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Studies on the Mechanism of the Filarial Periodicity
The Autofluorescence in the Microfilariae and Their Periodicity

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Introduction

There have been many hypotheses or assumptions on the mechanism of the filarial periodicity, since Sir Patric Manson, 1879 (Table 1). However, no decisive evidence has been shown to support any of them. The old assumptions on the simultaneous cyclical parturition and daily destruction of the larvae, by Lane (1926, 1933) and O'Connor (1931) have been clearly denied by Kume and Ohishi (1957, 1959) who have demonstrated a maintenance of the periodicity of *Mf. immitis*, even after the removal of the adult worms from the host. The site of diurnal concentration of *Mf. bancrofti* has been confirmed to be the lungs, by means of needle biopsies and surgical explorations of various organs and tissues by Masuya *et al.* (1957, 1958) (Table 2).

The negative heliotropism of the microfilariae has been experimentally demonstrated in case of *Wuchereria bancrofti* by Suganuma (1921) and in case of *Dirofilaria immitis* by Murata (1933). The most persuasive studies in this respect, would be of Yoshida (1966), who could show a complete inversion of the periodical appearance of *Mf. bancrofti* in the emigrants from Okinawa to Bolivia in 116 days, via Indian and Atlantic Oceans. The beginning of increase of microfilariae has been observed after the sunset, except 3 out of 15 examinations and far before the sleep in all cases.

The purpose of the present paper is to search for the presence or abscence of any photodynamic substance in the microfilariae, with different patterns of the periodicity, by means of fluorescence microscopy. Some approaches have been made to know the nature of the fluorescent substance(s) in the microfilariae.

Materials and Methods

The species and strains of microfilariae examined are as follows:

Wuchereria bancrofti from the Japanese (Kagoshima, Amami and Okinawa) and from the Indonesians, donated by Prof. Sri Oemijatti in Jakarta, both with highly nocturnal periodicity.

Polynesian strain of *W. bancrofti* from the Tahitians, confirmed to be subperiodic, not nonperiodic, by Rosen (1955), kindly endowed by Dr. Saugrain in the French Institute of Medicine, in Papete. The morphology of the larvae, stained with Giemsa solution, coincided with those of *W. bancrofti*.

Brugia malayi from the Koreans in Cheju Island, with low grade nocturnal periodicity, taken by Prof. Fukushima in Kagoshima University.

Brugia malayi from the Siamese, nocturnal, kindly endowed by Prof. Chamlong Harinasuta, Mahidol University, in Bangkok.

Brugia malayi in cats, low grade nocturnal, in London School of Tropical Medicine and Hygiene, taken by Masuya by courtesy

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Table 1 Theories on the mechanism of the filarial periodicity

Manson (1879, 1891)	Teleological theory
Lane (1929, 1933)	Synchronized, expulsive emptying parturition, impending larvicidal mechanism.
O'Connor (1931)	Simultaneous cyclical parturition and daily destruction of the previous night's brood of microfilariae
Manson (1883)	Microfilariae survive 100 hrs.
Taniguchi (1905)	Yes, including many other workers.
Hawking (1940)	14 days
Kume and Ohishi (1954)	Turnus continues, after the removal of adult canine heart worms.
MacKenzie (1881)	Inversion of the turnus, by inversion of activity and sleep.
Linstow (1892)	Sleep causes capillary dilatation, awakening causes constriction.
Scheube (1896)	Sleep, rest in horizontal position, activate lymph flow.
Ishiguro (1905)	No
Taniguchi (1905)	No
Rodenwaldt (1909)	Blood pressure theory
Yamada and Yamamoto (1916)	Tropism for CO_2
Lancereaux (1888)	Negative heliotropism, without evidence.
Suganuma (1921)	Negative heliotropism in *Mf. bancrofti.*
Murata (1939)	Negative tropism against UV-light, in *Mf. immitis.*
Masuya *et al.* (1958)	Site of diurnal concentration of *Mf. bancrofti* to be the lungs.
Katamine (1959)	Electroshock, forced labor disturb turnus, *Mf. immitis.*
Yoshida (1966)	Complete inversion of the turnus, (*Mf. bancrofti*) in the emigrants from Okinawa to Bolivia, in 116 days.
Hawking (1967)	Oxygen barrier theory
Masuya (1970)	Photodynamic substance theory

Table 2 Distribution of *Mf. bancrofti*, by means of biopsies and surgical explorations (Masuya *et al.* 1958)

	Daytime	Provocation	Night
Bone marrow	−	+	++
Liver	−	+	+
Kidney	−	+	+
Great omentum	−		+
Stomach	−		+
Intestine	−		+
Appendix	−	+	+
Subcutaneous tissue	−	+	+
Skeletal muscle	−		+
Lung	+++	++	++
Peripheral blood	−	++	+++

of Dr. Denham and in the Institute of Medical Science, Tokyo University, taken by Masuya by courtesy of Prof. Tanaka.

Brugia malayi in cats, nonperiodic, in the Institute of Medical Research in Kuala Lumpur, Malaysia, taken by Dr. Mak Joon Wah.

Brugia patei in cats, highly nocturnal, in London.

Brugia pahangi in cats in London and in a dog in the Insitute of Tropical Medicine, Nagasaki University, both subperiodic.

Dirofilaria immitis from dogs in Japan (Tokyo, Tokushima and Fukuoka), low grade nocturnal. Lung smears of the infected dogs and the smears of squeezed fluid out of the uterus of the adult heart worms were also

examined.

Dirofilaria uniformis in rabbits, prenocturnal, the peak of the microfilariae count being in 4–8 p.m. in Walter Reed Army Institute, Washington D. C., taken by Mr. Moon and later by Dr. Sadun.

Dipetalonema reconditum from dogs in Okinawa, the pattern of the periodicity being double peaked, at noon and after 6 p.m. according to Newton and Wright (1956). In each dog, the abscence of heart worm has been confirmed. Because of the fewness of the larvae, smears were made from the sediment of the centrifuged blood with cold H_2O.

Litomosoides carinii in cotton rats, nonperiodic, in the Institute of Medical Science, Tokyo University.

Onchocerca volvulus from the Congolese and the Ethiopeans, nonperiodic, taken by Dr. Kanekawa in Zaiire and Prof. Tada in Kanazawa Medical College.

Setaria digitata from the cattle in Gumma Prefecture, said to be nonperiodic.

Loa loa from the Congolese, diurnal. At first, sent by Prof. Fain in Antwerpen and Prof. Nelson in London, although they were too old for the present study. Thin, fresh blood smears were kindly endowed by Dr. Yamamoto in the Tenri Clinic in Brazaville Congo.

Icosiella kobayashii from a frog (*Rana limnocharis*) in Amami, caught by Prof. Miyagi, University of the Ryukyus, nonperiodic.

Mansonella ozzardi from the Brazilians, nonperiodic, kindly endowed by Prof. Dourado in Manaus-Amazonas.

A. Fluorescence microscopy:

The air dried and methanol fixed, thin blood or tissue fluid films were examined, without any staining, under fluorescence microscope. The wave length of the exciting rays ranged from 350 to 540 μm, according to the specification of the manufacturers. (Tiyoda, Olympus and Nikon in Tokyo) In most cases, Tiyoda FM 200 A was used, equipped with high pressure mercury lamp (Osram HBO-200), and with dark field condensor. The wave length of the exciting rays, most frequently used had the peak at around 410 μm. Fluorescence photomicrographs were taken, using Kodak High Speed Ektachrome, daylight, ASA 160. Although a minimal exposure is preferable, the weak autofluorescence of the microfilariae did not allow exposure, shorter than 120 sec, when the objective lense of 40× was used. The old specimens, even methanol fixed, were not suitable for the present study.

In case of *Dirofilaria immitis*, fluorescence microscopic cinematography was done, to show the autofluorescent granules in the living, wriggling larvae, with the cooperation of experts from Iwanami Productions Inc. Tokyo, movements of the larvae being suppressed.

B. Microfluorophotometry:

The relative fluorescence intensity in the microfilariae of several species and strains has been determined, using the automatic recording microfluorometer, Nikon SUR-F type, equipped with a high pressure mercury lamp-HBO Osram 200 W Toshiba. The spot illumination was done with the BV-filtered light, the wave length being aroud 400 μm. The penetrating light was removed by the special filter. The selected fluorescence was determined within a slit, 0.5×0.5 mm^2 in the viewer of the same magnification (objective lense 40×) passing through a photomultiplier. After some preliminary observations, the E max settled at 450 μm and the F max at 560 μm. Scanning speed was 1.1 sec/100 μm in most cases and occasionally 3.3 sec/100 μm. The intensity of the exciting light was adjusted to be constant by measuring the fluorescence of a piece of uran-glass as a standard. All the fluorometric data were expressed by the relative fluorescence intensity, which has not yet been corrected by the sensitivity of the photomultiplier. The smears of two or three different species have been examined on the same day, to obtain the values, as constant as possible.

Each segment of the microfilariae was put to lie diagonally in the visual slit, under the ordinary light. Then the illumination was

switched to the exciting light and the reading was done within 10 sec, adjusting the condensor as to obtain the maximum reading. The determinations were done at 3 to 4 segments per larva, apart from each other and was taken the highest value per each larva, taking into consideration, the distribution of the fluorescent granules and the fading effect of the previous illumination. As will be shown later, the relative fluorescence intensity of the microfilariae seemed to be dependent on the density of the granules, rather than on the breadth of the larvae.

At first, the determinations were done in the old specimens of *Mf. bancrofti* (7 to 11 months after preperation). However, as will be shown later, was significant the difference between the mean values in the old and fresh specimens of the same strain (examined on the 54th day of preparation). Hence, the determinations must be confined only to the species and strains, which could be examined within 50 days after taking samples. They were as follows: The ordinary and Polynesian strains of *W. bancrofti*, *B. malayi* from the Koreans, *Loa loa*, *D. immitis* and *B. malayi* from cats in Tokyo.

C. Scanning electronmicroscopy:

Heparinized blood was hemolyzed in the cold H_2O, with or without saponin, washed with phosphate buffer (0.1 M, pH 7.3). Fixation was done in 2.5% glutaraldehyde phosphate buffer, for 30 min. Then washed for 10 min, twice, with phosphate buffer. Dehydration was done in ethanol series, 50 to 90% and kept in 100% ethanol. The sediment was dispersed in 100% isoamyl acetate and a few drops were frozen in the liquid nitrogen. Some pieces of frozen, fractued particles were transfered into isoamyl acetate. Left in the air, at room temperature. One drop was applied to critical point drying. The dried sample was applied to a copper block and cemented with electroconductive dotite, then plated with carbon and gold in the ionization vacuum gauge (Model JVG-N1) for 15 min. Scanning electronmicroscopy was done using JSM-50 A.

Results

A. Flurorescence microscopic findings:

1. *Wuchereria bancrofti*, ordinary strain: In all cases, but one, examined, were observed in every larva, a diffuse autofluorescence and numerous autofluorescent granules. The color of the fluorescence varied according to the specificity of the barrier filter, gold brown, yellowish green or blue white. While in one case among those from Amami (islans between the main land of Kyushu and Okinawa), were seen very few granules. The daytime count of mf. in the last case was confirmed to be nil, by Prof. Fukushima. In the Indonesian strain, were seen the same findings with those in the other cases in Japan. (See Plate I and II)

2. *Dirofilaria immitis:* Less numerous granules were detected, not only in the dried, fixed smears, but in the living, wriggling microfilariae, which could be shown in the fluorescence microscopic cinematograph. The diffuse fluorescence was not observed in the living larvae, although it was marked in the dried smears. Among those in the peripheral blood, both taken by day and by night, were seen some larvae, without or with very few granules. In the lung smears of the infected dogs, were observed many shorter larvae without granule. In the smears of the squeezed fluid out of the uterus of the adult worms, were detected many coiled or stretched youngest microfilariae. None of them showed any granule.

3. *Litomosoides carinii:* In the freshly prepared blood smears from cotton rats, no fluorescence could be detected, neither diffuse nor granular, even in oil immersion magnification. Although only the contour of the larvae could be seen after 50 days of preparation, was seen a very faint diffuse fluorescence after 180 days of preparation.

4. *Brugia patei:* Numerous fluorescent granules, a little less than in *Mf. bancrofti*, were observed, together with a diffuse fluorescence. The night/day ratio of

micaofilariae was confirmed to be 30, by Dr. Denham, in London.

5. *Brugia pahangi* in cats: A diffuse fluorescence was seen in every larva. Fluorescent granules could be seen only in a few larvae and their density was very low. The night/day ratio of microfilariae was 3.0. *B. pahangi* has been transferred to dogs, in the Institute of Tropical Medicine, Nagasaki University. The night/day ratio of microfilariae was 2.8 (Dr. Aoki). The fluorescence microscopic findings were quite the same as those in cats.

6. *Brugia malayi* in cats in London: A diffuse autofluorescence was seen in all larvae. The fluorescent granules were a little less in some larvae and far less in the others, than in *Mf. bancrofti*. *B. malayi* in cats in Tokyo, showed a simillar finding. Very interestingly, the density of granules was definitely higher in the caudal side than in the cranial side of microfilariae.

7. *Brugia malayi* in the Koreans: The number of the fluorescent granules was somewhat less than in those in cats. The night/day ratio of microfilariae was 6, by Prof. Fukushima.

8. *Brugia malayi* in the Siamese: Both as for a difiuse fluorescence and fluorescent granules, the findings were almost the same as those in *Mf. malayi* in cats. While, the night/day ratio of microfilariae was confirmed to be 60, from the chart of Prof. Chamlong Harinasuta (1970). Considerations about the discrepancy of the pattern of the periodicity between these two strains will be done later.

9. *Dirofilaria uniformis:* In September, 1970, during his stay in Washington D. C. for the II International Congress of Parasitology, Masuya could examine the larvae of *D. uniformis* from rabbits, in Walter Reed Army Institute, by courtesy of Mr. Moon. Later, many blood smears were kindly endowed by Dr. Sadun in the same Institute. In addition to a diffuse fluorescence, very few granules were detected in most larvae.

10. Polynesian strain of *W. bancrofti*: A diffuse fluorescence was seen in every microfilaria. The fluorescent granules were detected only in very few larvae and their density was very low. Their morphology in Giemsa-stained specimens was quite the same with that in the ordinary strains from the Japanese and the Indonesians.

11. *Onchocerca volvulus*: Two strains from the Congolese and the Ethiopeans have been examined. Except a diffuse fluorescence, no fluorescent granule could be seen in any larva.

12. *Setaria digitata*: Ten blood smears from cattle have been examined. Only one microfilariae could be seen, in which no granule could be detected, except a diffuse fluorescence.

13. *Dipetalonema reconditum*: There were seen two kinds of microfilariae, with somewhat numerous granules and without any granule. Their periodicity has been described as double peaked, at noon and at night, by Newton and Wright, 1956. The examinations have been done in the afternoon.

14. *Brugia malayi* in cats in Kuala Lumpur: In October 1973, on the way home from Athenae (IX International Congress on Tropical Medicine and Malaria), Masuya could examine the nonperiodic strain of *B. malayi* in cats in the Institute for Medical Research in Kuala Lumpur, by courtesy of Dr. Mak Joon Wah. Immediately after preparation, a diffuse fluorescence could not be detected, even in the dried smears. In some larvae, there were detected a few granules. Diffuse fleuorescence was seen after 10 days.

15. *Loa loa:* At first, the very old specimens, endowed by Prof. Fain and Prof. Nelson, were examined. They showed a diffuse fluorescence, without any granule. The freshly prepared, thin blood smears were endowed by Dr. Yamamoto in Brazaville Congo, which could be examined 40 days after preparation. No fluorescence was detected, neither diffuse nor granular, in any larva. The Giemsa-stained larvae showed the distinguished excretory apparatus.

16. *Mansonella ozzardi*: Thin blood

Table 3 The autofluorescence in the microfilariae and their periodicity

Species/Strain	Host	Diffuse fluorescence	Granular fluorescence	Periodicity	Night/day ratio
Wuchereria bancrofti	Japanese*	+	++++	nocturnal	>100.
	Indonesians	+	++++	do.	
Polynesian strain	Tahitians	+	− or +	subperiodic	1.4
Brugia patei	cats, London	+	+++	nocturnal	30.
Brugia malayi	Siamese	+	+++	nocturnal	60.
	cats, London	+	+++	low nocturnal	8.
	cats, Tokyo	+	+++	do.	8.
	Koreans	+	++	do.	6.
	cats, Kuala Lumpur	+	− or +	nonperiodic	
Dirofilaria immitis	dogs	+	++	low nocturnal	6.
Dirofilaria uniformis	rabbits, Washington	+	+	prenocturnal**	10.
Dipetalonema reconditum	dogs, Okinawa	+	+ or −	curious***	3.
Brugia pahangi	cats, London	+	− or +	subperiodic	3.
	dog, Nagasaki	+	− or +	do.	2.8
Mansonella ozzardi	Brazilians	+	− or +	nonperiodic	
Onchocerca volvulus	Congolese	+	−	do.	
	Ethiopeans	+	−	do.	
Setaria digitata	cattle	+	−	do.	
Icosiella kobayashii	frog, Amami	+	−	do.	
Litomosoides carinii	cotton rats	−	−	do.	1.
Loa loa	Congolese	−	−	diurnal	0.1

* Several strains have been examined in Kagoshima, Amami and in Okinawa.
** The peak of the larvae in the blood, being seen in 4–8 p.m.
*** Two peaks of the larvae being seen at noon and after 6 p.m.
In the species, where the night/day ratio is not given, it has not been confirmed by the present author, or the reliable figures could not be available in the literature.

smears from the Brazilians, were kindly endowed by Prof. Dourado in Amazonas University, in November 1975. In most larvae was detected only a diffuse fluorescence, no granule. In a few microfilariae, were seen a few granules.

17. *Icosiella kobayashii:* In a frog (*Rana limmocharis*), caught in Amami by Prof. I. Miyagi, microfilariae were found. They were identified as *Icosiella kobayashii*, because of the long sheath, which differed from *Icosiella sasai* with short sheath. Except a diffuse fluorescence, was detected no granule.

The fluorescence microscopic findings of microfilariae, examined, could be summarized as shown in Table 3. As for a diffuse fluorescence, there may be a possibility of the artifact or the degradation of granules, since in the living larvae and in those, immediatly after preparation, there has been detected no diffuse fluorescence. However, the abscence of any fluorescence in *Mf. carinii* and *Mf. loa*, even in the specimens examined after 30 to 50 days of preparation, might suggest a possibility that even those with no granule but a diffuse fluorescence, might have had a very very few granules and the microfilariae of *Loa loa* and of *Litomosoides carinii* had no granule at all. In this connection, all species and strains must be examined in the living state. Even in the methanol fixed smears, immediately after air drying, the fluorescent grnules apt to

decrease or to become dim, after 30 days or so, while the relative fluorescence intensity of the larvae has not significantly decreased.

B. Microfluorophotometric findings :

Preliminary observations : The E max was settled at 450 μm and F max at 560 μm. From the decay curve of the fluorescence, it was considered the reading must be done within 20 sec. Most readings have been done within 10 sec. The color of the autofluorescence of the microfilariae, excited by the BV-filtered light of the mercury lamp (around 400 μm), was yellowish green, in all species and strains, examined. As mentioned above, the color under the ordinary fluorescence microscope was gold brown, yellwish green or blue white, according to the specificity of the barrier filter, for example barrier filter No. 4, 3 or 2 (Tiyoda).

In some of the microfilariae, the curves of the fluorescence intensity were compared, automatically recorded at 560 μm, excited by 450 μm, scanning speed being 1.1 sec/100 μm. The curve was the highest in *Mf. bancrofti* from the Japanese and the lowest in *Mf. loa* and *Mf. carinii*.

Semiquantitative determinations : The relative fluorescence intensity of the microfilariae, examined, could be summarized in Table 4. The mean value in each species or strain, seemed to be well corresponded to the density of the fluorescent granules in the larvae, under the fluorescence microscope.

Further, the difference of the mean values between each group, compared, was confirmed to be significant, by means of F-test or t-test. As mentioned above, the figures obtained in the very old specimens of *Mf. bancrofti* (a) showed a significant difference from the figures taken in the fresh specimens (b) of the same strain, in F-test at 1 % level. Therefore, the figures taken in the old specimens have been abandoned. It was significant by t-test, p=0.001, between *Mf. bancrofti* vs Mf. polynesian ; vs *Mf. loa* ; vs *Mf. malayi* Koreans : by F-test, 5 % level, *Mf. immitis* vs *Mf. bancrofti* ; vs Mf. polynesian ; by t-test, p=0.05, between Mf. polynesian vs *Mf. loa* ; vs *Mf. malayi* Koreans : t-test, p=0.001, between *Mf. loa* vs *Mf. malayi* Koreans : by F-test, 1 % level, between *Mf. loa* vs *Mf. immitis* and by t-test, p=0.01, between the caudal side vs cranial side of *Mf. malayi*, cats in Tokyo.

Table 4 Microfluorophotometry of the microfilariae

Species/Strain	N	Mean± SD
Wuchereria bancrofti (a)	13	76.85±27.37
Wuchereria bancrofti (b)	14	68.29± 9.93
Polynesian strain	12	25.59± 8.94
Brugia malayi, Koreans	13	33.85± 8.43
Loa loa	10	17.20± 6.24
Dirofilaria immitis	16	50.63±16.58
Brugia malayi, cats		
caudal	21	39.96±18.34
cranial	21	25.86±16.58

The relative fluorescence intensity of the larvae was recorded at 560 mu, excited by 450 mu, slit being 0.5×0.5 mm² in the viewer of the same magnification. The unit is arbitrary, however as the standard was used the reading of uran glass. The readings on each day have been corrected aocording to the standard value, taken each day.

The significance of the difference between the figures in the caudal side and the cranial side of *Mf. malayi* in cats, would suggest that the fluorescence intensity, thus determined, might be mainly dependent on the density of the fluorescent granules in the microfilariae. In some larvae of *D. immitis*, the recordings have been done at several parts of the larva, expecting to be able to show such relationship, comparing the chart with the fluorescence photomicrograph. However, in the photomicrograph of the same microfilaria, granules had been strongly decreased or disappeared, perhaps due to the previous illumination. (Fig. 1)

C. Scanning electron microscopic findings : (Monochro. Photo 1–10)

In the microfilariae of *D. immitis* in the peripheral blood, there were detected variable numbers of the spherical bodies, 5 to 50, of variable sizes, 0.1 to 0.35 μ, in each

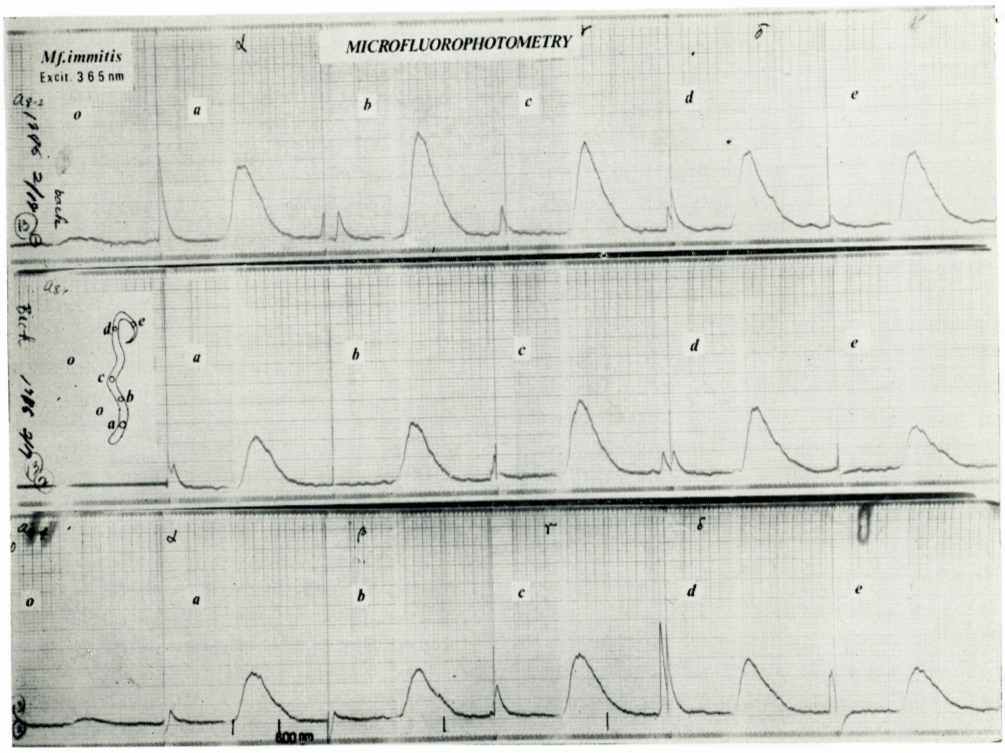


Fig. 1 Microfluorophotometric recordings in different parts of 3 larvae of *Dirofilaria immitis*.
"o" is outside of the larva, i.e. background.
"a, b, c, d and e" show the recording at each different part of the larvae.

fractured surface of all larvae, examined. Some globules were seen on the body surface and in the background near the larvae. They were regarded to be those spilt down at the time of fracture of the larvae.

In the larvae of *Dipetalonema reconditum*, some of them showed several globules of somewhat smaller sizes, in average, than those in *Mf. immitis*, and in the others could not be detected any such globule. Under fluorescence microscope, too, there had been two types of the larvae of this species, one with fluorescent granules and the other with no granule.

As mentioned above, the youngest larvae in the squeezed fluid out of the uterus of the adult heart worm (*D. immitis*), showed no fluorescent granule. Under scanning electronmicroscope, too, no spherical body could be detected in any fractured surface of many larvae, examined.

Although the examination on *Mf. bancrofti* has not yet been succeeded, bacause of the difficulty to catch the carriers and of the scarcity of larvae, it is very likely, such globules might correspond to the fluorescent granules under fluorescence microscope.

D. Microfluorophotometric and biochemical approach for the nature of the fluorescent substance :

Microfluorophotometry, done in Tokushima University, 1970, has suggested the fluorescent granules contained flavins, from E max and F max. The existence of flavins in the warm water extract of *Mf. immitis* was confirmed, using lumiflavin method by Yagi (1956), detecting the same excitation and fluorescence spectra with those of authentic riboflavin, as shown in Figs. 2 and 3.

On the silicagel thinlayer chromatography,

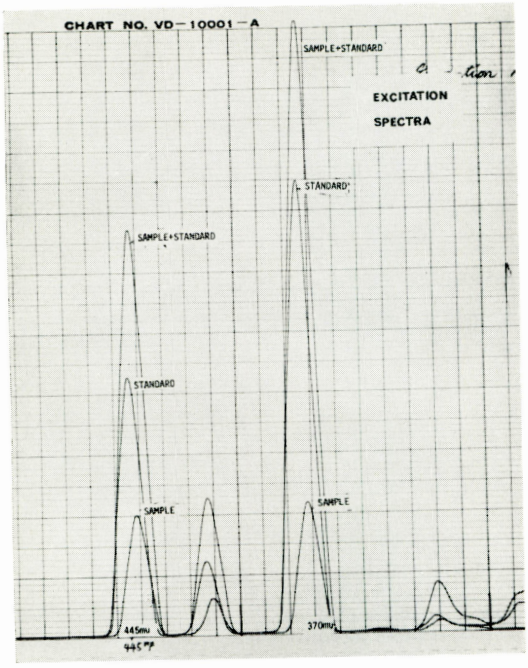


Fig. 2 Excitation spectra of the warm water extract of the mass of *Mf. immitis* (sample) with or without standard (authentic riboflavin) (see text).

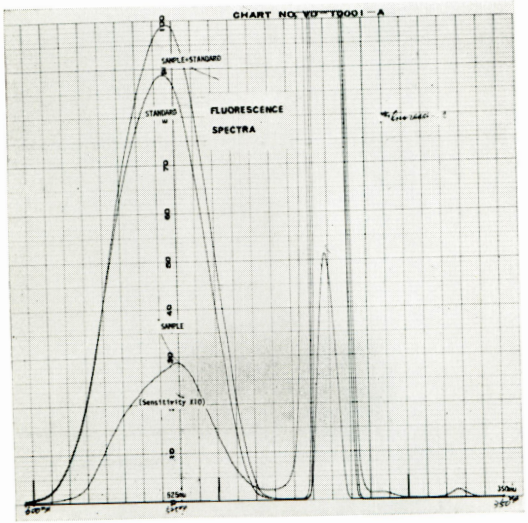


Fig. 3 Fluorescence spectra of the same materials, with those in Fig. 2 (see text).

the phenol fraction of the supernatant of ammonium sulphate-deproteinized warm

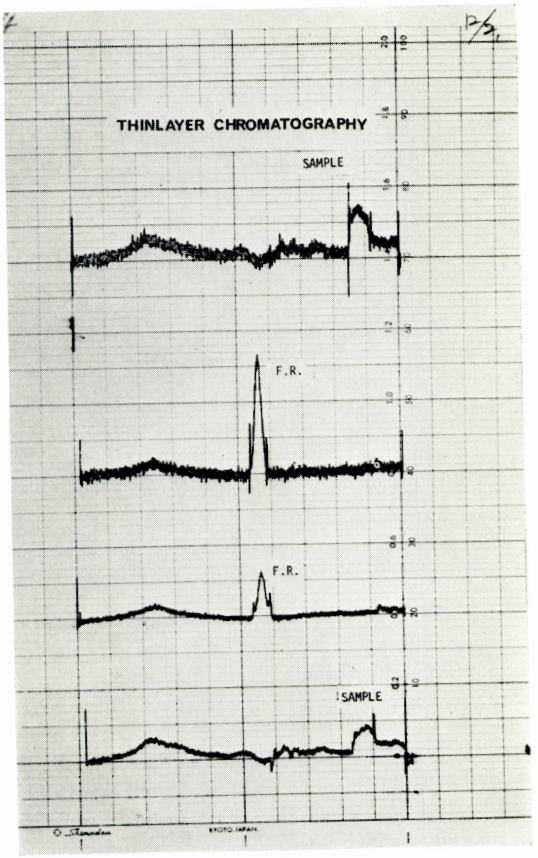


Fig. 4 Silicagel thinlayer chromatography of the extract of *Mf. immitis*, with butanol-ethanol-water (50 : 15 : 35) (sample) and riboflavin (see text).

water extract of *Mf. immitis*, showed similar Rf to that of FAD (0.13) and FMN (0.155), instead to that of free riboflavin (0.41) as shown in Figs. 4 and 5. The recent microfluorophotometry (1976) (the energy of the exciting rays being larger than that of the apparatus, used in 1970) has given the same F max (540 μm) in the larvae of *Dirofilaria immitis*, both when excited by 365 μm and 410 μm, while FMN and FAD did not fluoresce when excited at 410 μm, but fluoresced only when excited at 365 μm (Figs. 6 and 7).

Discussion

There had been two major problems on

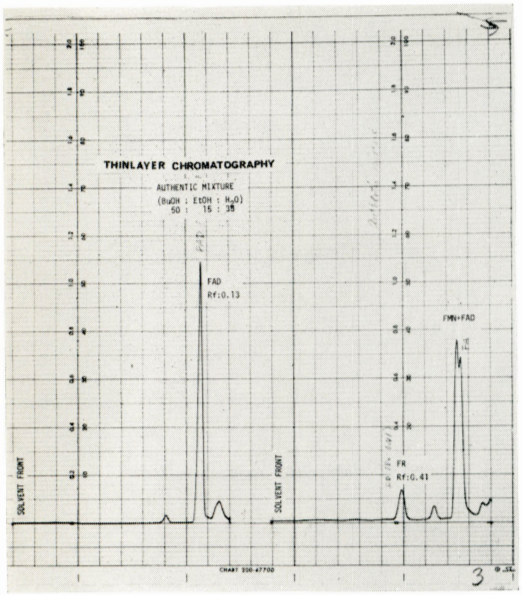
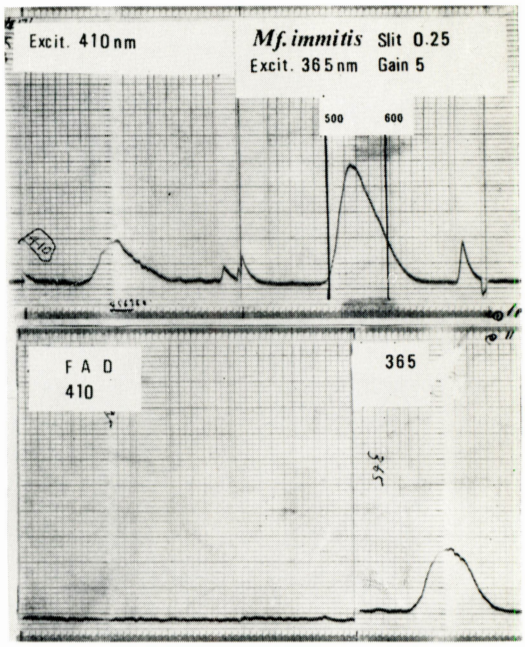
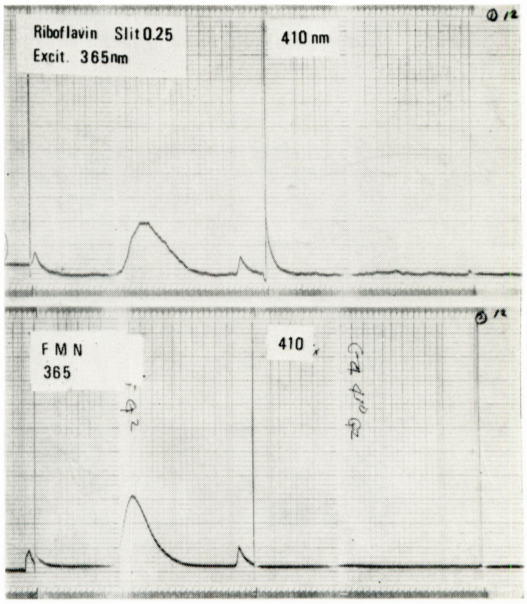


Fig. 5 Thinlayer chromatography of authentic FAD, riboflavin (FR) and mixture of FAD and FMN.

the mechanism of the filarial periodicty, especially of *Mf. bancrofti*. The first one is on the distribution of microfilariae in day time. The old studies on the distributions of *Mf. bancrofti* in the corpse, done by Rodenwaldt (1906) and others, might not be accepted to show real distribution of them in the living human body. As mentioned in the beginning of the present paper, the site of diurnal concentration of *Mf. bancrofti* has been confirmed to be the lungs, by means of needle biopsies and surgical explorations of various organs and tissues, in daytime and at night.

The second problem is how the microfilariae migrate into the peripheral blood at night, and what is the real cause of the negative phototaxis of the larvae with nocturnal periodicity. Since Strassburger (1878), the term "tropism" has been applied to movement of a portion of the living organism toward some focus of stimulation, and the term "taxis" to the movement of an entire organism. Hence, "negative heliotropism" of *Mf. bancrofti*, demonstrated by Suganuma (1921) may be better to be called "negative phototaxis".





Figs. 6 and 7 Microfluorophothmetric recordings of *Mf. immitis* and authentic flavins.

In the course of the clinical investigations on porphyrias (1969), has been surmised a possible presence of some photodynamic

substance(s) in the microfilariae with nocturnal periodicity and the larvae with different patterns of periodicity have been comparatively examined under fluorescence microscope.

A diffuse autofluorescence and numerous autofluorescent granules were detected in the highly nocturnal microfilariae of *W. bancrofti* (ordinary strains), *B. malayi* in the Siamese and *B. patei* in cats. In the low-grade nocturnal group of microfilariae of *D. immitis*, *B. malayi* in the Koreans and in cats in London and in Tokyo, were observed less numerous granules, although, somewhat numerous granules have been seen in *Mf. malayi* in cats. In prenocturnal larvae of *D. uniformis* in rabbits (the peak of microfilariae count being during 4–8 p.m. instead of midnight, Bray and Walton, 1961), granules were far less. In subperiodic group of microfilariae (the night/day ratio of microfilariae being 1.4 to 3.0) of the Polynesian strain of *W. bancrofti*, *B. pahangi* from cats in London and a dog in Nagasaki, most larvae showed only a diffuse fluorescence, in a few larvae being seen very few granules. In most species of nonperiodic group of microfilariae of *S. digitata*, *O. volvulus* (2 strains) and *I. kobayashii*, no granule could be detected, except a diffuse fluorescence. Heretofore, only two species or strain of the nonperiodic group—*B. malayi* in cats in Kuala Lumpur and *M. ozzardi*, showed a few granules (not so few in the latter) in some of the larvae. And in the nonperiodic microfilariae *L. carinii* and in the diurnal microfilariae of *L. loa*, was detected no fluorscence, neither diffuse nor granular. In the microfilariae of *D. reconditum* in dogs in Okinawa, there were seen two kinds of microfilariae with somewhat numerous granules and without any granule, in the blood smears taken in the afternoon (3–5 p.m.). Newton and Wright (1956) have described as to show two peaks of microfilariae at noon and at night, although in only a dog with single infection.

As shown in Table 3, there was noticed an approximate parallelism between the density of the autofluorescent granules in the microfiariae and the pattern of the periodicity—the size of the night/day ratioof microfilariae count—, so far as concerned with 21 species and strains, examined. The relative fluorescence intensity, measured by means of microfluorophotometry in 5 species and strains, showed a simillar tendency.

In cases of erythropoietic porphyrias, there are porphyrin rich erythrocytes, with red fluorescence, excited by UV- or BV-lights (Fluorocytes), in the peripheral blood. Such blood is subjected to photohemolysis *in vitro*. under exposure to the sunlight in 2 hr. Not only the fluorocytes are broken, but the nonfluorescent red cells are destructed, too. Since, hemolytic anemia is one of common symptoms in such patients, this photohemolysis is considered to occur in the subcutaneous capillaries, too. The red cell survival ^{51}Cr T 1/2 was shortened in the cases, examined. And in any type of erythropoietic porphyrias, the photosensitivity in the skin is one of main symptoms. These two phenomena are ascribed to the photodynamic activities of porphyrins in the red cells and in the skin. The fluorescent substances other than porphyrins are known to be more or less photodynamic. (Refer to color photo. 10–12 in plate II.)

The microfilariae with autofluorescent substance(s) might be more or less irritated in the subcutaneous capillaries under the sunlight. The degree of irritation must be dependent on the quantity of such substances and on the quantity of the exciting rays, penetrated into the subcutaneous capillaries. This irritation might be considered to be the cause of the negative phototaxis of the microfilariae, with nocturnal periodicity.

Thus, the microfilariae with numerous fluorescent granules, because of the potent irriation, would escape from the subcutaneous capillaries under the sunlight. There is no known apparatus, which can keep those larvae in the major circulation. It may be the reason, why *Mf. bancrofti* could not be detected in daytime, in the bone marrow, liver and in the other abdominal organs and tissues and in the kidney,

except in the lung, in spite of the frequent examinations by means of biopsies and surgical explorations (Masuya *et al.*, 1957–1961). The microfilariae, matured in the lung, must migrate into the peripheral blood in order to fulfil their instinct of race preservation. The larvae with much photodynamic substance(s) would prefer to migrate at night not in daytime. Thus, such microfilariae could be transmitted by the night suckers.

So far as concerned with 21 species and strains, examined, there seems to be applicable the "photodynamic substance theory" for the mechanism of the filarial periodicity, as a working hypothesis. In a case of bovine porphyria (erythropoietic), skin lesions were confined only within the area with white hair. The screening effect against the sunlight of the melanin pigment in the skin, would be well understood from the fact that the race with much melanin in the skin has been best adapted to live near the equator, without injuries. The scantiness of the photodynamic substance in *Mf. loa* and the abundance of melanin pigment in the skin of the host, might be the factors, which allow the diurnal periodicity and the transmission by the day sucker-*Chrysops*. Connal (1934) has described 45 cases of Loiasis among the whites in West Africa. However, no description has been done about the pattern of the periodicity. According to the personal communication from Prof. Nelson, there seemed no definite difference of the periodicity between the natives and the whites.

A discrepancy as seen in Table 3, between *B. malayi* in the Siamese and that in cats, might be understood from the fact that the man is not furred, the degree of irritation—the quantity of the exciting rays being more than in those furred.

Yoshida (1966) could show a complete inversion of the periodicity of *Mf. bancrofti* in the emigrants from Okinawa (E. 127°40′,

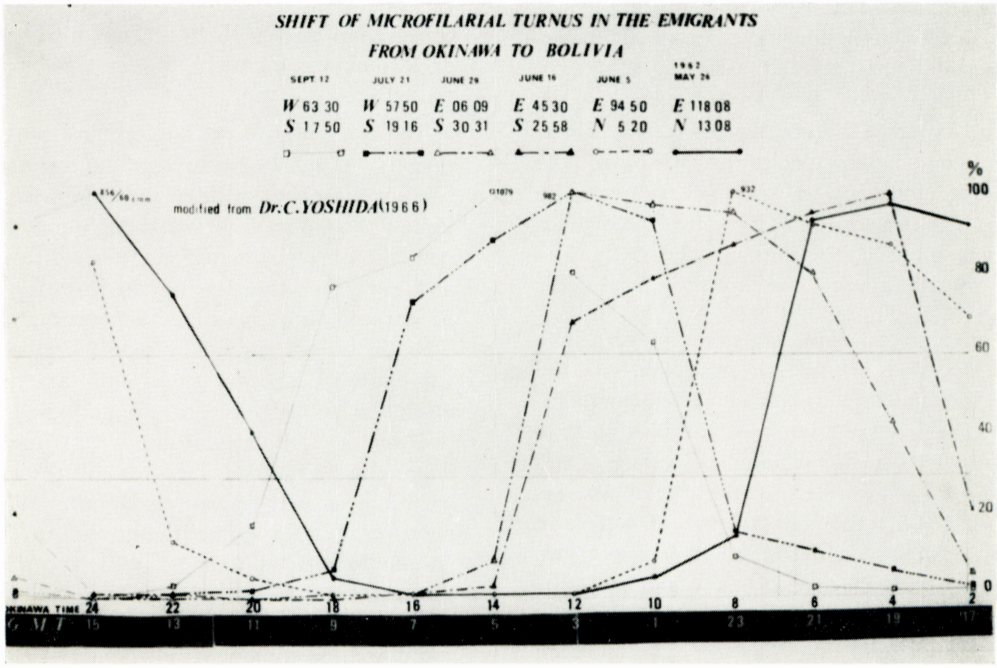


Fig. 8 Shift of the turnus of *Mf. bancrofti* in the emigrants from Okinawa to Bolivia, modified from the data of Dr. C. Yoshida (1966).

Table 5 Relationship between the rise-and-fall of microfilariae and the time of sunset-and-sunrise (derived from Fig. 8 and Nautical Almanac)

Date 1962	May 26	June 5	June 16	June 29	July 21	Sept. 12
Point of examination	E 118 08	E 94 50	E 45 30	E 06 09	W 57 50	W 63 30
	N 13 08	N 5 20	S 25 58	S 30 31	S 19 16	S 17 50
Sunset (LMT)	18 22	18 11	17 17	17 09	17 30	17 55
Duration of twilight	1 16	1 15	1 21	1 25	1 17	1 12
Complete darkness	19 39	19 26	18 38	18 34	17 47	19 07
Rise of miorofilariae count to 30 %	17 21	19 27	20 15	18 18	19 21	19 45
Lag from sunset	−1 01	1 16	2 58	1 09	1 51	1 50
Lag from darkness	2 18	01	1 37	− 16	34	38
Rise to 60 %	19 09	20 21	21 00	19 57	20 09	20 54
Lag from Sunset	44	2 10	3 43	2 48	2 39	2 59
Lag from Darkness	− 33	55	2 22	1 20	1 22	1 47
Sunrise (LMT)	5 32	5 45	6 42	6 57	6 24	5 58
Beginning of brightness	4 16	4 30	5 21	5 32	5 07	4 46
Fall of miorofilariae count to 60 %	4 48	5 51	6 15	3 54	3 24	5 36
Lag from sunrise	− 44	6	27	−3 03	−3 00	− 22
Lag from brightness	32	1 21	54	−1 38	−1 43	50
Fall to 30 %	5 36	6 30	7 09	4 33	4 18	6 33
Lag from sunrise	4	45	27	−2 24	−2 06	35
Lag from brightness	48	2 00	54	− 59	− 49	1 47

N. 28°30′) to Bolivia (W. 63°30′ S. 17°50′) in 116 days. Masuya has modified the chart of Yoshida and searched for the relationship between the rise and fall of microfilariae count and the times of the sunset and sunrise at the point of each examination. The times of sunset and sunrise, together with the duration of twilight, were cited from the Nautical Almanac. The rise to 60% of the peak of microfilariae count could be seen 44 to 223 min after the sunset. And the fall to 30% could be observed 4 to 45 min after the sunrise and in two points of examination before 144 to 126 min. In all examinations, microfilariae began to appear far before the sleep of the host (Fig. 8, Table 5).

Hawking (1967) has proposed his classification of microfilariae, based on his "oxygen barrier theory". However, in his second group, are included both diurnal *Loa loa* and the nocturnal species. In his first group, oxygen has caused a prompt fall of microfilariae count. Since Blum *et al.* (1953) and recently Rimington *et al.* (1967), the oxygen has been known to play a role in case of photosensitization reactions of the photodynamic substances. And the essential part of the process has been known to be a formation of the free radicals of peroxytype. The further observations tell us the oxygen and the photodynamic substances cooperate to cause a damage in the lipid rich membrane systems in the cell. Especially, the hydrolytic enzymes, released from the damaged lysosomes (with lipid rich membrane) are considered to cause cell injuries and photosensitization. Hawking's findings might be understood in part by "photodynamic substance theory".

Wan Chung Fan and his coworkers (1958) in Foochow (south China) have proposed an interesting theory. They offered that the larger the size of the microfilariae, the greater the periodicity. In their findings, on the size of *Mf. bancrofti*, the average size in daytime has been less than that at night. Masuya (1970) has already noticed, the shorter larvae in the lung semears of the infected dogs, had no or very few granules. And the youngest larvae, squeezed out of uterus of the adult heart worm showed no granule. Thus, the fluorescent granules seem to develop in the course of maturation of the microfilariae. It might be possible to imagine the smaller *Mf. bancrofti*, found in daytime, had less granules than those at night. Kume and Ohishi (1971) have noticed a nonperiodical appearance of *Mf. immitis*, during the first several months, in the newly infected dogs. These findings, too, would be well understood from the above mentioned facts.

As Turner and Edeson (1975) and some other authors have reported, different patterns of the periodicity are seen among the strains or varieties of the same species, such as some strains of *B. malayi* and the Polynesian strain of *W. bancrofti*. As mentioned above, the microfilariae of the Polynesian strain in the Tahitians showed by far the less granules than thsose in the ordinary, nocturnal strain. In the nonperiodic strain of *B. malayi* in cats in Kuala Lumpur, were seen a few granules in some larvae, far less than those in cats in London and Tokyo. Thus, on the larval side, the density of the fluorescent granules may vary from one strain to the other in the same species. However, it may be possible to consider the different environmental conditions, including the intensity of the sunlight and the factors on the host side, in relation to such phenomena. For example, alphatocopherol (vitamin E) is known to inhibit lipid peroxdation, which is considered to be the basic reaction of the photosensitization reactions.

Of course, further morphological and chemicl observations, including photochemical studies, must be done on the more species and strains. Especially, the observations in the living state of all species are most necessary. Here, the corroborative studies by the colleagues all over the world are cordially requested.

Addendum :

Jerome J. Wolken (1971) has reviewed the nature of photoreceptor molecules from the protozoa to the mammalia, although the informations have not been shown in the classes between the protozoa to mollusca. So far as concerned with his review, the common photoreceptor pigments are carotenoids, while flavoprotein has been regarded to act as accessory photoreceptor in the protozoa, for example, *Euglena gracilis*.

Looking back to the first appearance of life on the earth, all living organisms are dependent on the common biochemical processes, of unexpectedly few kinds. Geochemistry and biochemistry teach us, acetate and glycine, generated from mainly methane and NH_3 were the main materials of carbohydrates, fats, proteins, nucleotides and porphyrins. The photoreceptor in the microfilariae, if any, might not be widely different from those in the other species in the animal kingdom.

The existence of flavins in the nematoda has already been described by Yamao (1952) in tissues of the pig ascarid and by Obo and Tomohiro (1955) in the fluid of the body cavity of *Ascaris*, whose biological significance has not yet been shown. However, in case of microfilariae, microfluorophotometry along with the body length of *Mf. immitis* showed relative fluorescence intensity, which varied from one point to the other (from "a" to "e", in Fig. 1.—"o" being outside of the larvae). In cases of diurnal *Mf. loa* and nonperiodic *Mf. litomosoides carinii*, there could be detected no fluorescent granule under fluorescence microscope and no rise in microfluorophotometric curves. So far as concerned with flavin-containing fluorescent granules in the microfilariae, with more or less definite nocturnal periodicity, flavins

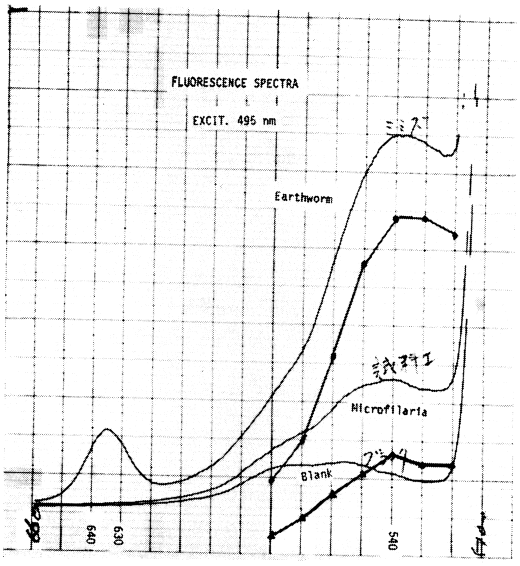


Fig. 9 Fluorescence spectra of the warm water extract of *Mf. immitis* and the earthworm epidermis. The second F max, 635 nm in the extract of the earthworm epidermis may be due to intermixture of porphyrins in the integument.

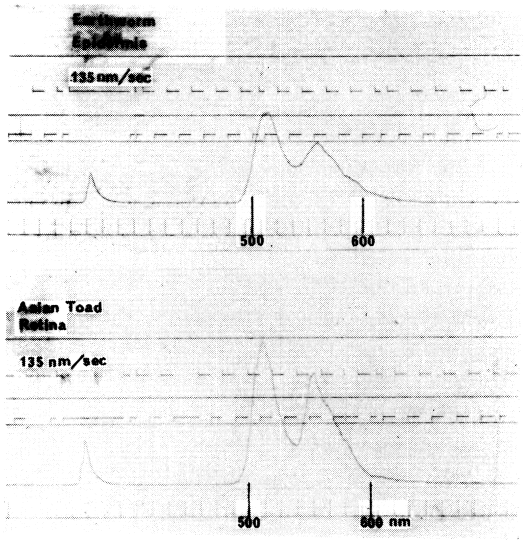


Fig. 10 Microfluorophotometric recordings of earthworm epidermis and of the retina of the Asian toad, in the yellowish fluorescent area apart from the carotenoid area.

seemed to play some role in their negative phototaxis. However, microfluorophotometry showed the existence of another (or other) substance(s) in addition to flavins.

Charles Darwin's experiment on the negative phototaxis of the earthworm has been cited in "The wonders of life on earth" of Time-Life Books, 1972. As early as 1896, Hesse has described "Lichtzellen" characterized with "Binnenkoerper" (phaosome), which did not associate to form visual organs but were disseminated in the epidermis and the small nerve branches at both ends of the worm and in the cerebral ganglion. Hesse's findings have been supported electronmicroscopically be Roehlich *et al.* (1970). Fluorescence microscopic observations have not yet been able to refer in the literature.

Nonstained, frozen sections of earthworm in Okinawa, whose species has not yet been identified, perhaps *Pheretima communissima*, have been examined under fluorescence microscope. In the epidermis or directly beneath the body surface, were observed numerous gold-yellow fluorescent granules, arranged in parallel with body surface, excited by BV-rays. The size of granules is larger than those in the microfilariae. Although further observations are necessary, it is very likely these granules act as photoreceptors. Of course, it has been well known the earthworm contained porphyrins. However, the red fluorescence of porphyrins is seen in the deeper parts of the body, than the layers of gold-yellow fluorescent granules. It may be difficult to consider the prophyrins play singly or more powerful role than the fluorescent granules in the epidermis, to cause negrative phototaxis of the earthworm.

The prominent peak of the two peaks of fluorescence in the warm water extract of the earthworm epidermis coincided with that in the extract of *Mf. immitis* (540 μm) (Fig. 9). The similar fluorescence has been detected in the retinae of the newt, *Triturus pyrrhogaster ensicauda* and the Asian toad, *Bufo bufo gargarizans*, in addition to that of the known carotenoids. Very interestingly, microfluorophotometry showed very similar two F max, 510 μm and 550 μm, both in the epidermis of earthworm and in

the region other than carotenoid in the retina of the toad (Fig. 10, Photo. 11).

Summary

A diffuse autofluorescence and numerous flurescent granules were detected in the highly nocturnal microfilariae, without any staining, of *Wuchereria bancrofti* (ordinary strain), *Brugia malayi* in the Siamese and *Brugia patei*. In low grade nocturnal larvae of *Dirofilaria immitis*, *B. malayi* in the Koreans, in cats in London and in Tokyo, were observed less granules. In prenocturnal microfilariae of *Dirofilaria uniformis*, granules were by far the less. In subperiodic microfilariae of the Polynesian strain of *W. bancrofti*, *Brugia pahangi* in cats and dog very few granules were detected in a few larvae. In three species of nonperiodic group of microfilariae of *Setaria digitata*, *Onchocerca volvulus* (2 strains) and *Icosiella kobayashii*, no granule could be dtected, except a diffuse fluorescence. Heretofore, only two species or strain of the nonperiodic group-*B. malayi* in cats in Kuala Lumpur and *Mansonella ozzardi*, showed a few granules in some of the larvae. And in the nonperiodic microfilariae of *Litomosoides carinii* and in the diurnal microfilariae of *Loa loa*, was detected no fluorescance, neither diffuse nor granular. In the microfilariae of *Dipetalonema reconditum*, whose periodicity being said to be double peaked, ther were seen two kinds of microfilariae with somewhat numerous granules and without any granule, in the blood taken in the afternoon.

There was noticed an approximate parallelism between the pattern of the periodicity (sizes of the night/day ratio of microfilariae count) and the density of the autofluorescent granules in the microfilariae, so far as concerned with 21 species and strains, examined. The relative fluorescence intensity of the larvae, determined by means of microfluorophotometry, showed a simillar tendency.

Refering to the photohemolysis and the photosensitivity in the skin in cases of porphyrias, a photodynamic substance theory has been proposed for the mechanism of the filarial periodicity, as a working hypothesis.

The fluorescent granules in *Mf. immitis* seemed to contain flavins, very similar to FAD and FMN. However, microfluorophotometry has suggested the existence of another (or other) substance(s) in addition to flavins. (The details on the nature of the fluorescent substance (s) in the microfilariae will be reported by Kodama, elsewhere).

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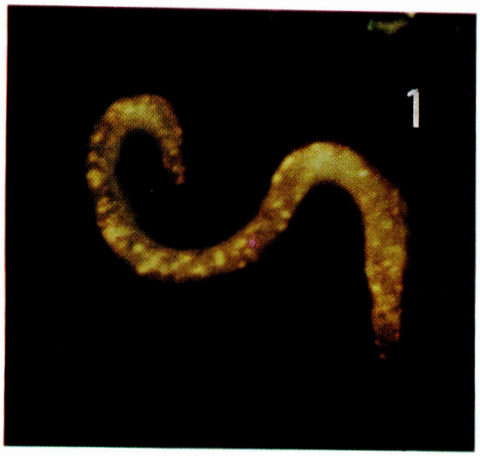
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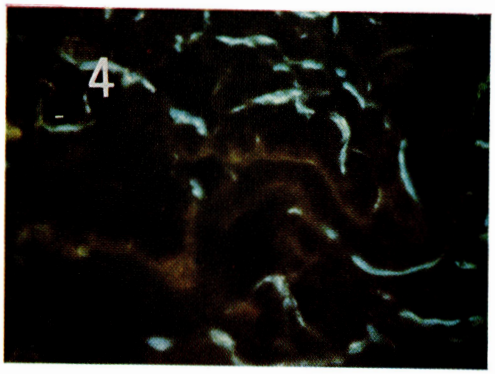
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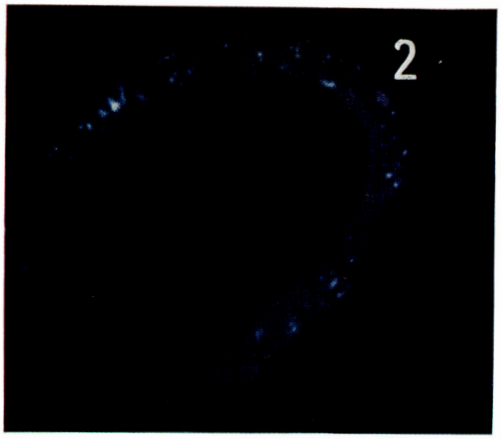
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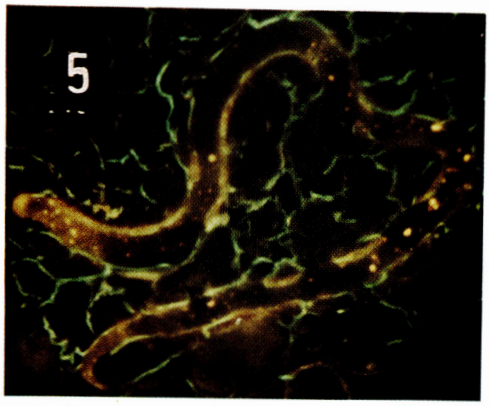
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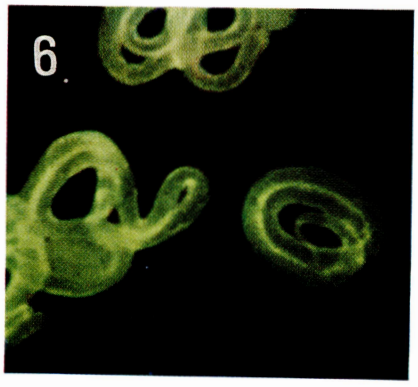
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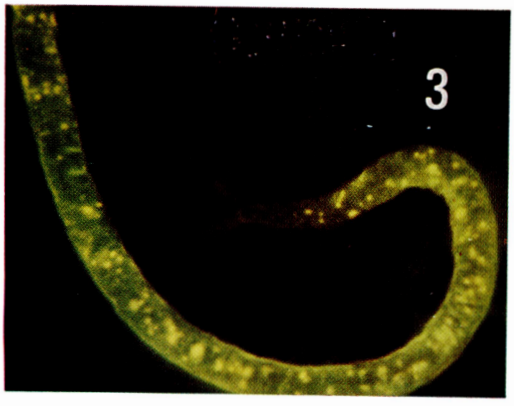
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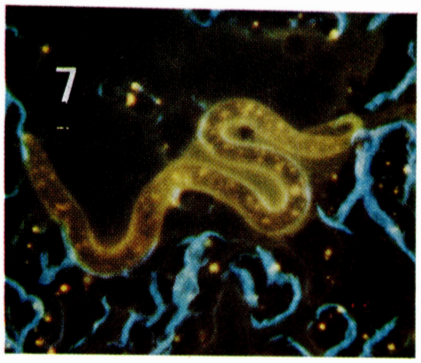
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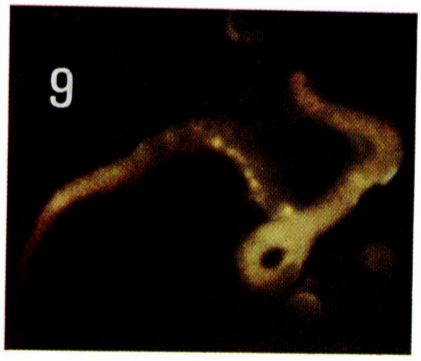
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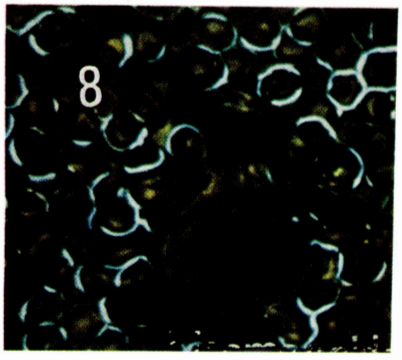
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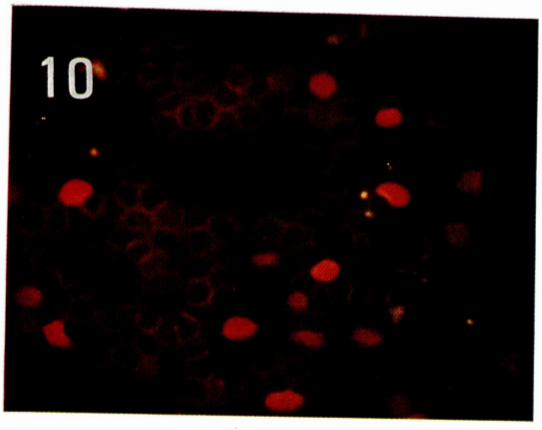
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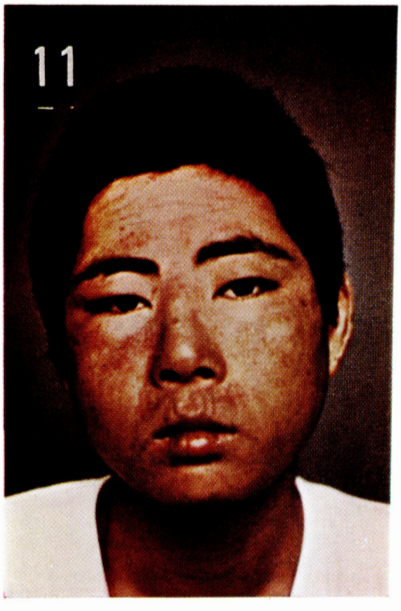
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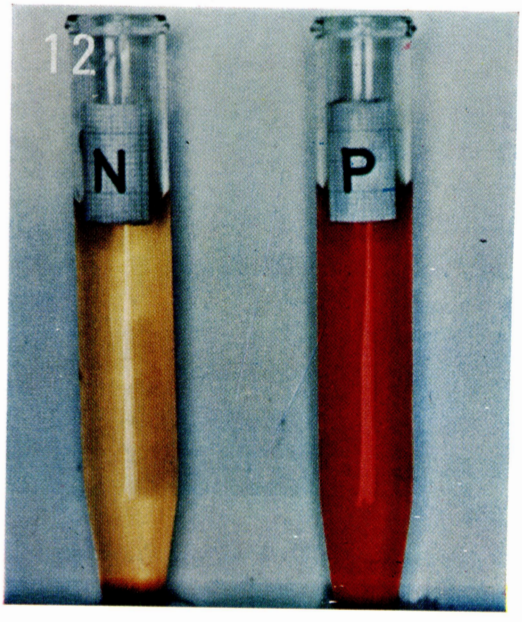
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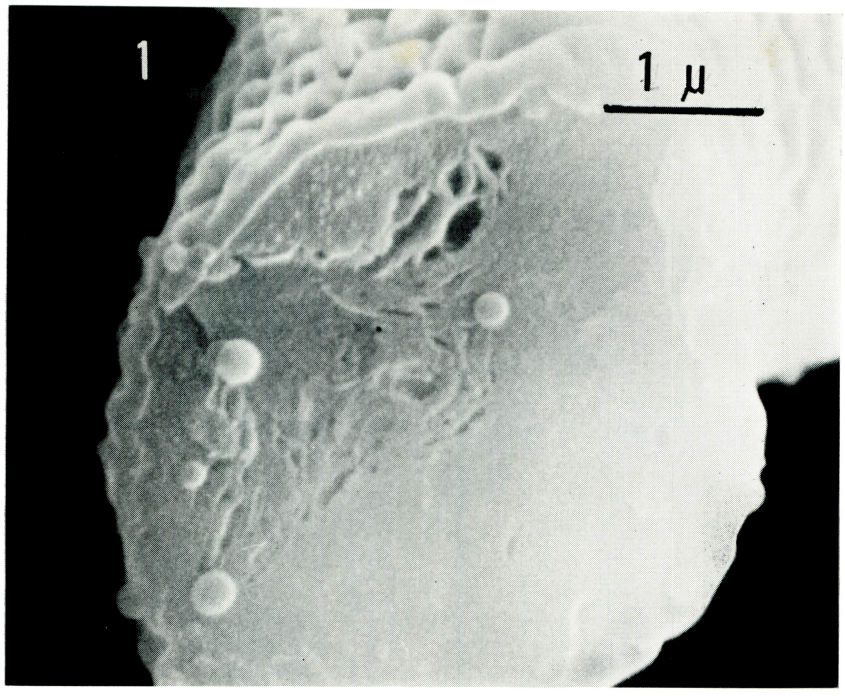
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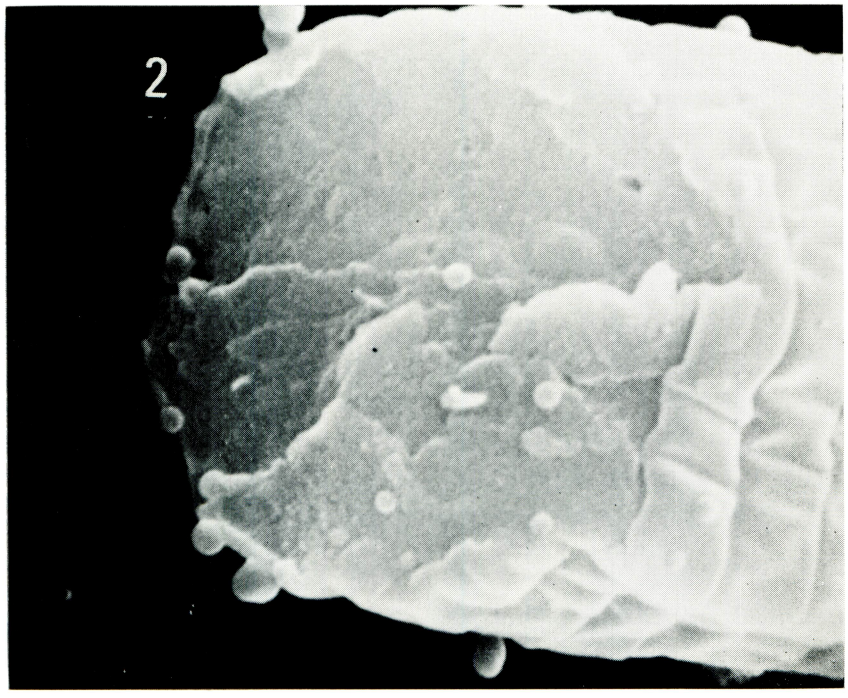
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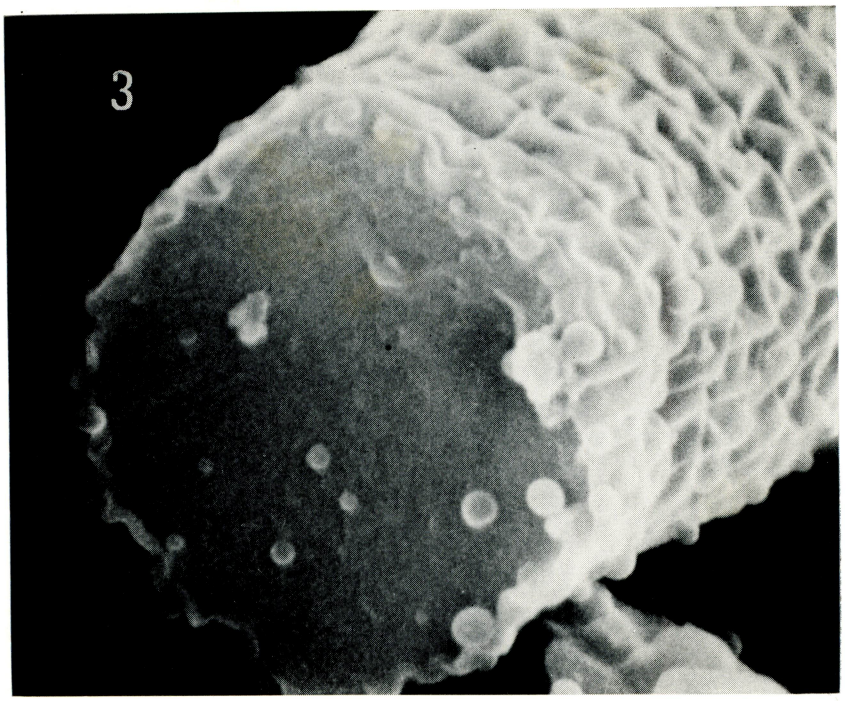
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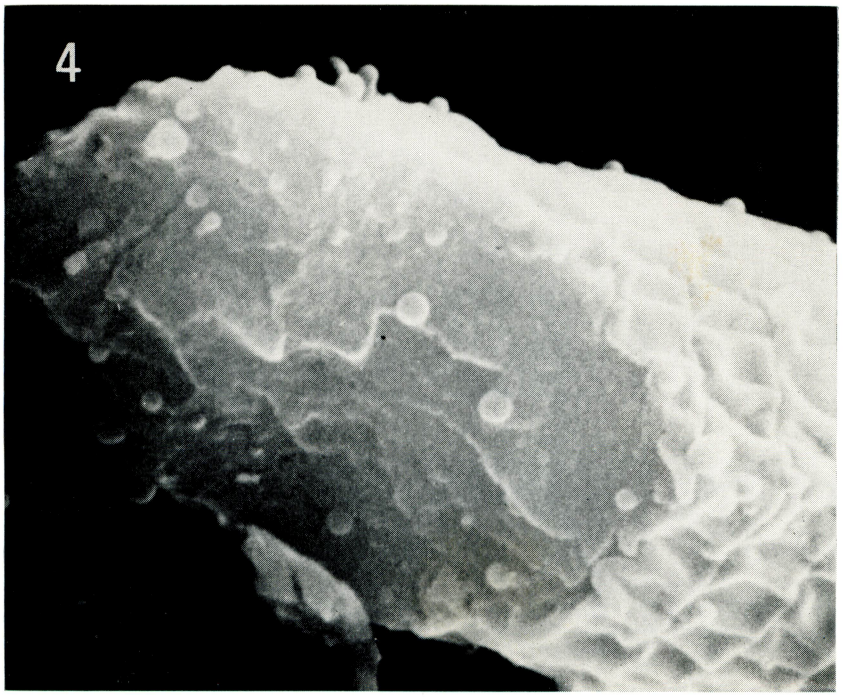
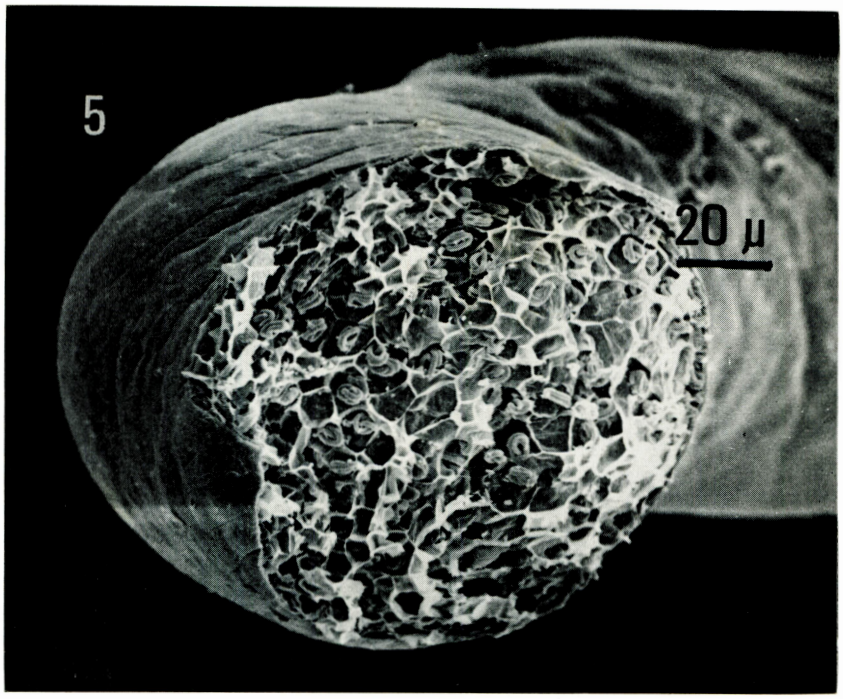
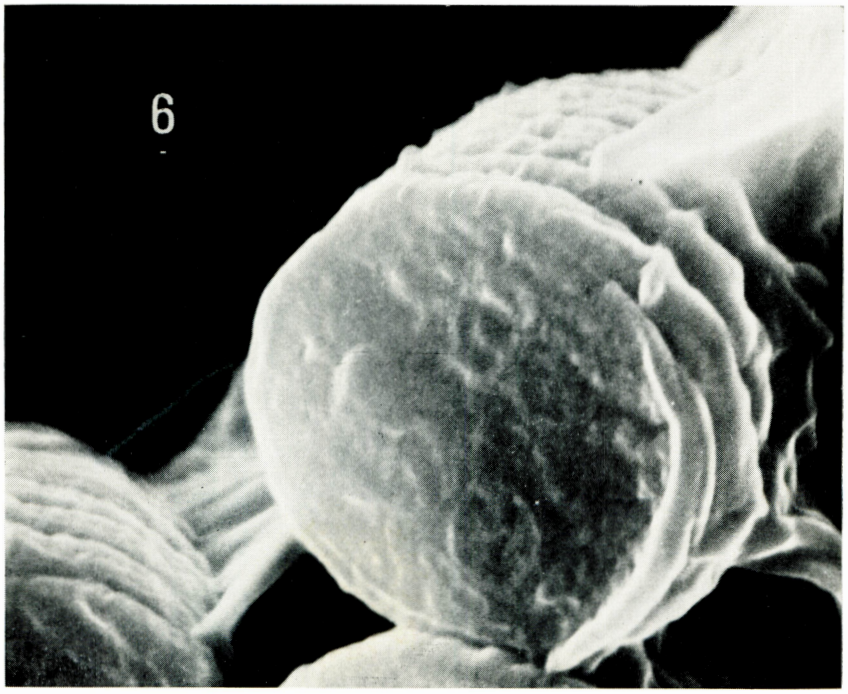
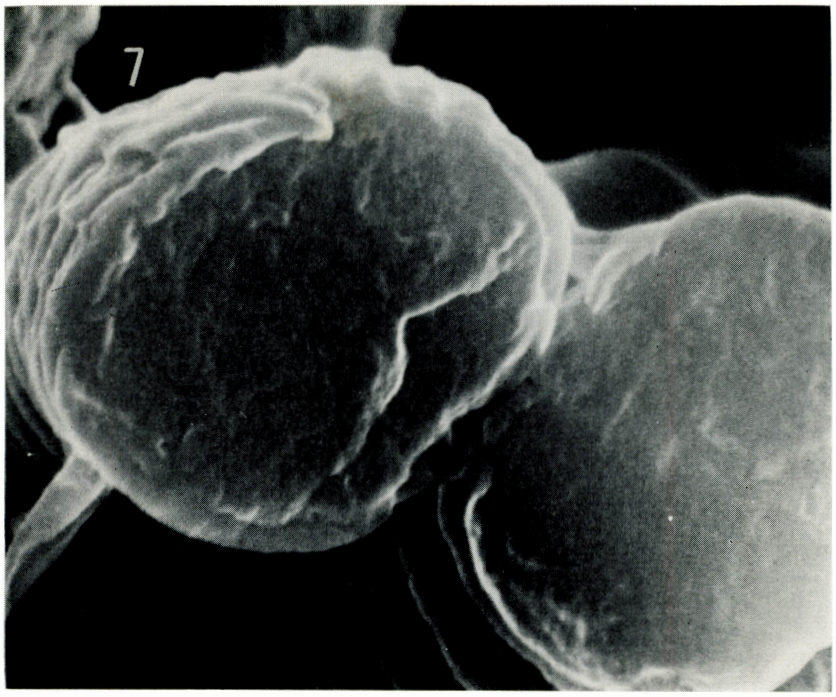
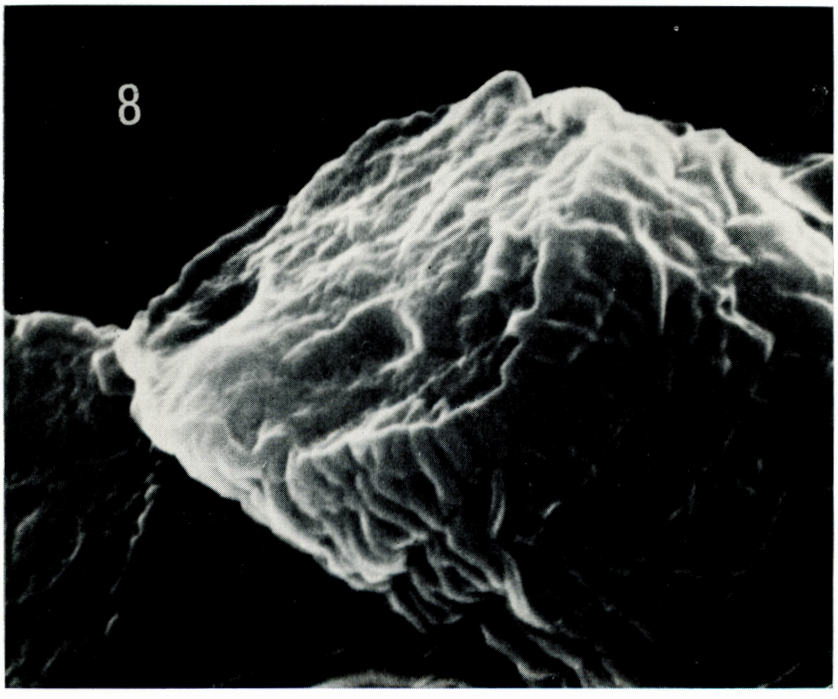
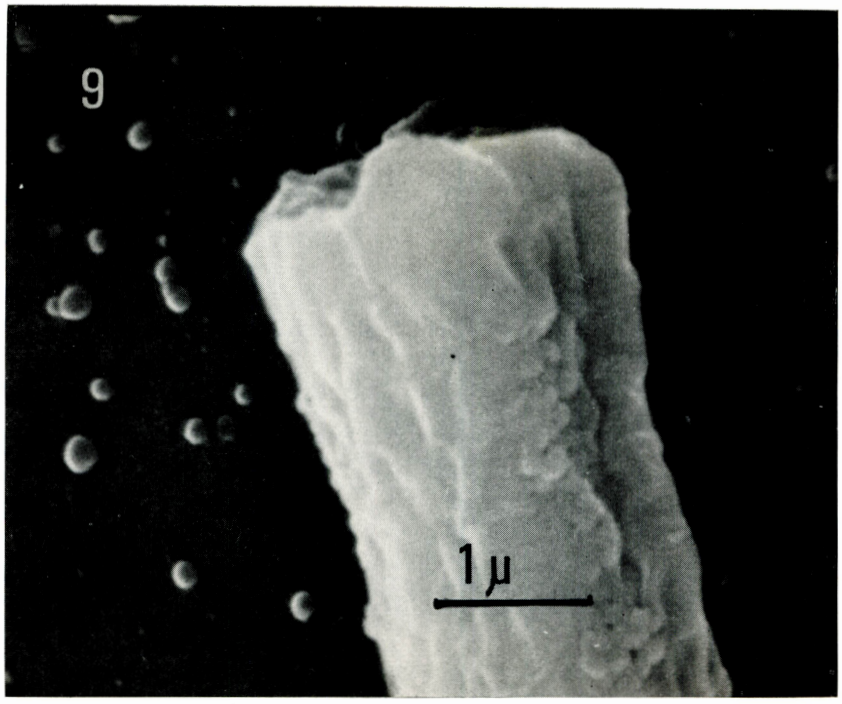

4


5
20 μ


6


7


8


9
1μ

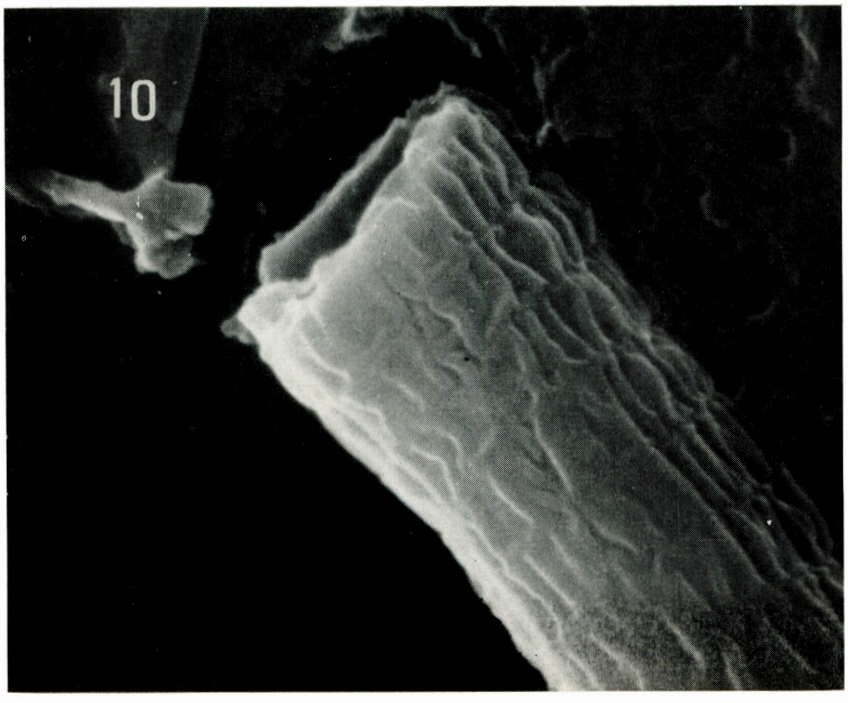
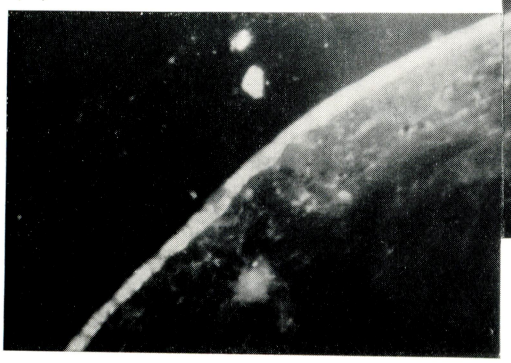
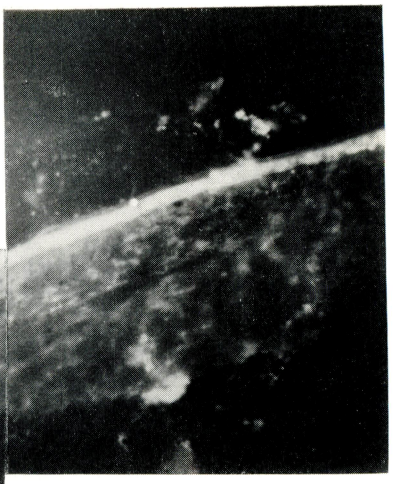

10

Photo. 11

(a)



(b)



Explanation of Photographs

Monochromatic Photo. 1 to 10. Scanning electronmicroscopic pictures.

Photo. 1–4 Fractured surfaces of *Mf. immitis*, in the peripheral blood. Magnification 20,000 ×

Photo. 5. Fractured surface of the uterus of the adult canine heart worm, showing many larvae in the honey comb like structure. 600 ×

Photo. 6–8 Fractured surfaces of the intrauterine larvae of *D. immitis*. 20,000 ×

Photos. 9 and 10 Fractued surfaces of *Mf. Dipetalonema reconditum*. 20,000 ×

In Photo. 9, globules of similar size with those in *Mf. immitis* are seen in the background.

In photo. 10, very minute granules are seen in the background. There may be possibility those granules have been spilt down at the time of fracture, which are so minute as not to be demonstrated under fluorescence microscope.

Photo. 11 (monochro) Earthworm integument, golden-yellow fluorescence in the epidermis, other than red one of porphyrin in the deeper layer.

Obj. ×10, 0cul. ×10

Enlargement of printing round ×11

(35×24⟶117×79 mm²)

80.

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Filarial periodicity の機序に関する研究
仔虫の自家螢光と定期出現性

桝屋富一
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高度夜間性の *W. bancrofti*, タイ国人 *B. malayi* およびネコ *B. patei* の無染色仔虫にビマン性自家螢光と無数の螢光顆粒をみとめた. 低度夜間性の *D. immitis*, 韓国人 *B. malayi*, London, 東京のネコ *B. malayi* 仔虫には稍々少ない螢光顆粒がみられた. 前夜間性の *D. uniformis* 仔虫の顆粒ははるかに少ない. 亜周期性の *W. bancrofti* のPolynesia 株, ネコ, イヌの *B. pahangi* 仔虫では一部のみに少数の顆粒を検出した. 非周期性の *S. digitata*, *O. volvulus*, *Icosiella kobayashii* 仔虫にはビマン性螢光のみで顆粒を検出し得ない. 非周期性仔虫のうち, Kuala Lumpur ネコの *B. malayi*, ブラジル人の *Mansonella ozzardi* の一部に少数の顆粒をみとめた. 非周期性の *L. carinii*, 昼間性のコンゴ人 *Loa loa* 仔虫にはなんらの螢光を検出し得なかつた. 二峰性といわれるイヌの *Dipet. reconditum* 仔虫には螢光顆粒を有つものを有たないものがあつた. 末梢血中仔虫数夜昼比と自家螢光顆粒の存否と密度の間には近似的平行関係が見られた. 顕微螢光分析による仔虫の相対螢光光度の値も同様の傾向を示した.

骨髄性ポルフィリン症における螢光赤血球, 皮膚ポルフィリン沈着, 日光溶血, 皮膚の日光過敏症と比較考察して filarial periodicity の機序に関する作業仮説として光力学物質説を提唱した.

末梢血中 *Immitis* 仔虫の凍結破断法による走査電顕で相当数の球状小体をみとめ, 螢光顕微鏡下全く顆粒を示さない心内成虫子宮内仔虫には走査電顕でもなんらの球状小体を検出できなかつた.

仔虫内螢光物質は F max, 薄層クロマトの Rf から FAD, FMN 類似の物質を含むと解されるが, 顕微螢光分析法は flavin 体以外の物質も含まれる事を示した.

〔附〕 ミミズの日光忌避は Darwin 以来知られるがその凍結無染色切片は表皮に無数の層状排列の黄色螢光顆粒を示した. ポルフィリンの赤色螢光はより深部に見られた. ミミズ表皮の温水抽出物の励起および螢光スペクトルは Mf. のそれと共通の E max, F max を示した. イモリ, ヒキガエルの網膜にカロテノイド螢光層の後方に Mf. 螢光類似の螢光を検出した. ミミズ表皮の螢光とヒキガエル網膜のカロテノイド螢光部以外の螢光部は共に 510 μm, 550 μm の2個の Fmax を示した.

Explanation of Plates

Plate I. Fluorescence microscopic findings (Photo. 1–6)

1. *Mf. bancrofti*, methanol fixed, nonstained blood film. Objective ×40, ocular ×10, barrier filter No. 4, Tiyoda.
2. *Mf. bancrofti*, filter No. 2.
3. *Mf. bancrofti*, objective ×100, oilimmersion, filter No. 3.
4. *Mf. loa* objective ×40, filter No. 4.
5. *Mf. immitis*, in the peripheral blood, objective ×40, filter No. 4.
6. *Mf. immitis*, in the fluid squeezed out of the uterus of adult canine heart worm. objective ×40, filter No. 3.

Plate II. Fluorescence microscopic findings (Photo. 7–12)

7. *Mf. malayi* in cat, Tokyo. objective ×40, filter No. 4.
   (Fluorescent graunles are more in the caudal side than in the cranial side).
8. *Mf. Litomosoides carinii*, objective ×40, filter No. 4.
9. *Mf. Dirofilaria uniformis*, objective ×40, filter No. 4.
10. The peripheral blood smear in a case of congenital porphyria, objective ×40, filter No. 4.
11. Phtosensitivity, in a case of erythropoietic protoporphyria.
12. Photohemolysis *in vitro* of the blood from a case of congenital porphyria (P) after exposure to the sunlight, for 2 hr, (N) being the control.