by Wilson (1965b) was consistent with the idea of a behavioural response having an underlying, sequential, physiological repertoire. (b) Overcrowding has been identified in this laboratory (unpublished data) as a potent cause of damage to S. ratti L3's. (c) Wilson & Dick (1964) reported that water was an unsuitable medium for the maintenance of suspensions of N. brasiliensis exsheathed juveniles. The comparative success of a balanced salt solution (Wilson, 1965a) does not prove that it mimics what is in the fluid in the sheaths of third stage worms. As reported here, skin application using that medium failed to reverse the damage that somehow results from artificial exsheathment, but the experimental design in this case could only answer the practical question. Nevertheless, there is a strong probability that the fluid film between the juvenile and its sheath contains solutes of various kinds.

The statistical benefits of a framework such as EDT20Nb were predicted by

Wilson et al (1978) and confirmed by Wilson & Simpson (1981) with Strongyloides ratti. Wilson et al (1978) found that, when doses were based on volume sampling of larval suspensions, the coefficient of variation, CV, of the doses themselves was 15%. In addition, a CV of 10% was attributable to observer error in the counting of parasites en masse in the intestine. The exact dose procedure eliminates both sources of variation and thus increases overall experimental sensitivity. Theoretically, however, the improvement is slight if other components of variance are dominant. For example, Twohy's (1956) data for N. brasiliensis in the intestine following skin application of L3's had a CV (derived from the within-sample-variance) of 46%. His doses were prepared by volume sampling and his counts in the intestine were of relatively large numbers of worms. The square of the coefficient of variation is a measure of the variance from which it is derived independent of the mean. Thus, if Twohy had used the EDT20Nb procedure, the predicted improvement in his overall experimental CV is given by:

$$\sqrt{46^2 - (15^2 + 10^2)} = 42\%$$

i.e. not a lot; for, clearly, additional components of variance were of greater importance in his study. This unexplained variance was much less obvious in our own work (Table 3). For the 73 rats involved in these experiments the within-sample-derived CV has a creditable value of 16%. The associated experimental error is thus at a level low enough to detect small differences between treatments within experiments. Of more importance in the long run is the fact that there was no added component of variance between experiments (Table 3), and the CV calculated from the total variation has the same value (16%) as that based on variance within experiments. Therefore the probability that replicate experiments using the method will be in quantitative agreement is high.

In conclusion, the EDT20Nb procedure in our hands has produced the highest mean p_f (0.784±0.015) for N. brasiliensis in a sizeable number of rats (73) on record. The method is offered as one to be preferred in any

context where the experimental goals can be achieved by the use of small numbers of parasites.

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Table 1. Establishment of exsheathed juveniles.
(\bar{p}_f = mean proportion of the dose
recovered as adults 10 days after skin-
application)

Number of rats	Mean exact dose	\bar{p}_f (\pm SEM)
4	18.5	0.628 \pm 0.044
10	16.8	0.311 \pm 0.046
7	17.7	0.537 \pm 0.067
4	18.0	0.667 \pm 0.074

Table 2. Comparison of establishment using sheathed and exsheathed juveniles from the same population.

Experiment	Number of rats	Sheathed		Exsheathed	
		Mean exact dose	\bar{p}_f^* (\pm SEM)	Mean exact dose	\bar{p}_f^* (\pm SEM)
C1	6	20.2	0.820 \pm 0.040	17.2	0.319 \pm 0.052
C2	10	18.8	0.836 \pm 0.057	16.0	0.379 \pm 0.069

*see Table 1

Table 3. Establishment of sheathed larvae.

Experiment number	Number of animals	Mean exact dose	\bar{p}_f^* (\pm SEM)
C1	6	20.2	0.820 \pm 0.040
C2	10	18.8	0.836 \pm 0.057
D1	5	18.4	0.683 \pm 0.052
D2	8	18.1	0.762 \pm 0.025
D3	5	19.6	0.681 \pm 0.082
D4	6	19.2	0.782 \pm 0.030
D5	6	20.2	0.771 \pm 0.057
D6	4	17.8	0.731 \pm 0.037
D7	5	18.8	0.802 \pm 0.042
D8	6	20.0	0.788 \pm 0.026
D9	6	21.2	0.846 \pm 0.061
D10 [#]	6	24.5	0.827 \pm 0.058
All data pooled	73		0.784 \pm 0.015

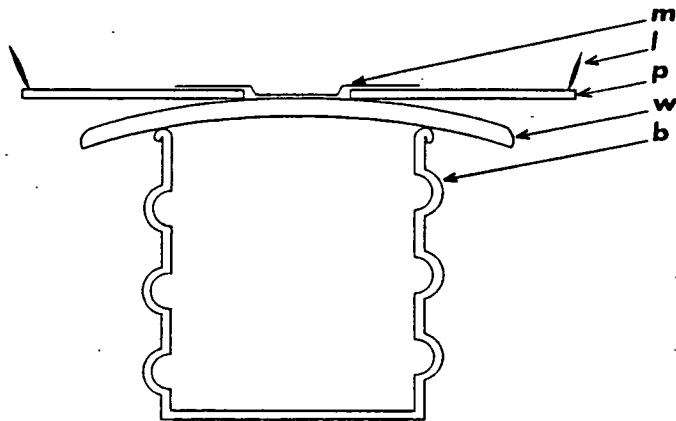
*see Table 1

[#]adults counted in intestine on day 8

LEGEND FOR FIGURE 1

Cross-section of the arrangement for counting juveniles on polythene:

m, masking tape; l, juvenile (not to scale); p, polythene film; w, watch glass; b, bottle screwtop. For dimensions, see text.



A basis to extend the proof of migration
routes of immature parasites inside hosts:
Estimated time of arrival of Nippostrongylus
brasiliensis and Strongyloides ratti
in the gut of the rat.

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SUMMARY

The time taken for larvae of Nippostrongylus brasiliensis and a homologous strain of Strongyloides ratti to complete their migration from the skin surface to the small intestine of the rat was estimated by a new method in which a mirror image of arrival times was created by counting mature parasites at day 8 (N. brasiliensis) or day 5 (S. ratti) in the intestines of rats that had received a single pulse of morantel tartrate by stomach tube at different times starting 10h after skin application of exact doses of infective larvae. It was confirmed that the effect of the drug was confined to parasites in the gut, so that the kinetics of migration were unperturbed. The persistence of the anthelmintic effect in the gut was shown by independent assays to be less than 8h for both species. The time-course of arrival calculated from drug pulse data corrected for persistent effects was compared with direct counts of larvae arriving in the intestines of rats not dosed with anthelmintic. Both methods agreed in all respects for N. brasiliensis, whereas the estimates for direct counts of S. ratti lagged 15-17h behind those from drug treated rats. In the discussion it is argued that the anthelmintic method provides a more correct picture for S. ratti and, on this basis, rates and synchrony of migration of the two species are compared. The role of these data as part of a proof of migration routes is explained.

Keywords: migration; proof; pathway; nematode; parasite

INTRODUCTION

The infective stages of many macroparasites migrate extensively inside the host to the site where they will mature and reproduce. Most of the evidence for the routes by which such invasive juveniles reach their destination has been gathered by "sampling at autopsy", i.e. determination of the numbers of parasites found in particular organs or tissues of animals experimentally infected and killed at different times afterwards. In the past no account has been taken of the fact that, if the maximum proportion (p_o) of the dose found in a putative transit site ('O') is less than the proportion which fails to reach the destination ($[1 - p_f]$, where p_f = the proportion that does reach the destination), then the parasites in 'O' are just as likely to be 'lost' as on their proper course (Figure 1). It follows that the categorical descriptions of such pathways to be found in textbooks have no rigorous factual basis, and could well be radically wrong.

A theoretical solution to the difficulty would be to devise a system in which all the parasites in an experimental dose always completed the journey (i.e. $p_f = 1.0$). Then $[1 - p_f]$ would be zero, and any juvenile found anywhere in the body except the destination must be on course for the destination.

Such a system cannot be sustained. However, a compromise is possible if values of p_f are raised experimentally to a level approaching 1. In that case an organ is proved unequivocally to be part of a pathway to the endpoint when $p_o > [1 - p_f]$, or $p_o + p_f > 1$ (Wilson, 1983)

(Figure 1). Using this principle, Tindall & Wilson (1988) presented the first rigorous proof applicable to any parasite's migration, and showed unequivocally that the nasofrontal region of the head was part of a pathway taken by Strongyloides ratti on its journey from the skin to the intestine of the rat.

Their study is conclusive in defining a major component of one route for S. ratti. For a complete description, however, it is necessary to establish if

a pathway is exclusive, and not merely one among a number of routes by which a given parasite may reach its destination. We address this problem in the present paper and its sequel, and deal with another skin penetrator of the rat, Nippostrongylus brasiliensis, as well as Strongyloides ratti.

This paper concerns an essential prerequisite for a complete description, namely, an accurate estimate of the time of arrival at the destination. In the sequel (Tindall & Wilson, 1990), this information is incorporated into a framework which proves that the pathways of the two parasites are mutually exclusive in their most important aspects.

The framework referred to requires a precise account of the time of arrival of these nematodes in the gut as a whole, which in practice means the stomach. Conventional techniques do not necessarily provide reliable relevant information. The problem was tackled by a novel method in which a mirror image of arrival times was created by counting mature parasites in animals that had received a single pulse of anthelmintic by stomach tube at different times after skin application of infective larvae.

MATERIALS AND METHODS

Rats used in this study were 3-week old Wistars of both sexes from an outbred colony kept in this department. Males and females were evenly distributed between treatments. The homogonic strain of S. ratti used is the one that figured in the initial proof for the involvement of the nasal region in the migratory pathway (Tindall & Wilson, 1988). It was cultured and maintained as described by Wilson & Simpson (1981). N. brasiliensis was cultured according to Tindall & Wilson (1989). The techniques for administering an exactly known dose of either species ('EDT20' and 'EDT20Nb') have been described in detail by Tindall & Wilson (1988, 1989). For S. ratti, a hand counted number in the order of 20 infective larvae was administered in 0.2ml of distilled water to a shaved infection site on the left flank of a rat. Rats were anaesthetised 1 hour before infection by an intra-peritoneal injection of pentobarbitone sodium (Sagatal, May and Baker). The amount administered was 3lug/g body weight, sufficient to immobilize the animal for 2.5 hours. Larvae not penetrating the skin 45 minutes post-infection, along with any remaining in glassware, were subtracted from the initial count. N. brasiliensis remains partially sheathed in the second stage cuticle attached to the substratum until contact is made with a host. To mimic the natural situation it is necessary to culture the parasite in a manner that allows the sheathed larvae to be counted without ambiguity. This was achieved by growing them in cultures containing transparent rings cut from sheet polythene, on to which some larvae migrate. An exact number of about 20 was counted on a snippet of polythene and applied to the dampened infection site still attached to the polythene. Any larvae remaining in their sheaths after 1 hour were subtracted from the initial count. For both species, although the dose was 'exact', it was actually 'in the order of 20'. That this is not the contradiction it seems to be is explained by the following two features common to both systems. In the processes by which larvae are obtained from faecal culture it is not possible to prepare in reasonable time many lots of exactly

20 larvae by handcounting. What is possible is to prepare samples of approximately 20, each of which is then counted exactly in its solid watch glass, or on its snippet of polythene film. The samples are used for skin application as described, but they are still not the actual 'doses'. The final exact dose for each animal is only known when the larvae that have not penetrated have been recovered after 45 (S. ratti) or 60 (N. brasiliensis) minutes. Thus every rat receives a unique exact dose in the order of 20 which is given by $d = n - w$, where n = the number handcounted initially and w = the larvae remaining outside the host after the time allowed for skin penetration. The number, ' n ', can be controlled better for S. ratti than for N. brasiliensis, consequently its range was 18 to 24 for the former and 18 to 28 for the latter. For S. ratti, $w = r + s$, where r are the larvae remaining in glassware and syringes and s are those washed from the skin after 45 minutes.

Establishment of the parasites at their destination was measured by a gut squash technique on day 5 for S. ratti and day 8 for N. brasiliensis (Tindall & Wilson, 1988, 1989). For the latter species this was judged by counting all worms present; invariably mature males and females. Parasitic individuals of S. ratti, when adult, are parthenogenetic females without male equivalents. Each worm of this species leaves a more or less straight track of eggs deposited in packets as it migrates through the intestinal mucosa. When numbers of worms are less than 100 the individual tracks are more obvious than the parasites themselves. Using a dose of one larva per rat we have shown that, in each of 56 animals in which a parasite established, there was a single egg track at day 8 (Tindall & Wilson, 1988). In unpublished observations using exact doses of the order of 20 we have verified that the number of tracks at day 5 is not significantly different from that at day 8. Consequently numbers of S. ratti were judged by counting egg tracks rather than worms. For both species establishment in any one animal was expressed as a proportion (p_f) of the unique exact dose.

Estimates of the time of arrival of larvae by direct observation were made in rats which were starved for 24h prior to killing to reduce debris in the intestine. When killed, the intestine was removed and divided into 100mm pieces. Each piece was slit open and squashed between two glass plates and examined under a stereo microscope at 25X magnification. The number of larvae found in each rat's intestine was first expressed as a proportion of the unique dose received by that animal. To standardise comparisons between experiments, and between direct counts and drug pulse data (see the following), these proportions were expressed as a fraction of the mean P_f determined for that population of larvae in 6 control rats killed on day 5.

A second technique for assessing the arrival time in the gut involved the use of drug pulses. This method is based on oral administration of a single dose of a non-systemic anthelmintic to the host at a particular time post-infection. If the drug has a short persistence it will remove larvae already in the gut but have no effect on subsequent arrivals. By comparing the takes in such hosts to those in untreated controls the number of larvae in the gut at the time of administration of the drug can be estimated. Preliminary experiments established that, with suitable controls, morantel tartrate (Pfizer) was effective at a dose of 0.02mg/g body weight for N. brasiliensis, and 0.06mg/g body weight for S. ratti. The drug was diluted to a concentration of 1mg/0.1ml distilled water, so that a 50g rat received a volume of 0.1ml if used against N. brasiliensis or 0.3ml against S. ratti. It was administered to a lightly etherized rat by a stomach tube constructed from a 1ml syringe (Sabre, Gillette) fitted with a blunt 19 gauge needle inserted into a 50mm piece of plastic tubing (PP80, Portex Ltd, Hythe, Kent). Rats were starved for 24h prior to administration, and fed 30 minutes afterwards in order to aid the passage of anthelmintic from the gut. Treated animals were kept in grid cages (Model R1, North Kent Plastics Ltd.), to reduce the possibility of their ingesting more of the drug

from contaminated faeces and urine. To obtain an estimate of the number of larvae present at the time of administration, and compare it to the control, rats were killed on day 5 for counts of S. ratti and day 8 for N. brasiliensis. The proportion of the larval dose establishing in drug-treated animals was termed p_m and, in controls, p_f . The proportion of p_f present when the drug was applied was therefore

$$\frac{\bar{p}_f - p_m}{\bar{p}_f}$$

where \bar{p}_f is the mean of 6 controls.

In some experiments larvae extracted from the head or lungs of donor rats were adoptively transferred orally to other rats. The donors for these experiments were infected by skin application of mean doses of 500 infective larvae measured by volume after the numbers in the suspension had been estimated by dilution sampling. In the case of N. brasiliensis larvae had first to be stimulated to exsheath by placing the polythene ring in distilled water at 37°C for 5 minutes. Infective larvae of S. ratti emerge from faecal culture already exsheathed and ready for use.

Preliminary experiments had shown that S. ratti larvae extracted from the nasal region of donors some 30 hours post-infection would develop to maturity in the intestines of recipients after oral administration. The same applied to larvae of N. brasiliensis taken from the lung at 40 hours post-infection. At the appropriate time donors were killed and the nasal region or lungs removed and rinsed in warm 0.9% sodium chloride solution to remove excess blood. The nasal region was then chopped coarsely with scissors, whilst a razor blade was used to mince the lung. Following incubation with 40ml of 0.9% sodium chloride solution in a petri dish at 37°C for 15 minutes, emerging larvae were counted into samples of the order of 20 and transferred to solid watch glasses with a drawn out pipette. The sample volume was reduced to 0.05ml, the number of larvae each contained was determined exactly, and

then it was incubated at 37°C until needed. The samples were administered to recipients with a stomach tube constructed as described above. 0.2ml of saline was taken up into the syringe, followed by the larvae in as small a volume of fluid as possible, to reduce the number passing up into the barrel. The larvae were then administered to the stomach of a lightly etherized rat. The syringe was flushed out several times with saline, and any larvae remaining therein were subtracted from the initial inoculum. The exact dose was calculated in a manner analogous to that already described for skin application.

EXPERIMENTS AND RESULTS

A. Arrival of S. ratti and N. brasiliensis in the small intestine

assessed by direct observation.

Four and five experiments respectively were done to chart the arrival in the small intestine of N. brasiliensis and S. ratti by direct counts. All rats were given an exact dose by skin application of larvae as described in the Materials & Methods section and killed sometime later, when the small intestine was removed and examined for the presence of larvae. Subjectively, it seemed that N. brasiliensis L4's could be counted without ambiguity as soon as they arrived in the intestine because of their relatively robust form (1 mm long) and opaque appearance. By contrast, counting of S. ratti L3's was much more difficult because they were smaller (0.65mm long), delicate, and virtually transparent. The results are shown in Figures 2a and 2b in comparison with those from the drug pulse method (see below). Where homogeneity ($P > 0.05$) exists at a particular sampling time the data from different experiments have been pooled to give an overall mean. If the data are heterogeneous each mean is recorded separately.

B. Drug pulses: persistence in the gut.

In order to interpret results from anthelmintic treatment it was necessary to know how long the effects of a pulse of morantel tartrate persisted in the gut. This was investigated as follows. Eighteen rats were given a standard dose of the drug at time zero. At 0h, and 4h and 8h later, groups of 6 rats were given an oral infection of N. brasiliensis larvae extracted from the lungs of donors infected some 45 hours earlier (see Materials & Methods section). A further 6 control rats were given larvae without anthelmintic. All animals were killed on day 8 and the worm burden in each was expressed as a proportion of that animal's unique exact dose of larvae. The results (excepting time zero, after which no worms developed) are shown in Figure 3a. Eight hours after administration of the drug the proportion of the larval dose which established was not significantly different from that in the

controls.

An experiment of identical design to that just described was conducted with S. ratti, with the exception that donor larvae were extracted from the nasal region 30h post-infection. Again, when larvae were fed immediately after drug administration, no worms developed. Other results are shown in Figure 3b. As in the case of N. brasiliensis, larval establishment in drug treated animals was indistinguishable from that in controls after 8h, even though the dosage was three-fold higher than in the earlier experiment (Materials & Methods).

For both parasites the effects of the drug wore off some time between 4 and 8 h after administration. It is tempting to interpolate a value of 6 h, but we have taken the safe course and applied the full 8 h in correcting the raw data to derive the estimated curves of time of arrival.

C. Drug pulses: ETA in the gut.

Forty six rats were infected by skin application of exact doses of N. brasiliensis, and then, in groups of 5 or 6, given morantel tartrate (1mg/50g body weight) orally at the following times post infection, 10h, 30h, 40h (5 rats), 50h, 60h (5 rats), 70h and 80h. The remaining 6 rats were left untreated as controls. All rats were killed on day 8 and the number of adult worms in the small intestine counted and expressed as a proportion of the larval dose (p_m for treated animals, p_f for controls). p_m was then translated to give the proportion of larvae in the gut prior to treatment (see Materials and Methods). The results are shown in Figure 2a in comparison with those from direct counts. The effect of the drug is first detectable statistically at 40h; the means of the arcsines of the raw data obtained prior to this time are not significantly different from the control mean (at 10h, $t_{[10]} = 0.905$, $P = 0.387$, and at 30h, $t_{[10]} = 1.438$, $P = 0.181$).

A similar experiment was carried out for S. ratti. Fifty three rats were infected, split into 8 groups of 5 or 6, and each group given the drug

(3mg/50g body weight) at one of the following times post-infection; 20h (5 rats), 30h, 40h, 50h, 60h, 70h, 80h and 90h. There were 6 untreated controls. The results are shown in Figure 2b. Analysis of the arcsines of the raw data for drug treatment at 20h compared with the controls reveals no statistical difference ($t_{[9]} = 1.217$, $p = 0.254$), but at 30h, the difference is significant ($t_{[10]} = 2.457$, $p = 0.034$).

In Figures 2a and 2b the uncorrected data are represented by solid black squares. Estimated times of arrival obtained by adding a delay of 8h to allow for drug persistence (section B) are represented by open squares. These corrected estimates fall on the same curve as those from direct counts for N. brasiliensis (Figure 2a). In contrast, the curve from direct counts of S. ratti lags 15h behind that derived from corrected drug pulses (Figure 2b).

D. The anthelmintic has no parenteric effects.

An essential feature of this method for analysing the kinetics of the system is that the drug exerts no influence on the migrating larvae. We can say, from the data already presented, that the earliest stages of migration are unaffected, since drug pulse data at 10h and 30h for N. brasiliensis, and 20h for S. ratti, are not significantly different from untreated controls (section B). The question arises, however, as to whether the significant differences that emerge after these times are really confined to effects on parasites in the gut. This was investigated as follows.

Four rats were infected by applying 500 N. brasiliensis L3's to the skin. At 40h, two of the rats were given a standard dose of morantel tartrate. All animals were killed at 45h, when the lungs were removed, and larvae extracted from them were used to infect 12 recipients by oral administration of exact doses as described in the Materials & Methods section. Six were given larvae from the treated donors whilst the others were given larvae from the controls. All were killed on day 8 and their worm burdens compared. The mean take of control larvae was 0.359 ± 0.028 (SEM) while larvae from the treated animals

gave a take of 0.416 ± 0.045 in the recipients. A t -value calculated from the arcsines of these data confirms homogeneity ($t_{[10]} = 1.069$, $p = 0.31$).

A similar experiment with the same numbers of animals given exact doses orally was carried out with S. ratti. The two donors were given drug at 30h, and recipients were infected at 35h with larvae extracted from the nasal region. The proportion of egg tracks present on day 5 was 0.624 ± 0.044 and 0.597 ± 0.050 for larvae from treated and untreated donors respectively. A t -test on the arcsines of these data gives $t_{[10]} = 0.409$, and probability, $p = 0.691$.

For both N. brasiliensis and S. ratti, these results show that larvae taken from the lung or the head 5h after oral administration of morantel tartrate are not impaired in their capacity to reach maturity.

The fact that the curve derived from corrected drug-pulse data for each species has the same shape and slope as that for direct counts of that species (Figures 2a & 2b), even though they are not superimposed for S. ratti (Figure 2b), corroborates the other experimental evidence that the use of this anthelmintic as described does not have any impact on larvae in transit.

DISCUSSION

An accurate picture of the overall kinetics of migration of these two nematodes is the starting point for the development of further proofs of the pathways involved (Tindall & Wilson, 1990). With the exception of the work of Love, Kelly & Dineen on N. brasiliensis (1974: see below), past estimates of the crucial parameter in such systems, namely, time of arrival in the gut, have focused on the intestine and have been based on direct observation (e.g. Twchy, 1956), radioactive tracking (Wilson, 1979), or methods of extracting larvae from intestinal tissue (e.g. Murrell, 1980). Each of these is suspect in one way or another, but all of them share the drawback that they take no account of transit time through the stomach. The drug pulse data supply what is lacking in those methods. In addition, it was desirable to correct a possibly important source of observer bias in direct counts which has its roots in the following. Newly arrived larvae in the intestine are small. As they increase in size they are more easily seen by the microscopist.

Consequently, 'time of arrival' curves based on direct counts are corrupted to an unknown extent by the growth component (Wilson Simpson & Seaton, 1986). The drug pulse procedure overcomes this bias because all counts are of worms at the same, late, stage of development, when individuals (or egg-tracks) are unambiguously visible in an intestinal squash preparation. Combined with an exact dose technique (Tindall & Wilson, 1988, 1989), and suitable controls to ensure that parenteric phases are unaffected (Section D), this procedure offers the possibility of a high degree of precision.

The results for N. brasiliensis from direct counts, and the corrected data from drug pulses for this species, fall on the same curve (Figure 2a). In this case, therefore, there is no observer bias in direct counts, nor is there a significant time delay in negotiating the stomach. Our results correspond well with those obtained by Love et al. (1974) using chronic catheterization of the oesophagus to collect hourly samples of N. brasiliensis larvae as they approached the stomach in non-immune rats. The

agreement between our two estimates, and that of Love et al. (1974), validates the method using anthelmintic and aids the interpretation of data for S. ratti. The curve derived from direct observation for the latter species (Figure 2b) lags 15-17h behind, and parallels that for the corrected drug-related counts. It seems improbable that such a large time difference is wholly due to delayed passage through the stomach. Transit of both species at this location is likely to be governed by the same forces. So, because N. brasiliensis takes no measurable time in the process, we conclude that S. ratti behaves similarly and that, therefore, the discrepancy between the two estimates for S. ratti is entirely a measure of observer bias in that case. This is corroborated by the fact that N. brasiliensis fourth stages in the lung are distinctly stouter and more opaque, and thus more visible, than the delicate third stages of S. ratti that leave the head on their way to the gut. In practice, the extent to which either explanation is valid is immaterial for the purpose in hand.

Accepting the curve from drug pulse data as the more correct picture for S. ratti, and comparing it with results from both types of evidence for N. brasiliensis, a distinct difference in kinetics emerges. At 40h a proportion of 0.20 of the total which will ultimately reach the gut has arrived in both cases. Following this, the increase to a proportion of 0.90 is achieved in 8 h by N. brasiliensis and in 28 h by S. ratti. Though the fastest migrators of both species take the same length of time to reach their destination, S. ratti is much less synchronised in its movements, with a time interval between early and late arrivals more than 3 times longer than that for N. brasiliensis. There is no rigorous evidence to support theories of how the lung or head is reached by these parasites (Wilson, 1983), but the kinetic differences we now identify are consistent with the possibility that third stage N. brasiliensis are bloodborne, whereas it is probable that those of S. ratti are not.

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LEGENDS FOR FIGURES

Figure 1: Idealised results from sampling at autopsy. Organ 'O' is a potential transit site for the parasite on its way to the destination. Proof of the involvement of 'O' is provided when $p_o > [1 - p_f]$, or $p_o + p_f > 1$.

Figure 2: Arrival in the gut of (a) Nippostrongylus brasiliensis and (b) Strongyloides ratti. Solid circles refer to direct counts; solid squares represent uncorrected data from the drug pulse technique; open squares are data from drug pulses corrected for drug persistence (see Figure 3). Morantel tartrate treated groups comprised 5 or 6 rats (see text). Direct counts were made on the following numbers:

N. brasiliensis; 17(35h), 11(40h), 6(45h), 19(50h), 5(60h).

S. ratti; 23(23h), 12(40h), 12(48h), 24(60h), 6(72h), 6(96h), 36(120h+).

Bars define 95% confidence limits.

\bar{p}_f 's & \bar{d} 's, S. ratti: 0.824 ± 0.014 ; 19.4 (direct),
 0.821 ± 0.048 ; 20.3 (pulse)

\bar{p}_f 's & \bar{d} 's, N. brasiliensis: 0.777 ± 0.020 ; 19.5 (direct),
 0.827 ± 0.058 ; 22.8 (pulse)

Figure 3:/

LEGENDS (continued)

Figure 3: Persistence in the gut of the anthelmintic effect of morantel tartrate administered by stomach tube. Larvae were taken from the tissues of donor rats and adoptively transferred by stomach tube to 6 recipients at times zero, 4 and 8 hours after a single dose of anthelmintic. The histograms record proportional development compared with those given to 6 controls without anthelmintic. (a) Nippostrongylus brasiliensis on day 8 ($\bar{d} = 21.2$), (b) Strongyloides ratti on day 5 ($\bar{d} = 22.0$). Bars define 95% confidence limits.

Figure 1.

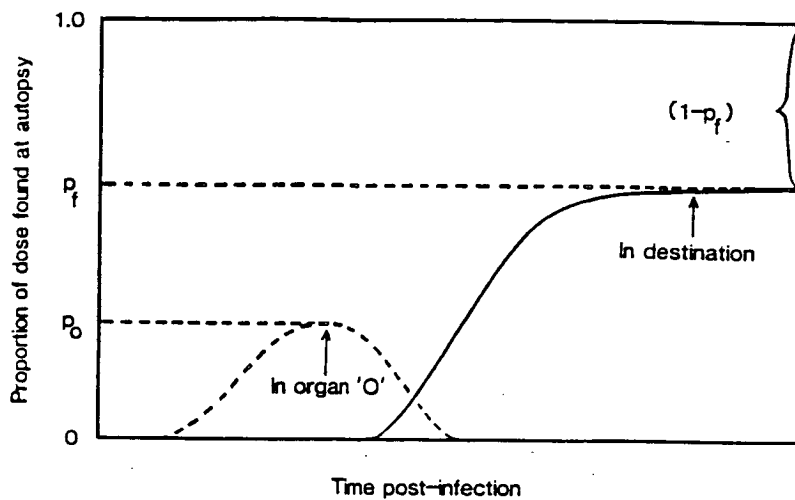


Figure 2.

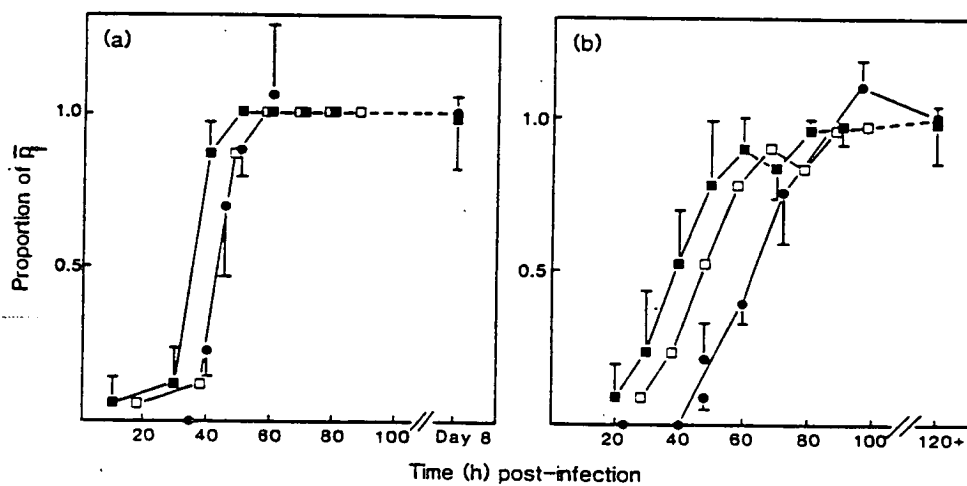
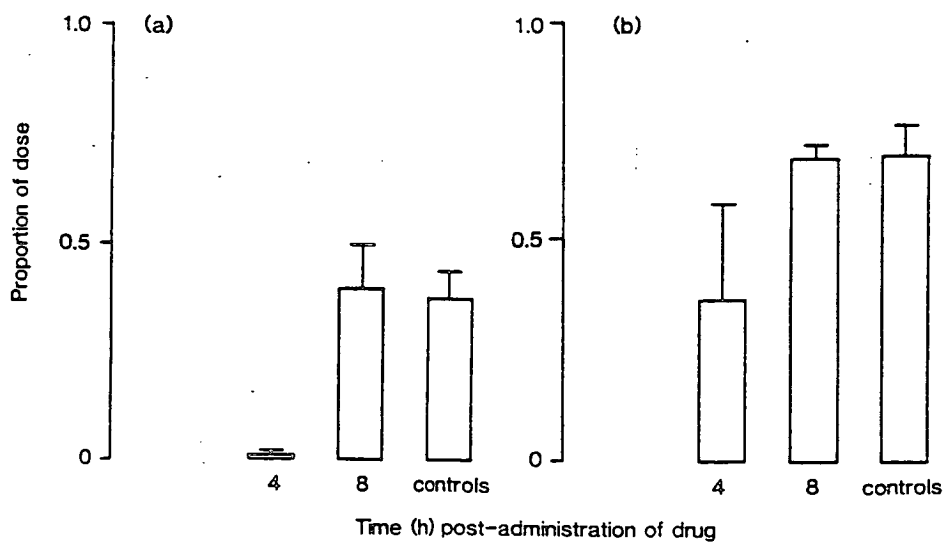


Figure 3.



An extended proof of migration routes of immature parasites inside hosts: Pathways of Nippostrongylus brasiliensis and Strongyloides ratti in the rat are mutually exclusive.

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SUMMARY

Rigorous proofs applicable to the routes of migration of Strongyloides ratti and Nippostrongylus brasiliensis skin-penetrating juveniles inside the rat are extended. By applying the inequality principle (Tindall & Wilson, 1988) it was confirmed with a probability of error of 1 in 10^{10} that N. brasiliensis larvae applied to the skin passed through the lungs on their way to the intestine. Taking the analysis further, migrating larvae of S. ratti or N. brasiliensis were extracted from the nose or lungs, respectively, of donor rats and transferred to recipients by stomach tube to assay their ability to colonise the intestine. Results showed that (a) changes undergone by each parasite in its proven, specific transit site were essential before larvae could establish in the intestines of recipients, (b) these changes could be monitored by morphological criteria, and (c) the changes were not completed until larvae had been in the nose or lung for a significant period. It follows from (c) that anywhere in the body of the host, termed a 'nursery', that supports a substantial amount of this mandatory development must be detectable by the conventional procedure of sampling at autopsy. Conversely, absence of parasites judged by sampling at autopsy is positive proof that a site is not a nursery when sampling is timed in relation to reliable estimates of overall kinetics (Tindall & Wilson, 1990), and with control information on the efficiency of sampling. Comparative data from sampling at autopsy using the same extraction techniques for both species met these criteria: they demonstrated that no part of the head of the rat was a nursery for N. brasiliensis, and that the lung did not serve in this capacity for S. ratti. The inability to extract parasites from the liver indicated that it was not a nursery for either species. It follows from these results that migratory routes of S. ratti and N. brasiliensis are fundamentally different and that the Looss- Fulleborn 'blood-lung route' must be rejected as a universal model to describe the behaviour of skin-penetrating nematode juveniles inside the host.

INTRODUCTION

The procedure of 'sampling at autopsy' routinely used in attempts to define the pathways of invasive stages of macroparasites inside hosts can only succeed if the natural route exhibits what has been called 'impedence' (Wilson, 1983, Tindall & Wilson, 1988). Impedence is necessary for parasites to accumulate in numbers sufficient to be observed by the sampling method. Even then, a critical concentration is required in a putative transit site before it can be claimed unequivocally as part of the route to the destination. This critical concentration is related to the initial infection dose, the proportion found in a site which could be on the route, and the proportion which completes the journey to the correct destination. The relationship that has to be proved is the inequality $p_o + p_f > 1$, where p_o is the proportion found at peak time in the candidate transit site, and p_f is the maximum proportion reaching the destination (Tindall & Wilson, 1990). Thus it is possible to achieve verification approaching certainty that particular locations are part of a route (Tindall & Wilson, 1988). The method is limited to this positive aspect, however. A site where no parasites are found by sampling at autopsy may be one which is not part of the pathway but, equally, it could be an essential transit point at which impedence is zero. In the light of this we need to examine more closely the components of 'impedence'. As originally presented (Wilson, 1983), the term was used to bring to notice assumptions implicit in the method of sampling at autopsy which had been routinely ignored. 'Impedence' is perhaps unfortunate in its implication that the migrating parasite struggles against the impediment presented by the host and that if this impediment were removed it would travel more rapidly to its destination. This is likely to be an oversimplification in many cases. It is probable that sites of impedence proven to be components of a route are also locations where the parasite undergoes development preparatory to its maturation at the final destination. This is the aspect we investigate in the present paper.

It has been demonstrated rigorously that a homogenic strain of S. ratti uses the nasofrontal region of the head in its migration from the skin to the intestine of the

rat and that, with some reservations, Nippostrongylus brasiliensis uses the lung in reaching the same goal (Tindall & Wilson, 1988). To what extent are these pathways mutually exclusive? As explained above, the inequality principle cannot serve if the lung is used by S. ratti, and the head by N. brasiliensis but that in these sections of each parasite's route impedance is zero. It may be tempting to assume that impedance for one parasite would apply equally to another in the same organ, but there is no logic in the assumption. We need to demonstrate that these migrating juvenile stages have a mandatory requirement for development before they can successfully colonise the intestine, and that this takes a substantial length of time. It then follows that any organ in the body which supports a significant proportion of the larval input at this stage of development must be identifiable by sampling at autopsy. Conversely, an organ which is not involved in development is also identifiable by the absence of parasites judged by sampling at autopsy.

The analysis presented here is predicated on reliable estimates of the overall kinetics of migration (Tindall & Wilson, 1990) and on proof that the head (S. ratti) and lung (N. brasiliensis) are parts of the relevant pathways. There is no doubt about the former, but some questions still remain unanswered as to the latter case. In our reappraisal (Tindall & Wilson, 1988) of Twohy's (1956) results, we took some liberties with the statistical treatment to offset limitations in the data. In addition, the proof then offered was confined to Twohy's experiments in which N. brasiliensis infections were initiated by subcutaneous injection of L3's. As a preliminary to the main study, therefore, we obtained experimental verification of the inequality and thus incontrovertible proof that this parasite uses the lung in its migration following the natural process of skin penetration.

MATERIALS AND METHODS

The animals and parasites involved in these experiments, the methods of culture, and means of percutaneous infection with exact doses were those described by Tindall & Wilson (1990).

Sampling for larvae outside the gut was done in the head, the liver and the lungs. The methods were based on the extraction procedure used for the head by Tindall & Wilson (1988). After killing, the head was separated from the neck, and the skin, mandible, muscles and eyes removed. The roof of the cranium was cut away, and the brain gently removed and washed in 50ml of warm Hanks balanced salt solution (HBSS, Gibco). The rest of the head was chopped coarsely and added to the washings. This was incubated in a 120ml centrifuge tube in a water bath at 37°C for 4 hours. After incubation, the liquid was aspirated to leave 15ml of tissue suspension which was poured through a domestic wire teastrainer. The material retained in the strainer was washed with 40ml of 0.9% sodium chloride, these washings being added to the original filtrate. The combined suspension was sedimented for 30 minutes at 37°C. After aspiration to 5ml, the sediment was transferred to solid watch glasses and examined under a dissecting microscope for larvae.

Livers and lungs were removed from the abdominal and thoracic cavities respectively and washed in 0.9% sodium chloride solution to remove excess blood. After being chopped finely on a glass plate with a razor blade, they were incubated as for the head.

A value of p_f for S. ratti in each animal was taken as the proportion of the exact dose which appeared as egg tracks in the small intestine on day 5 post-infection (see Tindall & Wilson, 1990). For N. brasiliensis, p_f was equivalent to the proportion of the dose reaching maturity in the gut on days 8 to 10. All counts were performed by the gut squash technique (Tindall & Wilson, 1988).

The following procedure was adopted to determine whether there is a mandatory process undergone by larvae in the head or lung which enables them to

establish successfully in the small intestine, and, if so, the time which such development takes. Parasites extracted from the tissues of donor rats over a selected period of time were administered by stomach tube to a recipient. The timing was based on the overall kinetics determined by Tindall & Wilson (1990). Donors were earlier infected percutaneously with a mean of 500 third stage larvae and tissue stages were extracted as described by Tindall & Wilson (1990).

Measurements of growth were obtained for larvae in either the lung or nasal region after extraction as in donor rats. Larvae were heat fixed at 60°C for 2 minutes and measured after they had been drawn with the aid of a camera lucida. Total body length (BL) and oesophagus length (OL) (S. ratti only) were obtained. The 'oesophagus index' (OI) for S. ratti was calculated as

$$\text{OI} = \frac{\text{OL}}{\text{BL} - \text{OL}}$$

(Nojima, Noda, Kawanabe & Sato, 1987)

EXPERIMENTS AND RESULTS

A. Proof of the use of the lung by Nippostrongylus brasiliensis.

Consistent with the doubts outlined in the introduction, studies were done to confirm that N. brasiliensis takes a path through the lung to the intestine following percutaneous infection. The protocol for the experiments was as follows. Rats were infected with larvae by the 'EDT20Nb' procedure (Tindall & Wilson, 1990) and killed in groups at different times to assay the parasite content of the lungs. Some were killed on day 8/10 to obtain a value for \underline{p}_f . Larvae were detected in the lung between 12 and 60 hours post-infection and the peak proportion of the dose (\underline{p}_o) appeared there at 35h. The lung was sampled at this time in four separate experiments, the results of which, along with their respective \bar{p}_f s, are shown in Table 1. The inequality, $\bar{p}_o + \bar{p}_f > 1$, is satisfied in every case and the \underline{p}_c 's (the excess of $\bar{p}_o + \bar{p}_f$ over unity) are all highly significant ($\underline{p} = 0.0015, 0.0062, 0.00094$ and 0.0059 : Table 1, col. 6). Analysis of variance of the arcsines of all the data for \underline{p}_o , on the one hand, and \underline{p}_f , on the other, reveals homogeneity in estimates of the same parameter in different experiments (variance between experiments was arithmetically less than the error variance in both cases). Pooling the data on this evidence gives a $t_{[42]}$ -value for the difference between the grand means of 8.61, with a probability of occurring by chance of 1×10^{-10} . This proves categorically that the lung is involved in the migratory pathway of skin penetrating N. brasiliensis.

B. Other possible transit sites for S. ratti and N. brasiliensis.

In the first paper in this series (Tindall & Wilson, 1988) we demonstrated the involvement of the nasal region of the rat head in the migratory pathway of S. ratti ($\bar{p}_o = 0.316 \pm 0.013$, $\bar{p}_f = 0.837 \pm 0.013$). The time when \underline{p}_o occurred was found to vary from 20-25 hours post-infection. Here we confirm that N. brasiliensis passes through the lung, with the peak at

35h post-infection. Although the proportions found were sufficient to fulfil the necessary criteria for a proof, p_o nevertheless falls well short of p_f in both cases. The explanation for the discrepancy need not be that alternative pathways are also involved (see Discussion), but the possibility remains, and was investigated as follows.

Experiments 5-8 were designed to look for larvae of S. ratti in the lung and liver after infection with exact doses of about 20 infective stages. The protocol for each experiment is outlined in Table 2. A single parasite was recovered from the lungs, and none from the liver, in a total of 12 (liver) and 49 (lung) rats. Larvae were confirmed in their ability to reach the destination in control animals in all 4 experiments

(Table 2, col 10)

The possibility that the liver and/or the head is part of the pathway of N. brasiliensis was examined in experiments 9 and 10. The protocols and results are shown in Table 3. No larvae were recovered in a total of 24 (liver) and 35 (head) rats, though they were normal in their migratory ability judged by controls (Table 3, col 8).

C. Development of S. ratti in the nasofrontal region.

Mimori, Korenaga, Chowdhury & Tada (1982) and Nojima et al. (1987) have shown that small changes in form of S. ratti L3's occur between the skin and the head. Kawanabe, Nojima & Uchikawa (1988) state, without supporting data, that larvae recovered from the skin were unable to infect by oral administration, whereas those from the nasal region could do so if the oesophageal index (OI) was greater than 1 (see Materials & Methods). Tindall & Wilson (1990) estimated that the first larvae to arrive in the small intestine had done so by 36 - 38h post-infection. If an obligatory process of development is a precondition for colonisation of the intestine, then that process has been completed by some parasites before 36h.

In experiments 11 - 15 inclusive exact doses of larvae extracted from the nasal region of donor rats were administered to experimental subjects by

stomach tube and the ability to mature in the intestine of larvae extracted at different times compared. Experiment 11 is described as an example of the general protocol applicable in this context. Four rats were infected with 500 larvae and killed at 23 hours (2 rats) and 48 hours (2 rats), at each of which times larvae were extracted from the nasal region and used to infect 6 recipients by the method described in Materials and Methods (mean exact doses 21.0 & 19.2 respectively). All recipients were killed on day 5 and their worm burdens assessed. In experiments 14 and 15, at the same time as the donors received their doses of 500 worms, six control rats were infected percutaneously with exact doses (means 21.7 & 22.0) to be sure that the \bar{p}_f 's attained by the particular larval populations were of a sufficiently high standard. The outline protocols and results of experiments 11 - 15 are given in Table 4. Larvae present in the nasal region at 30h had maximal potential for development in the intestine whereas those extracted at the earlier time of 20h were uniformly incapable of such development.

Measurements of body length, oesophagus length and the oesophagus index were made in experiment 16. Four rats were each infected with 500 larvae by skin application, and one was killed at each of the following times post-infection: 20, 25, 30 and 35 hours. Twenty-five worms were extracted from the nasal region on each occasion. They were fixed by heating and their morphological dimensions determined. The results are shown in Table 5. Analysis of variance of the data for body length prior to infection and the four sampling times reveals significant growth ($F_{[4,120]} = 16.17, P = 1 \times 10^{-10}$). However, this does not occur until 35 hours post-infection. Two similar analyses of oesophagus length and oesophagus index again reveal a change ($F_{[4,120]} = 129, P = 1.56 \times 10^{-42}$ and $F_{[4,120]} = 151, P = 6.5 \times 10^{-46}$ respectively). The change this time is apparent by 25 hours postinfection. Table 5, column 5, shows the proportion of the larvae in which the oesophagus index is greater than 1. By 25 hours 0.24 of the larvae have developed to this

degree, whilst by 30 hours virtually all have done so (0.92). Comparing the OI to the oral infectivity (Table 4) shows a correlation between the proportion of the larvae in which OI>1 and the establishment of the parasite in a donor rat. Larvae in the nasal region at 20 hours post-infection could not survive in the digestive tract. By 23 hours, a small proportion had developed to a sufficient degree, whilst by 30 hours the proportion that could develop to maturity after adoptive transfer was maximal.

D. Development of N. brasiliensis in the lung.

Good evidence has been presented by Twchy (1955, 1956) that N. brasiliensis L3's in the lung of the rat must moult before they continue their migration to the gut. The following experiments were designed to quantify the time course of this change. Tindall & Wilson (1990) have demonstrated that larvae first appear in the gut 40 hours post-infection, so that some individuals in the lung have completed their transformation in advance of this time. Experimental design was similar to that described for S. ratti, except for the donor tissue (lung) and the time when adoptive transfer was assessed (day 8). The results of experiments 17-19 are shown in Table 6. Although worms are found in the lung in quantities sufficient for this experimental framework as early as 20h postinfection, none survived the digestive tract if transferred earlier than 35 hours postinfection.

Table 7 shows the results of a study of growth of N. brasiliensis in the lung carried out by methods analogous to those used for S. ratti.

Analysis of variance identifies significant growth between the infective L3 and larvae in the lung at 45 hours postinfection ($F_{[7,150]} = 320$, $P = 1 \times 10^{-11}$), but those arriving in the lungs at 15 hours are not significantly longer than infective L3's. Maximum growth has been achieved by 35 hours, the earliest time at which some larvae are infective by adoptive transfer. All larvae extracted at 35h were undergoing a moult.

DISCUSSION

Our findings relating to Nippostrongylus brasiliensis reveal no surprises. It is now confirmed with a probability of error of 1 in 10^{10} that the lung is part of the natural pathway of this species, and past reports that a moult is initiated in this location (Yokogawa, 1922; Twohy, 1956) are also confirmed. These statements can be made with unprecedented rigour, but the real purpose of our study of N. brasiliensis lies in the insights it offers when compared with the homologous investigation of Strongyloides ratti, and vice versa. In effect, the two species are each other's controls in this experimental framework.

First, we have shown that sites of 'impedence' for both species are, in fact, locations where mandatory developmental changes are undergone. Larvae adoptively transferred from lung or nasal region when they first arrive in those tissues cannot develop in the gut, whereas larvae transferred later have acquired this capability. The relevant processes take over 20h for N. brasiliensis (Table 6). Tindall & Wilson (1988) found larvae of S. ratti in the nasal region as early as 16h postinfection and this, taken together with the data in Table 4, sets the limits for these events at 7 to 14h for this species. In each case a morphological marker identified the larvae competent to survive in the intestine. They were an oesophagus index greater than unity (S. ratti), and the onset of ecdysis (N. brasiliensis). Our morphological study of S. ratti agrees in essentials with that of Nojima et al (1987), though the times when changes in oesophagus index occurred were earlier in our system.

Second, our findings have major significance for testing the general hypothesis that either parasite reaches its goal by more than one route. Additional pathways that vary fundamentally will provide additional 'nurseries' in which larvae must prepare for the enteric environment. The possibilities are few, assuming the nursery has to give open access to the alimentary tract: they are the nasal region, the lungs, and the liver. The

time taken in this essential developmental change is substantial in both species, so 'impedence', in its wider connotation, would be sufficient to identify alternative nurseries by sampling at autopsy if the associated pathways were quantitatively significant.

Therefore we take our negative results in the head for N. brasiliensis (Table 3), and in the lung for S. ratti (Table 2), to mean that these sites do not function as nurseries for the species concerned. It follows that the pathways of the two parasites are fundamentally different. The fact that identical methods were used to extract S. ratti from the head (Tindall & Wilson, 1988) and N. brasiliensis from the lung in numbers sufficient for a rigorous proof in both cases reinforces the conclusion that negative results have real meaning.

There is no comparable control for assays in the liver, because no larvae of either species could be extracted from it. Findings in this context are therefore interpreted with some caution. However, the hepatic architecture is not intuitively of a type that would present a barrier to the method of extraction, bearing in mind the diversity of the other tissues in which this method succeeds; so our results imply a real absence of larvae from the liver.

The significance of negative results depends entirely on the times of sampling. These were defined by the overall kinetics determined earlier by Tindall & Wilson (1990). Thus the detailed contents of Tables 2 and 3, even though they generally record the absence of parasites from the tissues examined, are nevertheless central to the argument because they demonstrate the frequency of sampling as well as the critical relationship between the sampling times and the timecourse of concurrent and subsequent events in other organs of the host. The \bar{p}_f 's in Tables 2 and 3 also bear witness to the migratory ability of the larvae used.

The conclusion that no part of the head is involved in the pathway of N. brasiliensis is uncontroversial because, as far as can be discovered, no

other workers have investigated the possibility. That the lung is not a significant point of transit for S. ratti runs counter to the consensus view, past and present (see Dawkins, 1989). A substantial number of reports have recorded larvae of this parasite in rats' lungs (Spindler, 1958; Abadie, 1963; Wertheim & Lengy, 1963; Tada, Mimori & Nakai, 1979; Moqbel, 1980; Murrell, 1980; Bell, Adams & Gerb, 1981; Nojima et al., 1987) and the majority of those authors interpreted their findings as denoting a route (Tada et al., Murrell and Nojima et al. were exceptions). Most of these studies were reported in qualitative terms, and none gives a comprehensive quantitative picture of events in the lung. Using a system that differed in many respects from ours, Murrell (1980) found proportions of 0.011 and 0.017 of a subcutaneously injected dose of 1200 larvae per rat in the lungs of two groups of 6 sampled at 22 and 48h postinfection. Only one larva out of a total of 767 we applied to the skin of 39 rats was found in the lungs over four sampling times (23-48h inclusive, Table 2). The period chosen was that most likely to reveal sites of mandatory development judged by events in the head (Tindall & Wilson, 1988) and in the gut (Tindall & Wilson, 1990). Nojima et al. (1987) concluded that the pulmonary route was 'accidental' for S. ratti because the few larvae they found in the lung had a mean oesophagus index which was less than unity and unchanged with time.

The efficiency of the extraction procedure is an important factor. S. ratti is three-fold less synchronised in its migration than N. brasiliensis (Tindall & Wilson, 1990). The magnitude of asynchrony in the former case is such as to make it certain that, assuming the extraction of all individuals present, p_o 's would still not represent the total proportion which passes through a transit site. On this basis, and from the data of Tindall & Wilson (1988), we are sure that a conservative estimate of efficiency is in excess of 50%. Even if it were as low as 50%, the present data are categorical in demonstrating that the lung is effectively not a nursery for migrating S. ratti of the homogonic strain in Wistar rats of

the Edinburgh colony.

In conclusion, our results prove that pathways of S. ratti and N. brasiliensis in the natural host are mutually exclusive in important respects. Thus two nematodes, related in that they are both of rhabditid ancestral stock and having a host, point of entry, and destination inside the host all in common, nevertheless achieve their goal by fundamentally different routes.

This conclusion upholds the criticism by Wilson (1983) of the inductive ideas that have dominated the subject of migration in hosts since its inception, and which, to a significant extent, have led to the erroneous acceptance of a uniform model to describe such migrations.

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Table 1. Testing the inequality $\bar{p}_o + \bar{p}_f > 1$ for N. brasiliensis in the lung. \bar{p}_o estimated at 35 hours post-infection.

Expt	\bar{d}	\bar{p}_o	\bar{p}_f	\bar{p}_c	Probability that \bar{p}_c is due to chance	(df) [#]
1	18.1	0.484 ±0.054	0.782 ±0.030	0.266	0.0015	(10)
2	18.2	0.516 ±0.049	0.731 ±0.037	0.247	0.0062	(8)
3	19.2	0.508 ±0.034	0.802 ±0.042	0.310	0.00094	(8)
4	19.9	0.442 ±0.062	0.788 ±0.026	0.230	0.0059	(10)
All data pooled		0.487 ±0.025	0.779 ±0.016	0.266	1×10^{-10}	(42)

\bar{d} = mean exact dose

\bar{p}_o = mean peak proportion of \bar{d} 's in the lung.

\bar{p}_f = mean maximum proportion of \bar{d} 's in the intestine.

$$\bar{p}_c = [\bar{p}_o + \bar{p}_f] - 1$$

[#] numbers of rats equal in the 2 groups except expt 2, where $\bar{n} = 6$ (\bar{p}_o) & 4 (\bar{p}_f).

Table 2. Experimental protocols and results for migration of *S. ratti* through the liver and lungs.

Expt	\bar{d}	Tissue	Numbers of rats sampled at the following times (h) after infection with exact doses (\bar{d}) (a single larva was recovered from a single rat in the group marked *):						$\bar{P}_r \pm \text{SEM}$ (n)
			18	23	26	40	48	60	
5	18.8	lung	4	—	4*	—	—	—	0.819 ± 0.022 (4)
6	19.4	lung/liver	—	6	—	6	—	—	0.860 ± 0.032 (6)
7	19.8	lung	—	6	—	—	6	—	0.783 ± 0.017 (6)
8	19.4	lung	—	5	—	—	6	6	0.752 ± 0.031 (6)

Table 3. Protocols and results of migration of N. brasiliensis through the head and liver.

Expt.	\bar{d}	Larvae were absent from extracts of the heads and livers of rats sampled at the following times (h) postinfection:					$\bar{p}_f \pm \text{SEM} (n)$
		20	23	30	40	50	
9#	18.6	5	6	--	--	--	0.683 ± 0.052 (6)
10*	21.8	6	--	6	6	6	0.742 ± 0.049 (6)

\bar{d} = mean exact dose; n = sample size

#heads only; *heads & livers

Table 4. Establishment of S. ratti transferred from a donor to a recipient.

Expt	Mean proportion (\pm SEM) of larvae which established in the intestines of recipients after oral transfer from donors at the following times (h) post-infection of the donor (6 rats at each sampling time):							$\frac{\bar{P}_f}{\pm SEM}$ controls ($\Delta = 6$)
	20	23	25	30	35	40	48	
11	--	0.160 ± 0.038	--	--	--	--	0.743 ± 0.047	--
12	nil	--	--	0.648 ± 0.059	--	0.536 ± 0.041	--	--
13	nil	--	--	0.754 ± 0.048	--	--	--	--
14	nil	--	0.386 ± 0.045	0.820 ± 0.044	0.779 ± 0.020	--	--	0.809 ± 0.047
15	nil	--	0.124 ± 0.023	0.784 ± 0.037	0.790 ± 0.045	0.523 ± 0.056	--	0.778 ± 0.025

Controls were infected at the same time as the donors.

Mean exact doses ranged from 19.2 to 22.8.

Each group of 6 recipients was infected from the pooled worms from 2 donors.

Table 5. Growth of S. ratti in the nasal region of the rat head (expt 16).

Time (h) post- infection	Total body length (mm) mean±SEM	Oesophagus length (mm) mean±SEM	Oesophagus index (OI)	Proportion of larvae where OI>1
0	0.621 ±0.0036	0.272 ±0.0015	0.781 ±0.0075	nil
20	0.616* ±0.0022	0.274* ±0.0020	0.804* ±0.0085	nil
25	0.632* ±0.0028	0.309 ±0.0020	0.955 ±0.0095	0.24
30	0.630* ±0.0043	0.324 ±0.0035	1.066 ±0.0170	0.96
35	0.659 ±0.0065	0.351 ±0.0046	1.143 ±0.0018	0.92

* No significant change from L3 prior to infection (Time 0).
Sample size = 25 worms from 1 rat at each time.

Table 6. Establishment of N. brasiliensis after transfer from a donor to a recipient

Expt	Mean proportion of <u>N. brasiliensis</u> that established in the guts of recipients after oral transfer from the lungs of donors at the following times postinfection of the donor (6 rats at each time):					\bar{p}_f \pm SEM controls $n = 6$
	20	25	30	35	40	
	17	nil	nil	nil	nil	
18	—	—	—	0.073 \pm 0.030	0.700 \pm 0.020	—
19	nil	nil	nil	0.041 \pm 0.015	0.545 \pm 0.020	0.738 \pm 0.083

Controls infected at the same time as the donors.
 Mean exact doses ranged from 19.5 to 22.0.
 Each group of 6 recipients was infected with worms pooled from 2 donors.

Table 7. Growth of N. brasiliensis in the lung.

Time (h) post-infection	Total body length (mm) mean±SEM	Sample size
0	0.649±0.005	30
15	0.645±0.008*	20
20	0.757±0.010	20
25	0.918±0.011	21
30	0.962±0.007	21
35	1.029±0.011	20
40	1.021±0.014*	16
45	0.989±0.011*	10

* No significant growth over previous sampling time