

**"STUDIES ON THE ROLE OF TISSUE INVADING
HELMINTHS IN BACTERIAL INFECTIONS."**

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for the Degree of Ph.D.**

by

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CONTENTS

	<u>Page</u>
Review of the Literature.. .. .	1
The Parasite <u>Nippostrongylus brasiliensis</u>	47
Method of Culturing Larvae	49
Method for Collecting Maximum Number of Eggs	50
Method of Preparing Petri-dish Culture ..	50
Hatching of Eggs.. .. .	52
Method of Counting Larvae	55
Methods and Technique of Infecting the Experimental Animals	57
(1) Oral infection	57
(2) Percutaneous infection	58
(3) Mixed infection (percutaneous and oral)	58
(4) Infection through the intact skin of the tail	60
Method of securing animals in position	60
Exposure of intact skin of the tail to a mixed infection of <u>N. brasiliensis</u> larvae and <u>Str. pyogenes</u>	61
Experimental animals used	61
The micro-organism used	61
The parasite.. .. .	61
Technique employed	61
Experiment No. I.. .. .	62
Result	62

	<u>Page</u>
Experiment No. II.. .. .	63
Experiment No. III	64
Experiment No. IV.. .. .	66
Conclusion	67
(5) By injection	68
a) Subcutaneously	68
b) Intraperitoneally	68
c) Intrathoracically	69
Conclusion.. .. .	70
(6) Per rectum.. .. .	70
Factors Influencing the Invasion of the Experimental Host	71
Degree of Moisture Present on the Skin	71
Behaviour of the Parasite on Application to the Skin.. .. .	74
Duration of Penetration of the Skin	77
Route of Migration within the Experimental Animal	81
Personal observations	81
Procedure Adopted	81
Experiment No. I.. .. .	82
Experiment No. II	83
The Migration of the Larvae Inside the Body to the Lungs via the Lymph System	83
Experiment No. I.. .. .	84
Result of Examination	84

Page

Experiment No. II.. .. .	84
Result.. .. .	85
Experiment No. III	86
Experiment No. IV.. .. .	87
Experiment No. V	88
Result.. .. .	89
Conclusion	92
The Route of Migration of <u>N. brasiliensis</u> from the Lungs to the Small Intestine.. .. .	92
A Comparative Study of the Behaviour of <u>N.</u> <u>brasiliensis</u> in Different Kinds of <u>experimental Animals</u>	93
1) Longevity of the Parasite.. .. .	93
Technique.. .. .	93
Result	94
2) Route of Migration Inside the Body of Experimental Animals	94
Animals used	94
Mode of infection.. .. .	95
Larvae used	95
Technique used	95
Result of examination.. .. .	96
The Degree of the Reaction in the Tissues of the Under Surface of the Skin of Animals after the Application of the Infective Larvae	101
Animals used.. .. .	101
Helminth used	101
Technique employed	102

Result	102
Summary of the Investigations Carried out on the Parasite (<u>N. brasiliensis</u>)	103
Studies on the Role of the Tissue-Invading Helminth (<u>N. brasiliensis</u>) in Bacterial Infection	105
The Application of Pathogenic Micro- Organisms and Infective Larvae to the Intact Abdominal Skin and their Recovery from the Subcutaneous Tissues	105
Experimental animals.. .. .	105
Micro-organisms	105
The helminth.. .. .	105
Technique employed	106
Experiment No. I (<u>Staphylococcus aureus</u>)	108
Experiment No. II (<u>Streptococcus</u> <u>pyogenes</u>)	108
Conclusion	110
The Identity of Some of the Micro-organisms Inside and/or Adherent to the Surface of Infective Larvae from Normal Cultures	111
Technique employed	111
Recovery of the Micro-organisms from the Lymphatic Glands after the Application of the Helminth and Micro-organisms to the Intact Skin	112
Experimental animals.. .. .	112
Micro-organisms	112
Helminthic material	113
Technique employed	113
Procedure	113

Results	129
(3) The Simultaneous Application of Infective Larvae of <u>N. brasiliensis</u> and Bacterial Emulsion of <u>Sal. typhi-marium</u> to Unsoured Animals.. .. .	131
Experimental Animals.. .. .	131
The Micro-organisms	131
Helminthic Material	131
Technique Employed	131
Results	132
(4) The Simultaneous Exposure of Animals to an Emulsion of <u>B. anthracis</u> and Infective Larvae of <u>N. brasiliensis</u>	132
Experiment No.1	132
Results	133
Experiment No.2	134
Results	134
(5) The Oral Infection of a Rabbit and Guineapigs with Metacercariae of <u>Fasciola gigantica</u>	135
Experiment No.1	135
Experiment No.2	136
Results	137
Post-Mortem Findings.. .. .	137
Conclusion	138
Discussion	140
Summary and Conclusion	145
Addendum.. .. .	146
References	147

	<u>Page</u>
Experiment No.1	116
Experiment No.2	116
Result of the Experiments	116
Conclusion	116
The Simultaneous Application of Bacteria and Varying Numbers of Infective Larvae of <u>N.</u> <u>brasilensis</u> to the Intact Skin of Animals	118
Experimental Animals.. .. .	118
The Micro-organisms	118
The Parasitic Helminth	119
Technique Employed	119
(1) The Application of <u>Str. pyogenes</u> and Various Numbers of Infective Larvae to the Intact Skin	121
Experiment No.1	121
Results	121
Experiment No.2	122
Results	122
Experiment No.3	122
Results	123
Experiment No.4	124
Conclusion	126
(2) The Application of <u>Str. equi</u> and Various Numbers of Infective Larvae to Intact Skin	128
Experiment No.1	128
Results	128
Experiment No.2	129

Review of the Literature

Various investigators have from time to time attempted to prove or disprove that the penetration of the skin or the mucosa of the gastro-intestinal tract by parasitic helminths facilitates the entry of pathogenic bacteria or viruses into the body of the host.

Besnoit and Cuillé (1898) published a short account of a serious epizootic occurring in the South West of France amongst sheep and cattle. Deaths were chiefly due to fascioliasis and haemorrhagic septicaemia. These authors concluded that internal parasitism brings about a condition predisposing animals to microbial infection by causing local injury.

Lignières (1898) associated haemorrhagic septicaemia of sheep with verminous bronchitis and gastritis. The infection was supposed to be assisted by the injuries to the mucous membrane imposed by the worms.

Cay (1900) recovered varieties of organisms in seven cases of fistulous withers. These were a streptococcus and a micrococcus. In two cases of poll evil a streptococcus and a micrococcus were cultured respectively.

Faure and Marotel (1902) examining the caecal lesions produced by strongyles of equines stated that certain of these lesions are infected, and around them there are large inflammatory foci infected with bacteria - micrococci, streptococci, and ovoid bacteria.

Ketchnikoff (1906) suggested that helminths may inoculate the intestinal tract with bacteria thus setting up bacterial infection. At first his theory was met with strong opposition but he persisted in his researches and mentioned particularly the role played by intestinal parasites in causing appendicitis.

Basset and Carré (1907) demonstrated that the intestinal wall of the dog could be rendered permeable to the coccus of Mathis through a previous injury with drastic purgatives. From this Carré (1928) stated that similar effects may be produced through lesions caused by ascarids in the dog, and other intestinal parasites in sheep. He tried a series of experiments to reproduce Aynaud's "botriomycosis of sheep" by feeding them with large quantities of the causal organism. Eleven lambs born and reared at the

laboratory were used for his first attempt to prove this theory, but he was not successful. In a second attempt one of six lambs became infected. These six lambs were harbouring intestinal coccidia and worms, and it was thought that parasites may have been responsible for the successful infection.

Weinberg (1907) gave a detailed microscopic description of twenty-three cases of oesophagostomiasis in various species of apes. He maintained that sometimes ruptured cysts became infected with bacteria from the intestinal tract and ulceration followed. He was of the opinion that death was due to septicaemia, and not to substances secreted by the worms. He failed to recover any bacteria from the blood because he did not select the proper media for their cultivation.

Dutcher and Whitmarsh (1915) were successful in cultivating from the blood and exudate fluid in sixteen cases of filariasis (elephantiasis, lymphangitis, etc.) a bacillus resembling B. subtilis. Controls were negative. They proposed the name Bacillus lymphangitis for this organism and believed it to be the cause of diseases grouped under Filariasis.

Jacobsen in 1915 reported a case of a three-years-old girl who suffered from repeated attacks of ascariasis for six months. Anthelmintic treatment gave transient improvement. He performed laparotomy which revealed generalised peritonitis caused by the ascaris perforating the intestinal wall. The child died seven hours after the operation, and at autopsy he found that the common bile duct was occluded and the liver perforated throughout by the ascarids, causing abscesses and destruction of the tissues. He removed forty-nine worms from the liver tissue and ninety-four from the intestinal canal.

Leneveu (1915) noted that strongyles in the horse could produce the effects of a toxic nature characterized by an elevation of temperature sometimes more than 40°C, but he conducted no bacteriological examination.

Stephens (1916) quoting Dauernheim and Saltykow stated that when ascarid worms infect the biliary passages they introduce intestinal bacteria setting up liver abscesses.

Malvoz and Lambient (1918) stated that they produced a generalised tuberculosis infection in a guinea-

pig by placing on a shaved area of the skin a suspension of Ancylostoma larvae together with sputum containing the bacilli. In another experiment they used B. anthracis in the same way and infection was very readily produced. The authors are of the opinion that, in addition to the direct inoculation of bacteria such as was experimentally obtained by them, the Ancylostoma larvae may act in a more important way as carriers of bacterial diseases from the faeces of infected persons to other hosts.

Robson (1918) reported the presence of filarial worms of the withers of equines in Australia. He believed that some of the infected animals developed true fistulae of the withers by "lodgement of stray pus germs on the already inflamed, worm-infested area".

De Blicck and Baudet (1920) carried out some controlled experiments to ascertain whether Strongyloides larvae can take with them bacterial infection through the skin. In the first experiment the abdominal skin of a guinea-pig was shaved and a mixture of Strongyloides westeri larvae and Staphylococcus aureus were pipetted on to the skin. After five minutes they disinfected the site with soap and tincture of iodine. A piece of skin was excised under sterile conditions and

cultivated on three agar plates. Two days later they recovered colonies of Staphylococcus aureus in large numbers.

In a second experiment they removed hair from the back of two rabbits using barium sulphide in order not to damage the skin. The prepared site was treated with a suspension of fowl cholera bacillus and after quarter of an hour they applied larvae to the skin of one of the two rabbits. Later they disinfected the skin using soap, tincture of iodine, and collodion iodoform. The rabbit which was treated with bacteria and larvae died after forty-eight hours, while the control which had bacteria only remained alive.

In a third experiment two guinea-pigs were treated with anthrax bacilli taking the same precautions as in the previous experiment. Fifteen minutes later larvae were applied to the skin of one of the guinea-pigs, which died of anthrax two days later, while the control remained alive.

In a fourth experiment the hair on the back of two mice was clipped and the skin damped with a suspension of bacillus of swine erysipelas. Larvae were added to the site of infection of one of them, and the skin disinfected as before. Three days later

the mouse which was infected with larvae and bacteria died of swine erysipelas while the control remained alive.

From these experiments the authors concluded that the larvae during their invasion of the host carry pathogenic bacteria which could infect man and animals.

Hall (1920) in an article on parasites and parasitic diseases of sheep mentioned the presence of Oesophagostomum cysts containing yellowish or greenish necrotic material which may be of a caseous or calcareous nature. He ascribed the complications produced by the worms to large portions of the intestinal mucous membrane being rendered functionless by the lesions produced therein by the parasites and the absorption of toxic substances secreted by the worms and from the necrotic material of the lesions produced by the parasite.

Theiler (1921) described the post mortem appearances of oesophagostomiasis in sheep as those of a pronounced anaemia, hydraemia and serious atrophy of fat. Nodules were present in the small and large intestine. He classified the three types of nodules as (1) reddish nodule which is the young nodule, (2) green nodule which contains green pus, (3) the hard nodule in which

calcification has taken place. This author stated that there are two serious complications which may arise as a result of the nodular worm infestation, intussusception and septic infection of the serous cavities.

Brumpt (1922) recognized four types of pathological processes produced by the presence of Fasciola hepatica in the biliary passages:-

- (1) destructive
- (2) mechanical
- (3) irritative
- (4) toxic and bacterioferous action, due to general absorption into the system of toxic byproducts and invasion of bacteria into ulcerated areas.

Caslick (1922) examined the ligamenta nuchae of twenty-four horses, one male, and one colt. He found that twenty-four of these ligaments contained Onchocerca and showed calcification. Twenty-four of these ligaments were infested at the withers, sixteen at the poll and sixteen throughout the entire ligament. The most distinctive lesion of infested ligaments was calcification, a change which as well as parasitism always affected the funicular portion of the ligamentum nuchae.

Van Volkenberg (1923) studied calcification of the ligamentum nuchae of the horse. He recovered a parasite resembling Onchocerca cervicalis from twenty-two ligaments out of 102 cases examined. He made cultures from forty ligaments and B. coli, M. pyogenes albus and M. pyogenes aureus were isolated from thirteen cases. The rest were sterile.

Leiper (1924) commenting on present-day teaching of helminthology in relation to public health stated that although intense infestation does cause death, hookworm does most harm by lowering the working efficiency of the infected and rendering them more susceptible to intercurrent bacterial infection.

Aynaud (1926) recorded a Bradset tumour in a heifer belonging to a herd infected with verminous bronchitis. On cutting into the tumour he recovered nematode larvae. Between February and March he observed an epidemic disease of sheep, blackleg, and Braxy also known in Europe as "de Bradset" which was common in the North of Europe and Australia but not in France. In three animals which he was asked to examine he noticed the presence of strongyle as well as other larvae in the rumen. These findings led him to believe that blackleg and Bradset may be inoculated by worms.

Joest (1926) described the young nodule of oesophagostomiasis as consisting mainly of lymphocytes, together with the Oesophagostomum larva in a relatively small amount of broken tissue in which eosinophiles are present, fibroblasts surround this central mass. These young nodules are situated immediately under the muscularis mucosae. The larvae may migrate into the sub-mucous tissues and the nodules undergo caseation and necrosis, ulcers sometimes developing.

Koidzumi (1927) reported that Ugami et al. infected guinea-pigs with Ascaris larvae and seventeen days later gave them an intra-peritoneal injection of tubercle bacilli. The results which they obtained were that controls became more severely infected with tuberculosis than did those which had been infected with Ascaris.

Biester and Murray (1928) found that the lesions produced in healthy swine are less advanced than those in sub-normal subjects. They believed that the stunted growth of the pigs was due to Ascaris lumbricoides and that the parasite played a direct part in the production of advanced lesions.

Carre (1928) attributed a very important role to intestinal parasites in facilitating bacterial infection by producing injuries of the mucosa of the alimentary tract.

Davis (1928) observed that horses used continuously in cities are seldom affected with fistulous withers and stated "I should say that a hundred agricultural horses are affected for every one used constantly in town work, and never turned out to grass". He believed that the condition is infectious as glanders or tuberculosis.

Edgar (1928) demonstrated the presence of Cl. oedematiens in the liver of apparently healthy sheep in "Black disease" areas. He suggested that the movement of immature flukes damage the liver and set up the disease. The disease was produced experimentally in sheep by feeding them on toxin free spores of Cl. oedematiens, and the cercariae of Fasciola hepatica. The author concluded that the organisms probably penetrate the liver with the flukes in their migrations and that the bacteria multiply in the necrotic foci resulting from the parasitic invasion giving rise to a rapidly fatal bacterial toxæmia.

Bayon (1929) dealing with an outbreak among fowls suggested that massive infestation with Davainea proglottina brings about a condition similar to paralysis and leucaemia of fowls.

Joyeux and Baer (1929) carried out some experiments on the transference of the plerocercoids of Diphylobothrium ranarium and observed that in spite of the great injury which might have been caused by the larval stage of this parasite while passing through the walls of the stomach or the intestines, no bacterial invasion was noticed in the peritoneum. These authors claim to have proved that sparganum produces a bactericide which is capable of inhibiting the growth of bacteria in broth media.

Kawanishi (1929) reporting on the entrance of pathogenic bacilli incidental to percutaneous infection with hookworm expressed the opinion that in percutaneous infection with Ancylostoma larvae, bacilli adhering to the larvae may be carried into the human body and infections with the bacilli may result. He reported the frequent occurrence of infectious diseases as well as ancylostomiasis in Formosa. He recovered various bacilli such as B. coli communis, B. subtilis, B. pyocyaneus, staphylococci and streptococci from the surface of Ancylostoma larvae. He stated that the bacilli were adherent to the surface of the larvae as long as they were on the soil. He washed the larvae

with distilled water and claimed that the bacteria were not removed by washing in distilled water or by passage through agar or by their penetration through the skin. This author believed that the penetration of Ancylostoma larvae into the human body will carry the adhering bacilli with it. He could not find any bacilli within the larvae but he commented on the presence of a ferment with bactericidal properties in the Ancylostoma larvae.

Von Bülow (1929) described a case which was suggested by the circumstances to be an inactive pulmonary tuberculosis which was re-activated by the passage of hookworm larvae through the worms. He believed that the larvae may sometimes carry tuberculosis from infected faeces to a new victim.

Ackert and O'Neal (1930) examined twelve ligamenta nuchae from various animals and ten of the ligaments showed either portions of the parasitic worm Onchocerca cervicalis or calcified areas characteristic of previous infestations with this parasite. He did not mention bacteria in relation with the parasite.

Basset and Moulin (1930) observed the death within three days of young mules infected with strongyles. At the autopsies the deaths were found to be due to

coli bacillary septicaemia. Microscopic investigation confirmed the presence of considerable numbers of bacterial colonies in the abdominal cavities and different organs of the body. These authors stated that the presence of B. coli in the different organs is a proof of the role played by the parasite in invading the caecal wall and thus allowing the bacteria to invade the tissue via the lesions caused by the worms.

Fitch et al. (1930) examined samples of serum from seven cases of poll evil and thirty-seven of fistulous withers for agglutination against Brucella abortus. In all the cases of poll evil the titres ranged from 1 in 50 to 1 in 1000. Pus was not obtained from any of these lesions for bacteriological examination. Of the thirty-seven cases of fistulous withers examined, four showed no agglutinins at 1 in 50, but two of these had recovered two years or more; the remainder reacted at from 1 in 100 to 1 in 1000. In nine of these cases bacteriological examination of the pus was made and from three of them Brucella abortus was obtained.

Turner (1930) was able to show that in "black disease" the necessary conditions for the growth of Cl. oedematiensis organisms were found in the necrotic

areas produced by invasion of the liver with the wandering immature liver flukes, Fasciola hepatica. In these areas the organisms multiplied and gave rise to a toxin which was responsible for the disease.

Kinsley (1931) referred to oesophagostomiasis and haemorrhagic septicaemia in steers. He reported a steer which had died and at autopsy found extensive sero-gelatinous infiltration in the cervical region. The infiltration extended into the pectoral region and showed numerous small haemorrhages. There was some evidence of oesophagostomiasis. Laboratory tests of heart blood and sero-gelatinous material from the cervical region revealed the presence of bi-polar organisms. From the findings he assumed that intestinal parasitism was the primary cause of the disease and that haemorrhagic septicaemia caused the death of the animal.

Taylor and Purchase (1931) carried out some experiments to ascertain whether nematode larvae in penetrating the intestinal mucosa play any part in assisting pathogenic bacteria to invade the host from the lumen of the bowel.

In the first experiment they fed twelve guinea-pigs with a suspension of a vegetative form of B.

anthracis and Ascaris lumbricoides eggs containing infective larvae. Another twelve guinea-pigs received the same doses of bacteria only.

In the second experiment they used anthrax spores and Ancaris eggs.

In the third experiment they fed H. suispestifer and Ascaris eggs to rabbits and bacteria only to controls.

In the fourth experiment they increased the dose of bacteria used in the third experiment. They claimed that the results which they obtained were completely negative and that the conditions of their experiments were designed in such a way as to provide for the passage of pathogenic bacteria along the intestinal tract during the time large numbers of nematode larvae were actively penetrating the mucosa. The reason they suggested for this result was that the injury caused by the passage of the minute larvae through the tissues was insufficient to permit the ingress of sufficient bacteria to bring about infection.

Carne and Ross (1932) investigated the relation between caseous lymphadenitis and nodular worm O. columbianum in sheep. Five lambs were simultaneously drenched with bacterial cultures of Preisz Hoard

bacillus and larvae of O. columbianum. The bacterial organism was recovered from one mesenteric gland in one lamb only, although the nodular disease was well marked in the others. Twelve abdominal glands and three livers were invaded by the parasite but all of them were sterile. These authors believe that the injuries caused to the intestinal wall by the parasite are not a common way of entry of the organisms under natural conditions.

Bennetts and Filmer (1932) in Australia examined the serum of a chronic case of fistulous withers and found that it agglutinated Brucella abortus at 1 in 100. The serum of a second case with "bursitis at the withers" and a much enlarged un-opened bursa at the poll, agglutinated at 1 in 2000. As controls sera from three normal horses and from two affected with "quitter" were examined. Four agglutinated at 1 in 20 and the fifth was negative.

Dickmans (1932) has recovered a species of Onchocerca (O. flexuosa) in subcutaneous abscess in a deer.

Le Roux (1932) stated "Recently I observed cases of Quarter Evil in cattle which had evidently become

infected with the organisms of that disease through the agency of liver fluke found on the grass."

Kawanishi (1932) carried out percutaneous infection with Necator americanus (150-700 mature larvae) on three persons. A few minutes after the application of the larvae the site of infection began to itch and two hours later there appeared numerous red spots which turned into papules after several days. In one person a number of abscesses developed from these papules. The author maintains that this has an important bearing on the role that bacteria are introduced by the penetrating larvae. B. coli communis was always found adhering to the larvae and were recovered in pure cultures from the pus of these abscesses.

Basset and Moulin (1933) stated that Larotel on examining wounds caused by strongyles in the caecum of equines found that some of these wounds were infected with bacteria - micrococcus, streptococcus, and ovoid bacteria. They mentioned that in Germany numerous authors (Kasso, Otto, etc.) reported cases of septicaemia of a verminous origin in foals. They mentioned B. viscosum as one of the bacteria which they recovered. Basset and Moulin conducted some experiments on young

mules to study the role of septicæmia due to B. coli and B. viscosum. They concluded that in young equines strongyles of the intestine if present in large numbers cause inflammation of the mucosa of the intestinal wall thus permitting the invasion of these organs by bacteria causing septicæmia and death. In cases in which the parasitic helminths are very few they consider it very doubtful that the parasite predisposes the animal to septicæmia. They recovered B. coli and B. viscosum from the septicæmia noticed in parasitised mules.

Hetch (1933) fed large numbers of embryonated eggs of Ascaris lumbricoides and of Parascaris equorum, together with cultures of anaerobic pathogenic bacteria to guinea-pigs. It was found that these larvae during their usual migrations did not infect the tissues with these pathogenic organisms except in one animal.

Loffat Thomson, et al. (1933) stated "Onchocercosis appears to be very common in cattle. Two species of Onchocerca occur, one affecting the musculature and somewhat simulating cysticercosis and the other infesting connective tissues in the region of joints. There is a presumption that the joint swellings hitherto known as Lob-Knee Disease are to be ascribed to this

cause." Le Roux in a personal communication told me that Br. abortus was isolated from these lesions.

Steward (1933) found that twenty horses out of thirty suffering from fistulous withers and poll evil were infected with Onchocerca cervicalis. The results of the examination of fifty-three cases show that thirty-eight harboured the parasite. There was no mention of any bacteriological examination.

Steward (1934) examined thirty horses suffering from fistulous withers and poll evil and Onchocerca cervicalis was present in sixty-six per cent of them. He investigated the possible relation of Brucella abortus to the disease. The discharge from twelve cases were cultured and Brucella abortus was obtained from only one case.

Woodridge (1934) referring to fistulous withers mentioned that "in some instances, however, infection appears to have occurred by way of the blood stream with streptococci or with Brucella abortus, while the primary cause in other cases is a nematode worm, "Onchocerca cervicalis". (Onchocerca cervicalis).

Beveridge (1934) suggested in his preliminary work on foot rot in sheep that percutaneous infection with

larvae of Strongyloides papillosus may provide a means of entry for the causal organism Bacillus necrophorus. He considers that this may be the common way in which the disease is set up in the field and suggested that the skin lesions produced by the application of S. papillosus larvae indicate that percutaneous infection with this nematode may also provide a way of entry for bacteria causing other diseases of sheep. It is apparent from his observations on the epidemiology of the disease that near contamination with the causal organism, whatever it may be, is not sufficient to produce foot rot under all conditions. The disease has been produced experimentally by scarification of the skin of the interdigital space and application of pus from natural lesions, or cultures of B. necrophorus isolated from foot lesions, but where no scarifications were carried out the results were irregular.

Wooldrige (1934) describing the disease of foot rot in sheep blames the presence of a micro-organism in the sole which gains entrance to the subcorneal tissue and there produces its pathogenic effects. He suggests that a favourite habitat of the organism is

wet or marshy places, especially those soiled with an accumulation of excreta such as dirty folds. When one case of the disease appears in such a place it rapidly spreads through the flock. He mentions that B. necrosis is usually found in the lesion and he believes that its entrance is secondary.

Lusgrave (1935) stated that out of 242 children examined at the child clinic in Louth, Irish Free State, 151 were infected with Ascaris and/or Trichuris. The majority of the infected cases were under seven years of age. Lusgrave suggested that the larval Ascaris may aid the passage of tubercle bacilli through the intestinal wall at the time of migration.

Le Roux (1935) reported that "a species of Onchocerca which appears to be identical with the species considered to be the causal factor of Lobknee in cattle were collected from the connective tissue in the legs of two duikers, three reedbuck and one roan antelope bull. The latter was suffering from a well developed Lobknee lesion." From personal communication Le Roux has stated that Brucella abortus was isolated from these lesions.

Steward (1935) examined 49 cases of fistulous withers and poll evil and suggested the following classification of the types of such disease:-

(1) Onchocerca - infected cases without secondary infection. The secondary infection may occur subsequently and will almost certainly follow exploratory or operative interference.

(2) Onchocerca - infected cases plus Br. abortus infection. These may later develop into the third type of infection with pyogenic organisms occurring.

(3) Onchocerca - infection plus secondary pyogenic infections as with streptococci or staphylococci.

(4) Infection with pyogenic organisms alone. These are probably the superficial or non-typical cases, possibly of traumatic origin.

(5) A possible fifth type, infected with Br. abortus alone or complicated with secondary pyogenic infection.

Steward believes that examinations for microfilaria that give negative results can not be held to exclude the presence of the worms, because in his opinion these may be present as immature live specimens or dead adults.

Taylor (1935) carried out some experiments with parasites of Graphidium strigosum and Trichostrongylus retortaeformis, in order to produce extensive wounds in the tissues, and B. suispestifer of which a fatal infection can easily be produced by the administration of a sufficient dose of a broth culture per os.

In the first experiment twenty-four rabbits were divided into two equal groups. The first group received the Graphidium larvae on nine occasions spread over a period of eleven weeks. Fifteen days after the last dose of larvae all twenty-four rabbits received through the stomach tube, a dose of broth culture of virulent B. suispestifer.

In his second experiment fifty-one rabbits were divided into two groups, a test group of twenty-seven and a control group of twenty-four. The test group was sub-divided into three equal groups of nine which subsequently received doses of infective larvae of T. retortaeformis in the proportion of one, two, and four respectively. Three doses, at intervals of three days, were given to the rabbits in the test group. Eighteen days after the last dose all rabbits, in the test and control groups, received orally, a dose of broth culture of B. suispestifer.

The result he obtained indicated that the worms fail to play any part in introducing infection. Taylor concluded that "although the conditions in these experiments appear to have been very favourable for the bacteria present in the lumen of the bowel to invade the host through abrasions of the walls caused by the parasitic worms, the evidence of assistance given by the worms to the entry of bacteria has not been obtained." He thinks it highly probable that the injuries caused to the wall of the bowel by parasitic worms do not play any important part in creating bacterial infection from the lumen of the intestine.

Morales Otero and Pomales Lebron (1936) demonstrated the presence of agglutinins against haemolytic streptococci in 68 patients suffering from recurrent tropical lymphangitis. They demonstrated the presence of agglutinins in normal persons but in a lower concentration than in others infected with lymphangitis. Many cases of tropical lymphangitis gave allergic reactions to skin injections of haemolytic streptococcus filtrate.

Berny (1936) in French Guiana has observed that 30% of the cases of tuberculosis were carriers of hookworms. There was a marked eosinophilia in about

50%. The tubercle infection ran a similar course in parasitised and non-parasitised patients.

Poisson (1936) suggested that the markedly benign character of tuberculosis locally found in man and animals in Madagascar may in some way be associated with intense helminthiasis. He based that opinion on purely circumstantial evidence.

In Ray's experience (1936) a culture from acute filarial condition has been negative for streptococci or other bacteria.

Fourie (1937) carried out some experiments to investigate the lesions of oesophagostomiasis in lambs. The author stated that there is no evidence that worms carry pathogenic bacteria with them during primary parasitic migration. The primary migrating larvae may produce pathological changes in the mucous membrane especially in severe infestations, so that bacterial invasion of the mucous membrane can take place from the lumen of the intestine and produce various forms of enteritis. He believed that if the animals were exposed to continuous infection on the pasture primary and secondary parasitic migration would probably be present in many animals at the same time. In such

cases bacterial invasion of the sub-mucosa and the other tissues of the intestinal walls, deeper than the muscularis mucosae, can also occur and produce deep inflammatory processes in the intestinal wall and peritonitis in cases where the serosa becomes perforated. If enteritis is present the condition may be a contributory factor in the production of diarrhoea, a characteristic of the disease. It is his belief that: "it is very difficult to assess the importance or otherwise of the role played by bacteria in oesophagostomiasis. There is no doubt whatsoever that the parasites can and do produce the death of the animal, in cases where bacterial complications are not present, but to judge from the number of cases in which bacteria were shown to be present in the deeper portions of the intestinal wall, during the course of this study, there must be quite a number of cases in which bacteria are a considerable contributory factor in the production of symptoms and of mortality."

Le Roux (1937) in the annual Report (for the year 1936) of the Veterinary Department, Government of Northern Rhodesia, reported that a specimen of Onchocerca cervicalis was collected from the ligamentum nuchae of

a horse that was imported some years ago from Bechuanaland. The worms were extensively spread throughout the substance of the ligament and were not encountered in the loose connective tissues surrounding the structure. Le Roux believed that this species was responsible for the abscess-like formations, containing sterile fluid, which he observed in that country and elsewhere in East Africa.

Krober (1937) reported tuberculosis of the testicles in a young person brought into hospital for an operation. Preliminary investigation showed the presence of Ancylostoma eggs in the stool and a fair number of leucocytes in the sediment of the urine. This author, judging Sourur's opinion that tuberculosis and schistomiasis can be found in different organs side by side, suggested that in that case Ancylostoma was associated with tuberculosis of the testicles and neighbouring organs.

Martini (1937) suggested that ascariasis may predispose to poliomyelitis. He had noticed that children with infantile paralysis seemed to harbour round worms more frequently than did other children which are not infected with this disease.

Podyapolskaya and Dedova (1937) carried out some experiments to determine the relation between the infestation by ascarids and bacterial infection.

A. lumbricoides and A. suis were used with mouse typhoid bacteria (Bact. Berslau) and they concluded that the migrating larvae produced an earlier and more severe bacterial invasion than in the controls.

Rajahram (1938) reported that a six-year-old girl died of liver abscesses in which several ascarids were found. One abscess had opened in the epigastric region, leaving a sinus from which three ascarids had escaped while she was under observation in the hospital, at which time she had emaciation, fever, and an enlarged liver. At autopsy there were still two worms in the cavity into which the sinus ran. The dilated and inflamed bile ducts showed other worms and there was one in the duodenum. He did not mention any bacteriological examination of the abscess.

Stein (1939) et al., studying the possible role of endo-parasites in the transmission of infectious anaemia, stated that worms of the genus Strongylus from a case of equine infectious anaemia contained the virus of this disease. When they injected the saline

extract of the washed and macerated worms into a normal horse they reproduced the disease. Similar infections made from cylicostomes gave a negative result. Saline washings of the worms before extraction proved to be negative which indicated that the virus was completely absent from the external surface of the worms.

Baumann and Kment (1941) gave a brief account of the pathological significance of Onchoerca reticulata and fistulous withers. They concluded that O. reticulata causes damage to the tissues which when examined show either an inflammation or pus or both.

Shope (1941) demonstrated that the pig lung-worms Metastrongylus elongatus and Choerostongylus pudendotectus were capable of harbouring swine influenza virus and of transmitting it from animal to animal. The virus can remain dormant in the helminths for two years. The virus was present in a benign form and some means of provoking it into activity was required. He was not able to detect the presence of the virus by direct means in either the larval lungworm, in their annelid intermediate host, or in adult lungworm removed from swine lungs.

Spindler (1942) reported that Strongyloides ransomi may under certain conditions be responsible for the sudden death of pigs. He drew attention to the fact that Strongyloides larvae invade the myocardium of pigs, and that partial heart block may follow such infection. He thought that there was a possibility that larvae of S. stercoralis may penetrate the myocardium of infected persons and cause similar injuries. No mention was made of the presence of bacteria.

Fernando and Balasingham (1943) reported fourteen cases suggestive of encephalitis and one case of meningitis in a series of one hundred and sixty-two cases of acute ascariasis.

De Blicck and Jansen (1944) demonstrated that mice could be infected with Str. pyogenes on applying this bacteria and Strongyloides westeri larvae to their skin. Six minutes after the application of the larvae, Str. pyogenes was demonstrable in the sub-cutis. Eight out of nine mice infected simultaneously with the larvae died of pyo-septicaemia. They proved that it is possible to infect the sub-cutis of mice with

Shigella equili in the same manner. From their findings they suggested the possibility that the spontaneous infections with Str. pyogenes and Shigella equili occurring in colts are also brought about via the skin by means of S. westeri larvae. Canaries were found to be very susceptible to Shigella equili when bacteria and larvae of S. westeri were applied to their skin.

Gruttner (1944) found that seven of a herd of fifteen young cattle were carriers of Gartner bacillus (Salmonella enteritidis) and that all seven were in more or less advanced stage of liver fluke disease. He concluded that the liver fluke infection lowered resistance to bacterial infection although the general condition of the animals may still appear healthy.

Pollak (1944a) examined 1070 tuberculous patients for intestinal parasites. He stated that the incidence of parasites was high in the tuberculous group. He explained this as a reason for the reduced resistance to tubercle infection brought about by most parasites and by re-infection with tubercle through lesions of the intestinal wall.

Pollak (1944b) had administered Ascaris antigen and attenuated strain of Koch's bacillus sub-cutaneously

into guinea-pigs, the controls having either one or the other. After six days he demonstrated an allergic reaction to Ascaris in all guinea-pigs which had received antigen. The presence of Ascaris antigen delayed the onset of sensitivity to tuberculin although it increased the number of tubercular lesions which were apparent at autopsy.

Coppin (1945) stated that Dicrocoelium lanceolatum (D. dendriticum) often associated with Fasciola hepatica is of frequent occurrence in France and that it causes severe losses among sheep. He drew attention to the relation and association between Cl. oedematiens and Fasciola hepatica causing "black disease".

Abdallah (1946) reported that schistosomiasis predisposes the urinary tract to microbial infection. The most frequent organisms which he recovered were B. coli, although B. pyocyaneus, B. friedlander, B. typhosus and paratyphosus also occur. He found that these micro-organisms may be present in the absence of urinary bilharziasis but in a much smaller percentage of cases. He considered two years as the minimum period for the duration of bilharziasis infection which paved the way for microbial infection.

Black (1946) stated that eosinophilia is found in patients infected with tuberculosis as well as in patients infected with ancylostoma.

Gerbilski (1946) studied the possible role of the migrating larvae of Ascaris lumbricoides and A. lumbricoides var. suum as carriers of the bacterial flora of the intestine and of pathogenic forms in a series of experiments conducted on mice. He found that larvae of nematodes which mechanically injure the mucous membrane of the intestine do not inoculate normal bacterial flora into the tissues of the host. From his investigations he concluded that Ascaris larvae can introduce infection with Salmonella typhimurium from a non-infective dose and that the larvae of nematodes migrating via the intestine, liver, lungs, can introduce pneumococci which usually do not penetrate the intestine.

Jurny (1946) reported that out of 982 horses examined 137 had fistulous withers. In thirteen of the latter it was shown by X-rays that the condition was not due to Onchocerca infection. In half of the remainder, surgical operations revealed adult Onchocerca. In the rest larvae were found in the ligamentum nuchae. There was no mention of any bacteriological examination.

Gabathuler and Gabathuler (1947) in their routine examination of a large number of patients showed that onchocerciasis is widespread in Ulanga district and is present in 38.91% of the population. They noticed the presence of muscular abscesses in association with Onchocerca volvulus.

Granena (1947) reported the presence of hydatid and tuberculosis in the lungs of a 63 years old patient.

Maenhout (1947) recorded that Trichuris ovis in large numbers were pathogenic for eight-months old heifers. His clinical manifestations were marked emaciation, weakness, restlessness, staring coat, arched back, sunken eyes, hind quarters and tail soiled with faeces, slight oedema of the throat region and a profuse watery diarrhoea with air bubbles in the discoloured excreta. One animal died from peritonitis, the mucosa of which was red and slightly swollen, punctiform haemorrhages were present in the sub-mucosa and the parasite was present in large numbers. He mentioned no bacteriological examination.

Syverton, McCoy, and Koomen (1947) carried out some experiments on the association of Trichinella spiralis and lymphocytic choriomeningitis in guinea-

pigs. They claimed that their findings proved that guinea-pigs, which were infected with the virus and Trichinella spiralis, had acquired the virus and were capable of transmitting it to a new susceptible host. Transmission resulted both when living larvae were fed to normal guinea-pigs and when triturated dead larvae were injected subcutaneously. Control experiments and other tests which they carried out made it plain that the transmission of the virus was not due to mere adherence of it to the outer surface of the larvae but that these actually harboured the virus.

Tykcac (1947) reported that Ascaris lumbricoides was found in 82.5% of 135 farms where infectious paralysis of swine occurred. On 58 farms where there was no infectious paralysis less than half of the pigs harboured Ascaris lumbricoides. The author drew attention to the possibility that A. lumbricoides is transmitting the virus of swine infectious paralysis.

Voss (1947) described the pathology of fistulous withers in 68 horses produced by Onchocerca infection. Two types of swelling were observed, those predominantly of connective tissue in which fistulae developed only in the late chronic stage and can be distinguished

clinically from fistulas produced by Brucella or by Salmonella abortus equi infections, although mixed infection may occur.

Glasser and Weitzner (1948) showed that 1.2% to 1.6% of cattle infected with liver fluke were carriers of Gartner bacillus (Salmonella enteritidis). Cattle with unaffected bile ducts and gall bladders were much less likely to be carriers. They emphasized the necessity for eradication of liver fluke infestation from cattle in order to reduce the number of S. enteritidis carriers.

Stewart (1948) reported during the autumn of 1947 twenty sudden deaths among ewes and lambs which were investigated at Kings College laboratory, Newcastle-upon-Tyne. Clostridial organisms and immature Fasciola hepatica together produced the gross liver lesions characteristic of the "Black disease" previously reported from Australia and Scotland. The author pointed out that carbon tetrachloride was ineffective against the migrating immature flukes but that vaccination against the clostridium infection has proved valuable in Australia. However, the ultimate control of "black disease" was bound up with the control of molluscan vectors of the fluke.

Thomson and Ross (1948) in a letter to the editor of the Veterinary Record stated "There is, then, every reason to suspect that the disease (Black disease) has a wide distribution in fluke infested districts and its possible presence must now be considered in every investigation of sudden deaths among adult sheep during the autumn and winter seasons."

Wilson and Miles (1948) stated that "black disease" occurs amongst sheep in Australia, New South Wales, and in certain parts of Germany where it is termed braxy. In Germany two types were recognized - (1) Meadow braxy, affecting mainly ewes at pasture in well watered valleys; and (2) Stall braxy, affecting chiefly young castrated lambs that were being fattened for market. In Australia the meadow type alone seemed to be common. Well nourished animals were affected. Clinically the course in meadow braxy was usually very acute, and the animals were often found dead before suspicion of illness had been aroused, but in stall braxy symptoms might have been present for twelve hours before death. Examination of fresh carcasses revealed the presence of subcutaneous engorgement, which gave the skin a dark colour hence the term black disease. Bacterio-

logically the liver showed large anaerobic bacilli - Clostridium oedematiensis. By a careful series of observations and experiments Turner was able to show that in "black disease" the necessary conditions for growth of the causal organism were found in the necrotic areas produced by invasion of the liver with wandering immature liver specimens of Fasciola hepatica.

According to Davidov (1949) septicaemia developing during onchocerciasis is of chronic character. General intoxication is due to the presence of metabolic biproducts of the parasite, bacterial toxins, and disintegration of foreign protein in the blood.

Gebauer (1949) believes that fistulous withers due to onchocerciasis are less common than those due to injuries caused by harness or saddle.

Jamieson (1949) reported that immature Fasciola hepatica in their migrations in the liver activate the latent spores of Cl. oedematiensis in the necrotic foci and set up "black disease" in sheep.

Mönnig (1949) states that most nodules of O. columbianum are sterile. The larvae may carry bacteria into the intestinal wall and acute inflammation results, frequently followed by peritonitis if the resulting abscess opens into the peritoneal cavity.

Morgan and Hawkins (1949) state "Black disease is caused by Clostridium novyi. It has been shown that in areas where the disease occurs the organism may be present in the livers of healthy sheep without the animals suffering ill-effects. However, when the liver is damaged by the migrating trematodes the bacteria become active and the toxins are liberated which result in the rapid death."

Rasch (1949) thinks that liver fluke infection is a primary cause among cattle carriers of Gartner bacillus (Salmonella enteritidis). He states that changes in the bile ducts or gall bladder are not essential for the formation of a reservoir of bacteria in the gall bladder as Glasser and Weitzner (1948) have reported.

Gonzalez (1950) was in favour of the theory that there may be some relation between poliomyelitis and threadworms.

Ackert, Egerton and Hansen (1951) reported that chickens experimentally infected with A. galli and injected with botulinus toxin (type A) at the rate of 0.015 mg. per Kg. body weight showed that mortality was less in parasitised than in unparasitised birds. Birds which received A. galli and toxin had more worms

than those infested only with A. galli infection. The toxin apparently lowered the resistance to this nematode and the growth in length of the female worms was more retarded than that of males.

Beautyman and Woolf (1951) reported an encapsulated ascarid larva in the brain which was found in a child who died with clinical and morbid anatomical evidence of poliomyelitis. These authors are of the opinion that ascariasis may predispose to poliomyelitis.

Cameron (1951) reporting on the pathology of oesophagostomes states "The adults of these species live normally in the large intestine and appear to do comparatively little damage. The parasitic larvae of some species - perhaps of all - develop in the mucosa of the intestine and have a considerable pathogenicity. This probably, in part at least, is due to bacterial action, the microbes entering the lesion made by the migrating larva. Bacterial contamination is very common and the simple helminthic nodule becomes converted into a pyogenic abscess; this septic infection often spreads, rendering the condition one of the most serious of those initially produced by helminths."

Fathers (1951) stated that "Black disease is caused by young liver flukes (Fasciola hepatica) when they pierce the capsule of the liver. The disease is not recognizable before death as the affected sheep die suddenly. Animals in good condition are most frequently affected. At post-mortem examination the liver shows irregular pale grey or white necrotic patches varying in size up to the size of a two-shilling piece." He noticed that heavy losses among sheep do occur commonly about January and February.

Miller (1951) stated that there are three types of fluke diseases:

- (1) Acute fluke infection
- (2) Chronic
- (3) "Black disease".

He believes that the encysted flukes may remain on grass for many months and if picked up by sheep acute fluke infection and "black disease" may be seen in late winter.

Wohrlin (1951) described a case of unexplained meningo-encephalitis in a twenty-five years old man who was recovering from primary tuberculosis of the

lungs. He considered that ascariasis was responsible for both the meningeal symptoms and the lung infiltrations.

Innes and Thoho (1952) reviewed the work of some authors on nematode invasion of the central nervous system, and suggested that in addition to the production of disease by helminths, nematode invasion may facilitate neurotropic virus infection. In the foot note they stated that, "The fact that a nematode, and its lesions, are found in one part of the nervous system and evidence of virus infection is found in another would not necessarily exclude a connexion. A nematode could act as a destroyer of the blood-brain barrier in one region and thus facilitate virus invasion, and then wander out and away and penetrate another region when a stage of viraemia was over."

De Sa (1952) reporting his observation on cases of ascariasis, mentioned that obstruction is usually in the lower reaches of the ileum and may be associated with perforation in or above the obstructing mass of worms. Peritoneal reaction is slight, with a serous effusion in contrast to the purulent effusion in perforation of the appendix. He suggested that the perforation may be very minute and can occur apart from the obstruction and in some cases a localised abscess may form.

Cort, W.W. (in an introduction to nematology edited by Christie, Part VI sect. II p.315) deals with the pathogenicity of Trichuris trichiura and states "The adult worms produce some injury to the intestinal mucosa and when present in large numbers may cause considerable inflammation. Therefore in heaviest cases they may produce rather severe intestinal disturbances. There is no real evidence that they serve as a "lancoet of infection" for other diseases as suggested by many earlier workers (Guiart 1911) and their relation to the production of anemia is rather doubtful (Otto 1935; Swartzgelder 1939). In most cases their presence would pass unnoticed except for the finding of the characteristic eggs in faecal examination."

Le Roux, lecturing about the influence of the climate on the epizootology of animal diseases with special reference to parasitic affections, in the Royal Veterinary College, London, January 1953, mentioned that cases of Anthrax or Quarter evil, or both, occur on some farms while those adjoining escape infection. Some owners suffered losses from these bacterial diseases when they transferred their stock to swampy grazings during the dry season, and that

deaths occurred only while the herds were on the swamps. He mentioned one case in which an owner had his farm burnt out and his stock, which was free of liver fluke, was transferred to a grazing farm with a large swamp. Within sixteen days of transference several head of cattle were lost including trek-oxen aged 10 to 15 years. Blood, gland-smear as well as smears from discoloured muscles were sent to the laboratory for examination and Quarter evil was diagnosed. Post-mortem revealed that the animals contracted Fasciola gigantica when transferred to swamp grazing. He observed that there was no mortality in older animals immunised against Quarter evil and he suggested that the death of the before-mentioned old oxen resulted from the fact that years of grazing on liver fluke-free pastures had lessened their immunity against Quarter evil and the fact that there is no mortality amongst old animals grazed annually for a time on liver fluke pastures must be attributed to immunisation of the stock by natural infection acquired by the annual exposure to the disease by liver fluke infection.

From his African experience Le Roux emphasized the role played by the parasitic helminths in setting up bacterial diseases. He stated that liver fluke,

may predispose pig to anthrax; sheep to black disease; and immature wandering liver fluke in the lungs of cattle to contagious bovine pleuro-pneumonia. He drew attention to the fact that worms may, after all, do some good by trying to save their hosts for their own interests, and he believes that it remains to be ascertained whether the migratory larval stages of Fasciola hepatica and Taenia hydatigena and other larval helminths are nature's veterinary surgeons for the probable immunisation of animals against certain anaerobic infections.

The Parasite

Nippostrongylus brasiliensis

The reasons for selecting this parasite for this work are:-

- (1) Its infective stage larvae are skin penetrators.
- (2) It is readily maintained in rats and mice.
- (3) It is non-pathogenic to man.
- (4) Its life-cycle is reasonably short, allowing for rapid procedure of investigation.
- (5) Cost of experimentation is low.

A great deal of work has been published on the biology of this parasite and its behaviour in small laboratory animals.

After the investigation had been in progress for some time it was observed that some of the findings did not agree with recorded observations. Further investigations were made to determine the probable routes of migration of the parasite from its site of penetration to the intestine via the lungs.

This parasite was first described from wild rats by Yokogawa (1920) and he named it Heligmostomum muris. In 1922 he described in great detail the life-cycle, the free-living larval stages, method of culturing the

larvae, infection and migration in the host as well as the morphology of the adult parasite.

Lane (1923) renamed the parasite Nippostrongylus muris.

Travassos and Darriba (1929) recorded that Heligmostomum brasiliense Travassos 1914 is identical with Heligmostomum muris Yokogawa, 1920 and renamed it Nippostrongylus brasiliensis.

The live worm is a small, coiled or curved reddish-brown hairlike nematode found either free in the lumen or lying coiled between the villi of the mucosa of the small intestine. In the heavily infected rat it has been noticed that parasites are usually found in clumps at the anterior end of the duodenum and jejunum and evenly distributed in between them. In the moderately infected rat as well as in the mouse the parasites are usually evenly scattered throughout the duodenum.

Eggs are ellipsoidal in shape and have a very thin shell. Segmentation of the egg begins in the uterus. In freshly passed faeces the embryo is found usually in the 8-16 celled stage and exceptionally in the morula stage. Further development of the embryo occurs in the faeces.

Method of Culturing Larvae

Yokogawa (1920) mixed the faeces containing the eggs with animal charcoal. He found that liquid media were the best for the hatching of eggs and solid media for the study of larval development. He did not specify the optimum conditions for the hatching of the eggs. Luttermoser (1937) found that the optimum temperature for Hippostrongylus cultures was between 22°C. and 30°C.; hatching decreased at lower temperature, and at 35°C. or over the larvae died. Barakat (1948) stated that the optimum temperature for hatching, as well as for the maximum survival of the larvae, was at 27°C. He tried different absorbents, and found that a mixture of activated alumina and cellulose powder gave the best hatching results.

The method which has been used in this work for collecting infective larvae was the same as that of Barakat (1948) except that the eggs were incubated at 28°C. which gave a more rapid hatching than at 27°C. and cellulose powder was replaced by absorbent cotton wool owing to difficulty in obtaining the former.

Method for Collecting Maximum Number of Eggs

To obtain maximum amount of eggs from the faeces of infected rats the following procedure was used.

A tray is covered with a thick layer of absorbent cotton wool and a piece of newspaper spread over it. Filtered water is poured over this to keep the paper moist enough for the time required to collect the infected faeces and to prevent their destruction by desiccation. The cage with the infected rats is placed over the tray for 24 hours. The faeces required for culture are then collected from this tray and put into a beaker. To the 24 hours' collection of faeces from each rat 15 c.c. of filtered water is added and the mixture is broken up completely in the beaker with the aid of a glass rod.

Method of Preparing Petri-dish Culture

A piece of absorbent cotton wool the size of a shilling is placed in the centre of a 9 cm. wide Petri-dish and moistened with filtered water. Approximately 5 Gm. of activated alumina is placed over the cotton wool and moistened drop by drop with filtered water, then shaped into a small mound with a flattened summit. A number one filter paper 7 cm. diameter is placed over the alumina in such a way as to leave

the edge of the paper free from the Petri-dish and overlapping the alumina, Fig. 1.

The alumina serves to neutralise any acidity in the faeces, absorbs toxic matter and harmful metabolic biproducts of the larvae and serves as a bedding for the filter paper, while the cotton wool helps to retain the moisture and to keep the preparation fully saturated with water.

One c.c. of faecal emulsion is poured over the centre of the filter paper. This amount produces a reasonable number of hatching larvae which migrate to the edge of the filter paper. The Petri-dish is then covered and incubated at 28°C.

On the second day of incubation two or three drops of Shirlan solution (I.C.I.) 1:1000 are poured over the incubated faeces to inhibit the growth of fungi, which have been noticed to be harmful to the living larvae. Twice a week the filter paper is lifted with forceps and a few drops of water, enough to retain the same amount of moisture, is added to the alumina and the filter paper is replaced.

The use of activated alumina was later replaced by chicken grit which gave better and quicker hatching

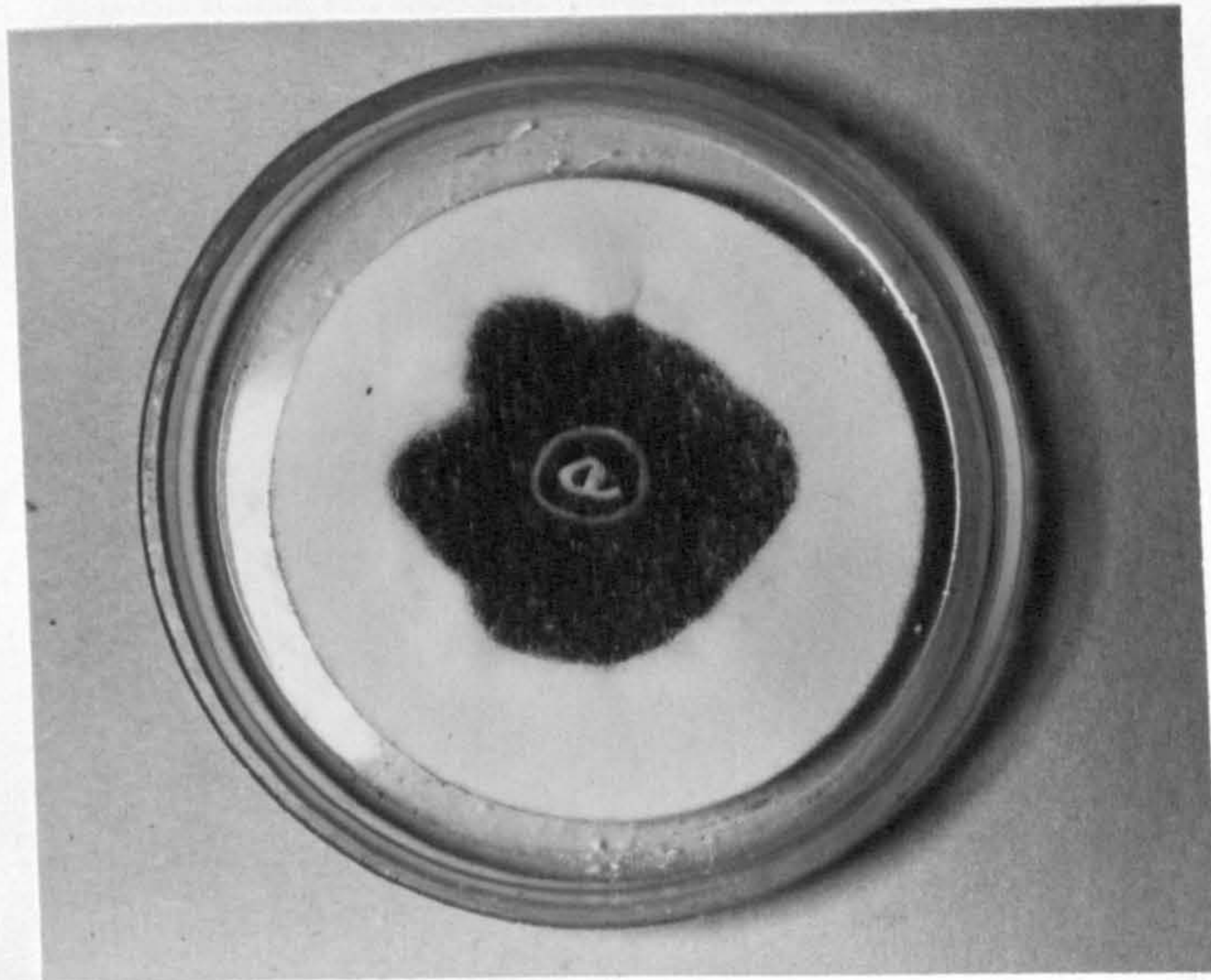


Fig. 1

Face on view of the Petri-dish culture of N. brasiliensis

a) Faecal emulsion on

b) Filter paper No. 1 (7cm.)

of the larvae than activated alumina. This may be due to the larger pieces of chicken grit providing more moisture but the larvae remained at the edge of the filter paper for a few days only, after which they migrated into the damp grit below the filter paper. When activated alumina is used the larvae migrate to the edge of the filter paper, stay there and are available to be collected for up to four weeks. The use of activated alumina has been employed in all the experiments in this work for obtaining infective larvae because it has the advantage that the larvae stay for a long time at the edge of the filter paper and also because they can be obtained free of the faecal debris.

Hatching of Eggs

Hatching of eggs occurs within one or two days depending on the degree of dampness of the media and the temperature. According to Lucker (1936) the first moult is preceded by an unusually early separation of the first cuticle, which is fine and unstriated, from the body tissue in the tail region, and later by the formation of an underlying new cuticle which is thicker and prominently striated. The writer has been unsuccessful in all attempts to confirm Lucker's findings. What is taken to be the second stage larvae of Lucker

has a prominently thick cuticle and a hair-like long tail. Along the middle of both sides of the cuticle there runs from head to tail two parallel lines not mentioned by Lucker. These parallel lines have also been observed in the infective stage larvae after shedding their sheaths. The infective stage larvae appear at the edge of the filter paper on the third day of cultivation. They force their way through the edge of the filter paper in such a way that the tail end remains embedded while the anterior part protrudes into the air. The larva is within the sheath in this position except for the head part which is usually projecting from the sheath, Fig. II. The cuticle at this stage is so thick and rigid that the larva cannot easily escape from it without mechanical assistance offered by some rough surface. When the larvae are attached to the edge of the filter paper they are in the infective stage and are ready to infect a host. The activity of the infective larvae was studied by Africa (1931) who observed their ability to effect a lateral migration to the edge of the filter paper as well as a vertical upward migration through soil. An optimum amount of moisture is essential for the migration of the larvae. Infective larvae can cross a water barrier of at least

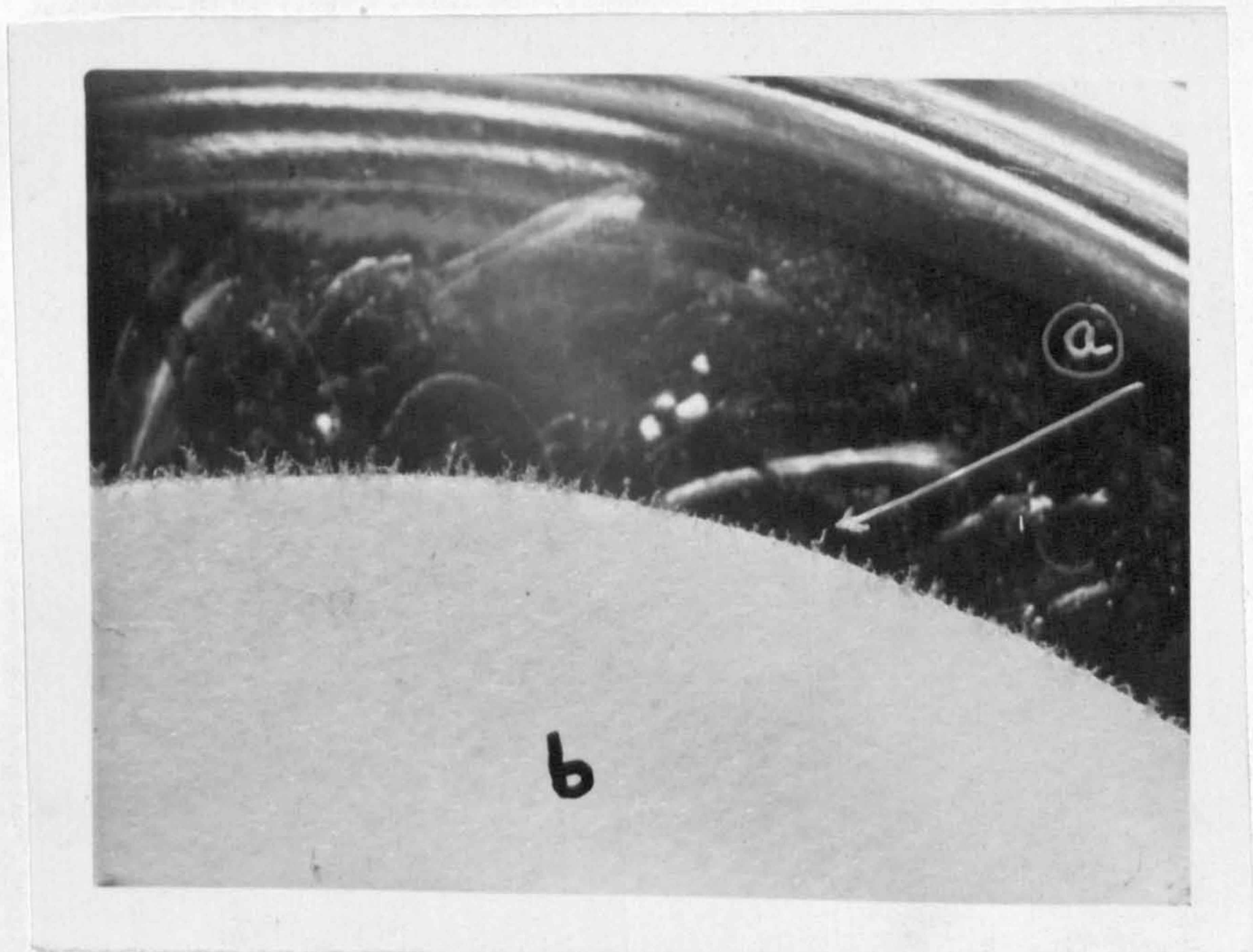


Fig. II

Showing larvae of N. brasiliensis (a) protruding beyond the edge of the filter paper (b) 4 days after the culture was prepared.

two inches and live under two inches of water for ten days without losing their migratory capability.

It appears that the manner in which the larvae of Hippostrongylus brasiliensis remain attached to the edge of the filter paper is a characteristic of this parasite. Eggs of Strongyloides, hookworm, and Rhabditis have been cultivated in the same way as those of H. brasiliensis and although all of them hatched, none of them showed this peculiarity of remaining at the edge of the filter paper with the anterior part protruding into the air.

It has been observed that the larvae appear on the edge of the filter paper three days after incubation irrespective of the diameter of the filter paper. This has been proved by preparing Petri-dishes for culturing with 7 cm., 11 cm., and 15 cm. diameter filter papers using the same quantity of faecal solution on each and incubated at the same time under the same conditions, and it was noticed that all the larvae appeared at the edges of the filter papers three days after incubation.

Method of Counting Larvae

The general procedure followed by all investigators working on nematodes has been to use the Baerman apparatus (Cort, et al. 1922) for the separation of larvae from charcoal cultures. They are then collected in the smallest volume of water, washed in saline, centrifuged repeatedly and counted per unit volume of suspension. Animals are infected subcutaneously by injection or percutaneously by placing the suspension on the shaved abdomen of the animal.

The viability of larvae separated in this way did not seem to be entirely satisfactory, for the cooling time of the larval suspension in the Baerman apparatus varied widely according to the temperature of the room. On a hot summer day it will take several hours to cool, after which time the larvae will be found mostly inactive. Africa (1931) and Chandler (1932) criticized this method and stated that larval counts in different samples of the same suspension varied by 10% and their viability, as measured by the number of adults that could reach the intestine after infection, varied between 10% and 50% of the initial dose of infection. Donaldson and Otto (1946) stated that 1% of the larvae remain behind in the syringe during their injection into the animal.

As the larvae migrate to the free edge of the filter paper in the culture dishes and taking into consideration the disadvantages of the Baerman apparatus, it was decided to count the number of larvae required directly on the filter paper under the dissecting microscope.

The cut edge of the filter paper carrying the infective dose of larvae was then placed directly on the clipped chosen site of the experimental animal.

The disadvantages of this method are:-

(1) When a large number of eggs are hatched the larvae at the edge of the filter paper will be seen clinging together in great masses making them difficult to count. This was overcome by using 1 c.c. of a solution of a 24-hourly crushed faecal sample from an infected rat (with 400 larvae counted on the filter paper) in 15 c.c. of filtered water.

(2) Larvae sometimes appeared at the edge of the filter paper on different levels and to overcome this difficulty the lens of the dissecting microscope was adjusted up and down to facilitate counting on different planes.

(3) Another disadvantage was that, after cutting the filter paper, more larvae may appear on the cut edge, and to eliminate this factor the paper was cut very close to the edge.

Methods and Technique of Infecting the Experimental Animals

Yokogawa (1922) states "when infective larvae are fed to rats only a small proportion are able to reach the intestine." In his opinion the percutaneous infection is far more effective than the ingestion of larvae.

(1) Oral infection

The infective stage larvae were put on a lettuce leaf and 100 larvae (counted on the edge of the filter paper) were fed to each of three rats (3 weeks old). Twelve days after infection the number of worms collected from the small intestine were 45, 33, and 36 respectively. This shows that the oral infection is effective. This finding conflicts with that of Yokogawa (1922) who fed the larvae in bread, and claimed that few reached the intestine. In the author's opinion this result was probably due to large numbers of larvae being crushed during mastication, or to their being destroyed in the stomach by the gastric juice. In the writer's opinion, lettuce, which requires

less mastication, gives the larvae a better chance to attach themselves to the mucous membrane of the mouth.

(2) Percutaneous infection

Three rats (three weeks old) were anaesthetised, secured abdomen upwards by tying gauze round the legs, and then pinned on to a board, Fig. III. This ensures that the animals remain still and stretched without being taut, during the experiment. The selected site was clipped with scissors, special care being taken not to injure the skin. The site of infection was thoroughly moistened with water and a piece of filter paper containing 100 larvae was applied to this spot on each rat. The animals were kept in position for one hour, after which they were released and each was kept in a separate jar. On killing them 60 hours later the larvae in the lungs of each rat were counted and were found to be 96, 75 and 72 respectively. This proves that infection through the skin is very effective.

(3) Mixed^{or combined} infection (percutaneous and oral)

The animal (mouse or rat) is placed in a glass jar of suitable size (height 12 cm., diameter 12 cm. for one or two animals, and height 12 cm., diameter 25 cm. for three to nine animals), containing infective larvae in a few c.c. of water, for one hour. By this method

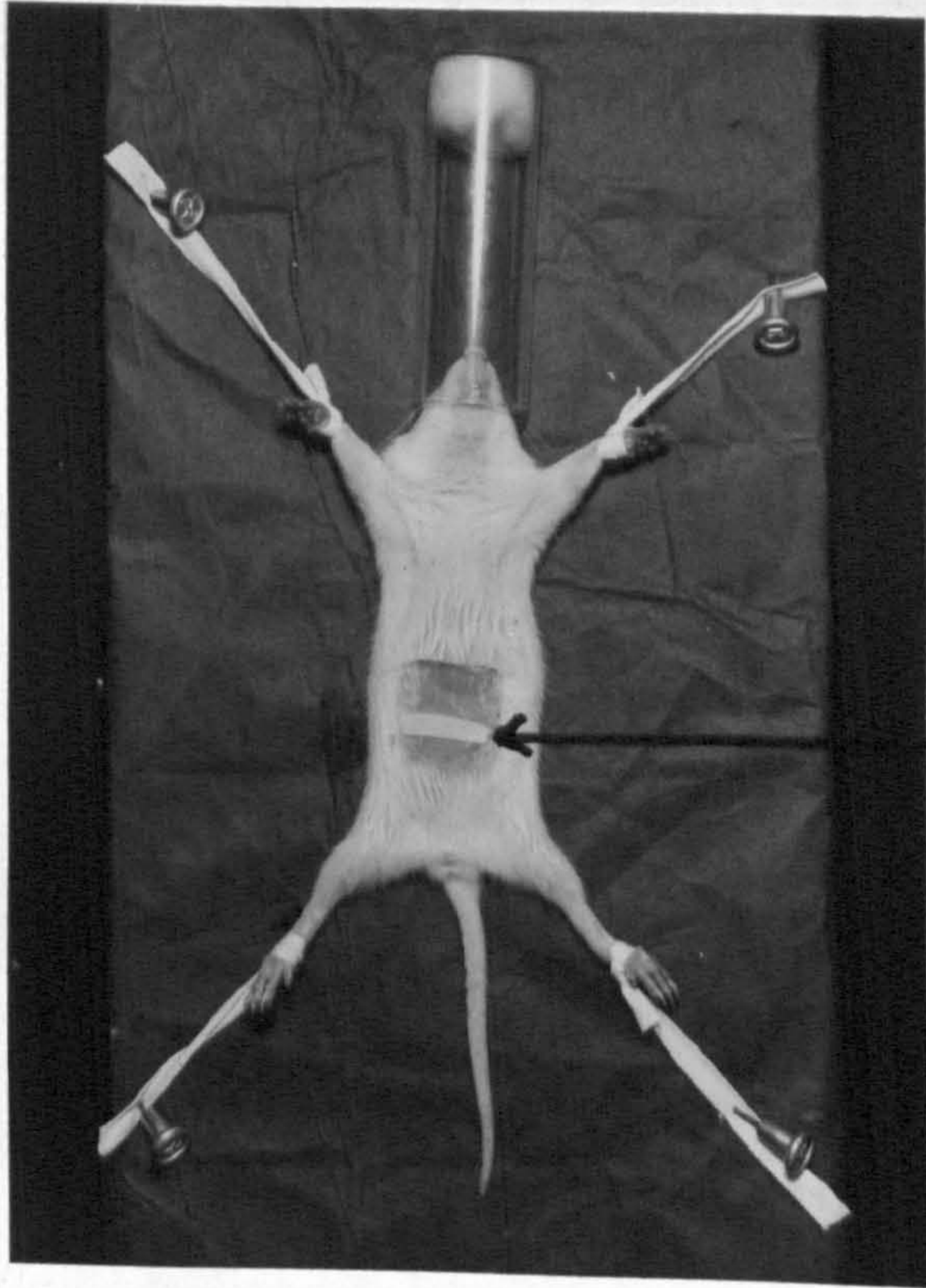


Fig. III

Animal secured on its back on board for percutaneous infection by the application of larvae adhering to the edge of the filter paper (a).

of exposure the parasites penetrate mainly through the skin of the hind limbs, especially between the claws, and as the body gets wet the animal usually licks its skin and infection also takes place through the mouth. A number of animals infected by this method were killed 12 hours after the beginning of exposure and the larvae were recovered from the precrucial lymphatic glands and from the submaxillary lymphatic glands. In the opinion of the writer this method resembles most closely the natural method of infection. It is of interest to note that four young rats (two weeks old) were exposed to infection with the parasite and then returned to the mother rat, which had been shown by repeated examinations of its faeces to be free of infection. On later examination of the faeces the mother was found to have a number of eggs, one week after the exposure of the young rats which had been found to have become infected. The experiment was repeated four times and all the mothers which had previously been shown to be free of infection, were found to have been infected. It seems that the infection may have been transferred from the young to the mothers, either by direct contact, or by the mothers licking their young or both.

(4) Infection through the intact skin of the tail

Louis Olivier and Stirwalt (1952) suggested that the cercaria of Schistosoma mansoni penetrate more readily through the intact skin of the tail of the mouse and that method of infection was proved by these authors to be more satisfactory than any other method of infection. For that reason the writer was interested to prove whether the parasite of N. brasiliensis could successfully penetrate through the skin of the tail of the mouse and migrate into the lungs. If that method would have proved to be satisfactory it would have been of great help in further experiments to prove or disprove whether the parasite during penetration of the skin introduces the pathogenic bacteria into the body tissues.

Method of securing animals in position

The mouse was immobilized in a glass tube (A) of 2-1/2 cm. diameter and 8 cm. length with a hole (C) to allow for breathing when the animal was fixed. The infective larvae were added to another tube (B) (closed at one end) the same calibre as the other tube (A) and of about 4 cm. long. The two tubes were connected by means of a cork with a hole in the middle through which the tail passed and was immersed in the infective medium as illustrated in fig. (IV).

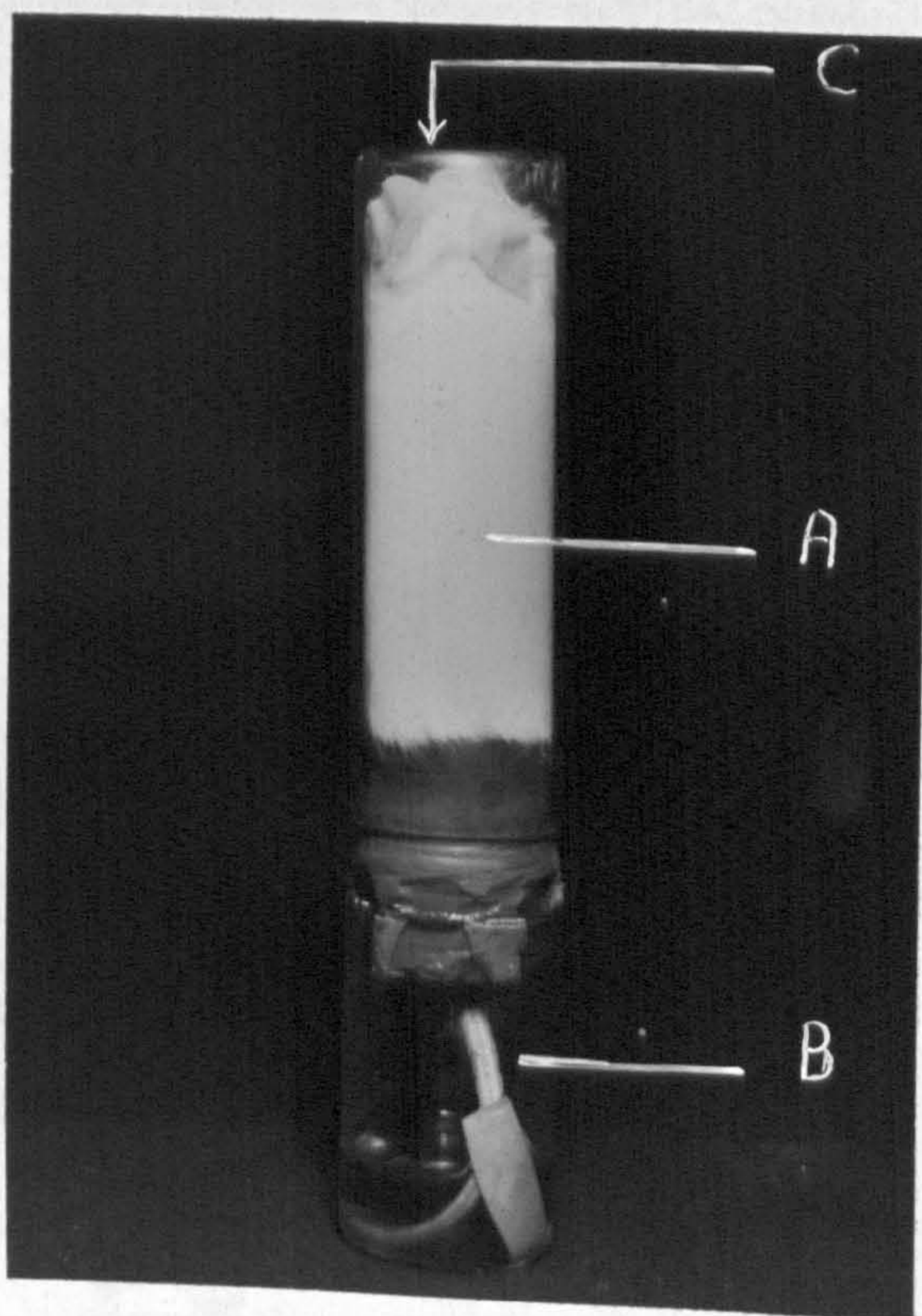


Fig. IV

Animal secured in tube (A) with a hole for breathing through (C) while the tail is being subjected to infection in tube (B).

Exposure of intact skin of the tail to a mixed infection of *N. brasiliensis* larvae and *Streptococcus pyogenes*

Experimental animals used:

Albino mice three weeks old, which have been proved by previous repeated examinations of their faeces to be free of *N. brasiliensis* infection, were used.

The micro-organism used:

Streptococcus pyogenes, a virulent strain which had been passaged three times in mice, cultivated in nutrient broth media and incubated at 37°C. for 24 hours, was used for this experiment.

The parasite:

The infective stage larvae of *N. brasiliensis* six days old and cultured as described on page 60 were used.

Technique employed:

Animals were secured in position as previously mentioned after smearing the base of the tail with vaseline. In tube B 2 c.c. of a 24-hours nutrient broth culture of *Str. pyogenes* and a piece of filter paper containing 200 infective larvae of the parasite was added in Group 1. The same dose of *Str. pyogenes* was added to tube B in Group 2. Animals in Group 1 and 2 were kept in position for half an hour, after

which the two tubes, A and B, were separated, and tube B was replaced by another empty one and animals left in position for ten minutes to allow for drainage of the fluid on the tail. Afterwards the animals' tails were dipped in another tube B containing 2 c.c. of a 5% solution of Dettol for five minutes, after which the animals were released free in separate glass jars and left for the rest of the experiment.

Experiment No. 1

Ten mice three weeks old were divided into two equal groups, 1 and 2. The first group (1) was treated with parasite and bacteria, and group 2 was treated with bacteria only. One mouse was injected subcutaneously with 1/2 c.c. of the broth culture used for the experiment and another one was injected with 1/2 c.c. of a sterile nutrient broth dose.

Result:

None of the animals either in Group 1 or in Group 2 died. Only the mouse which was injected subcutaneously with 1/2 c.c. of the bacterial broth culture died (the next day), and Str. pyogenes was recovered from the subcutaneous tissues at the site of injection. The faeces of the animals in Groups 1 and 2 were examined daily from the sixth to the twelfth day after the exposure of the animals to the infection and none of them

have proved to pass eggs of the parasite. Fourteen days after exposure the rest of the mice were injected subcutaneously with 1/2 c.c. of a 24-hours nutrient broth culture of Str. pyogenes and all animals were found dead the following day (see table I).

From this experiment it was noticed that in the animals in Group I no eggs of the parasite were detected in the faeces, so the writer considered it of some interest to investigate whether or not the parasite does in fact penetrate the skin of the tail of the mouse and migrate to the lungs, before continuing with any further experiments. The following experiments were accordingly carried out.

Experiment No. II

Five mice, three weeks old, free of infection of H. brasiliensis, were secured in position as previously described on page 58 for the percutaneous infection. The base of each tail was smeared with vaseline. The lower third of the free end of the tail of each of four mice was moistened with filtered water. A piece of filter paper containing 200 larvae (6 days old) was applied to the selected site of the tail in each of the four mice. The same dose of larvae (200) was applied to the damped abdominal skin of the fifth mouse. All animals were left in position for two hours, after

which the site of exposure was wiped thoroughly with an absorbent piece of cotton wool and the animals were released, each in a glass jar.

Forty-eight hours after exposure two of the four mice were killed and the lungs were examined, but no larvae were recovered in either of them. Seven days after exposure the faeces of the rest of the mice were examined and no eggs of the parasite were recovered, except from the mouse which had been exposed to infection via the intact abdominal skin. Ten days from exposure the remaining two mice, which were exposed to infection through the tail, were killed, and the small intestine opened and examined in saline under a binocular for the presence of the parasite. None of these animals proved to be infected.

Experiment No. III

Four out of five mice, four weeks old, were secured in position as previously described in Experiment I and the fifth mouse was secured as for infection via the intact abdominal skin and acted as a control. Two hundred infective larvae, six days old, counted at the edge of the filter paper, were added with one c.c. of filtered water to each of tubes B. The fifth mouse was exposed to the same dose of larvae (200) via the intact abdominal skin. All the animals were left in position

Table I

No. of House	Date of Infection	Kind of Infection	Date of Discovery of <u>N. brasiliensis</u> in Faeces	Subcutaneous inoculation of <u>Str. pyogenes</u>		
				Dose Used	Date of Inoculation	Date of Death
Experimental Group I	10-5-51	2 c.c. <u>Str. pyogenes</u>	Negative	1/2 c.c.	24-5	25-5-51
		+	Negative	1/2 c.c.	24-5	
		200	Negative	1/2 c.c.	24-5	
		larvae	Negative	1/2 c.c.	24-5	
			Negative	1/2 c.c.	24-5	
Control Group II	10-5-51	2 c.c. <u>Str. pyogenes</u>		1/2 c.c.	24-5	25-5-51
				1/2 c.c.	24-5	
				1/2 c.c.	24-5	
				1/2 c.c.	24-5	
				1/2 c.c.	24-5	
		1	1/2 c.c. <u>Str. pyogenes</u>		----	---
2	1/2 Sterile broth		1/2 c.c.	24-5	25-5	

for two hours, after which the site of exposure was wiped thoroughly with an absorbent piece of cotton wool and the animals were released, each in a separate jar. Forty-eight hours after exposure two out of the four mice were killed and their lungs were pressed between two slides and examined for presence of larvae. The result of examination did not prove the presence of the larvae in the lungs of these animals.

The faeces of the rest of the animals were examined on the sixth, seventh and eighth day after exposure and only the control mouse, which was exposed via the intact abdominal skin, was found to pass eggs of the parasite.

Experiment No. IV

The cut edges of filter papers containing a massive number of the infective stage larvae (these were not counted) were added to a beaker containing 6 c.c. of filtered water. The beaker was at 28°C. for half an hour to allow the larvae to swim into the water. Eight six weeks old mice, which had proved by repeated examinations of their faeces to be free of infection, were lightly anaesthetised with ether. The tail of each of the eight mice was injected subcutaneously with 0.1 ml. of the water in the beaker, and two other mice were exposed to the same dose of infection via the

intact abdominal skin for one hour. After exposure to the infection all the eight experimental animals were kept in a jar and the two controls in another jar. Forty-eight hours after exposure four of the eight mice were killed and their lungs were cut into pieces and pressed between two slides and examined under the low power of the microscope for the presence of larvae. None of them showed larvae in their lungs. From the sixth day up to the tenth day after exposure the faeces of the rest of the animals were examined daily for the presence of the eggs of the parasite and none of them except the controls were found to pass eggs of the parasite. On the tenth day after exposure the four experimental mice were killed and the small intestines were opened in saline and examined under the dissecting microscope and no parasite was recovered from either of them.

Conclusion

From the above experiments it was noticed that none of the infective larvae, when applied to the intact skin of the tail, will migrate to the lungs of the mice. This might be due to:-

- 1) Insufficient subcutaneous tissues underneath the skin of the tail of the mouse.

2) The tautness of the skin around the tail.

3) Insufficiency of lymph channels. (This route of migration was subsequently discovered to be the means by which the parasite travels to the lungs.)

(5) By Injection

a) Subcutaneously

Six mice and four rats about three weeks old, which had proved by repeated examination of their faeces to be free of infection, were anaesthetised with ether. Each animal was injected subcutaneously in the left axillary space with about 200 infective larvae in 1/2 c.c. distilled water. Forty-eight hours after injection, three mice and two rats were killed and the lungs of these five animals were examined under the microscope for the presence of larvae. The lungs of all these animals were found to be infected. One week after the injection of the parasite, the faeces of the remainder of the animals were examined separately and all of them were found to be passing the eggs of N. brasiliensis in their faeces.

b) Intraperitoneally

Six mice and four rats about three weeks old, which had previously proved by repeated examination of their faeces to be free of infection, were lightly

200 infective larvae in 1/2 c.c. of distilled water. Two days later three mice and two rats were killed and their lungs were thoroughly examined. Larvae of the parasite were present in the lungs of the three mice and the two rats. The other animals (three mice and two rats) were left until one week after the injection, when their faeces were examined separately for the presence of eggs. Result of examination proved the presence of eggs in the faeces of all the injected animals.

c) Intrathoracically

Twenty-six mice about three weeks old, free of infection, were anaesthetised with ether, and each of them was injected intrathoracically with 200 infective larvae in 1/4 c.c. of distilled water. Two hours after injection two mice were killed, and two were killed every two hours till the twenty-fourth hour. The lungs and bronchial lymphatic glands of these animals were examined carefully under the microscope between two slides.

The result of examination revealed that the parasite was present in the lymph glands after fourteen hours and in the lungs twenty-four hours after injection, but never earlier. The rest of the mice (two in number) died on the fourth day and the parasite was recovered from their small intestines.

Conclusion

1) The above experiments prove that when the infective stage larvae of N. brasiliensis were injected into the host subcutaneously, intraperitoneally, or intrathoracically, the larvae develop, after migrating through the lungs into the small intestine.

2) When the parasite was inoculated into the thoracic cavity it was noticed that the parasite, although in the chest cavity, had not penetrated into the lung tissue but had first been recovered from the lymphatic glands and later from the lungs, which shows that this parasite migrates to the lungs via the lymphatic system.

3) The time needed by the parasite to reach the lung was greater when the parasite was injected intrathoracically or intraperitoneally (24 hours), than in the subcutaneous or the percutaneous infection, whereby the parasite reached the lung in eighteen to twenty hours.

(6) Per rectum

The cut edges of filter papers containing a massive number (not counted) of the infective stage larvae were added to a beaker containing about 10 c.c. of filtered water. The beaker was left in a 28°C. incubator for half an hour to allow the larvae to exsheath and swim in the water.

Twenty mice four weeks old, which had proved by previous repeated examinations of their faeces to be free of N. brasiliensis, were used. Each mouse was lightly anaesthetised and held up high by its tail. The rectum was rubbed with glycerine. By the use of an eye-dropper 1/2 c.c. of the solution in the beaker was introduced into the rectum of each mouse. The mouse was kept held from the tail for about five minutes after which the area around the rectum was wiped and the animal kept in a glass jar. One animal was killed every hour from the time of the infection until the twentieth. The mesenteric lymph glands as well as the lungs of each animal were examined between two slides under the microscope. The result of examination showed that the parasite was present in the mesenteric lymph glands eighteen hours after exposure and in the lungs eighteen to twenty hours after exposure.

Factors Influencing the Invasion of the

Experimental Host

Degree of Moisture Present on the Skin

The following experiments were carried out in order to determine the importance of moisture in the penetration of the skin by infective larvae.

A one-inch layer of dry sand was poured into each of two glass jars, 5 inches in diameter and 5 inches high. The sand in one jar was moistened with water, sufficient just to cover the sand, while the sand in the second jar was left dry. Two pieces of filter paper each bearing approximately 200 larvae were placed on the sand in each jar.

Two young white mice (3 weeks old) were immediately transferred to each jar and left for one hour. They were then removed and kept in separate clean jars on which the date and time of exposure was marked. On removal of the mice from the jars two additional mice were put into each jar for one hour. This procedure was repeated for five consecutive hours giving an exposure of 24 animals. All the animals were killed 48 hours after being exposed to the infection (in the moist and dry sand), and the number of larvae present in the lungs of one of each pair was counted. (See Table II for numbers of larvae recovered from each animal). The results showed that of the mice which had been exposed to the filter paper placed on dry sand, only the first pair contracted infection and the number of larvae in the lungs of one of them was six. All the mice which had been exposed to the infection in the "wet-sand-jar" were found to be infected. The number of larvae recovered from the lungs of one of

each pair in this series were 15, 4, 20, 30, 10, and 6 respectively.

Each lobe of the lung was cut into small pieces and each piece was completely flattened by pressure between two glass slides, one of which was divided into two sections with a grease pencil in order to facilitate counting under the low power of the microscope, and the number of larvae was counted.

In another experiment two white mice (2 days old) were secured on a board, abdomen up, and their bodies were relaxed by means of ether anaesthesia. The edges of filter papers containing infective larvae were cut into pieces and immersed in a watch glass containing 2 c.c. of water. The watch glass was incubated at 28°C. for ten minutes to ensure the migration of the larvae into the water. The abdomens of the experimental animals (mouse no.1 and mouse no.2) were wetted with a thin film of water and 0.2 c.c. of the larval solution (2000 larvae) was syringed onto the abdomen of mouse no.1 every quarter of an hour for four hours after which the mice were left for one hour and their abdomens examined for larvae under a dissecting microscope. It was observed that very few larvae were to be seen on the skin of mouse no.1, while on the skin of the other

Table II

Number of Group	Number of Mouse	Time of Exposure to Infection	Result of infection and number of larvae counted 48 hours after infection	
			dry sand jar	wet sand jar
1	1	10.00 a.m. -	positive	positive
	2	11.00 a.m.	6	15
2	1	11.00 a.m. -	negative	positive
	2	12.00	negative	4
3	1	12.00 -	negative	positive
	2	1.00 p.m.	negative	20
4	1	1.00 p.m. -	negative	positive
	2	2.00 p.m.	negative	30
5	1	2.00 p.m. -	negative	positive
	2	3.00 p.m.	negative	10
6	1	3.00 p.m. -	negative	positive
	2	4.00 p.m.	negative	6

mouse most of the larvae were found together in clumps as in Fig. XI.(d)

Personal observations of other experiments showed that larvae penetrated the skin more readily in the presence of sufficient moisture than in cases with insufficient damping of the skin.

Behaviour of the Parasite on Application to the Skin

When studying the mode of entry of infective larvae in the early stages of penetration, prior to

skin was fixed either in absolute alcohol or in 10% formal saline solution. Sections were cut between five and ten microns. The stains used were either haematoxylin and eosin or Giemsa's stain.

When the larvae were applied to the damp skin they emerged from their sheaths, left the filter paper, and crawled along on the damp skin. The larvae were seen to attach themselves to the skin of the animal within a few minutes of exposure, and it was noticed that the first selected site did not always prove entirely satisfactory to the larvae and they sometimes moved to several sites before finally selecting the site of entry. Some larvae were observed to rest their tails on the skin while the rest of the body was more or less perpendicular to the surface of the skin, Figs. V, VIa. They remained in that position for some time after which they bent over in a semi-circle and forced the head into the stratum corneum, Fig. VIb. Other larvae were noticed to be parallel to the surface of the skin while the head of the parasite forced an entry into the stratum corneum, Fig. VIc. Still others attached themselves to hairs and forced an entry by way of the hair follicles.

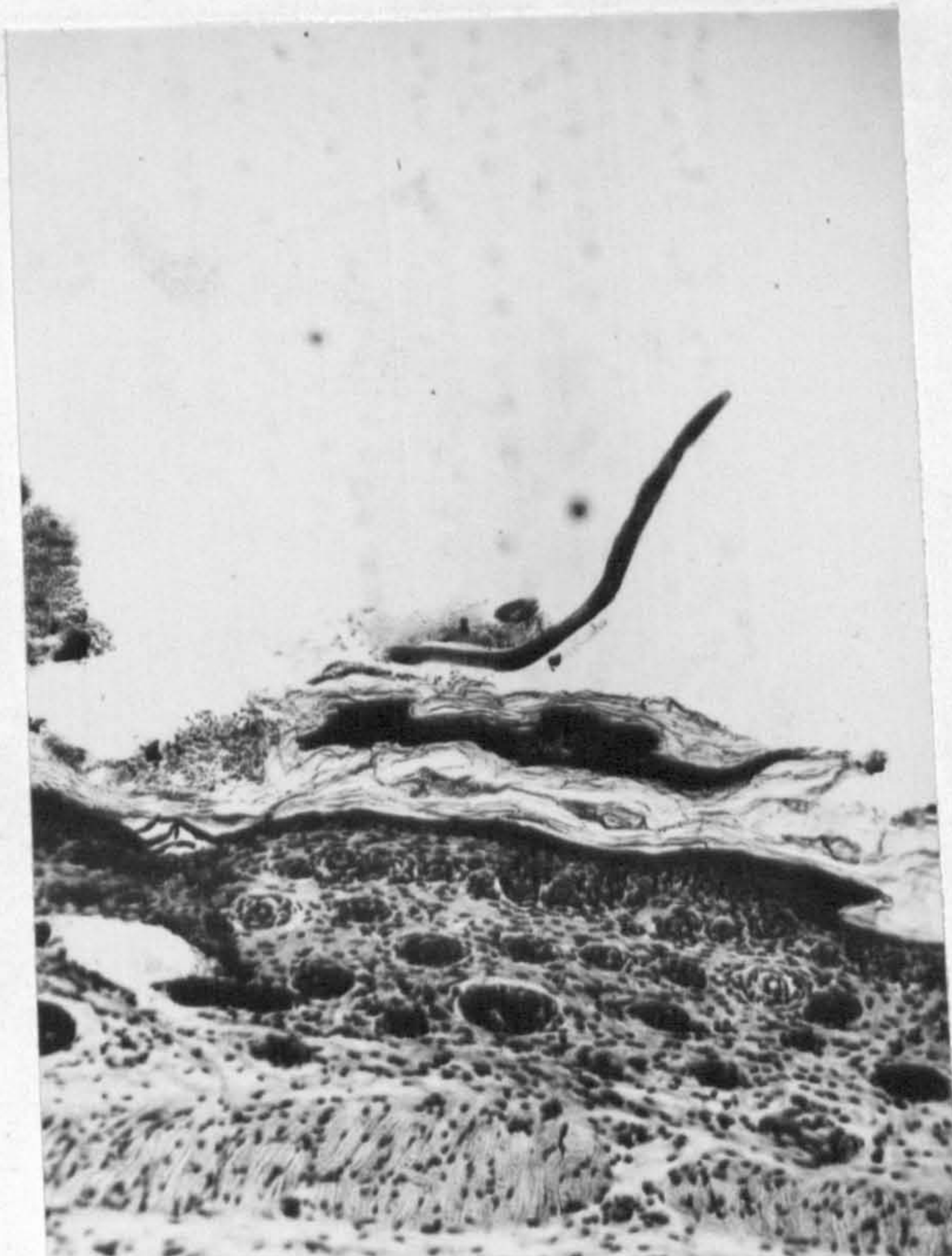


Fig. V

Cross section of the skin of a mouse showing tail of
the parasite resting on the skin before penetration
(15 minutes after exposure)

H.E (x100)

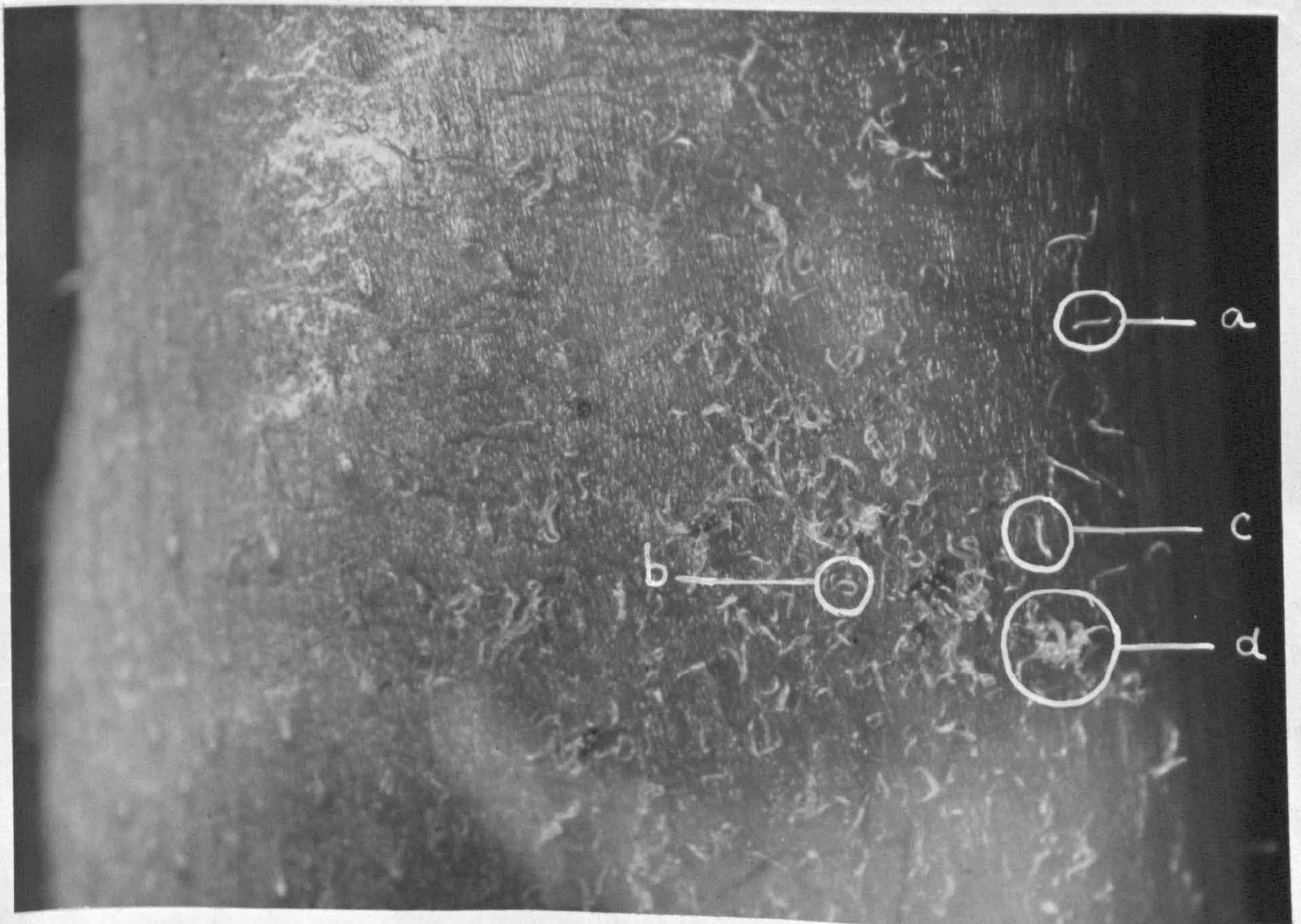


Fig. VI

Photograph showing positions assumed by the parasite on penetrating the skin. This plate is a photograph of the abdominal wall of a living mouse (less than 1 week old)

- a) Parasite resting tail on surface of skin. Body nearly perpendicular. See section 2.
- b) Parasite in a looped position, almost semicircular.
- c) Parasite almost parallel to skin.
- d) Accumulation of parasites ~~thus~~ preventing the entry of the larvae into the skin.

After the larvae have forced their heads into the stratum corneum they continue the invasion by separating the stratified layers of the cells by their activity and probably by some oral secretion, (Figs. VII and VIII). On their way through the stratum corneum the larvae were noticed to advance and retreat several times. They remain in the stratum corneum for periods varying from a few minutes up to two hours and sometimes even longer. In the stratum corneum the larvae were found lying parallel to the surface of the skin (Fig. IX) in a position similar to that of hookworm larvae (A. duodenale) observed in a study of the original specimens prepared by Looss (1905) when he investigated the penetration of the skin by hookworm larvae. After resting in the stratum corneum the larvae make their way down-wards into the epidermis. According to Taliaferro and Charles (1939) the infective larvae seem to penetrate directly only occasionally utilising a hair follicle (Fig. X). Thereafter they were found in the loose subcutaneous tissue as in Fig. XI.

Duration of Penetration of the Skin

Taliaferro and Charles (1939) studying the cellular reaction in the skin, lung, and intestine of normal and immune rats after infection with N. muris stated,

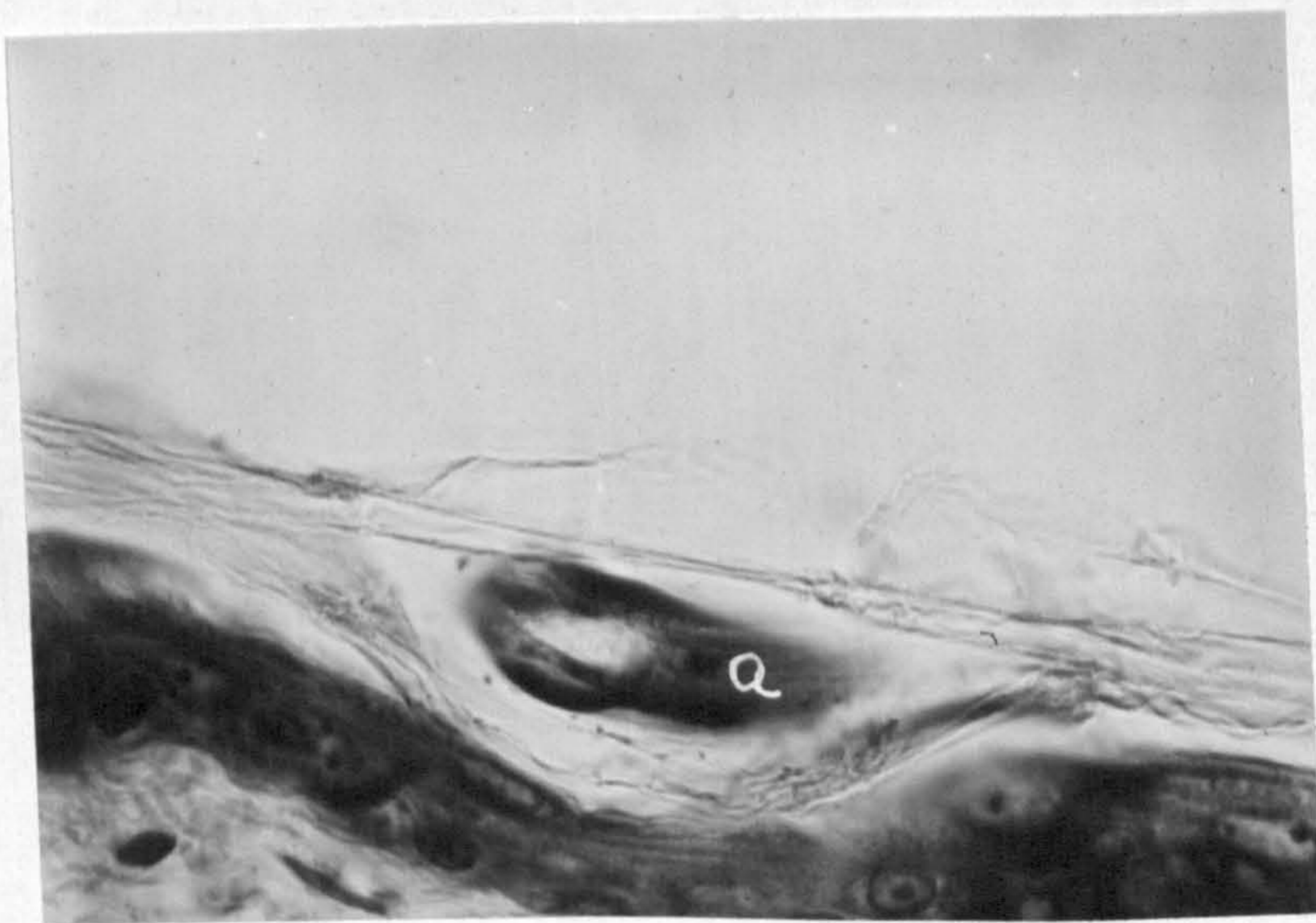


Fig. VII

Cross section of skin (rat) showing parasite (a) which just penetrated the cuticle (30 minutes after exposure).

(x700)

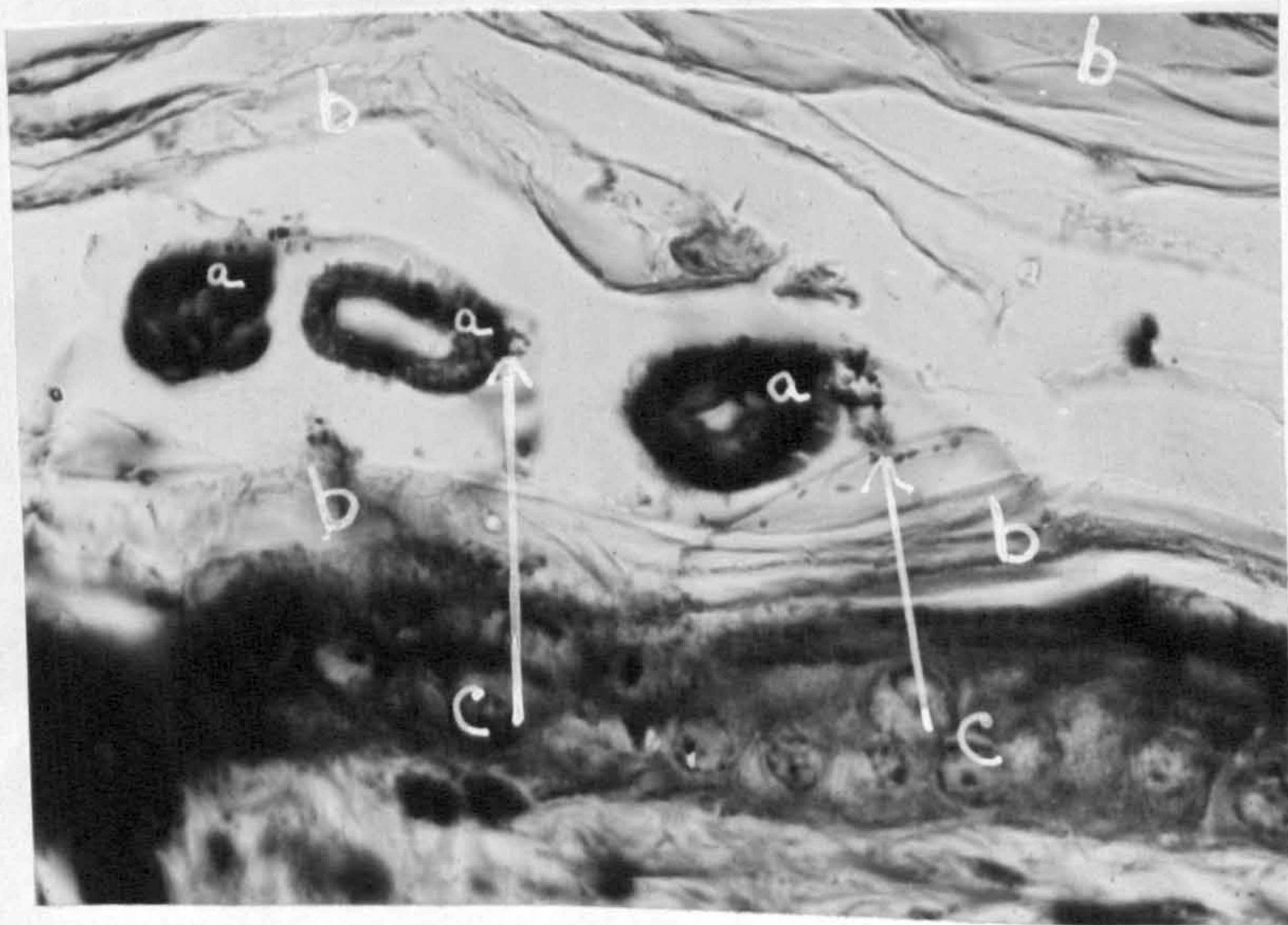


Fig. VIII

Section of skin (rat) showing larvae (a) of N.
brasiliensis separating the layers of the cuticle (b)
The micro-organisms around the parasite (c).

(x700)

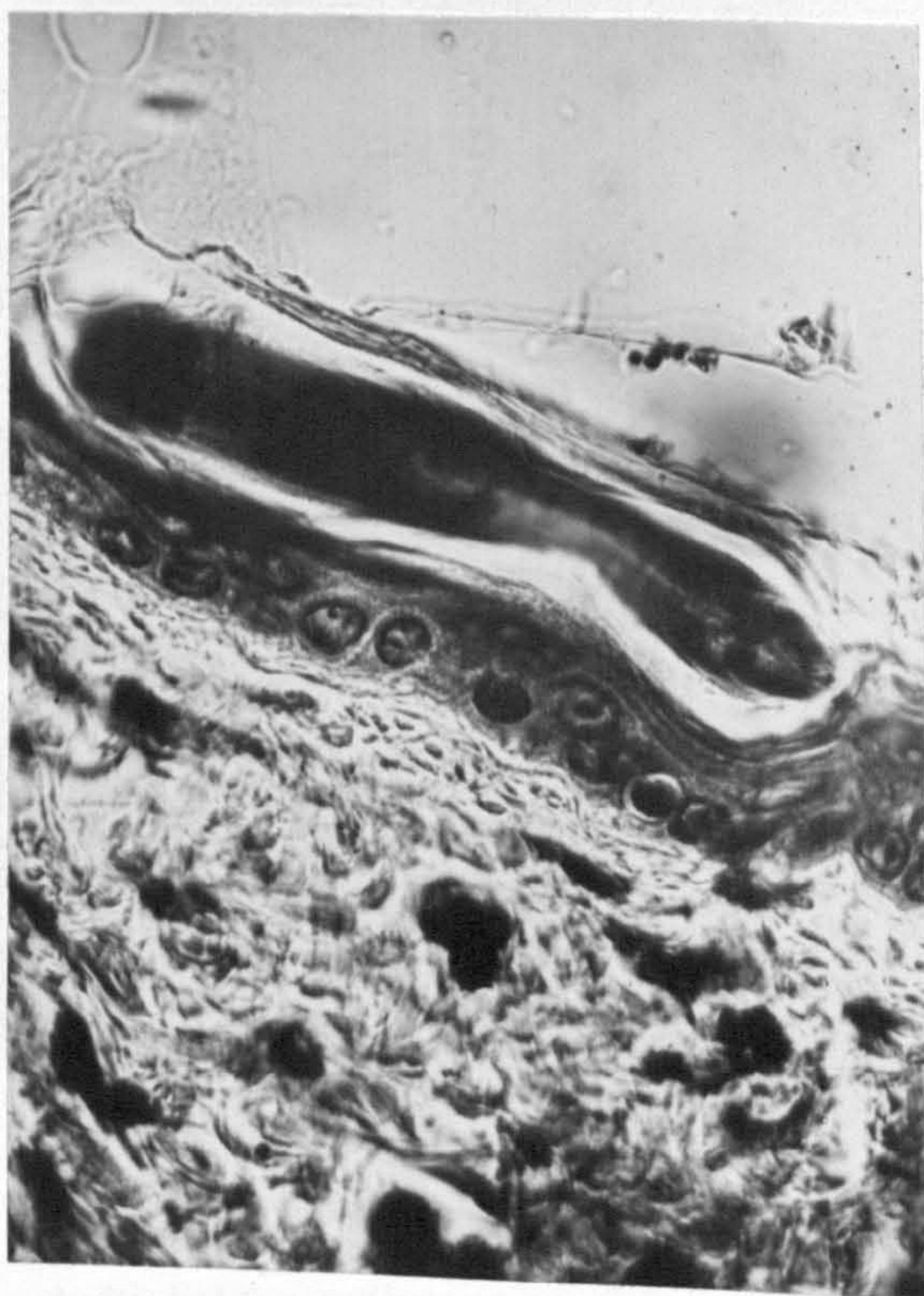


Fig. IX

Section of skin (rat) showing parasite after penetration of cuticle lying parallel to the cuticular layers (30 minutes after exposure).

(x700)

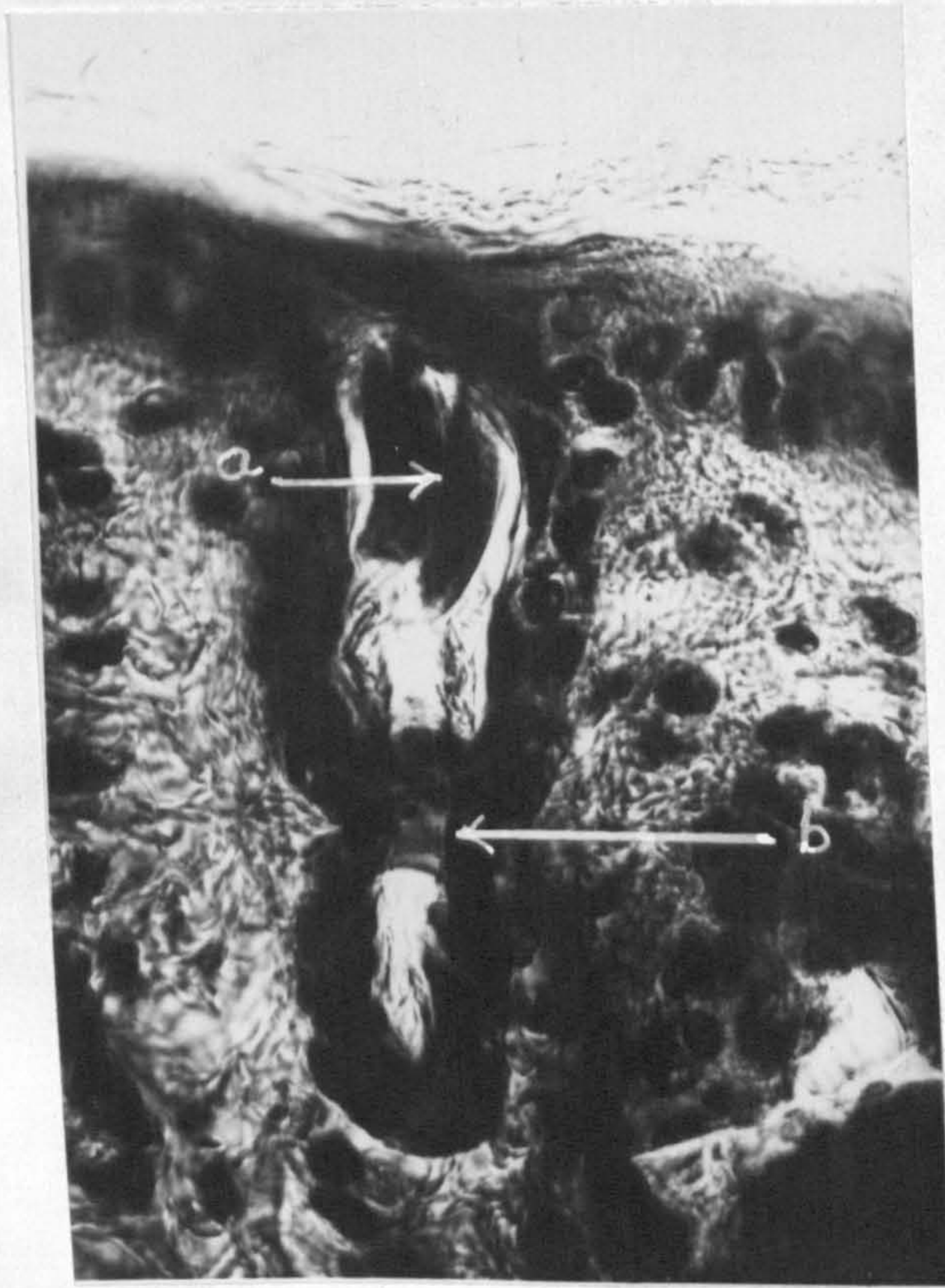


Fig. X

Section of skin (rat) showing parasite (a) migrating
down hair follicle (b). (30 minutes after exposure).

(x700)

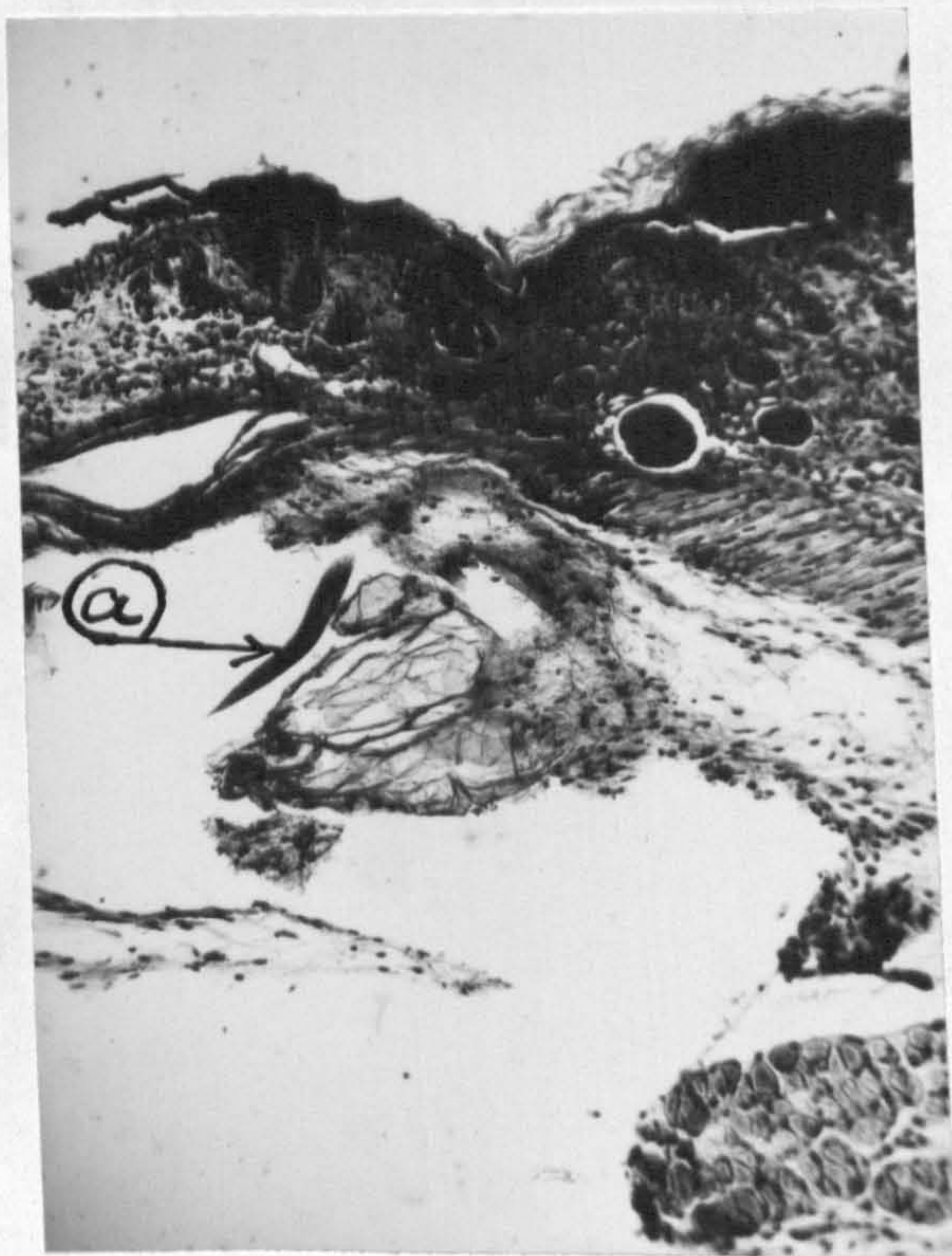


Fig. XI

Cross section of skin (mouse) showing parasite (a) in
loose connective tissue (15 minutes after exposure).

(x100)

"....., larvae were found penetrating the skin, (pl.1, Fig.1) from a half hour through two hours after they had been placed on the skin. They seemed to penetrate directly"

Yokogawa (1922) observed that this parasite when placed on the skin of rats, penetrated into the tissues very quickly. He did not specify any time for their entrance.

For further experimental work the writer found it was of importance to ascertain the time needed by the larvae to penetrate the skin, therefore eight mice, three weeks old, were fixed in position as for the percutaneous infection. An area in the middle of the abdomen was clipped with scissors, care being taken not to injure the skin. The site was damped with water and 200 larvae were applied to the clipped area of each mouse. The larvae were left for varying lengths of time on each mouse; 5, 10, 15, 20, 30 minutes, 1, 2 and 3 hours respectively. After the end of each of these periods the site of infection and surrounding area in each mouse concerned was wiped with 10% formalin solution, absolute alcohol, and then with a weak tincture of iodine (5%) to ensure that all larvae on the skin were killed. After disinfection each mouse was kept separately in a jar marked with the date and time of exposure to infection.

Forty-eight hours after exposure to infection all the mice were killed and the lymph glands draining the site of exposure, and lungs were examined for the presence of larvae and the results (table III) were as follows:-

(1) In the mouse exposed for 5 minutes dead larvae were found in the lymphatic glands of the axilla. One of these is shown in Fig. XII. The lung was not infected.

(2) In the mouse exposed for 10 minutes a few larvae were recovered from the lung and none from the lymphatic glands.

(3) In the mouse exposed for 15 minutes no larvae were recovered from the lymphatic glands or the lungs.

(4) In the mouse exposed for 20 minutes dead larvae were recovered from the lymphatic glands and none from the lungs.

(5) In all the mice exposed to the infection for 30 minutes, one hour, two hours, and three hours respectively, larvae were recovered from the lungs and none from the lymphatic glands.

These results suggest that some of the larvae may penetrate the skin within five minutes and the fact that they were recovered dead may be due to the larvae

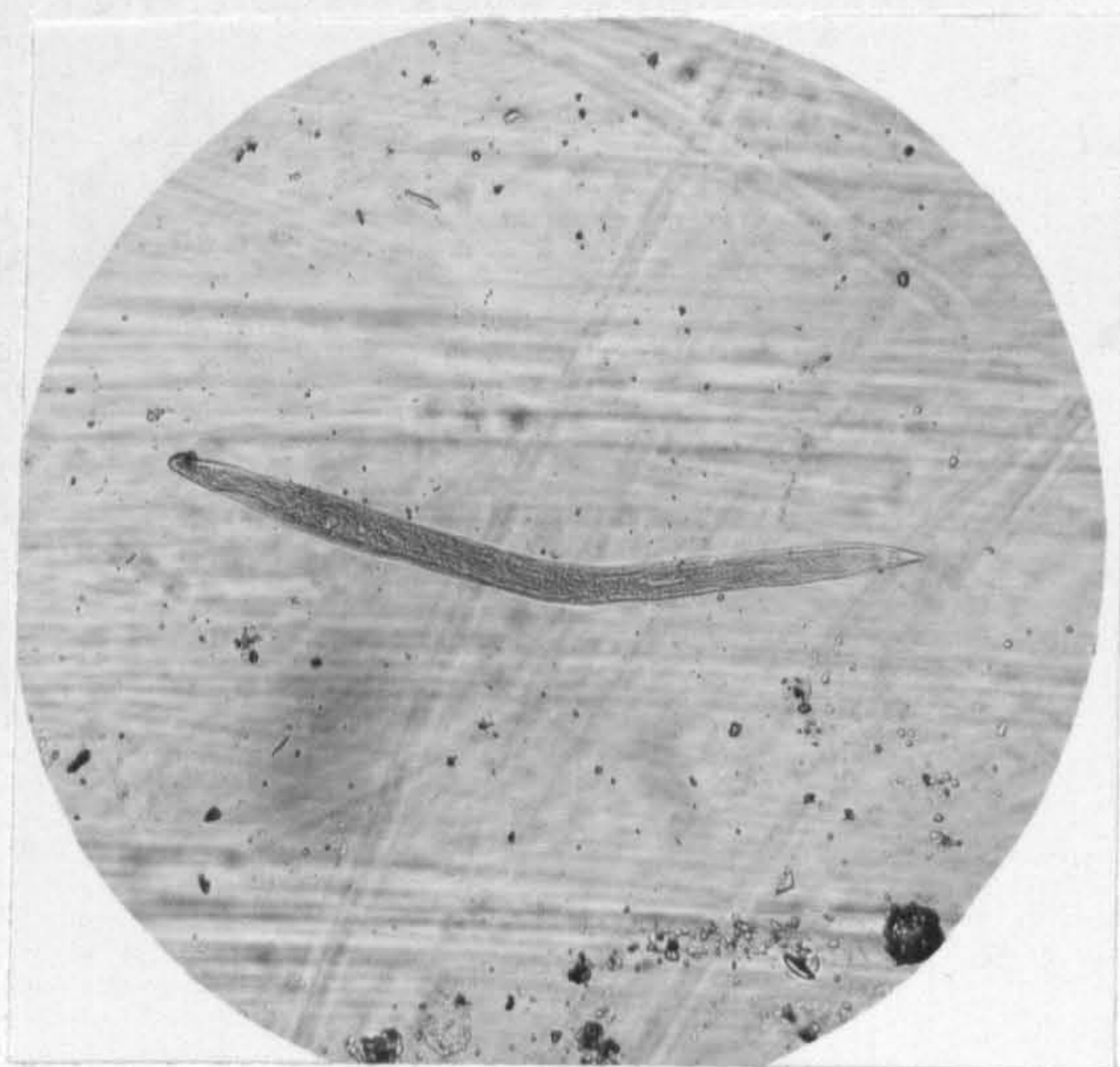


Fig. XII

A dead larvae in the precrural lymph gland of a mouse.

having been in contact with the applied disinfectants which killed them after penetration.

Table III

Time of Exposure	Result of the examination for the presence of larvae in	
	lymph gland	lung
5 minutes	dead larvae	negative
10 minutes	negative	positive
15 minutes	negative	negative
20 minutes	dead larvae	negative
30 minutes	negative	positive
one hour	negative	positive
two hours	negative	positive
three hours	negative	positive

This experiment was repeated on 15 mice (three weeks old) divided into three groups. The larvae were left on the skin for half an hour, one hour and two hours respectively and in all the animals larvae were recovered from the lungs 48 hours after exposure to infection.

These findings suggest that half an hour or more was sufficient for a number of larvae to penetrate the skin and reach the lungs.

Route of Migration within the Experimental Animal.

Yokogawa (1922) stated that the larvae penetrate the skin into the tissues and travel to the lungs, "..... by means of blood stream."

Taliaferro and Sarles (1939) studying the skin of rats during the initial infection with the larvae stated that "..... The third stage of inflammation was initiated at 16 hours by the movement of the larvae toward and through the larger subcutaneous veins during which small hemorrhages occurred....."

Barakat (1948) stated that, "..... The larvae after penetrating the skin are carried by the lymph or blood circulation to the lungs....."

Personal Observations

In trying to ascertain the sites likely to be affected by bacteria introduced by the infective larvae it was considered essential to confirm the observations of the above mentioned investigators.

Procedure Adopted

Animals used:- Albino rats and mice of ages varying from three weeks to two months. All animals had proved by previous repeated examinations of their faeces to be free from N. brasiliensis infection.

Method of infection:- The combined method of infection as previously described on page 58 was used.

The helminth used;- The infective stage larvae of N. brasiliensis, six days old, and cultured as previously described (see page 49).

Experiment No. 1

Fourteen rats were exposed to infection by the combined method in a glass jar and time of exposure was one hour, after which time the rats were removed to clean cages. It was arranged that one animal was anaesthetised and secured in position, abdomen up as in fig. III, at hourly intervals from one to fourteen hours. The abdominal skin of each rat was dissected and the blood vessels of the axilla were severed. The blood which oozed into the axillary space was sucked by a glass pipette into a test tube containing 5 c.c. of normal saline solution and then maintained at 28°C. for a quarter of an hour and the supernatant fluid was examined by a hand lens and afterwards under the binocular in a Petri-dish. The blood precipitate was examined under a binocular between two slides. The abdominal and thoracic cavities were opened separately and washed with normal saline. The washings from the peritoneal cavity were transferred into a Petri-dish and examined for the presence of larvae. The liver of

each animal was cut into pieces and each piece flattened between two slides and examined, under a dissecting microscope, for the presence of larvae.

- The above mentioned investigation was repeated on three occasions using fourteen albino mice.

The results were:- No larvae recovered from the blood, abdominal and thoracic cavities or from the liver of the exposed animals.

Experiment No.2

Fourteen mice were exposed to infection of infective larvae as previously described for the per-rectum method of infection (page 70). One animal was killed at hourly intervals from one to fourteen hours after exposure. The liver of each animal was cut into pieces and each piece flattened between two slides and examined under the low power of the microscope. The result of the examination did not prove the presence of the larvae in the liver.

The Migration of the Larvae Inside the Body to the

Lungs via the Lymph System

On failing to recover the parasite from the liver, blood, or body cavities of infected animals, the lymphatic glands were next examined for the presence of

larvae.

Experiment No.1

Fourteen young mice, three weeks old, were exposed to infective larvae by the application of the parasite to the intact abdominal skin. One animal was killed at one hour, two hours and so on until the fourteenth hour after exposure. To facilitate examination, these animals were not all exposed at the same time. The subcutaneous lymph glands, the axillary, precrural, popliteal, submaxillary, parotid and prepectoral lymph glands were examined between two slides under the low power of the microscope.

Result of the Examination:

Twelve hours after exposure, larvae were recovered from the axillary lymph glands (see table IV). No larvae were recovered before this period had elapsed.

Experiment No.2

Further experiments were conducted to prove or disprove the above findings using twenty-one white mice which were exposed to infection as above (experiment 1). Three mice were killed after twelve hours, three more an hour later and the rest in groups of three at the 14th, 15th, 16th, 17th and 18th hours after exposure. The axillary lymph glands and the lungs of all these

animals were examined for the presence of the larvae.

Table IV

Hours after Exposure	Lymph Glands					
	Axil- lary	Pre- crural	Popli- teal	Sub- maxillary	Parotid	Pre- Pectoral
1	Nega- tive	Nega- tive	Nega- tive	Negative	Negative	Negative
2	"	"	"	"	"	"
3	"	"	"	"	"	"
4	"	"	"	"	"	"
5	"	"	"	"	"	"
6	"	"	"	"	"	"
7	"	"	"	"	"	"
8	"	"	"	"	"	"
9	"	"	"	"	"	"
10	"	"	"	"	"	"
11	"	"	"	"	"	"
12	Posi- tive	"	"	"	"	"
13	"	"	"	"	"	"
14	"	"	"	"	"	"

Result:

Larvae were present in the axillary lymph glands in animals killed 12, 13, 14, 15, 16, and 17 hours after exposure but none were present in the lungs. In the group killed eighteen hours after exposure, larvae were

recovered from the lungs of all the animals but not from the axillary lymph glands (see table V).

Table V

Hours after Exposure	Number of Animals Used	Result of examining the	
		Axillary lymph glands	Lungs
12	3	Positive	Negative
13	3	Positive	Negative
14	3	Positive	Negative
15	3	Positive	Negative
16	3	Positive	Negative
17	3	Positive	Negative
18	3	Negative	Positive

Experiment No. 3

Six albino mice about four months old were secured in position as for the method previously described (page 58) for the percutaneous infection. The skin of the thigh of the right limb was damped with water. One hundred infective larvae on a piece of filter paper were applied to the selected site of exposure in each animal. The time for exposure was one hour, after which the animals were released. Twelve, sixteen, eighteen and twenty hours after exposure two animals were killed successively and the precrural lymph glands, popliteal

lymph gland of the limb exposed to infection, as well as the lungs of each animal, were examined under the microscope for the presence of larvae.

The result of examination was that larvae were recovered from the precrural and popliteal lymph glands in the animals killed after twelve and sixteen hours but none were recovered from the lungs. In the remaining animals no larvae were recovered from the precrural or popliteal lymph glands but larvae were present in the lungs (see table VI).

Table VI

Hours after Exposure of the Hind Limb	Number of Animals Used	Result of examining the		
		Lymph Gland		Lungs
		Precrural	Popliteal	
12	2	Positive	Positive	Negative
16	2	Positive	Positive	Negative
18	2	Negative	Negative	Positive
20	2	Negative	Negative	Positive

Experiment No.4

Seven mice of ages varying between two and three months were used. All animals were lightly anaesthetised and a piece of filter paper bearing about two hundred larvae was introduced into the buccal cavity of each

mouse. Eight hours after exposure one animal was killed and two hours later another one was killed and so on till the twentieth hour after exposure. The submaxillary and parotid lymph glands and the lungs of each animal were pressed between slides and examined under the low power of the microscope for the presence of larvae.

The result of examination proved that the parasite was present in the lymphatic glands twelve, fourteen and sixteen hours after exposure. In the lungs the parasite was present from the eighteenth hour onwards, as illustrated in table VII.

Table VII

Time of Examination after Exposure	Lymph Glands		Lung
	Submaxillary	Parotid	
8 hours	Negative	Negative	Negative
10 hours	Negative	Negative	Negative
12 hours	Positive	Positive	Negative
14 hours	Positive	Positive	Negative
16 hours	Positive	Positive	Negative
18 hours	Negative	Negative	Positive
20 hours	Negative	Negative	Positive

Experiment No.5

Thirty-two mice about four weeks old were divided into four equal groups. All animals proved by previous

repeated examinations to be free of H. brasiliensis infection. Each group was exposed to infection in the following manner:-

- Group I per rectum
- Group II subcutaneously
- Group III intraperitoneally
- Group IV intrathoracically

The technique used for exposure was carried out on the same lines as previously described (pages 66-67) for the different routes of infection. Each group was exposed to a large number of infective larvae.

Ten hours after exposure one animal from each group was killed and subsequently an animal from each group was killed at two-hourly intervals, i.e., at 10, 12, 14, 16, 18, 20, 22 and 24 hours after exposure. The lymph glands draining the site of exposure and the lungs of all the animals were pressed between slides and examined under the microscope for larvae to note the time of arrivals in these organs.

Result

The results of examinations as summarized in table VIII were:-

In Group I larvae were present in the mesenteric lymph glands 12, 14, 16 and 18 hours after exposure. Larvae first arrived in the lungs 18 hours after

exposure and were present 18, 20, 22 and 24 hours after exposure.

In Group II larvae first arrived in the axillary lymph glands twelve hours after injection and were present in the glands from the twelfth to the eighteenth hours after exposure. Larvae first arrived at the lungs eighteen hours after exposure and were present from eighteen to twenty-four hours.

In Group III larvae first arrived in the mesenteric lymph glands eighteen hours after exposure and were present from eighteen to twenty hours. In the lungs larvae first arrived twenty hours after exposure and were present 20, 22 and 24 hours after injection.

In the fourth Group larvae were first recovered in the bronchial lymph glands fourteen hours after injection and were present from fourteen to eighteen hours after injection. They first arrived in the lungs twenty hours after injection and were recovered 20, 22 and 24 hours after injection.

Sometimes more than thirty larvae were counted in one lymph gland. It was noticed that all larvae do not travel to the lymph glands or the lungs at the same time. It was observed that when the host was infected through the intact skin or injected subcutaneously the larvae reached the nearest lymphatic glands in twelve hours and

Table VIII

No. of Group	Method of Exposure	Time needed by the larvae to		
		Arrive in Lymph Gland	Recovery in Lymph Glands	Arrive in Lung
1	Per rectum	12 hours	12-18 hours	18 hours
2	subcutaneously	12 hours	12-18 hours	18 hours
3	Intra-peritoneally	18 hours	18-20 hours	20 hours
4	Intra-thoracically	14 hours	14-18 hours	24 hours

the lungs in eighteen hours, but when they were injected intraperitoneally or intrathoracically they took a longer time to reach the lymph glands draining those areas.

The arrival in the lungs was also prolonged compared with the time needed to reach them from the skin. This suggests that the infective larvae might need a longer time to penetrate through the serous tissues than that needed to penetrate the skin. When sections of infected lymph glands of rats, mice and guineapigs were studied, the larvae were noticed to be always present at the periphery of the glands as illustrated in Fig. XIV.

It is of interest to note here that Standen (1952) demonstrated that the cercariae of Schistosoma mansoni penetrate the lymphatic system of the mouse within twenty minutes.

Conclusion

From the results of all the above experiments it seems reasonable to conclude that the infective larvae of N. brasiliensis migrate to the lungs of the host via the lymphatic system and not by the blood stream as stated by Yokogawa (1922), Taliaferro and Sarles (1939) and Barakat (1948).

The Route of Migration of N. brasiliensis from the Lungs to the Small Intestine

Previous experiments by the author for determining the route of migration of the parasite inside the host (rat or mouse) have proved that the parasite first arrived in the lungs of all infected animals eighteen hours after percutaneous exposure. For the rat only this time was recorded to be fourteen to twenty hours by Yokogawa (1922), eighteen to forty-two hours by Schwartz and Alicata (1934) and twenty hours by Taliaferro and Sarles (1939).

The literature makes no mention of the time taken by the parasite to reach the lungs in mice.

In the lungs larvae feed and grow rapidly (Fig. X⁽¹¹⁾) and moult a few hours before leaving the lungs (Yokogawa 1922).

During some of the writer's experiments, two infected mice died about sixty-five hours after infection and on

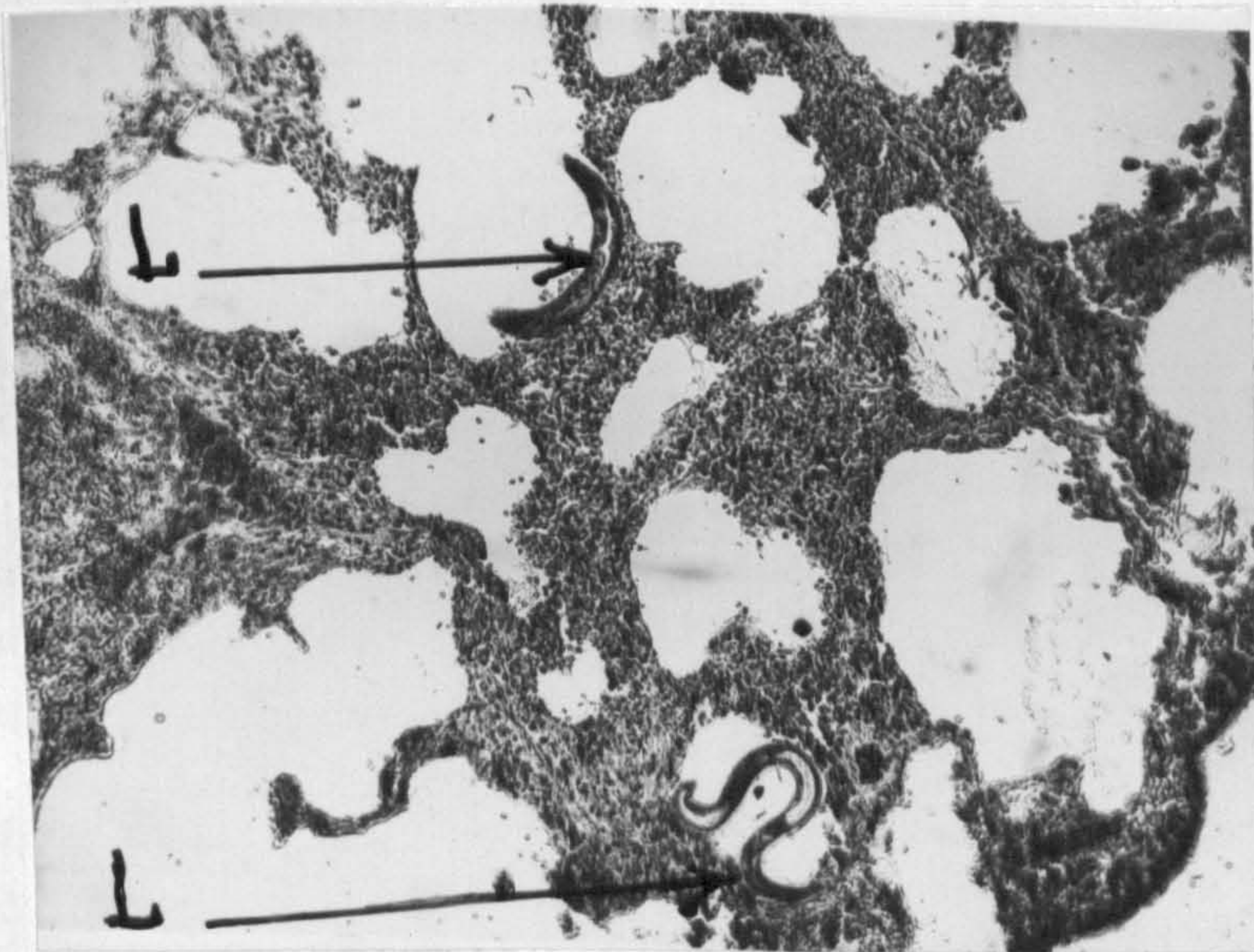


Fig. XIII

N. brasiliensis larvae (L) in the lungs of a rat (60 hours after exposure).

(x 60)

post mortem examination larvae were found in the oesophagus and trachea, as well as in the lungs of animals. As a result of this observation six mice, free of infection, were exposed by the combined method of infection (page 58). One animal was killed every hour from the sixtieth hour after exposure until the sixty-fifth hour.

The result of examination of the trachea, oesophagus and duodenum showed that the parasite was present in the trachea and oesophagus between sixty-three and sixty-five hours, and in the intestine sixty-five hours after exposure.

A Comparative Study of the Behaviour of *N. brasiliensis*
in Different Kinds of Experimental Animals

(1) Longevity of the Parasite

The following animals were used in groups of three:- albino rats, white mice, voles (*Microtus agrestis*) and guineapigs of ages varying between three to four weeks. Faecal examination of these animals for *N. brasiliensis* infection had proved them to be worm free.

Technique

All animals were lightly anaesthetised (anaesthetic ether) and fixed in position abdomen upwards (Fig. III). Each animal was exposed to about two hundred infective

larvae, six days old, and counted at the edge of the filter paper. All the animals were left in this position for one hour after which they were released and each group kept in a separate cage. From three days after exposure the faeces of each group were examined daily for the presence of eggs.

Result (see table IX)

The albino rats began to pass eggs five days after exposure and stopped passing eggs one hundred days after exposure.

The mice began to pass eggs six days after exposure and seven days later eggs ceased to pass in the faeces.

The hamsters were found to pass eggs six days after exposure and four weeks after exposure eggs ceased to be passed.

In the cotton rats very few eggs were passed seven days after exposure and ceased to occur from four days later.

The voles and guineapigs did not pass any eggs at all.

(2) Route of migration inside the body of experimental

animals

Animals Used

The following animals were used in groups of fourteen:- albino rats, albino mice, cotton rats, hamsters, voles and guineapigs of ages varying between three and six weeks.

Table IX

Animal	Date after Exposure when Eggs	
	were passed	ceased to be passed
Albino rats	5 days	100 days
Albino mice	6 days	13 days
Hamsters	6 days	28 days
Cotton rats	7 days	11 days
Voies	Nil	Nil
Guineapigs	Nil	Nil

Mode of Infection

Animals were lightly anaesthetised by anaesthetic ether and exposed to infection by the combined method described on page 58. The animals were exposed to a large number of infective larvae. To facilitate exposure and examination all animals were not exposed to infection at the same time.

Larvae Used

Infective stage larvae six days old cultivated on the Petri-dish were used for the infection of these experimental animals.

Technique Used

Two animals were killed from each group twelve, eighteen, twenty-four, forty-eight and seventy hours, and one and two weeks after exposure. The lymphatic

glands (submaxillary, parotid, preascapular, axillary, precrural and popliteal), lungs and small intestines were examined.

Sections were prepared from the precrural lymphatic glands of rats twelve hours after exposure and from the same glands of the exposed guineapigs forty-eight hours after exposure. These glands were fixed in absolute alcohol, sectioned five to seven microns thick, and stained either by Giemsa's stain or haematoxylin and eosin.

Result of Examination

1) Twelve hours after exposure larvae were present in most of the lymphatic glands of the experimental animals but not in the lungs or intestinal canal. In section the larvae were present in the periphery of the precrural lymph gland of the rat. It was noticed that larvae were to be found mostly in the cortex of the glands without or with very slight inflammation around them (see Fig. XIV).

2) Eighteen hours after exposure larvae were present in most of the lymphatic glands of all the experimental animals as well as in the lungs, but not in the alimentary canal. The cells of the intestinal walls of the parasite in the lymph glands (see table X) were observed to be:-

a) faintly pigmented in rats and mice

b) well pigmented in cotton rats and hamsters

c) highly pigmented in guineapigs and voles.

In the lungs the cells of the intestinal walls of the larvae were observed to be highly pigmented in all the experimental animals except the rats and mice in which they were not so markedly pigmented.

Table X

Animal	Degree of Pigmentation of the Cells of the Intestinal Walls of Larvae 18 Hours after Exposure	
	Lymph Glands	Lungs
Rat	Faintly pigmented	Pigmented
Mouse	Faintly pigmented	Pigmented
Guineapig	Highly pigmented	Highly pigmented
Vole	Highly pigmented	Highly pigmented
Cotton rat	Pigmented	Highly pigmented
Hamster	Pigmented	Highly pigmented

3) Twenty-four hours after exposure larvae were present in the lymph glands of the cotton rats, hamsters, voles and guineapigs but not in those from the rats or the mice. Larvae were present in the lungs of all the animals. No larvae were recovered from the small intestine of any of the animals (see table XI).

4) The result of examination of the lymph glands, lungs and small intestines forty-eight hours after exposure

was the same as for twenty-four hours. In the section made from the lymph glands of the guineapigs the parasite was seen to be surrounded by a zone of inflammation forty-eight hours after exposure.

Table XI

Animal	Presence of Larvae 24 Hours after Exposure in		
	Lymph Glands	Lungs	Small Intestine
Rat	Nil	Positive	Nil
Mouse	Nil	Positive	Nil
Cotton rat	Positive	Positive	Nil
Hamster	Positive	Positive	Nil
Vole	Positive	Positive	Nil
Guineapig	Positive	Positive	Nil

5) Seventy hours after exposure larvae were present in the lymph glands of cotton rats, hamsters, voles, guineapigs but not in the lymph glands of the rats or the mice. Larvae were present in the lungs of all the animals seventy hours after infection. The parasite was recovered in the small intestine of the rats, the mice, the cotton rats and the hamsters, but not from the voles or the guineapigs (see table XII).

6) One week after exposure dead larvae were present in the lymph glands of the guineapigs and the vole, but

Table XII

Animal	Presence of Larvae 70 Hours after Exposure in		
	Lymph Glands	Lungs	Small Intestine
Rat	Nil	Positive	Positive
Mouse	Nil	Positive	Positive
Cotton rat	Positive	Positive	Positive
Hamster	Positive	Positive	Positive
Vole	Positive	Positive	Nil
Guineapig	Positive	Positive	Nil

Table XIII

Animal	Presence of Larvae One Week after Exposure in		
	Lymph Glands	Lungs	Small Intestine
Rat	Nil	Nil	Positive
Mouse	Nil	Nil	Positive
Cotton rat	Nil	Positive	Positive
Hamster	Nil	Positive	Positive
Vole	Positive	Positive	Nil
Guineapig	Positive	Positive	Nil

not in the lymph glands of the other animals (see table XIII). The larvae were present in the lungs of the cotton rats, hamsters, voles and guineapigs, but not in the lungs of rats or mice. The parasite was recovered

one week after exposure from the small intestine of the rats and mice and recovered stunted in the hamster and cotton rats and was not recovered from the voles or guineapigs.

7) Two weeks after exposure few dead larvae were present in the lymph glands of the guineapigs but none were present in the lymph glands of the other animals (see table XIV). The examination of the lungs of the animals revealed that no larvae were present in the rats or the mice while in the lungs of the voles, hamsters, cotton rats and guineapigs dead larvae were present. No parasites were recovered from the intestinal canal of the mice, cotton rats, voles or guineapigs, but they were present in this organ in the rats and hamsters.

Table XIV

Animal	Presence of Larvae Two Weeks after Exposure in		
	Lymph Glands	Lungs	Intestinal Tract
Rat	Nil	Nil	Positive
Mouse	Nil	Nil	Nil
Cotton rat	Nil	Positive	Nil
Hamster	Nil	Positive	Positive
Vole	Nil	Positive	Nil
Guineapig	Positive	Positive	Nil

8) During the macroscopic examinations of the infected lymphatic glands of the different experimental animals it was noticed that these glands were congested in the guineapigs, voles, cotton rats and hamsters while those of the rats and mice appeared to be normal in colour.

Sections of the precrural lymphatic glands of experimentally infected rats and guineapigs were prepared respectively 18 and 48 hours after exposure. The result of examinations of these sections has shown a marked zone of reaction around the larvae in the lymph gland of the abnormal host, the guineapig (Fig. XV), and a very slight or no reaction around the larvae in the normal host, the rat (Fig. XIV).

The Degree of the Reaction in the Tissues of the Under-Surface of the Skin of Animals after the Application of the Infective Larvae

Animals Used

Albino mice, about four weeks old, and known to be free of N. brasiliensis infection, were used.

Helminth Used

Infective larvae of N. brasiliensis, from five days old cultures, were used. The edges of ten filter papers, bearing a large number of infective larvae

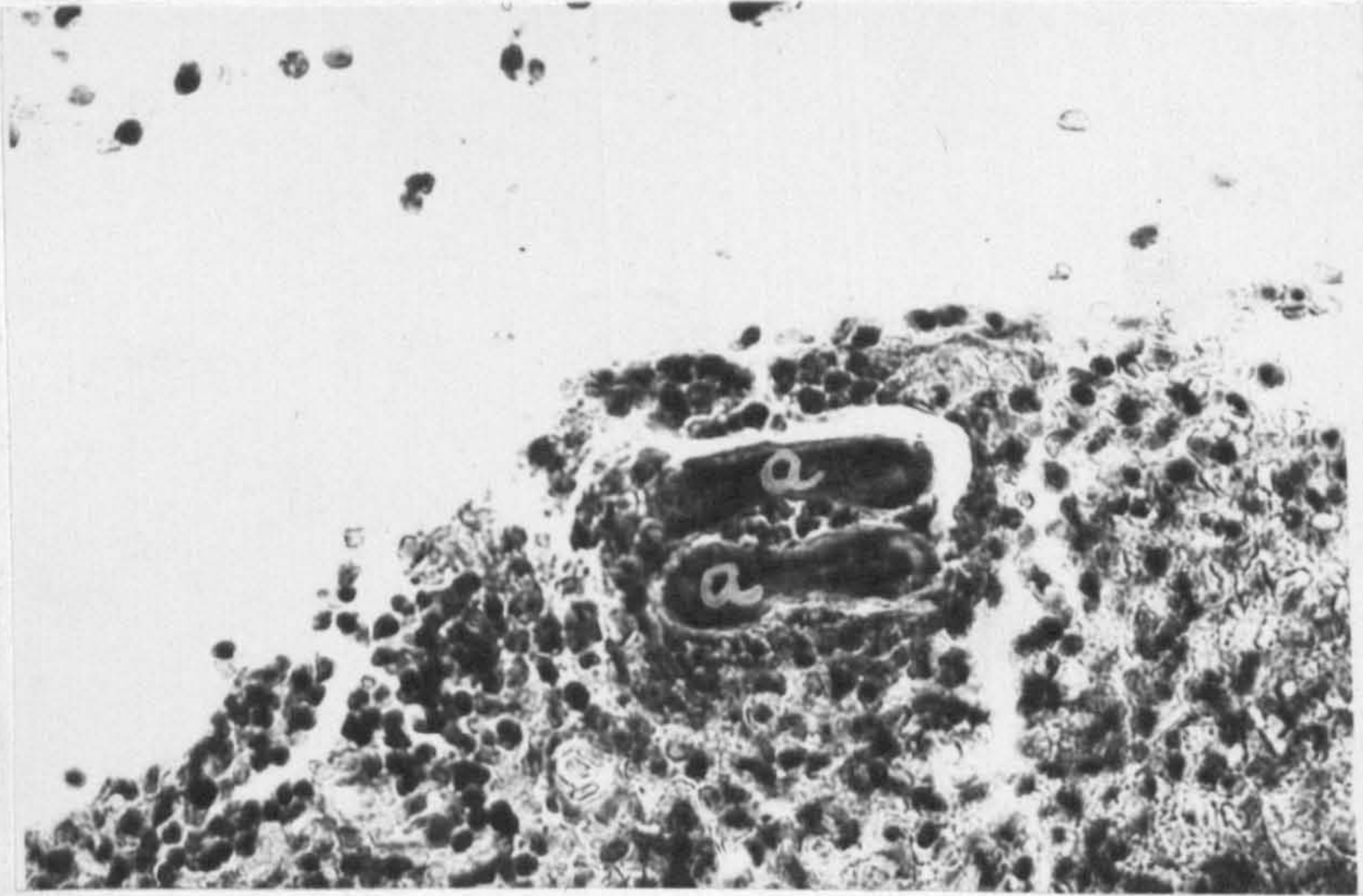


Fig. XIV

Cross section of the precrural lymph gland of a rat showing the larvae (a) of *N. brasiliensis* 12 hours after the exposure of the animal to infection.

(x400)

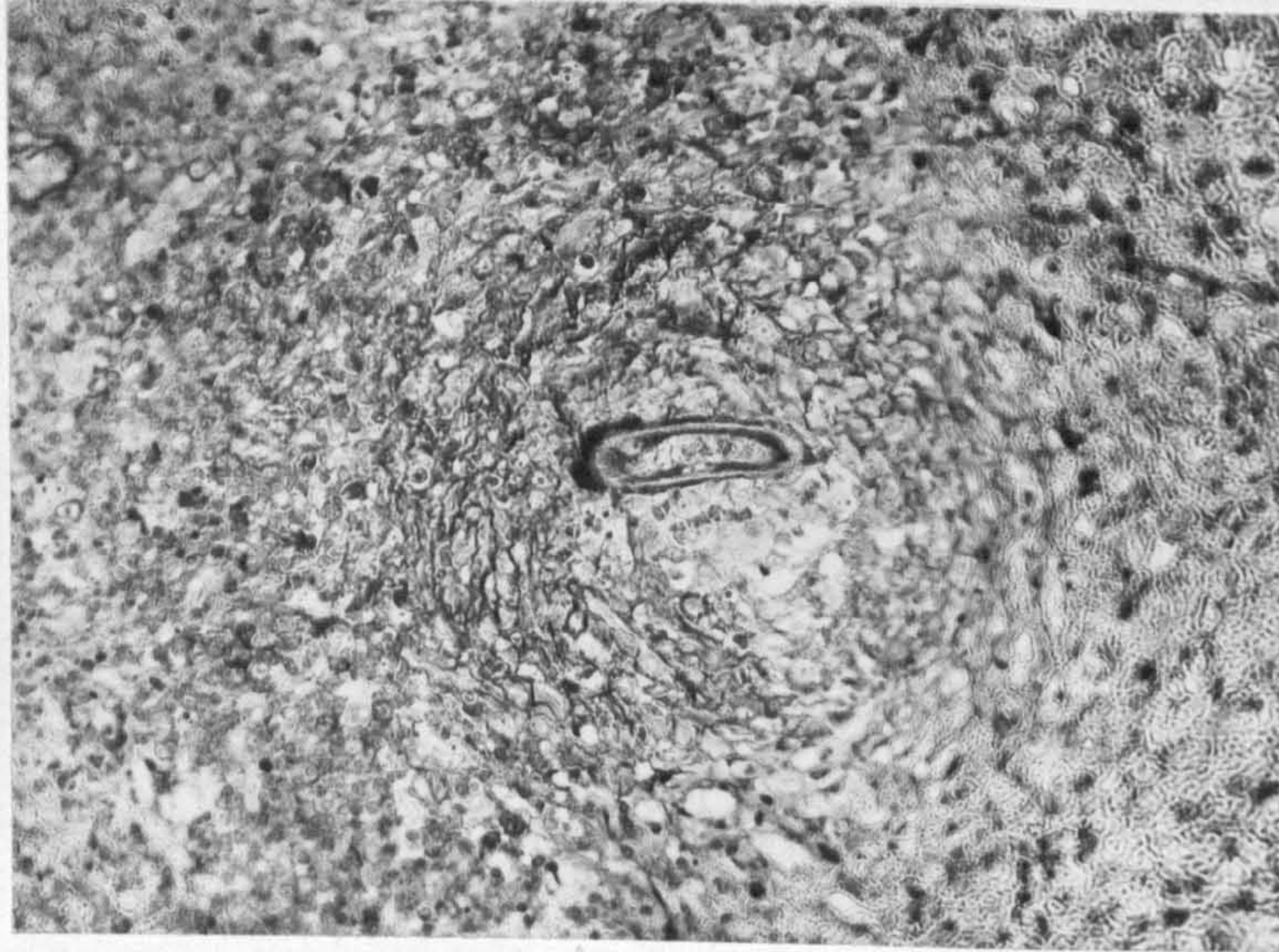


Fig. XV

Cross section in the precrural lymph gland of a guineapig showing the marked reaction around the larvae of N. brasiliensis 48 hours after exposure in that abnormal host.

(x200)

(each filter paper bore about 600 larvae) were put in a glass jar, as previously described in the mixed method of infection, containing about 10 c.c. of filtered tap water.

Technique Employed

Ten mice were dipped into the jar containing approximately 6,000 larvae and left there for one hour after which the mice were removed to a clean glass jar. Two animals were killed respectively 12, 16, 18, 24, and 48 hours after exposure. Each animal was pinned to a board and the skin was dissected and the body of the animal removed. A macroscopic examination of the undersurface of the skin was carried out by the naked eye and afterwards with a hand lens.

Result

Raised haemorrhagic spots were noticed on the undersurface of the skin in the animals killed after the eighteenth hour of exposure. A marked congestion of the tissues around the superficial lymphatic glands of the body was also observed (Fig. XVI). Twenty-four hours after exposure the spots on the undersurface of the skin and the congestion around the lymph glands were noticed to subside and at 48 hours the congestion had disappeared.

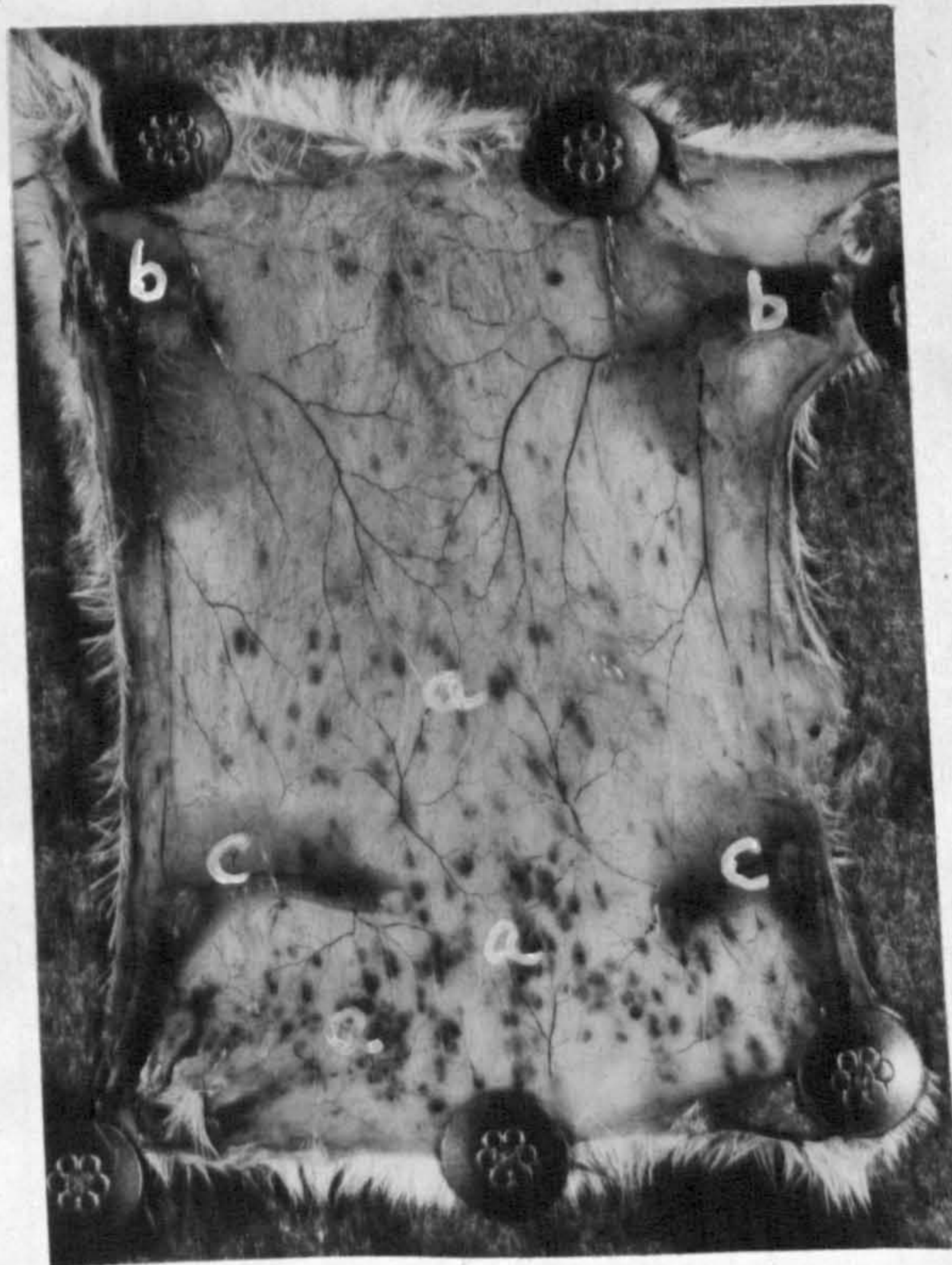


Fig. XVI

Under surface of the skin of a mouse (18 hours after exposure) showing marked reaction around:-

- a) sites of the penetration of the parasite,
- b) axillary lymph glands,
- c) precrucial lymph glands.

Summary of the Investigations Carried out on the
Parasite (N. brasiliensis)

1) The hatching of infective larvae on filter paper, using activated alumina as an absorbent, proved a satisfactory technique for obtaining and counting infective larvae for the experimental work.

2) Successful development of the parasite was demonstrated by the following techniques of infection; percutaneous, per os, per rectum, subcutaneously, intrathoracically or intraperitoneally, but not when larvae were applied to the tail of the mouse.

3) Dampness of the skin is an important factor in facilitating the infection with maximum number of larvae.

4) The behaviour of the larvae on application to the skin and during penetration was studied.

5) The greatest number of larvae was observed to penetrate the skin within half an hour of exposure.

6) It was proved that the route of migration of the parasite to the lungs was via the lymphatic system and not by the blood.

7) The larvae, after application to the surface of the skin, reach the lymphatic glands of all exposed animals not before 12 hours after exposure and the lungs 18 hours after exposure. If the larvae were introduced

into the body cavity by injection the time taken for them to reach the lymphatic glands and the lungs was usually longer than when percutaneous exposure was used.

8) The larvae did not progress further than the lungs in ^{abnormal} hosts such as guineapigs and voles.

9) The intestinal cells of the larvae in the lymph glands or lungs of abnormal hosts are highly pigmented.

Studies on the Role of the Tissue-Invading Helminth

(N. brasiliensis) in Bacterial Infection

The Application of Pathogenic Micro-organisms and Infective
Larvae to the Intact Abdominal Skin and their Recovery
from the Subcutaneous Tissues

Experimental Animals

Albino rats and mice, of ages varying between four and six weeks, were used. All animals had been proved by repeated examinations of their faeces to be free from N. brasiliensis infection. In each experiment three groups of animals were used. In Group No.1 infective larvae were applied to the skin, in Group No.2 bacterial emulsion was only applied to the skin and in Group No.3 bacteria plus larvae were applied.

Micro-organisms

The bacteria used in these experiments were Staphylococcus aureus and Streptococcus pyogenes and consisted of forty-eight hours nutrient broth cultures and cultures sown on blood agar incubated at 37°C. which were mixed together.

The Helminth

The infective stage larvae of N. brasiliensis used for the experiments were cultured as previously described on page 49. The infective stage larvae, as

recovered from the edge of the filter paper, were applied to the intact abdominal skin. They were not treated with any disinfectant.

The infectivity of the larvae was tested by exposing two rats to infection with the same culture of larvae as used in each of the experiments, and in each case the larvae proved to be infective, as eggs of the parasite were recovered when faeces were examined a week after exposure.

Technique Employed

Animals were anaesthetised with ether and secured, abdomen upwards, as previously described in the percutaneous method of infection (Fig. 11). The hair was clipped from a square in the middle of the abdomen of each animal and great care was taken not to injure the skin. The site of exposure was examined under a hand lens (x12) to make certain that the skin was quite intact and it was then damped with sterile filtered water. The bacterial culture grown on the blood agar was transferred to that in the serum broth and then to the prepared site in the animals of Group No.3. 0.3 ml. of the emulsion of the selected organism, and 500 infective larvae, were applied. The same dose of emulsion only was applied to the site in animals of Group No.2. To group No.1 only 500 infective larvae

were applied and 0.3 ml. of sterile filtered water was added. A quarter of an hour after exposure 0.3 ml. of the emulsion was added to the selected site in each animal of Groups No.2 and No.3, and 0.3 ml. of sterile filtered water to the animals of Group No.1. To insure the penetration of a maximum number of parasites into the subcutaneous tissues the animals were kept secured in position for two hours, after which the site of exposure and the area around it were disinfected by the application of 5% tincture of iodine. The animals were then killed by chloroformation and the abdominal skin was aseptically dissected exposing the under surface of the skin at the site of infection. All procedures were carried out as aseptically as possible. A platinum loop was used to make smears from the subcutaneous tissues under the site of infection in groups 2 and 3, and these were inoculated into nutrient broth media which was incubated at 37°C. for twenty-four hours. Subcultures were made from these broth cultures on serum and blood agar plates. Morphology of the colonies of bacteria and smears on slides were examined to note whether the bacteria used in the experiment had been conveyed by the larvae into the subcutaneous tissues.

Experiment No. I (Staphylococcus aureus)

Group 1 - Five rats (6 weeks old) were exposed to larvae.

Group 2 - Five rats (6 weeks old) were exposed to bacteria.

Group 3 - Five rats (6 weeks old) were exposed to bacteria and larvae.

This experiment was repeated on three subsequent occasions. The result of all the experiments showed that no bacteria were recovered from the subcutaneous tissues of the rats (group 2) exposed to bacteria only. In the twenty rats (group 3) which were simultaneously exposed to staph aureus and infective stage larvae the micro-organisms Staph. aureus were recovered from the subcutaneous tissues in nineteen cases out of twenty. In a few of these cases other bacteria (see table XV) were also recovered. A Gram-negative bacillus and a streptococcus were recovered in all animals of Group No. 1.

Experiment No. II (Streptococcus pyogenes)

This experiment using Str. pyogenes was conducted on the same lines as in the first experiment. Fifteen mice were used and this experiment was not repeated. The result (as illustrated in table XVI) showed that no bacteria were recovered from the mice in Group 2 while in Group No. 3 the micro-organism Str. pyogenes was recovered from each animal in the group. A Gram-negative bacillus and a streptococcus were recovered from all the mice in Group No. 1.

Table XV

Rat No.	Micro-organisms recovered from the subcutaneous tissues		
	Rats of Group 1	Rats of Group 2	Rats of Group 3
1			Staph. aureus
2			Staph. aureus
3			Staph. aureus
4			Staph. aureus
5	A Gram-negative	No bacteria	Staph. aureus
6	bacillus	were	Staph. aureus
7	and	recovered	Staph. aureus + Gram- negative bacilli
8	Streptococcus	from these	Staph. aureus
9	organisms	rats	Staph. aureus
10			Staph. aureus
11			Staph. aureus
12			Staph. aureus
13			Staph. aureus
14			Staph. aureus
15			Staph. aureus
16			Staph. aureus + Gram- negative bacilli
17			Staph. aureus
18			Staph. aureus
19			Staph. aureus
20			Negative

Table XVI

Number of Mouse	Micro-organisms recovered from the subcutaneous tissues		
	Mice in Group 1	Mice in Group 2	Mice in Group 3
1	A Gram-negative bacillus	Negative	Str. pyogenes
2	"	Negative	Str. pyogenes
3	A Gram-negative bacillus + Streptococcus	Negative	Str. pyogenes
4	"	Negative	Str. pyogenes
5	"	Negative	Str. pyogenes

Conclusion

From the above mentioned experiments it seems reasonable to conclude that the larvae during penetration of the skin introduced the pathogenic bacteria which were recovered from the subcutaneous tissues after the application of bacteria and infective stage larvae to the intact abdominal skin. The fact that bacteria other than the one used in the experiment (Experiment I, Group No.3) were recovered suggests that these bacteria were adherent to the cuticle of the parasite and were either of faecal origin or were present on the skin. The animals which were exposed to the parasite only (Group 1) have proved to favour the penetration of micro-organisms into the subcutaneous tissues.

The Identity of Some of the Micro-organisms Inside and/or
Adherent to the Surface of Infective Larvae from Normal
Cultures

Technique Employed

The infective stage larvae, five days old, used for this study were cultured as previously described on page 49. A piece of the edge of the filter paper, containing larvae, was cut and held by a pair of forceps in such a way as to leave the larvae at the edge of the filter paper free. The edge of the paper bearing the larvae was exposed to the surface of a blood agar plate and only the anterior part of the larvae which protruded from the sheath was allowed to touch the medium. When the larvae were in contact with the surface of the agar, they migrated onto the medium, which was afterwards incubated at 37°C.. The larvae were noticed to move actively on the surface and into the agar. Another piece of the filter paper, cut nearer to the edge, was inoculated into a cooked meat medium. All the media were incubated at 37°C. for twenty-four hours. The micro-organisms which were recovered were kindly identified by Dr. McCloy of the Bacteriology Department, London School of Hygiene and Tropical Medicine, London. These organisms were:-



Fig. XVII

Longitudinal section of N. brasiliensis larva, stained with Giemsa's stain, showing highly pigmented bodies which appear to be inside the body of the parasite and which have the appearance of micro-organisms.

(x600)

- 1) B. coli (5 strains)
- 2) Haemolytic streptococcus (2 strains)
- 3) Streptococcus faecalis
- 4) Cl. tertian (non-pathogenic)
- 5) Cl. oederatiens
- 6) One of the Clostridium group (not identified)

The above mentioned organisms were also demonstrated in direct smears and cultures which were sown from the faeces of rats infected with N. brasiliensis.

This finding suggests that the faecal bacteria recovered from the surface of the larvae may have been utilized as food by the larvae (Fig. XVII) or they may have been carried mechanically from direct contact with the faeces.

Recovery of the Micro-organisms from the Lymphatic Glands after the Application of the Helminth and Micro-organism to the Intact Skin

Experimental Animals

Albino mice, ages about four weeks, and free of N. brasiliensis, were used.

Micro-organisms

Twenty-four hours broth cultures of Staph. aureus and B. cereus were used. These organisms were kindly supplied by the Bacteriology Department, London School of Hygiene and Tropical Medicine, London.

Helminthic Material

The infective stage larvae of H. brasiliensis (5 days old cultures) used for the following experiments were cultured as previously described.

Technique Employed

As it had been previously noticed by the investigator that infective larvae obtained from Petri-dish cultures were contaminated with different strains of micro-organisms it was decided to wash the larvae in different chemical solutions to minimise the number of bacteria adherent to the surface of the larvae. The following chemical disinfectants were used:-

- a) Mercuric chloride solution 0.002% in distilled water (1 in 50,000).
- b) Potassium permanganate solution 0.02% in distilled water (1 in 5,000).
- c) Acriflavin solution 0.02% in distilled water (1 in 5,000).

All solutions were sterilized by autoclaving at a pressure of 1-1/2 atmosphere for 20 minutes as also was the filtered water used for washing the larvae.

Procedure

- 1) Large numbers of infective larvae were suspended in a centrifuge tube in 5 c.c. of filtered water. The larvae were washed three times with the filtered water

by centrifuging at 1,300 revolutions per minute for three minutes.

2) Supernatant fluid was discarded and 10 ml. of mercuric chloride solution (0.002%) was added to the deposit and the centrifuge tube was rolled gently between the palms of the hand and left to stand for 10 minutes after which the tube was centrifuged (1,300 r.p.m.) for three minutes and the supernatant fluid was discarded.

3) The deposit was washed with filtered water and centrifuged (1,300 r.p.m.) for three minutes and the supernatant fluid discarded.

4) To the deposit potassium permanganate solution (10 ml.) was added and the solution mixed and suspended for ten minutes, after which the tube was centrifuged (1,300 r.p.m.) for three minutes and the supernatant fluid was removed.

5) The deposit was washed with filtered water and the suspension centrifuged for three minutes (1,300 r.p.m.) and the supernatant fluid removed.

6) The deposit was mixed with 10 ml. of acriflavin solution (0.2%) and suspended for ten minutes.

7) The tube was centrifuged for three minutes (1,300 r.p.m.) and the deposit was washed three times in filtered water.

8) The last washing was discarded and to the final deposit 5 ml. of filtered water were added to make a uniform suspension. All procedures were carried out as aseptically as possible using a sterile pipette for each washing.

The animal was secured to a board, abdomen downward, as for the percutaneous infection illustrated in fig. III. The anterior part of the body was raised higher than the rest of the body by the help of cotton wool. The toe of the left hind limb of the animal was dipped in a glass capsule (diameter 4 cm., height 1 cm.) containing a mixture of 1/2 c.c. of the washed larval suspension and 1/2 c.c. of a nutrient broth culture of the bacteria used for each experiment. The animal was left in that position for two hours after which the glass capsule was removed and the toe dried with absorbent cotton wool and then dipped in 5% Dettol solution for five minutes, and afterwards the animal was released in a clean glass container. Twenty-four hours after exposure the animal was killed and the skin of the abdomen dissected and the right and left procrural lymph glands were removed separately and each inoculated into a nutrient broth culture and incubated at 37°C. for 24 hours. All aseptical precautions were taken during the dissection and different instruments were used for each lymph gland.

Experiment No.1

1/2 c.c. of a 24 hours nutrient broth culture of Staph. aureus and about 500 infective larvae (washed) of N. brasiliensis were applied to the left foot of the hind limb of each of five mice.

Experiment No.2

1/2 c.c. of a 24 hours nutrient broth culture of B. cereus and about 300 infective larvae of N. brasiliensis (treated with antiseptics) were applied to the left ^{foot} toe of the hind limb of each of five mice.

Result of the Experiments

In both Experiments 1 and 2 smears were prepared separately from broth cultures of the left and right precrural lymph glands of each animal and stained with Gram's stain. The result of the examination, as illustrated in Table XVII, has shown that the corresponding micro-organisms were recovered from the precrural lymph gland of the left hind limb in all the animals, while the right precrural lymph gland remained sterile in every case. Pieces of the lungs of each animal were flattened and examined under the microscope and all the animals proved to be infected with the helminth parasite.

Conclusion

The recovery of the micro-organism from the lymph gland draining the site of infection suggests that the

parasite during penetration may have facilitated an entry for the bacteria into the body tissue or the bacteria may have been carried in by the parasite during migration to the lymph gland.

Table XVII

No. of Experiment	No. of Animal	Result of the smear examination made from cultures of the precrural lymph glands 24 hours after exposure	
		Left lymph gland of hind limb	Right lymph gland of hind limb
1	1	Staph. aureus	Negative
	2	Staph. aureus	Negative
	3	Staph. aureus	Negative
	4	Staph. aureus	Negative
	5	staph. aureus	Negative
2	1	B. cereus	Negative
	2	B. cereus	Negative
	3	B. cereus	Negative
	4	B. cereus	Negative
	5	B. cereus	Negative

The Simultaneous Application of Bacteria and Varying
Numbers of Infective Larvae of N. brasiliensis to the
Intact Skin of Animals

The procedure in all the experiments in this investigation was as follows:-

Experimental Animals

All the experimental animals (mice and rats) had been proved by repeated examinations of their faeces to be free of N. brasiliensis infection and were divided into three equal groups.

Group I was exposed to simultaneous infection of infective larvae and bacterial emulsion.

Group II (control) was exposed to the same dose of bacterial emulsion as used in Group I.

Group III (control) was exposed to the same number of larvae used in Group I.

The Micro-organisms

Virulent young broth cultures, of 24 hours growth, of the following micro-organisms were used:-

- 1) Streptococcus pyogenes
- 2) Streptococcus equi
- 3) Salmonella typhi-murium
- 4) Bacillus anthracis
- 5) Clostridium chauvoei

The virulence of the cultures was achieved by passing the strain of bacteria through a series of individuals of the same species, inoculating the animals one from another in succession. The pathogenicity of the emulsion was tested by inoculating it subcutaneously into animals using different doses. The dose of bacterial emulsion to which the skin of each animal was exposed was never below that of the minimum lethal dose.

The Parasitic Helminth

Infective stage larvae of N. brasiliensis were hatched on filter paper as previously described. These larvae were not treated with any disinfectant and were applied to the skin after being counted on the edge of the filter paper. It was arranged that one animal from Group I and another from Group III were exposed to equal numbers of larvae from the edge of the same filter paper.

Technique Employed

All the animals of the three equal groups were lightly anaesthetised with ether and secured to a board as illustrated in Fig. III. The hair on a square of the middle of the abdomen or the back was clipped carefully. The site was afterwards damped with distilled water. To the site of exposure in each animal of Group I and

Group II a dose of a 24 hours broth culture was applied and an equivalent volume of distilled water equal to that of the dose of bacterial emulsion was applied to the site in each animal of Group III. An equal number of infective larvae was then applied to the site in each animal of Group I and Group III. To keep the site of exposure damp and to facilitate the entry of a large number of larvae, the dose of bacterial emulsion used or its equivalent of distilled water was applied to the site on four times successively at intervals of about ten minutes. The animals were kept in position for two hours, after which the site of exposure and the area around it were first wiped with 5% Dettol solution, then ten minutes later with a 5% solution of Tincture of iodine, after which the area of exposure was left to dry and the site was covered with adhesive plaster to prevent the animals becoming infected with bacteria via the mouth. Each animal was released in a clean glass container. Different numbers of larvae were applied with each bacterial emulsion, namely, 200 in the first experiment, 400 in the second experiment, 600 in the third and 800 in the fourth experiment.

It was noticed that some of the experimental animals in Group I, a fortnight after exposure, did not die when injected subcutaneously with a dose of bacterial emulsion,

of 24 hours' growth, of the same organism used in the experiment, while most of the controls died when injected subcutaneously with the micro-organism. It was considered advisable subsequently to inject as a routine, all the animals which were still alive in each experiment, 15 days after original exposure, with the same micro-organism used in the various experiments.

(1) The Application of Str. pyogenes and Various Numbers of Infective Larvae to the Intact Skin

Experiment No.1

Fifteen young mice, about 4 weeks old, were divided into three equal groups.

The total dose of bacterial emulsion applied to the site of exposure in each animal of Group I and Group II was 2.0 ml.

Two hundred infective larvae, 5 days old culture, were applied to the site in each animal of Group I and Group III.

Results

1) There was no mortality amongst the animals in the three groups but when they were inoculated subcutaneously each with 1/2 c.c. of a 24 hours broth culture of Str. pyogenes, a fortnight after the original exposure, they all died.

2) All animals of Group I and Group III were examined daily from the fifth day after exposure and all were found to be infected with N. brasiliensis and eggs were recovered in the faeces six days after exposure.

Experiment No.2

Experiment No.1 was repeated on fifteen mice, 4 weeks old, using the same technique except that the number of larvae was increased to 400 per animal in Group I and Group III.

Results

1) No mortality resulted until 15 days when each animal in the three groups was inoculated subcutaneously with 1/2 c.c. of a virulent 24 hours broth culture of Str. pyogenes, when they all died.

2) All the animals of Group I and Group III were found to be infected with N. brasiliensis, 6 days after the original exposure.

Experiment No.3

The same procedure as described before was carried out on fifteen mice of four weeks old. A 2 c.c. dose of the bacterial emulsion Str. pyogenes, a 24 hours virulent broth culture, was applied to the site of exposure in each animal of Group I and Group II. Six hundred infective

larvae, 5 days old culture, were applied to the site in each animal of Group I and Group III. Each animal in the three groups was injected subcutaneously with 1/2 c.c. of a virulent, 24 hours growth, broth culture of Str. pyogenes fifteen days after the original exposure.

This experiment was repeated twice making the total number of mice used in Experiment (III) 45 (15 mice in each group).

Results

The result of the experiment as summarised in table XVIII was:-

1) No mortality occurred within 14 days after the original exposure.

2) Eggs of N. brasiliensis were recovered in the faeces of all animals in Group I and group III six days after exposure.

3) The result of the subcutaneous injection of the bacterial emulsion 15 days after the original exposure was:-

a) Two animals out of fifteen died within 24-48 hours, after injection, in animals of Group I.

b) All 15 animals of Group II died within 24 hours after the subcutaneous inoculation of the micro-organism.

c) Fourteen mice out of 15 in group III died within 24 hours after the subcutaneous inoculation of the bacterial emulsion.

In all animals which died after the subcutaneous inoculation of the bacterial emulsion, the organism was demonstrated by smears made from the subcutaneous tissue at the site of inoculation.

Experiment No.4

The same procedure as described above was carried out on eighteen mice (divided into three equal groups) of about four weeks old. A 2 ml. dose of the bacterial emulsion Str. pyogenes, a 24 hours virulent growth, was applied to the site of exposure in each animal of group I and Group II. Eight hundred infective larvae, five days old culture, were applied to the site in each animal of Group I and Group III. Each of the animals remaining alive fifteen days after the original exposure was injected subcutaneously with 1 ml. of a 24 hours growth culture of Str. pyogenes.

The result of the experiment as summarised in table XIX was:-

(1) No mortality occurred within the fourteen days after the original exposure in Group II. In Group I five animals out of six died between 60 hours and 3 days. In Group III three animals died between 3 and 4 days after exposure.

Table XVIII

Number of Group	Number of Animals in each Experiment	Date of Original Exposure	Date of the Death of Animals after Original Exposure	Date of the Recovery of Eggs of <u>N. brasiliensis</u>	Date of the Subcutaneous Inoculation of <u>Str. pyogenes</u>	Number of Dead Animals	Date of Death
I	5	5-9	Nil	11-9	20-9	1	20-9
	5	7-9	Nil	13-9	22-9	Nil	Nil
	5	19-9	Nil	25-9	3-10	1	5-10
II	5	5-9	Nil	-----	20-9	5	20&21-9
	5	7-9	Nil	-----	22-9	5	22&23-9
	5	19-9	Nil	-----	3-10	5	3&4-10
III	5	5-9	Nil	11-9	20-9	5	20&21-9
	5	7-9	Nil	13-9	22-9	5	23-9
	5	19-9	Nil	25-9	3-10	4	4-10

(2) The result of the subcutaneous injection of the bacterial emulsion (1 c.c. broth culture) fifteen days after original exposure was:-

- a) The remaining mouse from Group I did not die and it was discarded fifteen days after injection.
- b) All animals of Group II, 6 mice, died between 24 and 48 hours after the subcutaneous inoculation of the micro-organism.
- c) The remaining 3 mice of Group III died within 24 hours from the time of injection.

Conclusion

These results, although they have been obtained from a few animals, suggest that the amount of bacteria which might have entered the body of the animal through the invasion of the larvae was not enough to cause death when the number of larvae applied was 200 and 400. When 600 larvae were applied an immunity was set up against the micro-organism used in the experiment. When 800 larvae were applied a large number of animals died which may be due to the toxins produced by the invasion of a large number of bacteria, or may be due to the damage caused by the parasite during migration into the tissues, or both.

Table XIX

Group No.	No. of Animal	Date of Original Exposure	Date of Death of Animals	Total Number of Animals Dead in the Group	Date of Subcutaneous Inoculation	Date of Death	Number of Animals Dead
I	1		Nil	5	26-7	Nil	Nil
	2		14-7				
	3		14-7				
	4	11-7	14-7				
	5		14-7				
	6		14-7				
II	1		Nil	Nil	26-7	27-7	6
	2		Nil				
	3		Nil				
	4	11-7	Nil				
	5		Nil				
	6		Nil				
III	1		14-7	3	26-7		3
	2		15-7				
	3		15-7				
	4	11-7	Nil				
	5		Nil				
	6		Nil				

(2) The Application of *Str. equi* and Various Numbers of Infective Larvae to Intact Skin

The same technique as mentioned on page (119) was carried out on the following experiment.

Experiment No.1

Fifteen mice, about 4 weeks old, were divided into three equal groups. The total dose of bacterial emulsion applied to the site of exposure in each animal of Group I and Group II was 2.0 ml. Six hundred infective larvae from a 5 days old culture were applied to the site in each animal of Group I and Group III. Each of the animals remaining alive in the three groups was injected subcutaneously with 1.0 ml. of a virulent 24 hours growth, culture of *Str. equi* fifteen days after the original exposure.

This experiment was repeated twice making the total number of mice used in the experiment forty-five mice, 15 animals in each group.

Results

1) No mortality occurred, within 14 days from the time of the original exposure, in any of the animals in the three groups.

2) Eggs of *N. brasiliensis* were recovered in the faeces of all animals in Group I and Group III six days after exposure.

3) The result of subcutaneous inoculation of the bacterial emulsion 15 days after the original exposure was as follows:-

- a) One mouse out of fifteen died within three days of the inoculation, in the animals of Group I.
- b) All the mice of Group II died 3 to 4 days after inoculation.
- c) All the mice of Group III died 3 days after the inoculation.

In all the animals which died after the subcutaneous inoculation of the bacterial emulsion, the organism (Str. equi) was demonstrated by smears made from the subcutaneous tissues at the site of inoculation.

Experiment No.2

This experiment was carried out on a total of 45 mice (divided into 3 equal groups) on the same lines as Experiment No.1, except that the number of larvae applied to each animal in Group I and Group III was increased to about 800 infective larvae from the 5 days old culture.

Results

- 1) No mortality occurred within the 14 days after the original exposure in any of the animals in Group II.
- 2) In Group No.I twelve mice died between three and

four days after the original exposure. Cultures were made from subcutaneous tissues at the site of application of the larvae and bacteria from each dead mouse and each culture was inoculated into an uninfected mouse. All twelve mice thus inoculated died with streptococcus infection. A control mouse was inoculated with the same dose of sterile nutrient broth solution and the mouse did not die.

3) In Group No. III nine mice out of fifteen died between 3 and 5 days after exposure. Cultures were made from the blood of these dead animals and were inoculated into uninfected mice in doses of 1/2, 1 and 2 ml. of a 24 hours growth culture and all the injected mice died. Smears made from these animals demonstrated various groups of organisms which were not identified. Control animals were inoculated with sterile broth media, equivalent to the dose of bacterial emulsion used and none of them died.

Fifteen days after the original exposure all living animals in the three groups were inoculated subcutaneously each with 1 ml. of a 24 hours virulent growth and the result was:-

- a) None of the remaining three mice of Group I died and they were discarded two weeks later.
- b) All mice in Group II (15 mice) died within 3-4 days from the time of injection.

c) The five remaining mice in Group III died within 3-4 days after the injection.

(3) The Simultaneous Application of Infective Larvae of *N. brasiliensis* and Bacterial Emulsion of *Sal. typhi-murium* to Unsecured Animals

Experimental Animals

Eighteen young mice of about four weeks old, and free of *N. brasiliensis* infection, were divided into two equal groups. Group I was exposed to simultaneous infection of bacterial emulsion and infective larvae in a glass jar. Group II was exposed to the same dose of bacterial emulsion only.

The Micro-organism

A twenty-four hours growth culture, broth meat medium, of *Sal. typhi-murium* incubated at 37°C. was used.

Helminthic Material

The infective stage larvae of *N. brasiliensis* (5 days old culture) were cultured as previously described. The edges of filter papers bearing about five thousand infective larvae were added to 10 c.c. of distilled water.

Technique Employed

Two sterilised glass jars (A & B) of height 14 cm. and diameter 12 cm. were used as containers for the animals during exposure.

To jar (A) 10 c.c. of distilled water and 3 c.c. of the bacterial emulsion were added.

Ten c.c. of the distilled water bearing the 5000 larvae and 3 c.c. of the bacterial emulsion were added to the jar (E). Nine mice were placed in each jar and left there for two hours, after which each group was transferred to a clean glass receptacle.

Results

Two mice from Group B died 60 hours after exposure and the rest of the animals died a day later. Cultures were made from the lungs of these animals and Salmonella organisms were recovered in smears prepared from these cultures. Six mice were inoculated subcutaneously with varying doses of the bacterial emulsions (from the lungs of the dead mice) 0.1, 0.2, 0.3, 0.5 and 1 ml. respectively. Another two mice were each inoculated with 1 ml. of sterile cooked meat broth. The result was that all the six mice died the next day and the two control mice did not die and they were discarded two weeks later.

No mortality in Group A occurred and the mice were discarded three weeks after exposure.

(4) The Simultaneous Exposure of Animals to an Emulsion of B. anthracis and Infective Larvae of N. Brasiliensis

The same procedure and technique described on page(119) was used in the following experiments.

Experiment No.1

Nine mice of ages about 4 weeks old were divided into three equal groups. All animals were lightly anaesthetised and secured abdomen downwards. A full platinum loop of a twenty-four hours growth culture, broth media, of B. anthracis was applied to the site of exposure in each animal of Group I and Group II. To the site of exposure in each animal of Group I and Group III eight hundred infective larvae were applied at four occasions (200 infective larvae each). The sites of exposure in all the animals in the three groups were kept damp by pipetting distilled water on to them. This was carried out for about one hour after which the animals were kept secured in position for another hour. The sites of exposure were disinfected with 10% formalin solution for five minutes and then with 5% tincture of iodine for another five minutes. The sites were covered with adhesive plaster and the animals were released and kept individually in a clean glass experimental container.

The Result

No mortality occurred in any of the animals of the three groups within two weeks after exposure. Two weeks after exposure all the animals (in the three groups) were then injected subcutaneously with 1/2 c.c. of a 24 hours broth culture of B. anthracis. The results were:-

1. All animals in Group II and Group III died within less than 24 hours from the time of the subcutaneous inoculation. As the animals died blood films, obtained from the tails, were immediately examined for the presence of B. anthracis. The blood smears from all the animals proved positive.

2. No mortality occurred among the animals in Group I during the 2 week period and they were destroyed.

Experiment No.2

Experiment No.1 was repeated on nine black hooded rats, aged about 4 weeks old, divided into three equal groups. Twenty-four hours after the original exposure the animals were re-exposed to infection in the same manner and kept under observation.

The Result

1. All animals in Group I died within 48 hours after original exposure and B. anthracis was demonstrated in the blood films and in 24 hours broth media cultures.

2. No mortality occurred amongst animals of Group II and they were discarded and destroyed.

3. Two animals in Group III died three days after the original exposure and the third died three days later. Blood was inoculated into broth media and the following organisms were demonstrated:-

- (a) Gram-negative bacilli
- (b) Organisms morphologically resembling B. tetani
- (c) Streptococcus organisms.

(5) The Oral Infection of a Rabbit and Guinea pigs with Metacercariae of Fasciola gigantica

The metacercariae used in this experiment were supplied by Dr. P.L. Le Roux.

Experiment No.1

An English rabbit was fed at 1 p.m. on 3-11-52 with 8 metacercariae, shed at 7.30 p.m. on 4-11-52 and encysted to a blade of grass, of Fasciola gigantica. The rabbit died on 15-1-53 and the post mortem findings were:-

Liver flukes were recovered from the liver and peritoneal fluid. There were adhesions between the small intestine and the abdominal peritoneum. At the sites of adhesions there were abscesses. Abscesses were also noticed in the mesenteric lymph glands. Peritonitis was well marked and there was an accumulation of serous fluid in the abdominal cavity. The liver showed marked changes from the normal. There were many scattered abscesses in the liver and the hepatic lymph glands. The liver contained necrotic tracts winding in all directions. Cultures were prepared from the liver, in broth meat media, and smears were prepared and a clostridium organism was recovered from

these cultures. These findings were kindly confirmed by the Department of Bacteriology at this Institute.

Experiment No.2

Nine guineapigs about one month old were divided into three equal groups.

Group I

Each animal of this group was fed with 6 metacercariae of F. gigantea encysted on blades of grass.

Group II

Animals of this group were not fed with metacercariae.

Group III

Each animal in Group III was fed with 6 metacercariae of F. gigantea encysted on blades of grass as in group I.

Material Used

Cl. chauvoei organisms, kindly supplied from the Bacteriology Department of this Institute, were used in this experiment.

To obtain toxin free micro-organisms, a 48 hours growth culture (on blood agar) was washed and centrifuged twice with sterile normal saline for 1/4 an hour at 3000 revolutions per minute. To the residue, sterile normal saline was added to make an emulsion of 10 c.c. To ascertain the presence of Cl. chauvoei a platinum loop of this emulsion was sown on blood agar media. Cl. chauvoei organisms were recovered after 24 hours culture.

Each animal of Group I and Group II was inoculated intraperitoneally, 24 hours after the feeding of the metacercariae, with 1/2 c.c. of this emulsion and each group was kept separately in a special cage.

The Result

(1) Two animals from Group I died 3 weeks after the date of injection. The third one died 23 days after the injection. Necrotic lesions and tracts winding in all directions in the livers of these three animals were noticed. Sections were prepared from one of these livers and the presence of necrotic tissue was confirmed. Cultures were made from these livers and Cl. chauvoei and streptococci organisms were demonstrated.

(2) No mortality occurred among animals of Group II and Group III within this period.

The animals were kept under observation for two months and the result was:-

No mortality occurred among the animals of Group II. One animal from Group III died five weeks after the feeding of the metacercariae while the rest of this group died two weeks later.

Post Mortem Findings

The livers of these animals (Group I and Group III) were found to be necrosed. Multiple abscesses and necrotic

tissues were also noticed as illustrated in Fig. XVIII .

Clostridium organisms were recovered from cultures prepared from these livers. No attempt was made to identify them.

In other observations on guineapigs experimentally infected with metacercariae of F. gigantea, Dr. P.L. Le Roux noticed that whenever these animals died, Cl. chauvoei organisms were present in these infected livers.

Conclusion

Although the number of animals used in these experiments was few, yet the results suggest the following:-

(a) The reason why Cl. chauvoei organisms, toxin free, when inoculated intraperitoneally into the animals of Group II did not cause mortality, may be because conditions were unfavourable for their multiplication. But in Group I larvae migrating into the tissues of the liver and there causing tissue necrosis, might have brought about the optimum conditions for the multiplication of these organisms, thus causing the mortality in the guineapigs.

(b) When animals died which were fed only with the metacercariae, Clostridium organisms were recovered from their livers. This may have been due to the natural occurrence of these organisms in the liver where they had



Fig. XVIII

Liver showing abscesses (indicated by arrows),
necrosis and cirrhosis in a guineapig that died 5
weeks after having been infected with Fasciola
gigantica.

the opportunity of multiplying when necrotic lesions were caused by the migration of the larvae.

Discussion

On various occasions several investigators, such as De Blicq and Baudet (1920), Kawanishi (1929 & 1932), Malvoz and Lambert (1918), Koydzumi (1927), Fourie (1937), Podyapolskaya and Dedova (1937), Stein et al. (1939), Shope (1941) and De Blicq and Jansen (1944), have succeeded in their attempts to prove that some parasitic helminths facilitate the entry of pathogenic bacteria or viruses into the body tissues of animals either by direct transmission or as a result of the damage they cause. Other workers, however, namely, Joyeux and Baer (1929), Taylor and Purchase (1931), Carne and Ross (1932), and Taylor (1935) could find no evidence that skin lesions which might have been caused by the helminth played any part in the entry of pathogenic bacteria into the host.

It is well known that the first two larval stages in the life cycle of nematodes belonging to the superfamily STRONGYLOIDEA, have a free-living existence. During this time, the larva which hatches from the egg feeds actively, undergoes two moults and grows considerably before reaching the infective stage, when it is ready to invade a definitive host. Under natural conditions this internal development takes place in the faeces, which have been deposited by the infected host on ground likely to be

contaminated with various bacteria. In the laboratory McCoy (1929) succeeded in hatching eggs of Ancylostoma caninum in agar cultures of 22 different kinds of bacteria, and the larvae developed to the infective stage. Lapage (1933) has pointed out that infective larvae reared on cultures of B. coli may contain, in their intestines, bacteria picked up from those cultures before they have become unshathed. Kawachi (1929) recovered various bacteria from the surface of Ancylostoma larvae. Hope (1941) demonstrated that the pig lung-worms Metastrongylus elongatus and Choerostongylus pudendotectus were capable of harbouring swine influenza virus and of transmitting it. The author has recovered different strains of micro-organisms from the body surface of infective larvae of N. brasiliensis.

If, therefore, pathogenic bacteria are present in faeces in which infective larvae develop, these bacteria might remain alive long enough, inside or on the surface of the larvae, to be inoculated directly into the body tissue of the host during the invasion either via the skin or mucous membranes.

The natural barriers (skin, respiratory tract, alimentary tract and genito-urinary tract) of the body of a healthy host are usually contaminated with bacteria which do not normally gain access into the body tissues

unless one of these natural defence barriers is damaged and rendered susceptible to invasion.

If bacteria are present on the skin of, or within, the infective larvae during the penetration of the host's skin, the bacteria may gain access to the body tissues of the invaded host. The detrimental effect on the health of the animal in such a case depends upon the number of larvae penetrating the skin or the tissues and on the virulence of the micro-organisms which gained access with the helminth larvae. If the number of bacteria is small they may be eliminated by the natural defences of the body. If the bacteria are not effectively opposed, they may break down the natural defence mechanism of the body and cause disease. In some cases the number of bacteria and their virulence may be such that the host does not show any ill effects but develops for a time an immunity against further invasion.

Larvae which mechanically injure the mucous membrane of the intestines may cause the inoculation of normal bacterial flora or pathogenic bacteria, if present, into the tissues of the host. The bacteria which enter the body through the intestinal tract, if introduced into the lymphatic vessels, must pass through at least three groups of lymphatic glands. In the case of N. brasiliensis my

findings are that migration of the larvae is via the lymphatics. This suggests that bacteria which are introduced by helminthic larvae may be arrested and destroyed in the lymph nodes and that their destruction there may result in the production of an immunity. If the number of inoculated bacteria is great some of them may not be destroyed and disease results.

Parasitic helminths whose larval stages migrate in the body tissues e.g., Ascaris, Schistosoma, Fasciola, Ancylostoma, H. brasiliensis, etc. are thus all likely to cause lesions which may bring about favourable conditions for bacterial invasion and development. Thus we have the examples of black disease in sheep, haemorrhagic septicaemia in animals and contagious bovine pleuro-pneumonia in cattle which are associated with Fasciola invasion. Again there are the examples of parasitic larval helminths migrating in the lung, damaging the mucosa and allowing pathogenic bacteria to gain access to that organ to set up such diseases as tuberculosis, contagious bovine pleuro-pneumonia, etc., through inhalation of droplets containing bacteria, virus, or fungi.

The adult parasitic helminth in the alimentary tract of the host is naturally surrounded by bacterial flora and it is difficult to say whether these helminths cause any damage which might allow pathogenic bacteria to gain

entrance through the lesions. If it is true that these parasites secrete an anti-coagulum, a further study is needed to discover whether that secretion or other secretions, if present, have an antibiotic action against the pathogenic bacteria. If that is so this may explain why some workers maintain that the parasite does not play any role in setting up a bacterial infection. But these workers' conclusions are invalidated by the fact that none of them considered the possibility of the bacteria having been destroyed and an immunity established. They did not test their experimental animals for an immunity against the micro-organisms used, after subjecting them simultaneously to larval helminths and bacteria.

While investigating the fate of the larvae of N. brasiliensis in abnormal hosts it was noticed that although the infective larvae invaded such animals they did not reach the intestine and complete their life cycle. Is it not therefore possible that this invasion of an abnormal host by infective larval helminths, which must often occur under natural conditions, may be responsible for the introduction of bacteria or the exposure of the abnormal host to bacterial or virus infection? For example, the damage caused to the lungs of children by the larvae of Toxocara canis may predispose their lungs to bacterial or virus infections. Moreover, this invasion of the host by abnormal parasites, accompanied by disease-producing

agents, may establish an immunity against these diseases. In nature, as the abnormal helminth does not reach maturity in the host it is difficult to demonstrate its presence and the part it plays in the production of disease or immunity.

Summary and Conclusion

From the result of my experiments it seems that the parasites do play a role in facilitating pathogenic micro-organisms in gaining access to a susceptible host. It seems reasonable to accept that the causation of disease in the invaded host will depend on the numbers of larvae gaining access and on the number of and the virulence of the bacteria introduced by them.

Addendum

The author now wishes to confirm Lucker's findings (1936) that the first moult of the larvae of M. brasiliensis does occur at about 52 hours after the hatching of the eggs incubated at 28°C.

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