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DEVELOPMENTAL STAGES OF FILARIAE IN MOSQUITOES

by

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Introduction

This paper has been prepared in pursuance of Recommendation No. 10 of the Conference of Experts on Filariasis and Elephantiasis held under the auspices of the South Pacific Commission at Papeete (Tahiti) in August, 1951. Recommendation No. 10 reads as follows:

> "That a collection of photomicrographs showing the various larval stages in the development of filarial nematodes in mosquitoes as well as photographs of other forms with which they could be confused, be assembled and distributed by the South Pacific Commission".

Four plates of photomicrographs are here reproduced. Plates 1 and 2 show the developmental stages of Wuchereria bancrofti (non-periodic form) from Samoa. These are U.S. Navy photographs from Byrd, E.E., St. Amant, L.S. and Bromberg, L., "Studies on Filariasis in the Samoan area", U.S. Naval Medical Bulletin. 44 (1), 1945. Plate 3 comprises photographs of developmental phases of W. bancrofti (nocturnal periodic form) from Sorong, Netherlands New Guinea, taken by Dr. H. de Rook. Plate 4 comprises photographs of developmental stages of Dirofilaria immitis taken by Dr. L. Kartman.

Photomicrographs often do not show morphological details of diagnostic importance in the differentiation of species of filariae found in mosquitoes. To supplement these plates, notes illustrated by pen and ink drawings are presented in this paper. The information has been collected partly from published literature and partly from the author's studies. The sources of the information are acknowledged at the appropriate places. All the drawings illustrating this paper were prepared by Mr. M.A.U. Menon, Technical Assistant, section of mosquito-borne diseases, South Pacific Commission.

In connection with epidemiological investigations on human filariasis (Wuchereriasis), wild caught mosquitoes are examined for determining the local vectors of the infection and the natural infection rates in them. Two species of Wuchereria, namely W. bancrofti and W. malayi, have a wide distribution in the tropical and sub-tropical regions of the world. Infection with Dirofilaria immitis, a common parasite of the dog, often co-exists in areas with human filariasis*. All the three filarial parasites mentioned above undergo their

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* Other filariid nematodes parasitic in animals are not included in this study. Although quite a number of these are known to occur in various animals, domestic as well as wild, little is known of their intermediate hosts or of their developmental stages.

larval development within the body of the mosquito. Consequently, in areas where two or more of these infections are prevalent, there is the possibility of their larval phases occurring in wild caught mosquitoes. In order to avoid erroneous conclusions it is essential that, in every case of positive finding of filaria larvae in mosquitoes, the larvae are examined carefully to determine the type of infection present in the mosquito. A precise knowledge of the distinctive characters of these parasites in their different developmental stages within the mosquito host is therefore of considerable importance. The object of this paper is to furnish notes of assistance to field workers in the recognition of the three species, Wuchereria bancrofti, W. malayi and Dirofilaria immitis in their different stages of development within the mosquito host.

Two forms of Wuchereria bancrofti are known, the nocturnal periodic form and the non-periodic form. In the former, the microfilariae in the human host show characteristic nocturnal periodicity in the peripheral blood stream, whereas those of the non-periodic form exhibit no such periodicity and occur in the peripheral blood of the human host in more or less equal numbers at all hours of the day and night. The nocturnal periodic form of W. bancrofti has a wide distribution in the tropics. Within the South Pacific region it occurs only in the western section comprising New Guinea, Solomon Islands, New Hebrides, Gilbert Islands, Caroline Islands, Marshall Islands and Marianas Islands (Iyengar 1954). The non-periodic form of W. bancrofti has a very limited distribution and is not known to occur outside the South Pacific region. Even within this region, its range of distribution is restricted to New Caledonia and the islands extending from Fiji and the Ellice Islands on the west to the Tuamotou Archipelago on the east. On the basis of certain biological characteristics the name Wuchereria pacifica was proposed by Manson-Bahr (1941) for the non-periodic form of W. bancrofti. In the present paper, the two forms are grouped under W. bancrofti because the microfilariae and the larval phases of the two forms do not present any differences as regards morphological characters.

Wuchereria malayi infection is endemic in many countries of south-east Asia. It is not known to be endemic in any part of the South Pacific region, which extends from New Guinea on the west to the Tuamotus on the east. Imported cases of W. malayi infection have, from time to time, been observed from among Indo-Chinese and Indonesian labour in certain island groups chiefly in the western part of the South Pacific region, but no authenticated cases of endemic infection have so far been recorded.

Dirofilaria immitis infection has a wide distribution in the tropics and often occurs in areas with W. bancrofti infection. Within the South Pacific region, it has a very wide distribution.

Technique

For the study of microfilariae in the undigested blood meal within

the midgut of a mosquito, the following procedure is recommended: The gut contents are separated, mixed with about an equal volume of normal saline and a smear preparation made of the mixture. When the smear is dry, it is fixed with absolute alcohol, dried, and then stained with Giemsa solution (one drop to one cc. of distilled water) for 20 minutes. This procedure would bring out the characters of diagnostic value in the microfilariae that may be present in the midgut.

For the study of filaria larvae, the author employs the following technique: After dissecting the mosquito, the debris is cleared, leaving only the larvae on the slide. Excess of saline is removed, a drop of the following fluid added, and a coverslip applied.

Azur-II	0.04 gramme
Distilled water	96 cc.
Sodium chloride	0.8 gramme
Formalin	4 cc.

In a few minutes, the larvae are immobilised and some of the internal structures of the larva take up the stain.

The length of the worm in the microfilaria stage as well as in the third instar stage is of importance in specific determination. For measuring the length of the worm, a camera-lucida drawing is first made on a sheet of paper. using the Abbe camera-lucida or the Zeiss-Winkel drawing apparatus. For making measurements of third instar larvae, only freshly immobilised larvae in a wet condition should be used; specimens fixed with alcohol or by heat, or those mounted in canada-balsam or euparal would not be suitable in view of the shrinkage likely to result from such a procedure. A projection of the markings of a stage micrometer is then made on the sheet of paper, using the same magnification as that employed for the drawing of the worm. With a pair of fine dividers set to a conveniently small division on the scale, the length of the worm is measured by counting the steps of the divider legs along the median line of the worm (see fig. 1).

Comparative Morphology of the Microfilariae of Wuchereria bancrofti, W. malayi and Dirofilaria immitis.

The microfilariae which normally occur in the blood of the vertebrate host are here discussed to facilitate the identification of any that may be found in the undigested blood meal within the midgut of a mosquito.

The characters by which the microfilariae of the three species can be differentiated are the following:-

1. Presence or absence of a sheath.

2. Length of the microfilaria in dried smear preparations.

3. Cephalic space (area devoid of nuclei at anterior end of the worm).

4. Position of the excretory cell in relation to the excretory chamber.

5. Character and position of the anal pore.

6. Character of the tail end.

The microfilaria of W. bancrofti, like that of W. malayi, is enclosed in a sheath. That of D. immitis is devoid of a sheath. This character by itself would not be of much value in the identification of the species because the microfilariae of the sheathed forms tend to lose the sheath after entry into the midgut; the sheath is also liable to rupture during the procedure employed for making a smear preparation of the contents of the midgut.

The average lengths of the microfilariae in dried smear preparations are as follows:-

W. bancrofti: 270 μ, range 245 to 296 μ, according to Feng 1933.
289 μ, range 256 to 322 μ, according to Iyengar 1939.

W. malayi: 201 μ, range 178 to 231 μ, according to Feng 1933.
186 μ, range 135 to 238 μ, according to Iyengar 1939.

D. immitis: 265 μ, range 238 to 294 μ, according to Kartman 1953.

The cephalic space is short in W. bancrofti, generally less than the width of the worm in that region. It is about as long as broad in D. immitis, and twice as long as broad in W. malayi (figs. 2 and 3).

In well-stained smear preparations, the excretory chamber and the excretory cell are clearly seen. In W. bancrofti, the excretory chamber is usually small, and the excretory cell is situated just behind the posterior wall of the excretory chamber. In W. malayi and D. immitis, the excretory chamber is larger and the excretory cell is well separated from the excretory chamber and placed at a distance posterior to it (fig. 3).

The anal pore is small and inconspicuous in W. bancrofti. It is conspicuous in W. malayi and in D. immitis. The position of the anal pore in W. bancrofti and W. malayi is at 80 to 83 per cent of the total length, whereas in D. immitis, it is at 75 per cent (Fülleborn 1929, & Saisawa 1913). The part of the tail beyond the last nucleus of the nuclear column in W. malayi attennuates irregularly, with two small bulbous swellings, one at about middle

and another at tip. These two bulbous swellings enclose each a small nucleus or nucleus-like body which takes stain. These nuclei are well separated from the nuclei of the nuclear column and present a different appearance. The terminal part of the tail (beyond the last nucleus of the nuclear column) in W. bancrofti and in D. immitis is devoid of any nuclei. This part in D. immitis is long (about one-tenth of the total length) and tapers to a fine tip (fig. 3); in W. bancrofti, it is short (about one-twentieth of the total length).

The differences in the morphology of the microfilariae of the three species as seen in stained smear preparations are summarised in table below.

Differentiation of the microfilariae of Wuchereria bancrofti, W. malayi & Dirofilaria immitis (stained smear preparations).

Character	W. bancrofti	W. malayi	D. immitis
Microfilarial sheath	present	present	absent
Total length	260 to 320 μ.	150 to 240 μ.	240 to 300 μ.
Cephalic space	short, less than breadth	long, about twice as long as broad	about as long as broad
Position of excretory cell	adjoins excretory chamber	well separated from excretory chamber	well separated from excretory chamber
Anal pore	not conspicuous	conspicuous	conspicuous
Nuclei at tip of tail	absent	present	absent
Tail end	broadly tapering	irregular with two bulbous swellings	Finely tapering

Developmental Phases Within the Mosquito of the Three Species of Parasites

The filaria in the mosquito host passes through three larval instars. A moult occurs at the end of the first instar and another moult at the end of the second instar. The three instars can be recognised by the general appearance of the larva and the character of the tail end.

The first and second instars are spent in a more or less quiescent state in a special niche suitable for their development. In the case of W. bancrofti and that of W. malayi, this site is within the muscle bundles of the thorax; in the case of D. immitis, it is within the malpighian tubules. Larvae of the third instar of all the three species move about actively within the haemocele of the mosquito, principally of the thorax and head.

The first instar larva has a long and thin tail. The larva soon

after it has entered its resting site within the body of the mosquito (the thoracic muscles in the case of W. bancrofti and W. malayi, or the malpighian tubules in the case of D. immitis), is similar in appearance to the microfilaria. Its initial development consists of a reduction in length and an increase in thickness, until the larva reaches the shortest phase of its development in what is often called the "sausage stage". Its subsequent development consists of a progressive increase in length. Thus the first instar larva may be differentiated into two phases, the early phase which is long and thin like the microfilaria, and the later phase which is short and stumpy.

In the second instar, which follows the first moult, the larva is longer and thicker than the sausage form and is characterised by the presence of a short conical papilla at the posterior extremity. This papilla appears to be absent in the second instar larva of D. immitis.

After the second moult the larva enters the third instar. The larva of this instar is worm-like, long and thin, and devoid of what may popularly be called a tail, the posterior end being broadly rounded or truncated. There are, however, one or more short rounded papillae at the tip of the posterior end of the worm. The larva which at this stage is usually actively mobile, emerges from its hitherto resting site and moves about within the body cavity of the mosquito through the blood spaces in between the various organs and tissues.

The following notes would help in the recognition of the three species in their different larval instars.

In W. malayi, the first instar larva is characterised by the presence of two small nuclei in the terminal part of the tail (fig. 4, a.b. & c). In the second instar the larva has an anal prolapse which is more marked in this species (fig. 4, d & e) than in the corresponding phase of W. bancrofti. The third instar larva (fig. 4, f) has an average length of 1304 μ which is inferior to the average length of the third instar larva of W. bancrofti. Its average width is 20 μ (Feng 1936). The posterior end of the larva is somewhat truncated, and has three minute rounded papillae which are placed apart from one another (fig. 4, g). The arrangement of the papillae is different from that in the third instar larva of W. bancrofti.

In the first instar sausage form of W. bancrofti, the tail is short, drawn out to a point and devoid of nuclei (fig. 5, a, b, & c). In the second instar larva the anal prolapse is not as conspicuous as in W. malayi larva of this instar (fig. 5, d & e). The third instar larva (fig. 5, f) is markedly longer than the corresponding larva of W. malayi. Its average length is 1617 μ and its average width is 23 μ (Kobayasi 1940). Larvae of this stage may sometimes reach a length of 1800 μ. The tail end is characteristic, with three globular caudal papillae placed symmetrically around the rounded tip of the tail (fig. 5, g).

In Dirofilaria immitis, as stated previously, the first and second

instar larvae are lodged within the malpighian tubules of the mosquito (fig. 6,a) whereas in the other two species, they occur within the muscle bundles of the thorax. The first instar larva (sausage form) is characterised by a long and thin tail, which is devoid of nuclei (fig. 6, b, c, d, e, & f). The tail in D. immitis larva of this stage is much longer than that of the W. Bancrofti larva of the same stage. In the larva of the second instar the posterior end is broadly rounded and the terminal papilla is absent (fig. 6, h, and fig. 7, a). The third instar larva is short (fig. 7, b,c, & d), with a length ranging from 700 to 1100 μ and a width of 23 to 26 μ. The average measurement for larvae of this instar is 850 μ by 25 μ (Kartman 1953). The tail of the larva has normally a single rounded terminal papilla (fig. 7, e); very rarely an additional but smaller papilla may be seen just behind the terminal papilla (fig. 7, f).

The following table furnishes the differentiation of the developmental phases of the three species discussed in this paper.

Character	W. malayi	W. bancrofti	D. immitis
First instar sausage form			
Situation	Thoracic muscles	Thoracic muscles	Malpighian tubules
Tail nuclei	Present	Absent	Absent
Tail end	Irregular with two bulbous swellings, one at extreme tip & another halfway	Broadly tapering	Finely tapering
Second instar larva			
Situation	Thoracic muscles	Thoracic muscles	Malpighian tubules
Anal prolapse	Marked	Not marked	Not marked
Third instar larva			
Length & breadth	1304 μ x 20 μ (average according to Feng 1936)	1617 μ x 23 μ (average according to Kobayasi 1940)	700 μ to 1100 μ. x 23 to 26 μ average 850 x 25 μ (according to Kartman 1953)
Tail end	Truncate	Rounded	Rounded
Tail papillae	Three papillae or less, placed wide apart at tip of posterior end	Three papillae placed symmetrically around posterior tip.	One terminal papilla, sometimes an additional small papilla behind tip.

Larvae of Dirofilaria immitis can be recognised by the following: The first and second instar larvae are found only within the malpighian tubules of the mosquito. The first instar is characterised by a long and tapering tail. The second instar is devoid of a terminal papilla. The third instar larva has a length range of 700 to 1100 μ, averaging 850 μ. The tip of the tail of the third instar larva usually has a single terminal papilla.

The first instar larva of Wuchereria malayi can be recognised by the presence of bulbous swellings in the tail, containing minute nuclei. The second instar larva has fairly conspicuous anal prolapse. Average length of the third instar larva is about 1300 μ; its posterior extremity is different from that of W. bancrofti.

The larval stages of W. bancrofti can be recognised by: absence of nuclei in the tail in the first instar larva; absence of a marked anal prolapse in the larva of the second instar; length of the third instar larva which is over 1600 μ on the average; the presence of three tail papillae placed symmetrically around the tip of the caudal end.

Structures Likely to be Mistaken for Filaria Larvae.

A brief mention is here made of certain artefacts, parasites and pseudo-parasites likely to be mistaken for filaria larvae. In dissections of mosquitoes, a long muscle fibre from the thorax may sometimes simulate the very early stage of the first instar larva. A careful examination would however reveal the striae of the muscle fibre.

Non-striated muscle fibrils at the ventral attachments of the vertical muscle bundles of the thorax of the mosquito are fused to form solid, hyaline, and glistening structures. In dissections of the thorax of mosquitoes, some of these muscle fibril masses assume the size, shape and hyaline appearance of the sausage stage filaria larva. Such structures have been mistaken for filaria larvae.

Contamination of distilled water and of normal saline solution with certain free-living nematode larvae is a possible source of error in reports of filarial infection in mosquitoes. As shown by Manson-Bahr (1952), larvae of species of Aphelenchoides may occur as contaminants in distilled water. It is stated that these larvae are particularly resistant and can live in 2.5 per cent salt solution. They could live unaffected in normal saline solution used for dissecting mosquitoes. Aphelenchoides parietinus is a very common species in soil, fresh water and in decaying plant tissues. There is the possibility of contamination of distilled water and of normal saline solution with these organisms. Young larvae of Aphelenchoides measure 280 μ by 16 μ, and 320 μ by 20 μ (Manson-Bahr 1952). These may be mistaken for early stage filaria larvae. Later larval forms of Aphelenchoides measure 800 μ by 30 μ.

Larvae of Aphelenchoides can be recognised by the double bulbous oesophagus, the posterior bulb being characteristically globular. Fig. 8, a, b. and c (after Manson-Bahr 1952) show this feature of the larva of Aphelenchoides. Fig. 8,d is an illustration (after Franklin 1955) of Aphelenchoides parietinus.

Immature stages of certain worms belonging to the family Mermithidae are known to be parasitic in adult mosquitoes. When a mosquito parasitised by such a worm is dissected, a long glistening white worm emerges. The worm is easily recognised by its enormous dimensions (8 to 17 mm. long and 138 to 174 μ thick, according to Iyengar 1929). Mermis infestation in adult mosquitoes is of comparatively rare occurrence.

Norman and Donaldson (1955) have reported on the finding of spores of helicosporous fungi in blood smears. These spores resemble coiled nematodes and look much like microfilariae. They are air-borne contaminants and may occur on slides and fluids left exposed to air. Although in general appearance they look like microfilariae, they can be recognised by their small size, their length being usually less than 100 μ and their thickness about 2 μ. Fig. 8, e & f show these bodies as observed in blood smear preparations (after Norman and Donaldson 1955).

Acknowledgements.

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Ric. Lab. Anat. Norm. Univ. Roma, 3 : 275-353, 1901.

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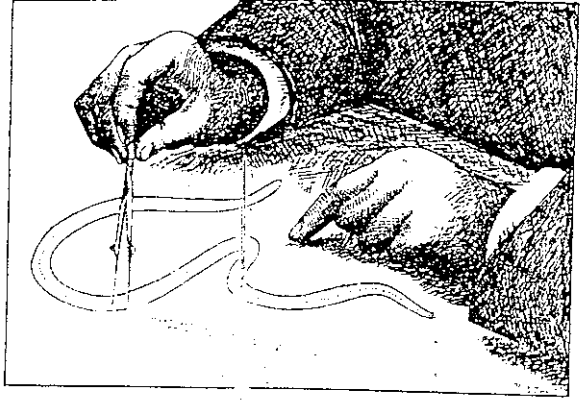


FIG. 1—Procedure for measuring the length of a worm from a camera-lucida drawing.

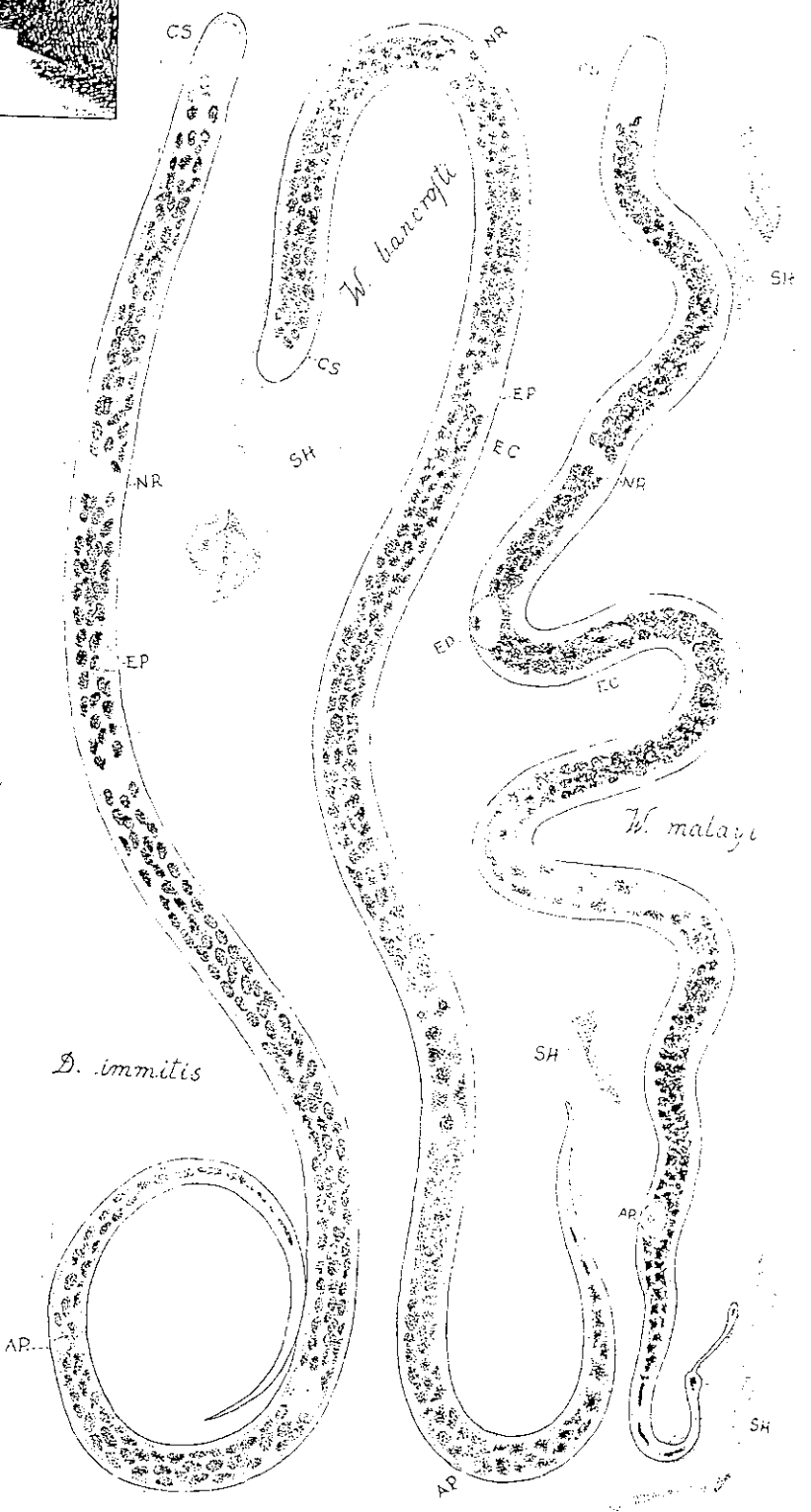


FIG. 2—MICROFILARIAE OF—

1, *Dirofilaria immitis*, 2, *Wuchereria bancrofti*, and 3, *W. malayi*. (Semi-diagrammatic.)

AP—Anal pore
CS—Cephalic space
EC—Excretory cell
EP—Excretory pore
NR—Nerve ring
SH—Microfilarial sheath

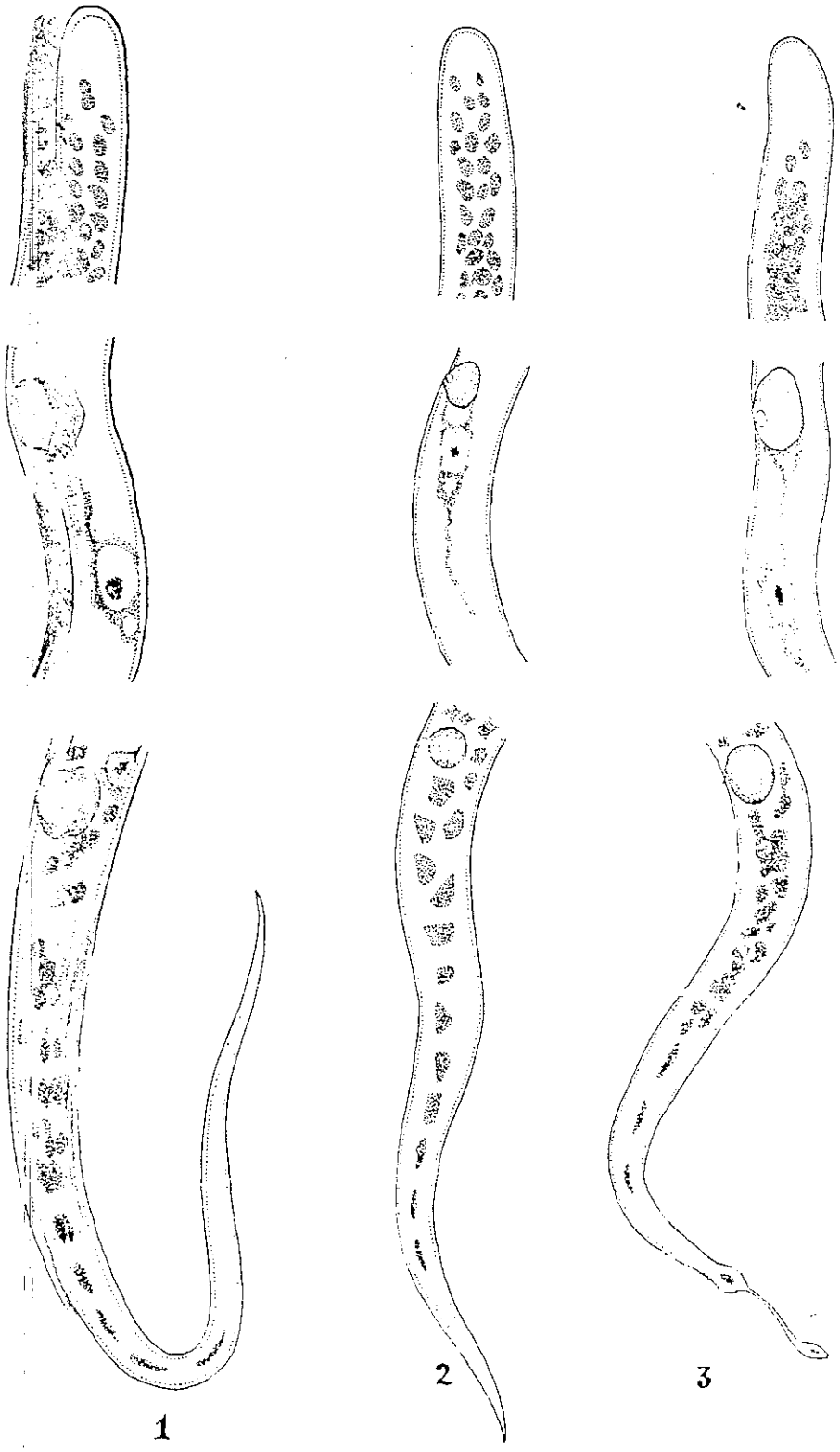


FIG. 3—HEAD END, EXCRETORY PORE AND CELL, AND TAIL END OF—
1, *Dirofilaria immitis*, 2, *Wuchereria bancrofti*, and 3, *W. malayi*.

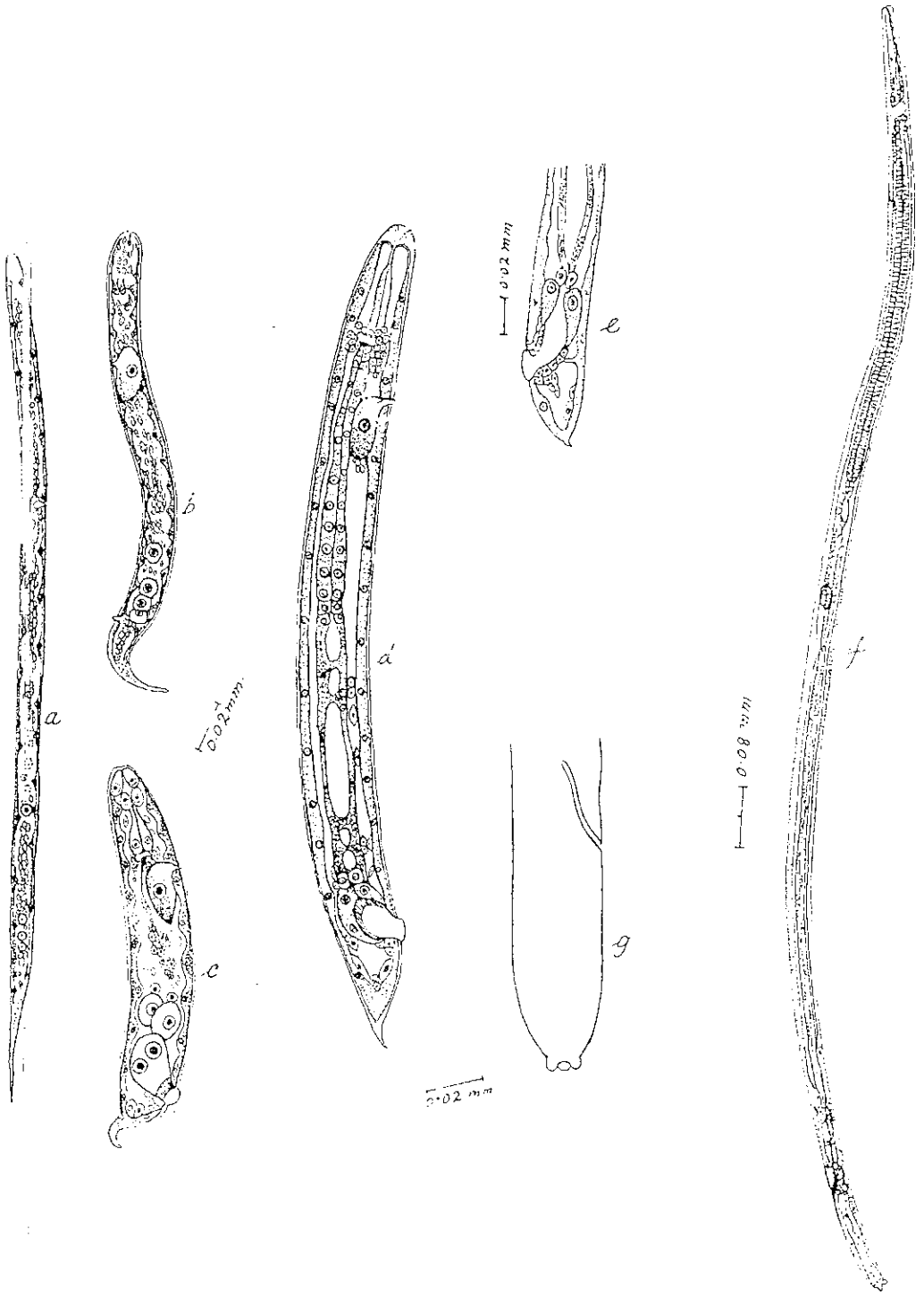


FIG. 5—DEVELOPMENTAL STAGES OF *Wuchereria bancrofti*. ("a" to "f", after Kobayasi 1940, and "g", after Lebredo 1905.)

a, b, c. First instar larva in different phases of development.

d. Second instar larva still within the first moult skin.

e. Posterior end of second instar larva.

f. Third instar larva.

g. Posterior end of third instar larva showing terminal papillae.

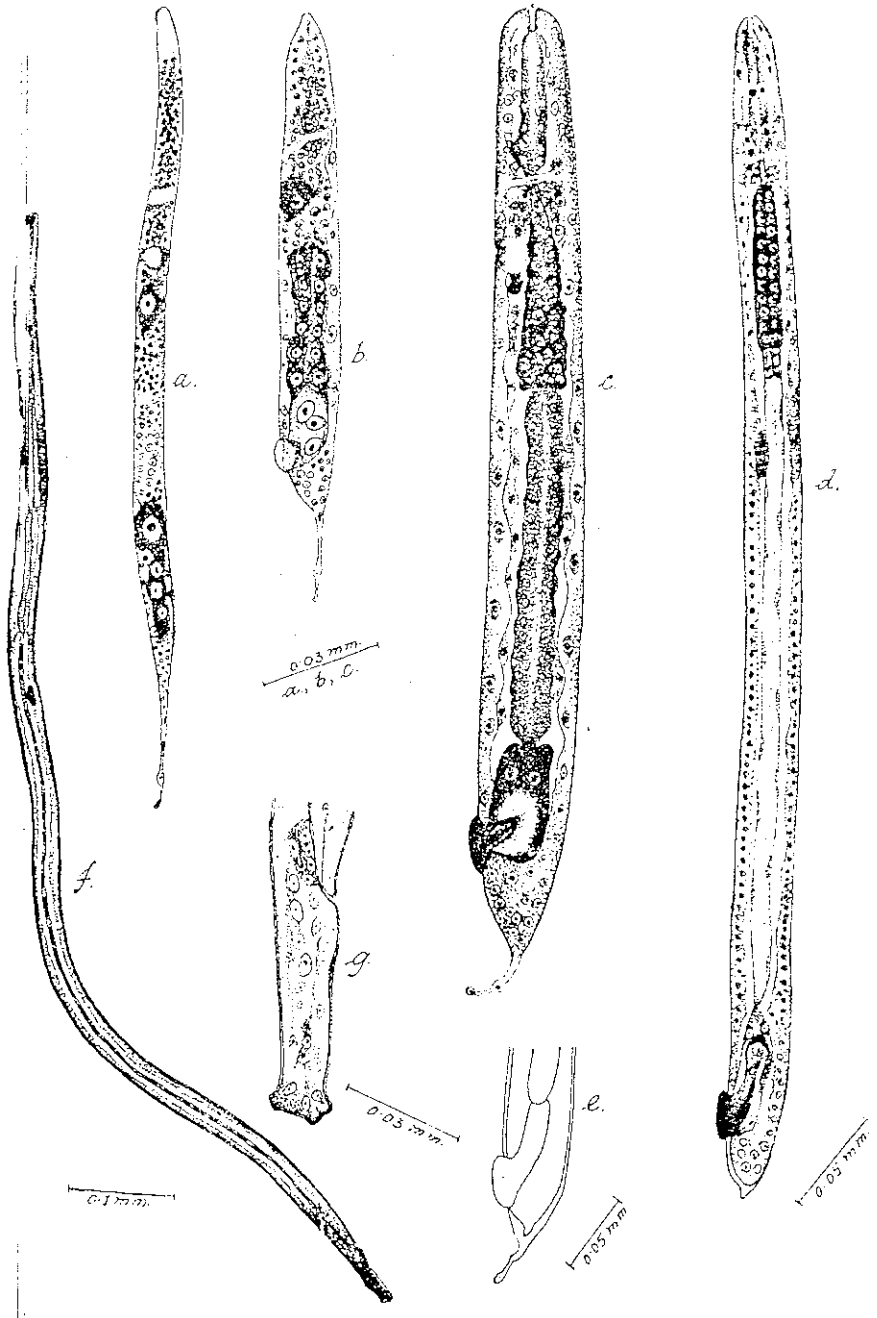


FIG 4—DEVELOPMENTAL STAGES OF *Wuchereria malayi* (AFTER FENG 1936).

a, b, and c. Larvae in different phases of the first instar.

d. Second instar larva.

e. Posterior end of second instar larva with moult skin of first instar larva still attached.

f. Larva of third instar (infective stage).

g. Posterior end of third instar larva.

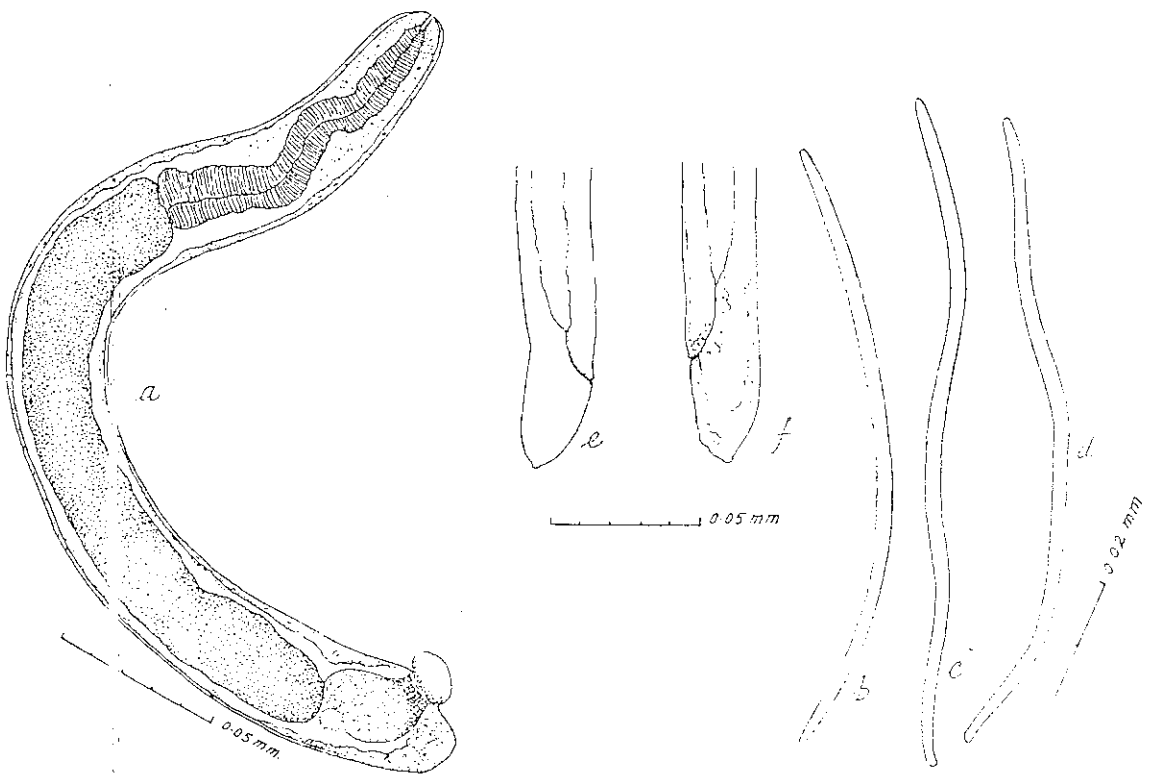


FIG. 7—*Dirofilaria immitis*.

a. Late second instar larva. Camera-lucida. Original.

b. c. d. Third instar larvae. Camera-lucida. Original.

e. Tail end of third instar larva showing short terminal papilla. Camera-lucida. Original.

f. Tail end of third instar larva showing terminal papilla and a suggestion of a smaller papilla higher up. Camera-lucida. Original.

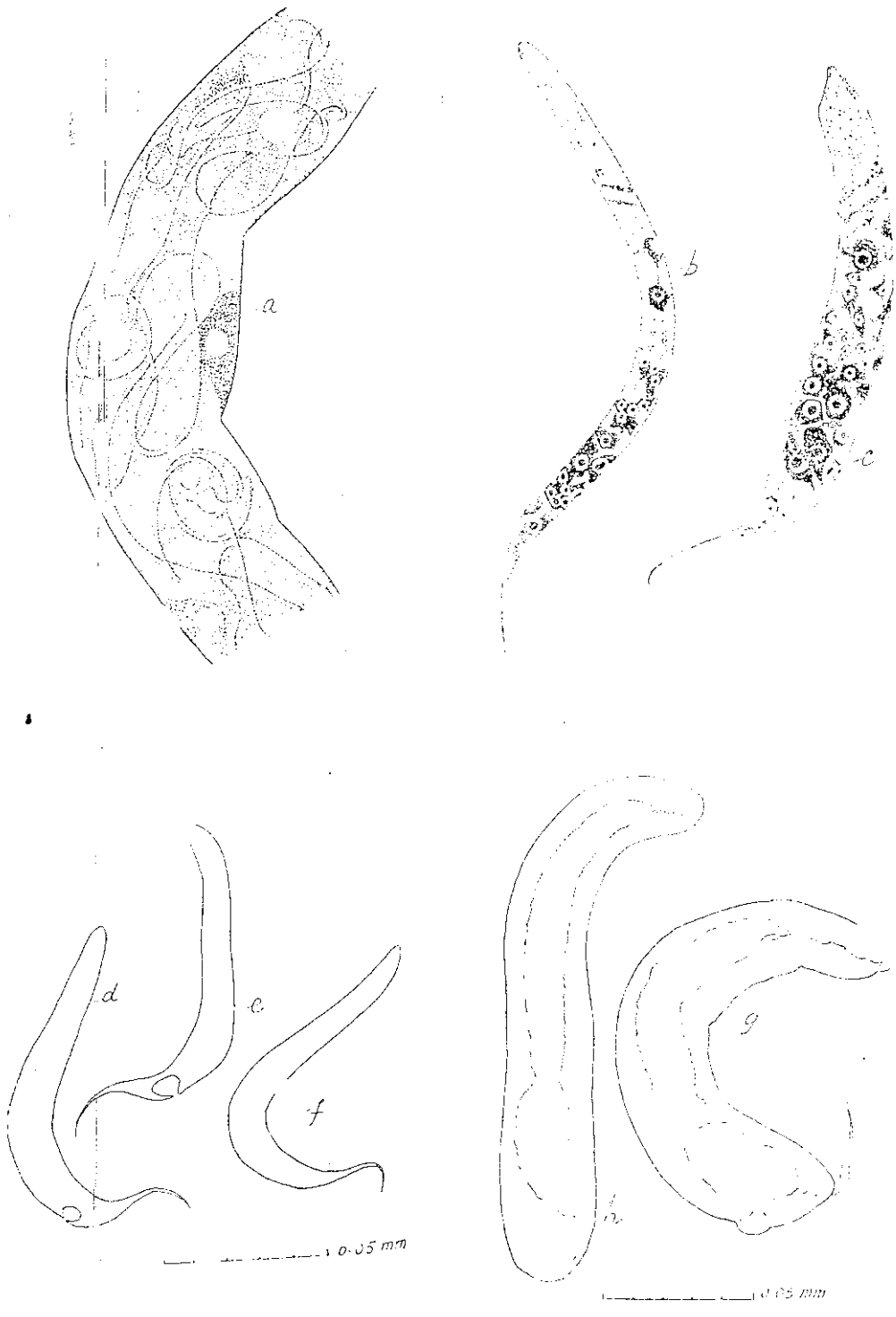


FIG. 6—DEVELOPMENTAL STAGES OF *Dirofilaria immitis*.

a. Part of a malpighian tubule showing young first instar larvae (microfilaria-like) within the tubule. After Noé, 1901

b. Early first instar larva. After Saisawa, 1913

c. First instar larva, "Sausage" form. After Saisawa, 1913.

d, e., and f. Sausage stage larvae. Camera-lucida. Original.

g. Full grown first instar larva moulting. Camera-lucida. Original.

h. Early second instar larva. Camera-lucida. Original.

FIG. 8—*Pseudoparasites.*

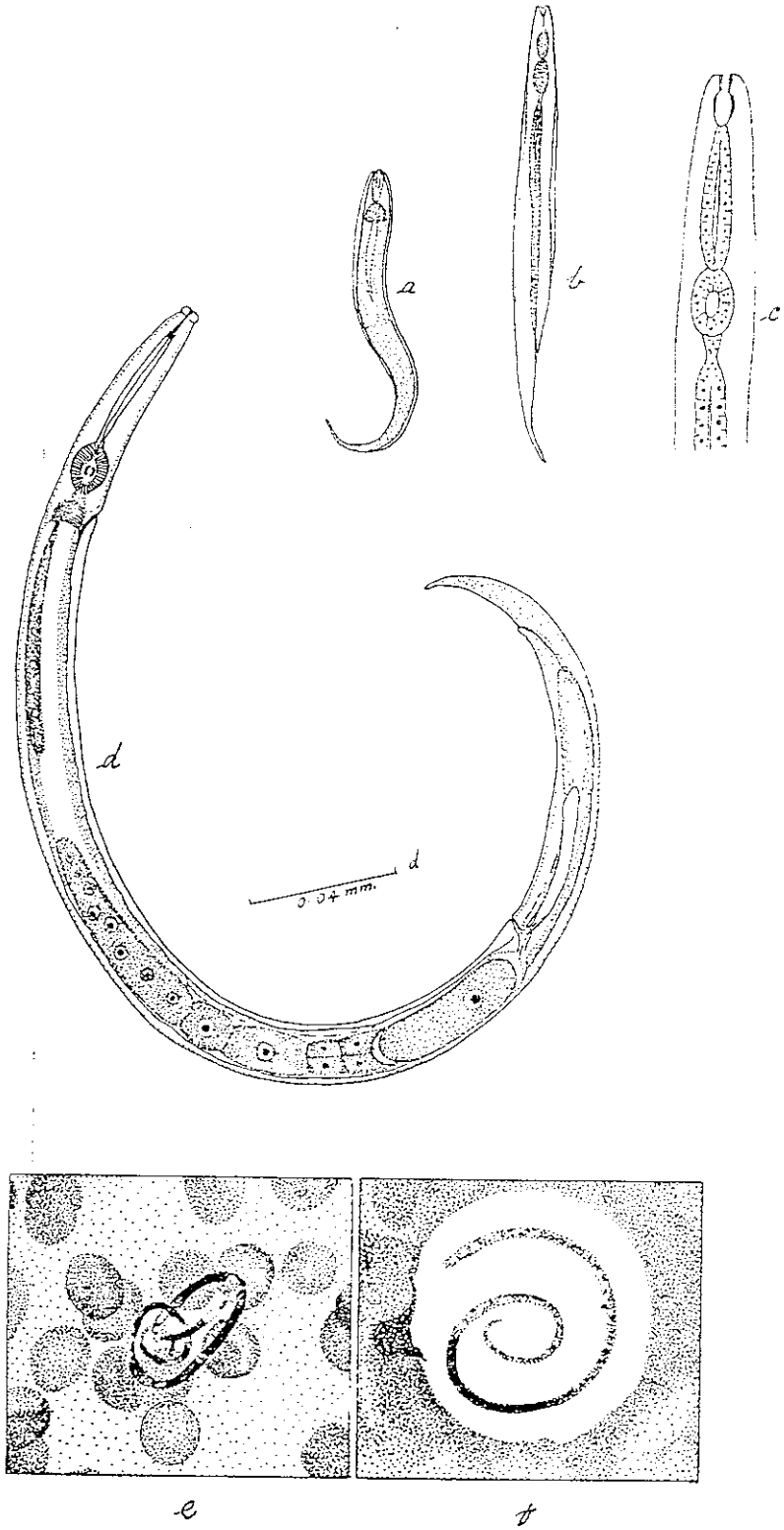
a. Larva of *Aphelenchoides*, 280 μ by 16 μ (after Manson-Bahr 1952).

b. Later stage larva of *Aphelenchoides*, 320 μ by 20 μ (after Manson-Bahr 1952).

c. Head end of a larva of *Aphelenchoides* showing the oesophagus (after Manson-Bahr 1952).

d. *Aphelenchoides parietinus* (after Franklin 1955).

e, f. Spores of helicosporous fungi simulating nematodes in blood smears (after Norman and Donaldson 1955).



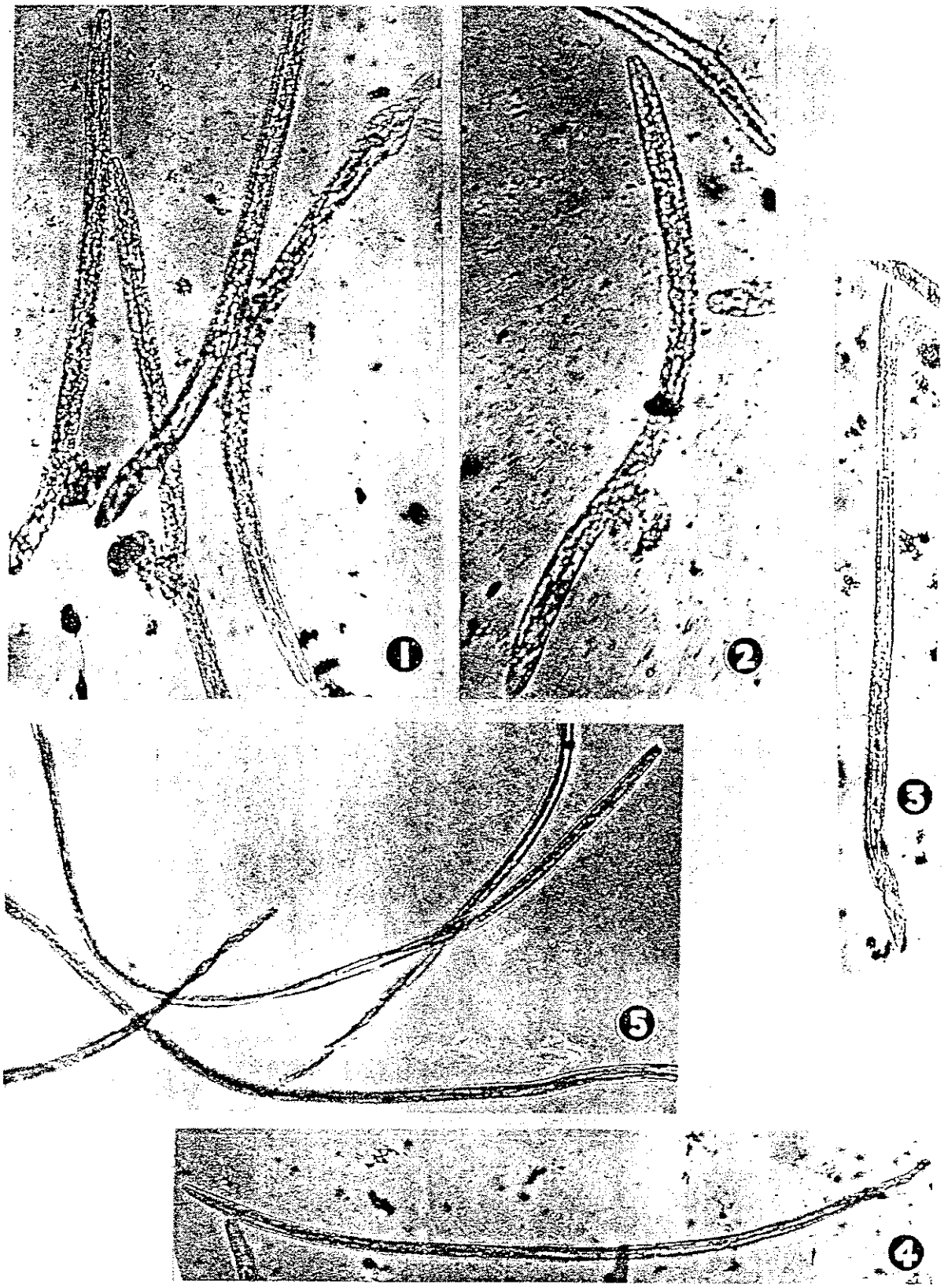


PLATE 2. PHOTOMICROGRAPHS OF *Wucheria bancrofti*, NON-PERIODIC FORM.
(U.S. Navy photographs from Byrd, St. Amant & Bromberg, 1945.)

1. Second instar larvae, 9 days old.
2. Second instar larva, 10 days old.
3. Late second instar larva, 11 days old.
4. Early third instar larva, 13 days old.
5. Late third instar larvae, infective stage, from head and labium of mosquito.

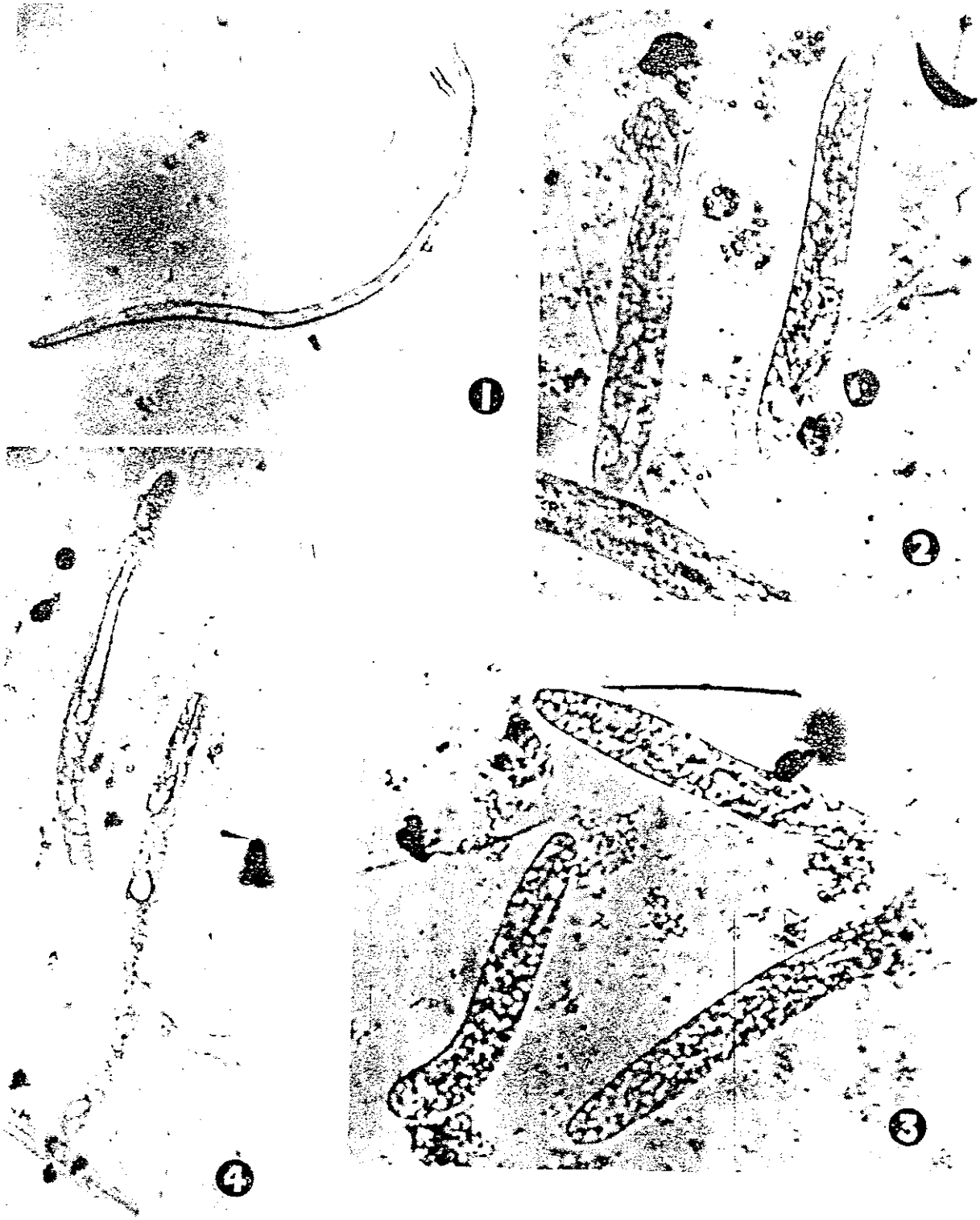
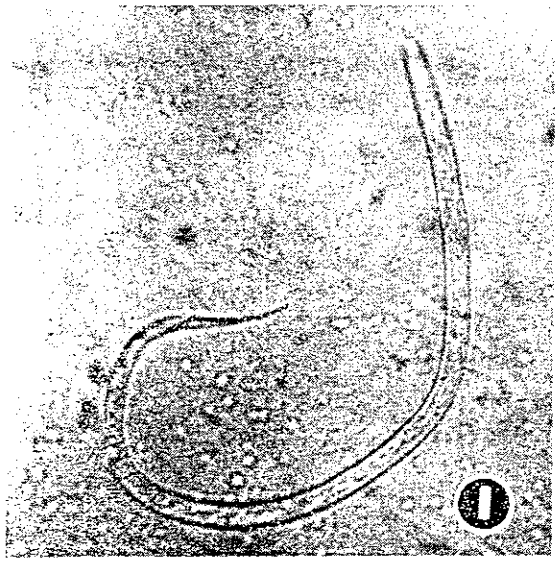
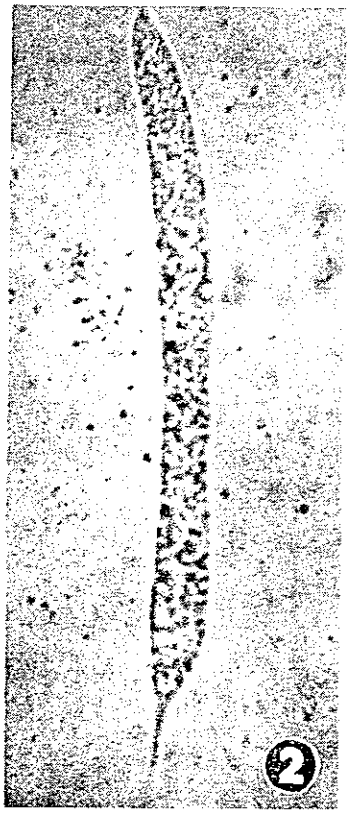
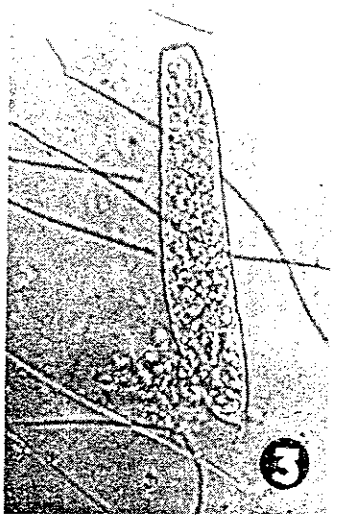


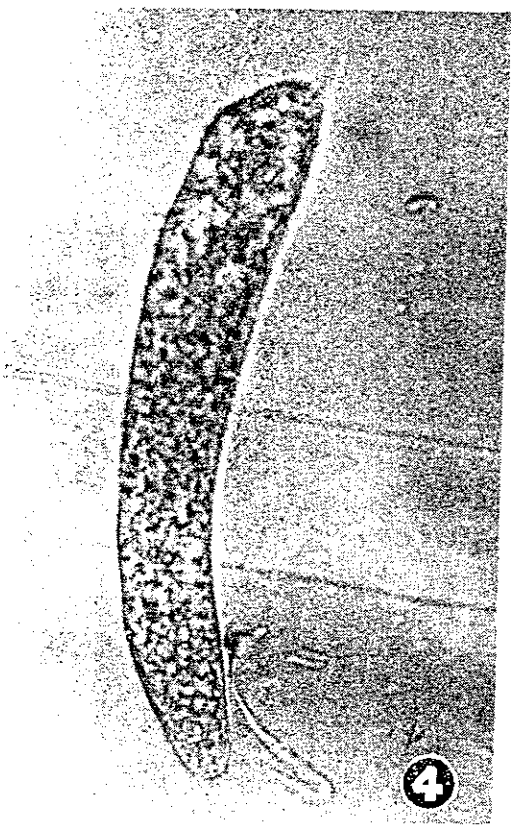
PLATE 1. PHOTOMICROGRAPHS OF *Wuchereria bancrofti*, NON-PERIODIC FORM.
(U.S. Navy photographs from Byrd, St. Amant & Bromberg, 1945.)

1. Early first instar larva, one day old.
2. First instar larvae, sausage form, 5 days old.
3. First instar larvae, sausage form, 6 days old.
4. Early second instar larvae, 8 days old.









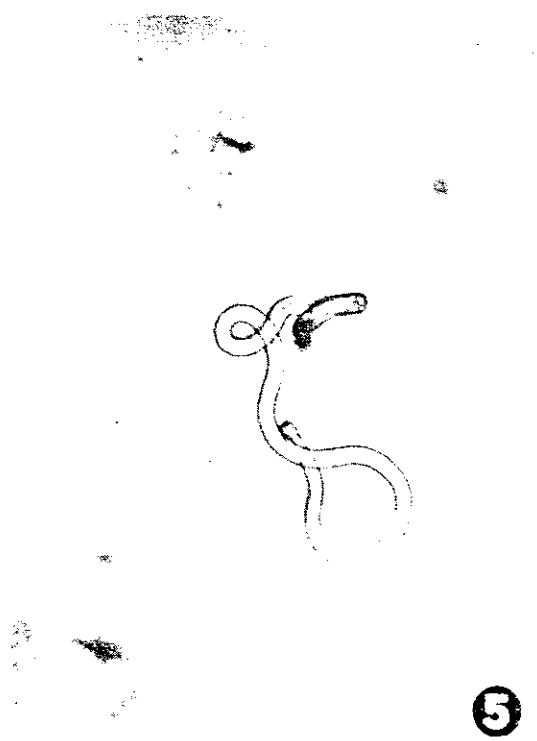


PLATE 3. PHOTOMICROGRAPHS OF *Wuchereria bancrofti*, NOCTURNAL PERIODIC FORM.
(Photographs taken by Dr. H. de Rook.)

1. Early first instar larva, microfilaria form.
2. Early sausage stage.
3. Sausage stage, first instar.
4. Later sausage stage, first instar.
5. Infective stage third instar larva, photograph of live specimen.

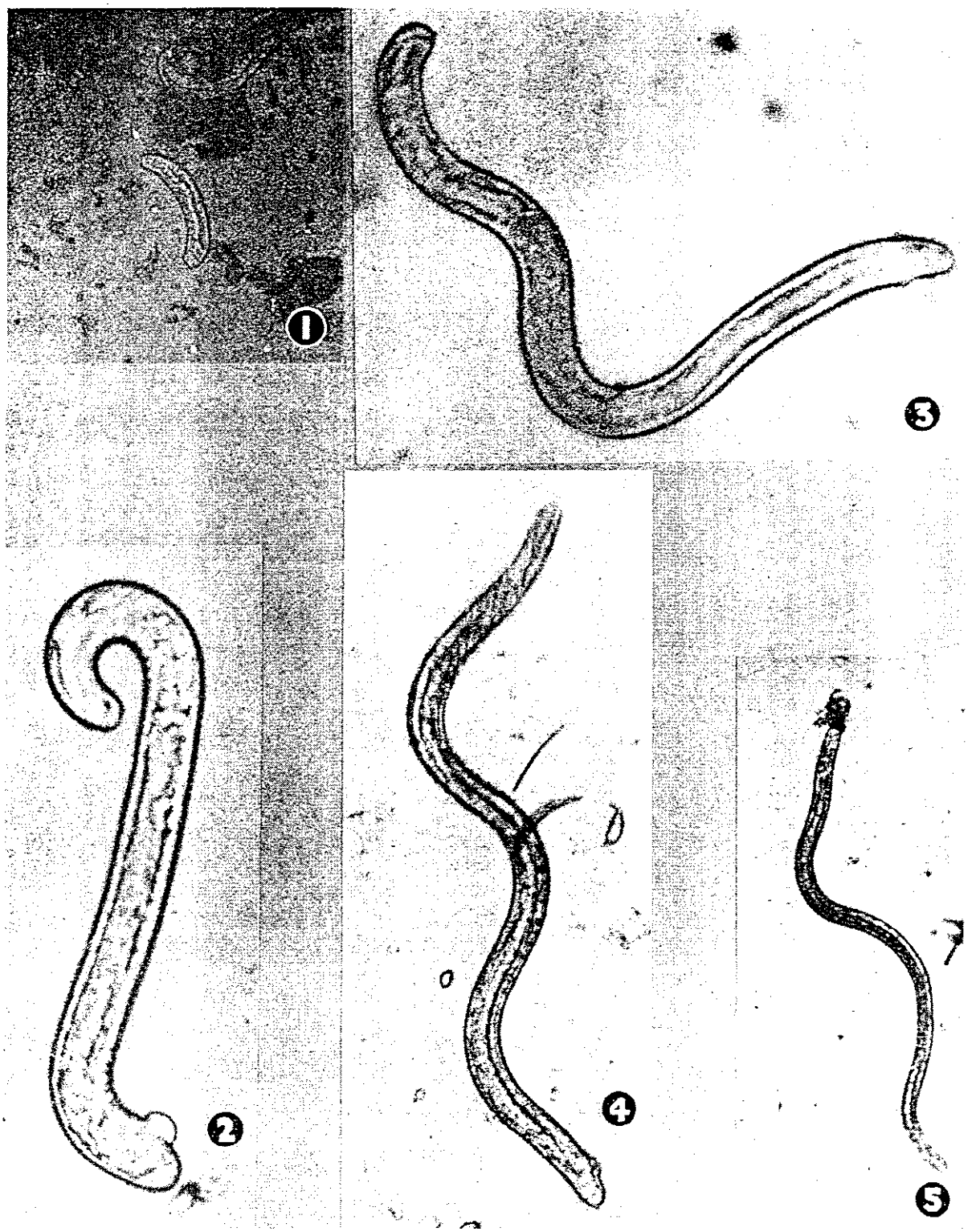


PLATE 4. PHOTOMICROGRAPHS OF *Dirofilaria immitis*.
(Photographs taken by Dr. L. Kartman)

1. Sausage stage larva, first instar, 3 days old.
2. Second instar larva, early stage.
3. Second instar larva, later stage.
4. Third instar larva, early stage.
5. Third instar larva, infective stage.