MALARIA OF THE ORANG-UTAN (*PONGO PYGMAEUS*) IN BORNEO

By W. PETERS
Department of Parasitology, Liverpool School of Tropical Medicine

P. C. C. GARNHAM, F.R.S., R. KILLICK-KENDRICK
Department of Zoology and Applied Entomology, Imperial College, London

N. RAJAPAKSA†
Department of Health, Sabah

W. H. CHEONG AND F. C. CADIGAN‡
Institute of Medical Research, Kuala Lumpur

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[Plates 1–6]

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† Present address: Commonwealth Department of Health, P.O. Box 100, Woden, Canberra ACT, Australia.
‡ Present address: Director of Medical Research, HQ, U.S. Army Medical Research and Development Command, Washington DC 20314, U.S.A.

The primary objective of this project was to study the life cycle and ecology of *Plasmodium pitheci*, a malaria parasite of the orang-utan. The field work was based on the orang-utan rehabilitation centre in the Sepilok Forest Reserve of eastern Sabah. Two visits were made to Sepilok, the first in February and March, 1972, and the second (by W. P.) in January 1974. On the first visit two species of 'surrogate host' were taken to Sabah, i.e. chimpanzees and *Aotus* monkeys for experimental work.

The arboreal habitat of the orang-utan in the dipterocarp forests of eastern Sabah is described. In the Sepilok Forest Reserve dwell gibbons and leaf-monkeys, in addition to a small population of semi-domesticated and wild, free-ranging orang-utans of various ages.

Although numerous species of anopheline mosquitoes have been collected in eastern Sabah, longitudinal studies are not available. *Anopheles balabacensis* was caught both attracted to orang-utans and to man at Sepilok. This species which is the main vector of human malaria in the north of Borneo, is suspected also of transmitting orang-utan malaria in this part of Sabah.

Repeated blood examinations have been made on a number of orang-utans in the centre since 1966 and a high prevalence of infection was recorded with *Plasmodium pitheci*. In 1966 10 out of 19 animals had demonstrable parasitaemia. Detailed case histories are presented to show the course of parasitaemia in several orang-utans. Infections of *P. pitheci* were found to run a very chronic course. During the 1972 expedition a second, previously undescribed malaria parasite of the orang-utan was discovered, and was named *P. silvaticum*. The new parasite was successfully transmitted both by blood inoculation and, later, by sporozoite inoculation, into splenectomized chimpanzees.

Although both species of malaria parasite may cause transitory signs of illness, orang-utans in general appear to be little discomforted by the infection. The animals do however suffer from other infectious diseases such as amoebic and balantidial dysentery, and melioidosis is a serious natural hazard which may have accounted for

several deaths of wild orang-utans. An unidentified, intraerythrocytic structure that appeared in the blood of one chimpanzee, which had been inoculated with blood from an orang-utan, may have contributed to its death.

Detailed descriptions and illustrations of *P. pitheci* and *P. silvaticum* are given. All stages of the life cycle of *P. silvaticum* are known (the tissue stages having been described in the liver of a 'surrogate host', the chimpanzee) but only the blood and sporogonic stages of *P. pitheci* have been seen. This species was not infective to a chimpanzee, although there is an earlier report of a transient infection in this host by other workers. In the blood both parasites showed a tertian periodicity. From the appearance of the tissue schizonts on the seventh day it was estimated that the complete pre-erythrocytic cycle of *P. silvaticum* in the chimpanzee would occupy 8 days.

P. pitheci is readily distinguished from *P. silvaticum*, and most closely resembles *P. hylobati* and *P. youngi* of the gibbon. The sporogony of both orang-utan parasites in anopheline mosquitoes is described. Although *P. pitheci* produced a transient parasitaemia in the splenectomized gibbon following blood inoculation, *P. silvaticum* did not do so, and neither parasite was infective to *Aotus* or *Macaca*. *P. pitheci* was also non-infective to man.

P. silvaticum clearly belongs to the *vivax-cynomolgi* group and resembles *P. eylesi* of the gibbon. Several morphological and other features distinguish it from *P. vivax* with which a close comparison was made. Based on the new knowledge acquired of the two malaria parasites of the orang-utan and existing knowledge of other primate malarias, a new plan of the evolution of the primate malarias in relation to their hosts is proposed. This agrees well with other evidence linking the orang-utan with the gibbons, rather than with the other anthropoid apes, *Pan*, *Gorilla* and *Homo*.

In considering the epizootiology of orang-utan malaria attention is drawn to the high prevalence of infection in relation to the relatively solitary habits and low population density of these apes. The hypotheses that *P. silvaticum* may produce zoonotic infections in man or, alternatively, that orang-utans may contract *P. vivax* malaria are considered, and rejected.

The logistics of using exotic animals in this investigation are reviewed, and an outline of the complex technical procedures followed are provided in the appendix.

1. Introduction

Of all the hominoid apes, the orang-utan (*Pongo pygmaeus*) is in the greatest danger of extinction. It originally occupied a wide area of eastern Asia extending from 'Peking to the Celebes' (Harrisson 1962), but in historical times its distribution has been restricted to Borneo and Sumatra. In this century the ape has been found only in limited parts of these islands, north of the Mahakam River in Borneo and in the northern half of Sumatra. Even in these areas the population is shrinking rapidly, and more than half the total is believed to be in the northeast corner of Borneo in Sabah (Eastern Malaysia). Little is known about the situation in Kalimantan (the southern and largest portion of Borneo). Two subspecies of orang-utan are recognized by Napier & Napier (1967), *Pongo pygmaeus pygmaeus* (Linnaeus, 1760), and *Pongo pygmaeus abelii* Lesson, 1827. The nominate subspecies occurs on the island of Borneo to which our own observations are restricted. *P. p. abelii* is the Sumatran subspecies.

A rehabilitation project of de Silva (1971) in Sabah is showing signs of considerable success and, while this energetic game warden remains in charge, the clock may be turned back and the numbers of orang-utan may even be augmented. His scheme is based on strict legislation which (*a*) totally restricts the killing or capture of the animal (including its retention as a pet) and (*b*) establishes a rehabilitation area of *ca.* 400 km^2 (10000 acres) in the Sepilok Forest Reserve (figure 1). Illegally held animals are confiscated, treated for any injuries, malnutrition or obvious illness by veterinarians and are then set free in the forest beside a permanent encampment

(plate 1, top) of the game rangers. The animals gradually become adjusted to life in the wild and, after a longer or shorter period, gradually go further afield where the females mate with wild males (a few natural inhabitants having remained in this forest reserve and in nearby forests such as Gomantong). Between 1964 and 1969, 41 animals were absorbed into the project; during the succeeding 5 years, a further 10 were introduced and two births have been recorded.

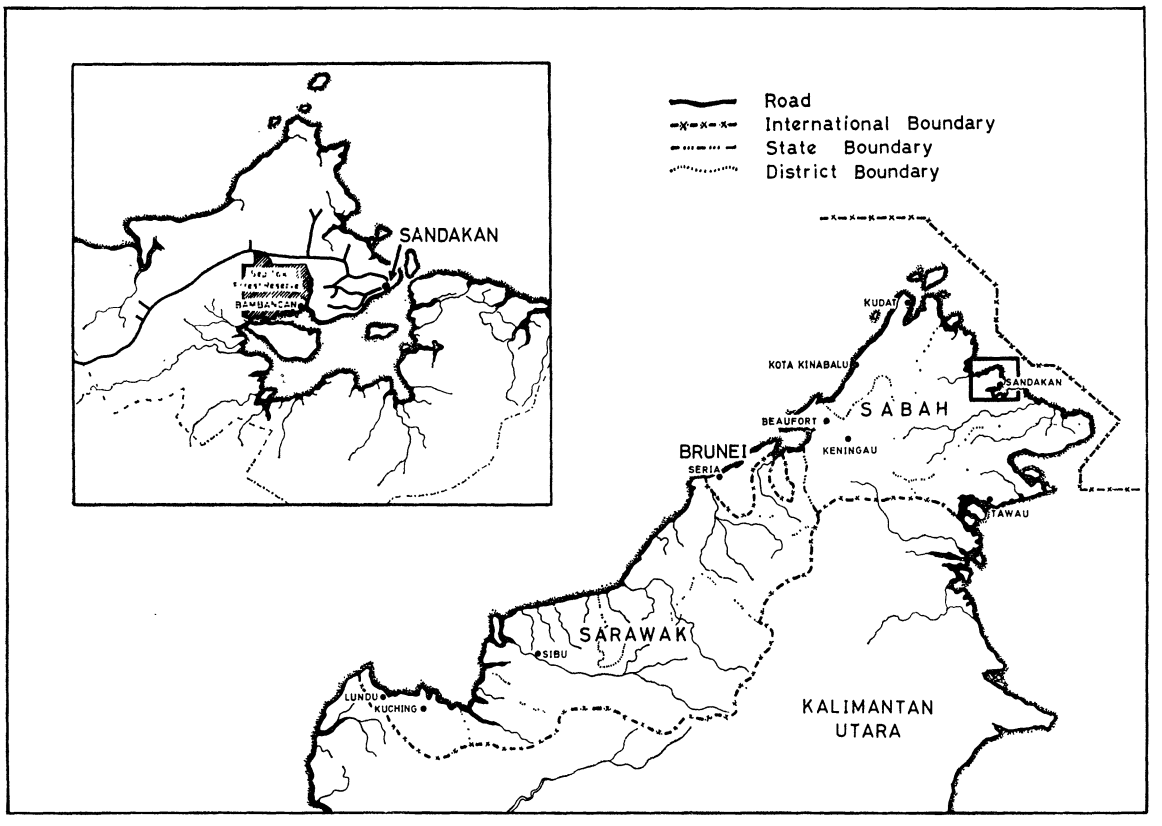
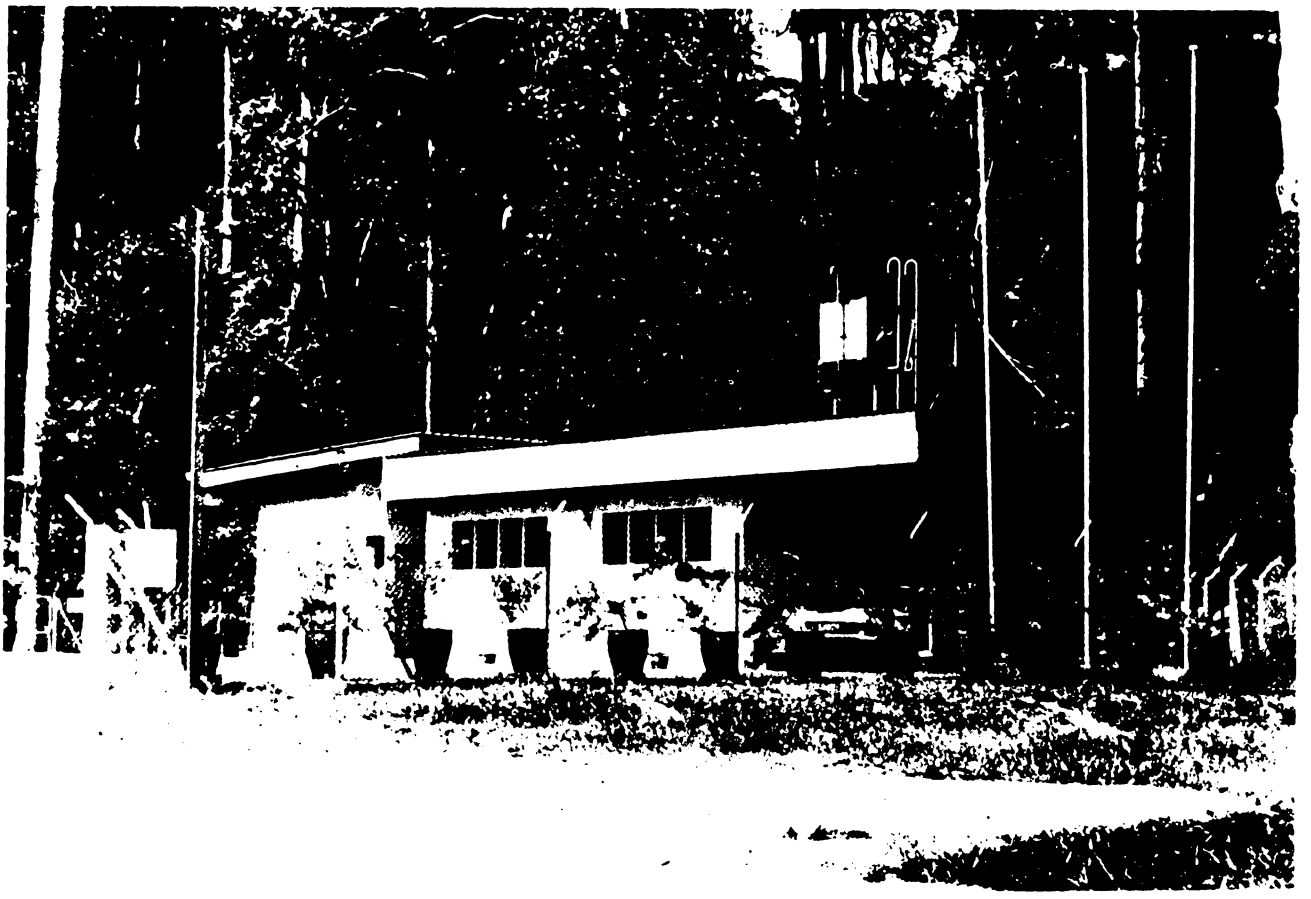


FIGURE 1. Map of East Malaysia, Brunei and Kalimantan Utara to show the locality of the Sepilok Forest Reserve.

Stanley de Silva's eventual policy may be to seed depopulated forests elsewhere with surplus orang-utans from Sepilok, selecting particular forests which are already 'Reserves' or are not likely to be destroyed in the near future by the timber trade.

On the southern coast of the island of Borneo the Indonesian Forestry Service has established another orang-utan rehabilitation centre in the 2000 km² Tanjung Puting Reserve of Kalimantan. Both wild and once captive animals are the subject of a long-term observation project by Galdikas-Brindamour & Brindamour (1975) together with a group of Indonesian students.

The situation in Sumatra appears to be worse than in Borneo and a survey of the Sumatran primates by Wilson & Wilson (1974) indicated that a very low population existed in primary lowland forest in Aceh Province in the northern part of the island. A rehabilitation centre has recently been set up there (in the Loeser Forest Reserve, 60 km from Medan) on the lines of de Silva's in Sabah, and further stations have been recommended for conservation of the subspecies



Top. Accommodation for sick animals and veterinary laboratory at the entrance to the Sepilok Forest Reserve; the main footpath into the reserve lies immediately behind these buildings. (Photograph by courtesy of Mr Stanley de Silva.) *Bottom left.* Typical tropical rain forest at Sepilok containing numerous species of dipterocarps, the crowns of which form the forest canopy at a height of 30–40 m (photograph by courtesy of the Sabah Forestry Department). *Bottom right.* The female orang-utan Joan aged 15 years (photograph by courtesy of Mr Stanley de Silva).

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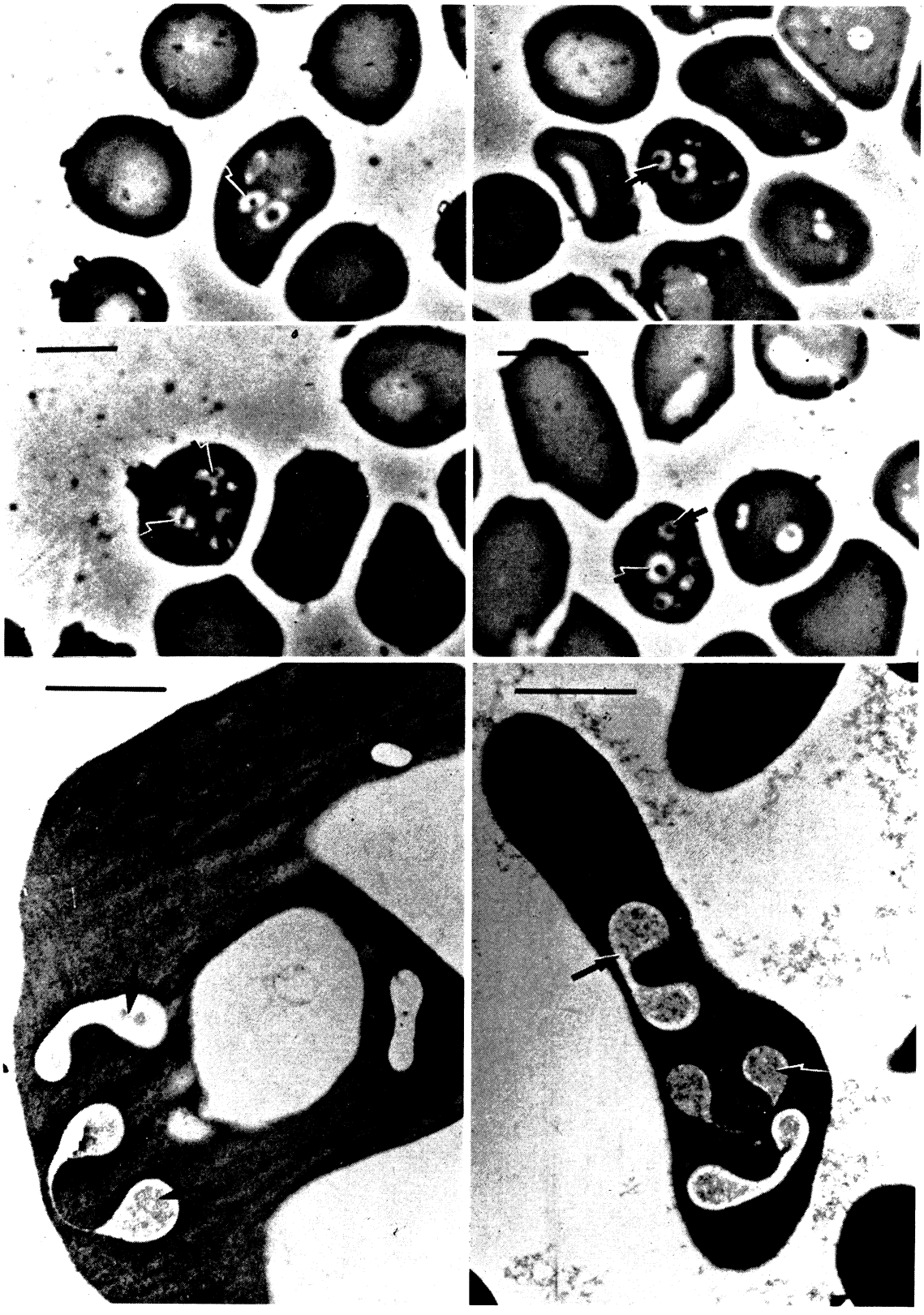

Top and centre. Unidentified structures (arrowed) in erythrocytes of the splenectomized chimpanzee Khan. The blood films which were stained with Giemsa's stain were photographed with phase-contrast optics (bar = 5 μm).

Bottom. Electronmicrographs of erythrocytes of the splenectomized chimpanzee Khan showing unidentified structures corresponding to those seen in top and centre figures (bar = 1.0 μm). (Blood prefixed in glutaraldehyde and post-fixed in osmic acid – electronmicrographs by courtesy of Professor V. Zaman.)

Pongo pygmaeus abelii and for studies on its ecology. MacKinnon (1973) estimated an approximate density of 1 orang-utan per km^2 in various forests in North Sumatra where they ranged about half a kilometre a day. There was more clumping of groups in this island than he (1971) noted in Borneo.

Estimates of present numbers of orang-utans in the world vary from 3800 to 4500; there are, in addition, more than 500 in captivity, and the animal is now being bred with some success in zoos and in primate centres. According to the studbook compiled by the Yerkes Regional Primate Research Center (1969, 1971) 139 animals were born in captivity in the years 1967–71 inclusive. The secret of success is apparently to transfer the baby at birth immediately into an intensive care unit.

In Sabah as elsewhere, exploitation of forests is leading to the rapid destruction of the silvatic habitat of the orang-utan, and unless the ape can adapt itself to greatly altered forests, or large tracts of suitable forest are set aside as properly administered reserves, it seems unlikely that it will survive in its natural state beyond the end of the present century.

Hypotheses on the evolution of the malaria parasites of man are based on the zoogeography, morphology, life cycles and infectivity to vertebrate and invertebrate hosts of the whole range of species of *Plasmodium* of primates. One species was described in the orang-utan, namely *Plasmodium pitheci* Halberstaedter & Prowazek, 1907, in the blood of an unspecified number of animals from Borneo which were examined in Berlin. Although this was the first malaria parasite of anthropoids other than man to be described and named, the only information on it available up to 1972 were somewhat conflicting accounts of the morphology of its blood forms based on a few scattered records. It was, therefore, difficult to compare *P. pitheci* with the malaria parasites of other pongids or of man.

The extinction of the orang-utan would be a grave loss to workers engaged on comparative studies of the few surviving hominoids. Similarly, there would no longer be opportunities to study its parasites. With the mounting concern for the fate of this ape, it became clear that an attempt had to be made to discover as much as possible about its little known malaria parasite before it was lost with its only known vertebrate host.

An opportunity to study malaria of the orang-utan arose in 1966 when McWilson Warren (Coatney, Collins, Warren & Contacos 1971) discovered malaria parasites in the blood of orang-utans which were being rehabilitated in the Sepilok Forest Reserve, Sandakan, Sabah. Only single observations were made. An expedition to Borneo was therefore arranged by us with the express purpose of attempting to elucidate the life cycle of *P. pitheci* in full and establish its relationship to the malaria parasites of other apes and man. During field work in Sabah, undertaken during February and March 1972, a second, previously undescribed parasite of the orang-utan was discovered and subsequently named *P. silvaticum* Garnham, Rajapaksa, Peters & Killick-Kendrick, 1972.

In the present paper *P. pitheci* and *P. silvaticum* are fully described. An account is also given of the general environment in which the animals live, for without this knowledge it seemed unlikely that we should fully understand the enzootiology of the infection. The biocoenosis comprises the vertebrate animal itself, its ecto- and endoparasites, its neighbours in the forest, the trees and their fruits, the seasons and the rainfall, humidity, temperature and winds, the geology of the forest floor and cliffs and the course of the rivers. Also included in the complex are the invertebrate hosts (anopheline mosquitoes) of the parasites and their specialized breeding places.

In the event these two objectives proved to be not wholly compatible. The investigation on the malaria parasites entailed a number of sophisticated research procedures, and although we tried to establish an insectary in the forest itself (see appendix, p. 481) we were obliged to abandon it for more controlled conditions in Sandakan. Eventually we moved to the Medical Research Institute, Kuala Lumpur, where the techniques of artificial mating of mosquitoes were in full operation. Moreover, laparotomy and removal of pieces of liver of our experimental animals (splenectomized chimpanzees) could not be undertaken without the proper conditions of an operating theatre.

Nevertheless, we managed to obtain at least a superficial idea of the biocoenosis by working in the Sepilok Forest Reserve for about a month in 1972, and briefly visiting neighbouring forests where orang-utans live. One of us (W.P.) revisited Sepilok in January 1974 and stayed in the new forest rest house for a further period of three weeks.

2. The habitat of the orang-utan with special reference to conditions in the Sepilok Forest Reserve

Fortunately, the zoological literature contains much information about the orang-utan and its habitat, particularly the works of Wallace (1898) and Hornaday (1880) in the last century, and of Schaller (1961), Stott & Selsor (1961), Harrisson (1961), Yoshiba (1964) and MacKinnon (1974) in recent years.

The orang-utan is an arboreal primate living in tropical rain forest at an altitude of below a thousand metres, though it may extend its range higher in mountainous regions, e.g. on the slopes of Mount Kinabalu. Similarly it may descend into the swampy land bordering the mangroves of the coast and rivers. It prefers primary forest, though by force of circumstances it may become adapted to second growth vegetation (Stott & Selsor 1961). The essential needs of the animal are an adequate food supply and freedom from excessive disturbance by man as in logging operations.

The apes live in the middle stratum of the forest rather than in the canopy itself (Davis 1962), but occasionally descend to the forest floor. Their existence is almost semi-aquatic, with water dripping from the leaves, in nearly 100 % humidity in hot, steamy conditions and in perpetual twilight.

The Sepilok Forest Reserve comprises sandstone ridges rising in steep scarps up to 150 m above mudstone beds and descending towards the south to the alluvial formations around the river mouths draining into the sea. Much of the forest grows on the slopes of undulating small hills (Fox 1969).

The timber trees of the Sepilok Forest largely comprise dipterocarps (see Fox 1970), including numerous species of *Shorea* ('Serraya'), *Dipterocarpus* ('Keruing'), *Vatika* ('Resak'), and *Hopea* ('Selangan'). The canopy reaches a height of 30–40 m (plate 1, bottom left). There are also figs, laurels, myrtles, and leguminous species mixed in profusion, and *Pandanus* in the ground vegetation. The favourite places for the orang-utans to rest in are the dipterocarps. For food, however, they seek out the fruit trees, particularly species of *Durio* ('durian'), *Nephelium* ('rambutan' and 'maeritan'), *Langsium* ('langsat'), *Euphoria* ('mata kuching') and *Artocarpus* ('tarap' and breadfruit). They also raid plantations of mangos and mangosteens when their fruit are in season. MacKinnon (1971) gives a much more complete list of plants eaten by the orang-utan in Sabah, and Galdikas-Brindamour & Brindamour (1975) have 'catalogued 200 types of orang-utan food'.

Many streams and ravines intersect the forest, and morasses increase the water area, providing breeding places for anopheline mosquitoes and a nidus for *Pseudomonas pseudomallei* (the causative organism of melioidosis, an infection which causes some mortality in orang-utans as well as in man). At the beginning of the rainy season, the stream flow reached a discharge of 0.94 m^3/s (3.3 cusec) (in the Sepilok Besar, September 1968). On the southern boundary, the forest abruptly terminates in the mangrove swamps of the Bay of Sandakan.

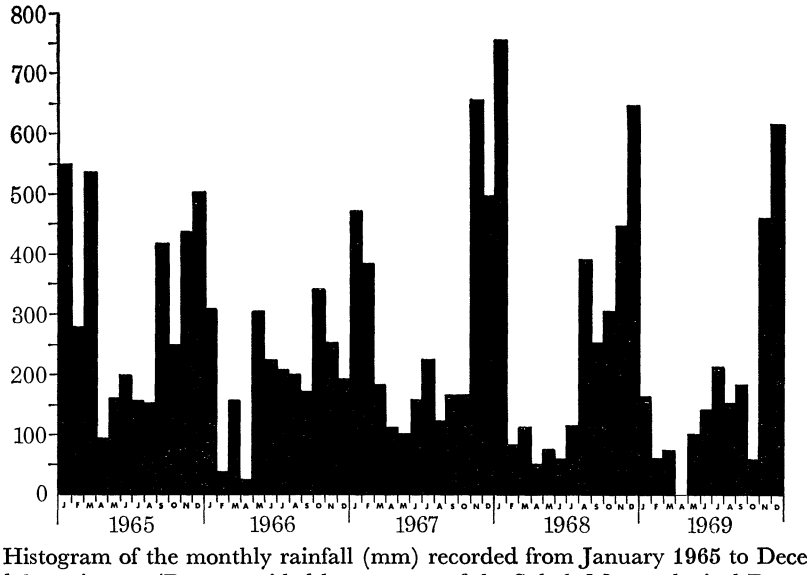

FIGURE 2. Histogram of the monthly rainfall (mm) recorded from January 1965 to December 1969 at Sandakan airport. (Data provided by courtesy of the Sabah Meteorological Department.)

The Sepilok Forest supports a considerable population of mammals other than the orang-utan. No human beings live in the reserve except for the forest guards at Sepilok Kesho, while there are a few habitations immediately beyond the periphery. Other primates however, are numerous; they include gibbons (*Hylobates moloch* (Audebert, 1797–8)), macaques (*Macaca nemestrina* (Linnaeus, 1766)) and leaf-monkeys (*Presbytis cristatus* Raffles, 1821, *P. aygula* (Linnaeus, 1758) and *P. rubicundus* Müller, 1838). All these monkeys harbour malaria parasites here, and elsewhere in Malaysia. Mouse deer (*Tragulus javanicus klossi* Chasen, 1934) are common at Sepilok and in adjacent forests, and are known hosts of malaria parasites, as are the bats *Pteropus vampyrus* (Linnaeus, 1758), *Miniopterus australis* Tomes, 1858 and *Rhinolophus* spp. Furthermore, a variety of other arboreal mammals has been found infected here or in other parts of Malaysia. They include the colugo (*Cynocephalus variegatus natunae* Miller, 1903), flying squirrels (*Petaurista petaurista rajah* Thomas, 1908) and tree squirrels (*Callosciurus notatus dilutus* Miller, 1913), and *C. nigrovittatus* (Horsfield, 1823). A Malayan honey bear, *Helarctos malayanus euryspilus* Horsfield, 1825, also occurs in the Sepilok Forest. An unidentified body resembling a piroplasm was found in the red blood cells of a young female bear that was being cared for at the Rehabilitation Centre. The animal showed no signs of illness. Medway (1963, 1969) gives the distribution of these animals in his useful field key and check list.

Climate. Sepilok lies 6° north of the Equator and has a typical tropical rain forest climate. Meteorological records are available for the Sandakan airport (rainfall) and for the arboretum at Sepilok (maximum and minimum temperatures and wet and dry bulb daily readings). The rainfall figures probably approximate to those prevailing in the forest, but the temperature and

humidity records in the arboretum are unlikely to be strictly comparable; the maximum and minimum extremes would be less in the forest and the humidity would be higher. Figure 2 shows that in the 5 years 1965–69 no month passed without some rain. The annual rainfall for the 5 years was as follows:

1965	1966	1967	1968	1069
3700 mm	2400 mm	3200 mm	3250 mm	2130 mm

The wettest months are December, January and February, though rainfall can be quite heavy at other times of the year.

The lowest temperatures occur during the seasons of heavy rain, when the minimum may fall as low as 18 °C, the maximum on the same day being 6–7 °C higher. In the drier months, the maximum temperature is seldom lower than 32 °C, and may reach 37 °C. The relative humidity is often close to 100 % and is rarely under 70 %. Microclimatic data in the old nests of orang-utans could prove useful and, even more important, would be records taken in the resting places of fed *Anopheles balabacensis* Baisas, 1936, the probable vector of the malaria parasites.

3. *Anopheles* of East Malaysia

Forty-three species and subspecies of the mosquito genus *Anopheles* are known to occur in Sabah (table 1). Ramalingam (1974) reported that he and his associates had collected 32 of these. In Sandakan Presidency they found the following during a general mosquito survey carried out over a three-month period in 1970 (S. Ramalingam, private communication):

A. (*A.*) *aitkenii*
A. (*A.*) *donaldi*
A. (*A.*) *hodgkini*
A. (*A.*) *montanus*
A. (*A.*) *vanus*
A. (*C.*) *balabacensis*
A. (*C.*) *stookesi*

On two nights in 1966, Cheng (private communication) collected mosquitoes in the Sepilok orang-utan reserve. He surrounded 14 young animals held in a large cage, with a very large mosquito net left open at the base. On four occasions during each night, all the resting mosquitoes between the net and the cage were caught. The following *Anopheles* were collected:

A. (*A.*) *pollicaris* 4 unfed ♀♀
A. (*C.*) *balabacensis* 2 engorged ♀♀
A. (*C.*) *philippinensis* 1 engorged ♀
A. (*C.*) *tessellatus* 12 ♀♀ (3 engorged)

In 1972 at Sepilok we collected mosquitoes that rested on the outside of another large cage that contained four young orang-utans during the early hours of several nights. The following *Anopheles* were found:

A. (*C.*) *vanus*
A. (*C.*) *limosus*
A. (*C.*) *kochi*
A. (*C.*) *subpictus*

During a visit to Sepilok in January 1974, the senior author (W.P.) collected mosquitoes attracted to light in the resthouse recently erected on the very edge of the rain forest, close to the

site of the cage used in 1972. The following *Anopheles* were caught resting on walls inside the house:

A. (*A.*) *albotaeniatus* *A.* (*A.*) *donaldi* *A.* (*A.*) *balabacensis*

A. balabacensis breeds in shallow temporary pools of rainwater in the spongy, moisture-absorbent humus material that constitutes the forest floor (Rajapaksa 1971). Random collections unfortunately tell us little about the anopheline fauna of Sepilok Forest, and even less about the species that feed on the orang-utan in nature. Although we do not know whether the

TABLE 1. LIST OF ANOPHELINE MOSQUITOES OF SABAH†

genus *Anopheles* Meigen, 1818

subgenus *Anopheles*

laticorn section

Myzorhynchus series

hyrcanus species group

1 *nigerrimus* Giles, 1900
2 *nitidus* Harrison, Scanlon & Reid, 1973
3 *lesteri paraliae* Sandosham, 1959
4 *peditaeniatus* (Leicester, 1908)

barbirostris species group

5 *donaldi* Reid, 1962
6 *hodgkini* Reid, 1962
7 *pollicaris* Reid, 1962
8 *vanus* Walker, 1859

albotaeniatus species group

9 *albotaeniatus* (Theobald, 1903)
10 *montanus* Stanton & Hacker, 1917

umbrosus species group

11 *umbrosus* (Theobald, 1903)
12 *baezai* Gater, 1933
13 *brevipalpis* Roper, 1914
14 *letifer* Sandosham, 1944
15 *collessi* Reid, 1963
16 *roperi* Reid, 1950
17 *separatus* (Leicester, 1908)

angusticorn section

Lophoscelomyia series

asiaticus species group

18 *interruptus* Puri, 1929

Anopheles series

lindesayi species group

19 *gigas crockeri* Colless, 1955

aitkenii species group

20 *fragilis* (Theobald, 1903)
21 *aitkenii* James, 1903
22 *bengalensis* Puri, 1930
23 *acaci* Baisas, 1946
24 *borneensis* McArthur, 1949

subgenus *Cellia* Theobald, 1902

Neomyzomyia series

25 *tessellatus* Theobald, 1901
26 *kochi* Dönitz, 1901
27 *watsonii* (Leicester, 1908)
28 *stookesi* Colless, 1955
29 *saungi* Colless, 1955

leucosphyrus species group

30 *leucosphyrus* Dönitz, 1901
31 *balabacensis* Biasas, 1936
32 *hackeri* Edwards, 1921
33 *pujutensis* Colless, 1948
34 *riparis macarthuri* Colless, 1956

Myzomyia series

minimus species group

35 *minimus flavirostris* Ludlow, 1914

Pyretophorus series

36 *subpictus* Grassi, 1899
37 *vagus* Dönitz, 1902
38 *limosus* King, 1932
39 *sundaicus* (Rodenwaldt, 1925)
40 *litoralis* King, 1932

Neocellia series

41 *maculatus* Theobald, 1901
42 *karwari* (James, 1903)

annularis species group

43 *philippinensis* Ludlow, 1902

† List based on collections by T. J. Chang, F. Y. Cheng, K. F. Chin, T. K. Chung, D. H. Colless, W. Peters, N. Rajapaksa, S. Ramalingam and J. A. Reid.

anophelines that transmit orang-utan malaria feed on their hosts in the canopy while the animals are sleeping, this appears to be most likely. So far no collections have been made in the canopy in Sabah. However, in West Malaysia, it has been shown that a number of anophelines are preferentially canopy feeders and it is suspected that they may transmit various species of simian malaria. A technique has been developed by Muul & Lim (1970) in the latter region for studying the fauna in or just below the canopy of tropical rain forests by means of transect walkways, 250 m or more in length; observations from these transects will revolutionize knowlege of the habits of acrodendrophilic species of *Anopheles*.

Species of the *A.* (*C.*) *leucosphyrus* group are known to transmit simian malaria over a large part of southeast Asia and to be preferential canopy feeders (Reid 1968). In the island of Borneo, *A. leucosphyrus* occurs commonly in Sarawak but is replaced largely by *A. balabacensis* in Sabah where it was the main vector of human malaria prior to the intensive DDT-based malaria eradication campaign of recent years. *A. hackeri*, another canopy feeder that occurs in Sabah, is a proven vector of five species of simian malaria in West Malaysia, and unidentified sporozoites have been recorded in *A. pujutensis* and *A. riparis* (Reid 1968). These two also occur in Sabah.

Of the species recorded in the Sandakan area or in Sepilok itself: (i) *A.* (*A.*) *aitkenii*, *A.* (*A.*) *hodgkini*, *A.* (*A.*) *pollicaris*, *A.* (*A.*) *montanus*, *A.* (*C.*) *stookesi*, *A.* (*C.*) *philippinensis* and *A.* (*C.*) *tessellatus* are not regarded as vectors of human malaria in Borneo, (ii) *A. donaldi* is suspected to be a minor vector of human malaria in Borneo and Malaya; (iii) *A. vanus* has been found infected with malaria in the Celebes but it is probable that the sporozoites were of non-human origin and the species is unlikely to be a vector of human malaria; (iv) *A. balabacensis* is the major vector of human malaria parasites.

The strongest suspect for the rôle of vector of the orang-utan malarias in nature in Sabah appears also to be *A. balabacensis*, which may be replaced further south in Borneo by the closely related *A. leucosphyrus*. The canopy feeders, *A. hackeri* and *A. pujutensis*, and perhaps *A. riparis* are also possible vectors. Intensive entomological studies will be needed to resolve this question. Such work should include canopy collections using orang-utans as bait, dissections for oocysts and sporozoites, and precipitin tests on blood meals of wild anophelines to determine the host species. The experimental aspect of the problem is described in detail in the appendix.

4. History of the orang-utans examined at the rehabilitation centre, Sepilok

Since 1966, when McWilson Warren first took blood films from several orang-utans of Sepilok, specimens from the Rehabilitation Centres have been examined on a number of occasions. The 1966 films were stained and examined at the Medical Research Institute, Kuala Lumpur by Yap Loy Fong (private commun.) who found that ten animals out of the 19 studied were infected with malaria parasites. The names of the animals with parasites were as follows: Joan (plate 1, bottom right), Coco, Cynthia, Kira, Simbo, Jippo, Paul, Molly, Liza* and Spencer*. The negative animals were named: Dinky, Baby, Henry, Stella, Kiddie, Clementine*, Winnie, Billy* and Cholmondeley*. These animals are all listed in de Silva's Table 3 (1971) according to age and location (i.e. whether still maintained at the Sepilok Station or permanently living in the forest; the names marked with an asterisk refer to the latter group, though since 1971 more animals have returned to the forest and some of course have died). Yap Loy Fong noticed that in at least one animal, two types of parasites appear to have been present, the second one produc-

ing Schüffner's dots in the infected erythrocyte. Coatney *et al.* (1971) base their description of *P. pitheci* on this material, but apparently were not convinced of the existence of a second parasite as they made no reference to it. Prolonged investigations were necessary to confirm the presence of the latter and these were not undertaken until our expedition to Sepilok in 1972.

Additional material from the orang-utans at Sepilok was obtained by one of us (N.R.) from 1968 to 1971 and examination of the blood films of three animals gave the following results:

An 8 year old female orang-utan (Stella) showed a scanty infection of an unidentified malaria parasite in 1969 and 1971. (Her blood was free of malaria when examined in 1966.) A 2 year old male animal (Rajan) was captured on the Sungai Manila, near Sandakan in February 1968. In November of the same year his blood showed a moderately heavy infection of *Plasmodium pitheci*. Further samples of blood were examined in September and December 1969, in October 1970 and in December 1971; *P. pitheci* was present in declining numbers in those blood smears, while in January 1972 no parasites were detected. We continued to investigate this animal after our arrival and five further specimens of blood remained negative (up to March 1972). Rajan died of an unknown cause in June of that year. The third animal was brought to Sepilok from the Chia Hwa estate at Sikan in May 1967; he was a male orang-utan (Chip) about 6 months old on arrival and weighing less that 1.5 kg. A fairly heavy infection of *P. pitheci* was found in his blood in February 1969 and again in December 1971. His subsequent history is shown on page 450.

Subsequent to our visit to Borneo in 1972, Stanley de Silva and Peter Govind provided us with blood films from a few more animals. Other orang-utans were examined by W.P. in January and February 1974. The details are as follows:

A female (Grace) about $6\frac{1}{2}$ years old was found to have no malaria parasites in thin and thick blood films taken on two evenings in mid-January 1974, nor were parasites seen in the blood of a $2\frac{1}{2}$ year old male (Rejevan), examined at the same time (Rejevan arrived at Sepilok in September 1973). Four days later however, scanty trophozoites of *P. silvaticum* were seen in his blood and this infection persisted for one more day. Subsequent blood examinations during the next 2 days revealed no more parasites.

Three other male orang-utans brought to Sepilok in 1973 showed malaria parasites when examined in January 1974. A one year old specimen (Muhammed Ali) from Ulu Dusun had *P. pitheci* in the blood, and ova of *Ancylostoma duodenale* (Dubini, 1843) and trophozoites of *Balantidium coli* (Malmsten, 1857) were found in the faeces. Another year old male (Ken Norton) was picked up on the road near the Sepilok Forest in April 1973 and a fairly heavy infection of *P. pitheci* (rings, trophozoites, schizonts and rare gametocytes) was seen in his blood films. The third animal (Joe Frazier) was an infant which was caught in May 1973 during tree-felling operations at Ulu Dusun. *P. pitheci* was present in the blood of this animal also.

The history of the orang-utans first examined by us at Sepilok in 1972 is given below in individual detail as these animals were kept under observation for a month or longer. The clinical features and concomitant infections are described on p. 450.

Barbara. She was caught at Lugga, Second Division, Sarawak in July 1970 and was about 2 years old on arrival in Sepilok a month later. She was in poor condition and weighed only 5 kg; 6 months later, she weighed $8\frac{1}{2}$ kg.

Blood films were examined systematically from 5 February 1972. They first showed a highly synchronous infection of *P. silvaticum* which disappeared after 9 days to be replaced by *P. pitheci*. Later the former parasite returned and a mixed infection persisted until March, though

P. pitheci remained dominant. On 8 February when an apparently pure infection of *P. silvaticum* was present, 6 ml of blood were inoculated intravenously into the splenectomized chimpanzee Khan. During the course of the malaria, Barbara became listless and obviously unwell; these symptoms may have been due to intestinal infections (q.v.) which she developed at this time. A light infection of *P. pitheci* was still present in January 1974.

Martin. He had been caught in 1969 near the Sungai Manila (Sandakan) and arrived at Sepilok in September 1970, aged about $2\frac{1}{2}$ years. In February 1972, he weighed 10 kg. A mixed infection of *P. pitheci* and *P. silvaticum* was present in the blood, with the former much predominating and persisting into the first week of March. This animal also had intestinal infections which necessitated chemotherapy, though the malaria, as in all the animals, remained untreated. Martin died (? from snakebite) in June 1972.

Chip. The early history of this animal is given above. When we first saw him in February 1972 he weighed nearly 17 kg and exhibited a low grade infection of *P. pitheci* which persisted for the duration of our stay. A moderate infection of *P. pitheci* was found by W.P. in January 1974.

Wallis. She had been captured originally in Kalimantan, whence she was taken in July 1971 over the border into Sarawak. Dr D. Kok found her in poor condition which he thought was due to malaria (a *P. vivax*-like infection was found in her blood). Her weight then was 5 kg but had doubled by the time we saw her in 1972, when her estimated age was about $1\frac{1}{2}$ years. Her blood film was negative on 2 February 1972, but on 22 February small rings and trophozoites of *P. pitheci* were discovered, and as in the other orang-utans, quite severe intestinal infections followed shortly afterwards. *P. silvaticum* appeared to be absent from this animal when we examined her, although the original report of the presence of a *vivax*-like parasite by Dr Kok suggested that this species was present. Wallis died of melioidosis in January 1974.

Cyril. A male orang-utan from Papar was brought in a poor condition to Sepilok on 17 February 1972. His weight was 8 kg and the blood film was negative. He died 2 months later.

Roberta. This animal originated near the Kinabatangan River and arrived at the Rehabilitation Station on 2 March 1973. Her weight was 6 kg and estimated age one year. A light infection of *P. pitheci* was observed, which declined during the following days, but on March 18 she died from suspected melioidosis.

5. Clinical features of malaria in the orang-utan and concomitant infections

We had the opportunity of watching young orang-utans during the course of malaria, though it was impossible to determine when or where the infection had been acquired. There was little evidence that much morbidity was caused by the malaria and we could see no obvious differences between infections due to *P. pitheci* and those due to *P. silvaticum*. The rectal temperature was seldom above normal, and surprisingly no splenomegaly could be detected even in animals like Barbara or Martin who were several years old, and had had malaria for at least 3 or 4 weeks without specific treatment.

Barbara had to be removed to the veterinary clinic on several occasions, and on one of the last was found to be listless, unwell, slightly anaemic and with an exacerbation of the (*P. pitheci*) malaria. This time she seemed to be undergoing a typical attack, with the cold phase occurring at 09.45 and the hot phase (accompanied by the major rupture of the schizonts) at 12.15; the temperature then had risen to 38 °C. She recovered within 5 days when the parasitaemia had

markedly declined to the chronic level. Although in their original description Halberstaedter & Prowazek (1907) commented on the absence of fever or other obvious signs of illness in *P. pitheci* infected orang-utans, the spleen of one animal at autopsy was said to be somewhat enlarged and heavily pigmented. On the other hand, Dodd (1913) attributed the death of an orang-utan in the Sydney Zoological gardens to a heavy infection with *P. pitheci*.

The chief causes of morbidity and possibly of mortality in the wild were probably intestinal infections, and it is possible that the effects of the latter were accentuated because the animals also had malaria. Two animals in our series probably died from melioidosis; this infection is known to cause high mortality in the orang-utan (de Silva 1971). The usual sequence of events during our investigation was that a malaria infected orang-utan (under daily observation for 1 or 2 weeks) would be noticed to be ill and taking little food. The illness was accompanied by diarrheoa (including a 'strawberry-coloured discharge' – as earlier reported by Patten 1939) and the passage of blood and mucus in the faeces. The sick orang-utan would then be taken to the veterinary clinic in Sandakan for further examination and treatment.

The dysentery was due to two protozoal infections – *Balantidium coli* and *Entamoeba histolytica* Schaudinn, 1903, and enormous numbers of trophozoites of both species were found in the faeces.

The former was treated with oral metronidazole (20 mg/kg daily for 5 days) and the latter with emetine hydrochloride (1 mg/kg given by intramuscular injection daily for 3–5 days). These drugs produced a rapid cure, though in two instances the amoebic dysentery relapsed after a few weeks. Four animals (Barbara, Martin, Wallis and Muhammed Ali) suffered from these double infections, which were probably widespread throughout the group at Sepilok.

Hookworm ova were found in the faeces of one orang-utan and probably this nematode is responsible for some morbidity.

The origin of the protozoal infections in the alimentary tract is interesting. The colony of orang-utans live in the vicinity of the quarters of the forest guards at Sepilok and it is probable that the animals acquired their infections of *E. histolytica* from the latter; trophozoites appeared to be identical in size and morphology with the human species.

The balantidial infections in the Sepilok orang-utans are unlikely to have arisen from a human source as the ciliate is rare in man. On the other hand, *Balantidium coli* is common in pigs, including the wild pig. Many of the latter exist in the Sepilok Forest and herds of these pass along the paths or stream beds near the Resettlement Scheme; their excreta would provide a ready source of infection in the ape. Possibly the ciliate may be transmitted from ape to ape as cysts were seen in the dejecta of one orang-utan. The size, shape, cytostome and arrangement of cilia all corresponded with the morphological details of *B. coli* of man.

Balantidium coli is occasionally seen in orang-utans in zoos and has been reported as a cause of death. An animal died in the London Zoo in May 1957; the rectal contents were examined and the stained fixed film showed numerous ciliates, 50–70 μm in diameter and oval or circular in outline. The macronucleus was a prominent bean-shaped structure, about 17 μm in length. A cytostome could sometimes be seen; at the mouth it was 3 μm across. The basal granules of the cilia were also visible in some specimens. The cytoplasm was much vacuolated and was usually surrounded by a thin cyst wall, the cilia protruding through the latter. Probably the cyst had begun to form in this specimen shortly before the death of the host, for in our fresh preparations in Borneo, practically only trophozoites were seen in the young animals; cysts were seen in specimens of normal appearance in one of the older animals (Rajan). Patten (1939) and Mortelmans, Vercruysse & Kageruka (1971) describe fatal cases of balantidiasis in orang-utans in zoos

where the origin of the infection may have been from cysts produced by other primates in the vicinity. Ruch (1959) comments on an earlier epizootic which was thought to have originated from giant tortoises, but this seems to be unlikely as the species in the latter is clearly distinct from those found in mammals.

Unidentified intraerythrocytic bodies in the chimpanzee

Strange bodies were found in the blood of chimpanzee Khan who had been inoculated with blood from orang-utan Barbara. They are described here because they may have originated in the latter animal. They became manifest in the abnormal host which, because it had been splenectomized, would have had a lower resistance to infections in general. The reduction of immune protection is of course non-selective so that the animal is rendered unusually susceptible not only to *Plasmodium* but to whatever infective challenge it may encounter, e.g. bacterial or viral infections from the donor animal, or from its environment. The case history of the chimpanzee is as follows:

The chimpanzee Khan was splenectomized in London on 10 January 1972 and made an uneventful recovery. This animal received blood from orang-utan Barbara 29 days after splenectomy and *P. silvaticum* appeared in its blood 23 days later. The parasitaemia increased rapidly over the next week and about 4000 laboratory-reared mosquitoes were then allowed to feed on it. On the 12th day of patency the level of parasitaemia began to fall and there was a sharp drop on the 13th day. From the 10th day Khan had shown signs of malaise and on the 14th day he developed a fever which reached 40 °C the following day. By this time parasitaemia was very scanty. The pyrexia was interpreted as probably being due to a secondary viral or bacterial infection, and Khan was promptly given therapeutic doses of penicillin and chloramphenicol. In spite of this treatment and a continuing decline of the malaria parasitaemia, Khan's fever steadily increased and on the 15th day (40 days after he had received the inoculation of orang-utan blood, or 69 days after splenectomy) the animal died. Because of the fear that Khan might have been infected with a primate virus that could be highly pathogenic for man (there were several precedents, e.g. the history of Marburg disease, to make this feasible), no post-mortem examination was carried out. Serum had previously been collected for virological examination.

Examination of the serial blood films taken from Khan revealed that from the 23rd day following inoculation with orang-utan blood onwards (i.e. the first day of patent malaria parasitaemia), unusual features were present in the red blood cells. Some of these, such as evidence of a younger population of red cells (anisocytosis, poikilocytosis and the presence of Howell–Jolly bodies and other chromatin residues) were readily accounted for because they are features of the blood picture of any splenectomized primate. However, in addition, unusual structures were found in red cells uninfected with *Plasmodium*. These gave the appearance of colourless vacuoles, i.e. they failed to take up the Giemsa stain which coloured the rest of the red cells a mauve or reddish mauve tint. The bodies were best seen with phase contrast illumination (plate 2, top left and right). No such structures were ever observed in any of the blood films made from orang-utans. They varied in shape from simple circles to crescentic or bean-shaped bodies, the sizes of which also varied from about 1 to 3 μm across at the maximum. Frequently several bodies appeared in a single red cell, but rarely more than 4 or 5. No internal details could be detected in these bodies even with phase optics. In the most heavily infected blood film the infection was approximately 10–20 % of the red cells.

Blood had been collected, fixed and sectioned for the examination of the ultrastructure of *P. silvaticum* from Khan on the 9th day of patency. Unfortunately the fixation of this material under the conditions in which we were working was imperfect. Nevertheless, a retrospective study of the electronmicrographs made by Dr F. Colley and Professor V. Zaman at the University of Singapore, revealed the presence of numerous unidentifiable structures within uninfected erythrocytes. These structures resemble in shape, size, number and location the 'Khan bodies' that could be seen in air dried, Giemsa-stained thin blood films (plate 2, bottom left and right).

The serum taken from Khan was sent to the W.H.O. Regional Reference Centre for Simian Viruses, San Antonio, Texas for investigation. No bacteria were present in the serum and no evidence of viral antibodies was obtained (Kuntz, private communication). Since it seemed possible that the infection in Khan could have been due to a *Mycoplasma*, the serum was also subjected to a complement fixation test for primate mycoplasmal antibodies. Again, no evidence of infection was found (Kalter, private communication).

The nature of the 'Khan bodies' remains undetermined. Dodd (1913) drew attention to the presence of 'nuclear fragments' in the red cells of an orang-utan that died with a heavy infection of *P. pitheci* in the Sydney Zoo. He found similar bodies in blood taken from this animal's bone marrow, and in peripheral blood of another orang-utan that 'was ill at the same time, but recovered after a radical change of diet. It died six months later'. The blood of two other orang-utans (both of which died soon after arrival in Sydney) contained no 'chromatin bodies'. The red cell inclusions described by Dodd were clearly Howell–Jolly bodies and appear to bear no relation to 'Khan bodies'. Organisms of the genus *Eperythrozoon* Schilling, 1925 occur in the blood of the owl monkey (*Aotus trivirgatus* Humboldt, 1811). They are ring-shaped structures that appear both on the surface of, and between the erythrocytes (Peters, Molyneux & Howells 1974) and are quite distinct in shape, size and staining characteristics from the 'Khan bodies'. So too is *Haemobartonella* Tyzzer & Weinman, 1939 which has been described from rhesus monkeys by the same authors and from South American monkeys by others (Pessôa & Prado 1927; Aikawa & Nussenzweig 1972). *Haemobartonella* has a superficial resemblance to *Bartonella bacilliformis* (Strong *et al.* 1913), a bacterial infection of human red cells, and to the red cell inclusions that were recently described in the blood of a patient with malignant melanoma by Clark (1975). None of these organisms resembles the 'Khan bodies'. A comparison has also been made with photographs (kindly provided by Professor D. Weinman) of *Bartonella*-like red cell inclusions found in patients with haemolytic anaemia in northern Thailand by Whitaker *et al.* (1966). These too are quite different from the 'Khan bodies'.

If the structures found in the red cells of Khan are indeed organisms and not artefacts, they may have arisen from organisms originally present in its own blood or in the blood of the donor orang-utan. They may also have been acquired when Khan was bitten by large numbers of colony-bred mosquitoes in Kuala Lumpur. They may too have been acquired through the bites of wild mosquitoes, either in Sabah or Kuala Lumpur, or simply been contracted from the environment. No structures corresponding to the 'Khan bodies' were seen in the blood of chimpanzee Sandy or in at least ten other splenectomized chimpanzees that we have examined in the course of various malaria experiments. Whether the 'Khan bodies' were in fact in any way related to the death of this chimpanzee unfortunately will have to remain an enigma.

6. Epizootiology and zoonotic potential of orang-utan malaria

Most observers (see, for example, de Silva 1971) comment on the small groups in which the orang-utans move around; two or three seems to be the usual number in a density of less than 2 per square mile, and this sparse distribution in the forest must be of considerable importance in the transmission of malaria (see p. 455). Harrisson (1962), however, stated that after leaving the mother young animals live with companions, often in groups of 3–6 individuals whom she terms 'teenagers'. Galdikas-Brindamour & Brindamour (1975) remark that, in southern Kalimantan, although adult males are invariably solitary, adult females with an infant or two may travel and forage in groups of two or three, although rarely for more than a few days at a time. They state that, compared with the adults, the immature orang-utans are almost gregarious. MacKinnon (1973, 1974), who also commented on the essentially solitary habit and nomadism of Bornean orang-utans, suggests that the smallness of the groups is a consequence of the limited and specialized food that forms the staple diet of the orang-utan, namely forest fruits, and their scattered distribution. A group of two or three animals will strip a tree very quickly. On the other hand, quite large groups of orang-utans are said (Harrisson 1961) to congregate in the vicinity of trees with ripening fruits and remain there for some time until the fruit (e.g. durian) is completely eaten. During this interval the sporogonic cycle in the mosquito could be completed and the infection transmitted. The supply of food also governs the nomadic behaviour as the animals must travel long distances in search of the strictly seasonal fruit.

In this way, a high proportion of the mosquito population of the forest has a good chance of picking up a malaria infection; if the apes were more static, infection would be limited to the special localities where the animals congregate.

The density of the population of orang-utans is nevertheless very low in these forests, and this is a factor that has been shown in human malaria to influence the incidence of disease. Thus, one of us (W.P.) noted that in the upper reaches of the Fly River in the Western District of Papua New Guinea, where the human population was distributed very sparsely, the prevalence of malaria indicated only a hyperendemic pattern of transmission as compared with the holoendemicity encountered in the more populated, but otherwise ecologically comparable delta area of the same river system.

Malaria might therefore be expected to be uncommon in the orang-utan, but this is not so, as has been demonstrated by the high prevalence reported by various workers and by ourselves in animals brought to Sandakan after confiscation or capture. How then is the infection acquired? We know that the parasites persist in the blood for 7 years or longer, so that an infected animal can act as a carrier for a long period. We know also that young animals are often infected. The infant may have contracted malaria congenitally through the placenta, although such a mode of transmission is rare in other forms of the infection, e.g. in human malaria (Covell 1950) and in rodent malaria (Gunders 1957). Prolonged contact of the infant with an infected mother provides an opportunity for mechanical transmission to take place, via scratches or orally. These abnormal routes of infection are, however, unlikely to contribute much to the high prevalence of the disease in young animals. It is most probable that they are bitten by mosquitoes which had become infected from earlier feeds on the infected mother. If subsequently the mother and infant remain in the locality for a further 10–12 days (MacKinnon 1971 gives a figure of 11 days' stay in Sabah), transmission could occur. In the ideal climate provided by the tropical rain forest, infected mosquitoes may live for a month or more and during this time they may feed on the

animals. The suspected vector (*Anopheles balabacensis*) dwells in the canopy of the forest where the orang-utans sleep in nests high in the trees; contact between infected mosquitoes and apes thus readily occurs.

Malaria infections of the orang-utan seem to have been found easily by most observers who have studied the blood of these animals, though no exact figures were given except by Coatney *et al.* (1971) who stated that 10 out of 18 animals were infected with *P. pitheci* in a single examination. This is certainly a minimal figure and repeated examination would have shown a higher prevalence. We found that 11 out of 13 orang-utans were infected with malaria. Six showed *P. pitheci* only, 1 *P. silvaticum* only, 3 a mixed infection, and 1 an infection with an unidentified malaria parasite.

Although the conditions prevailing at the Sepilok Rehabilitation Settlement have resulted in a concentration of the population of orang-utans, this does not fully explain the high rate of infection: many newcomers were already infected with malaria on arrival.

The lower prevalence of *P. silvaticum* may be due either to the shorter life of this species in blood as compared with the more chronic *P. pitheci* or to its being a less common parasite. There is some evidence (p. 449) that *P. pitheci* may temporarily suppress *P. silvaticum* and this partial inhibition may be responsible for the relative infrequency of the latter. Moreover, *P. pitheci* parasitaemia persists at a low level for long periods of time, whereas *P. silvaticum* produces more acute but transient peaks of parasitaemia, which are almost certainly followed by relapses. However, *P. silvaticum* infects anopheline mosquitoes readily and there seems no reason why transmission should be different.

One of the main lines of our enquiry was the possibility that malaria of the orang-utan may be a zoonosis, i.e. capable of being transmitted in natural conditions to man. The likelihood of malaria parasites of apes infecting man has long been considered, and several of the early workers in Africa attempted to investigate the question by inoculating themselves with malaria parasites of the chimpanzee. Thus Blacklock & Adler (1922) inoculated themselves with blood from a chimpanzee heavily infected with *P. reichenowi* without success, an experiment which was repeated by Rodhain, Van Hoof & Muylle (1938) and Rodhain (1939), in patients with neurosyphilis, with the same result. Man was shown by Rodhain & Dellaert (1955) to be susceptible to the blood forms of *P. schwetzi* and finally Contacos *et al.* (1960) demonstrated the susceptibility of Caucasian (but not Negro) man to the sporozoites of this parasite, grown in *Anopheles balabacensis*. The latter mosquito does not exist in Africa (the home of *P. schwetzi*) and the natural vector is unknown, though it must be a sylvatic species, probably with acrodendrophilic habits. Bray (1963) pointed out that a zoonosis involving *P. schwetzi* was improbable because man is unlikely to be bitten by the forest vector, while the predominantly domestic mosquito, *A. gambiae* Giles, 1902, is unable to transmit the ape parasite.

The potentialities of simian malaria affecting man in southeast Asia are greater and three genuine human cases of *P. knowlesi* malaria have been reported in North Americans working for a short time in the forests of Western Malaysia (Chin, Contacos, Coatney & Kimball 1965; Yap, Cadigan & Coatney 1971; Dissanaike, private commun.). *P. knowlesi* is readily transmissible by blood inoculation to man, as was first shown by Knowles & Das Gupta in 1932 and, later, by many other workers. Ciucă, Tomescu & Badonski (1937) used this parasite on a large scale for malaria therapy in cases of cerebral syphilis. *P. cynomolgi* and *P. inui* are also common simian parasites in Malaysia. Both are transmissible without difficulty by mosquitoes, either deliberately or, in the case of the former, accidentally in the laboratory, and it might be thought

that zoonoses would occur in Nature. None however has been reported and Warren, Cheong, Fredericks & Coatney (1970) had entirely negative results following the inoculation into rhesus monkeys of samples of blood from over a thousand indigenous inhabitants of the forests of Pahang.

In South America, there is some evidence (see, for example, Dunn 1965; Garnham 1967, 1973 *a*) that *P. brasilianium* and *P. simium* Fonseca, 1951 of the New World monkeys may represent a 'zoonosis in reverse', in which the origin of the parasites is thought to be from human infections of *P. vivax* and *P. malariae* in the Conquistadores or their negro slaves. Both simian species are today readily transmissible back to man (Coatney *et al.* 1971) in whom they produce benign tertian and quartan malaria respectively.

The susceptibility of man to *P. silvaticum* of the orang-utan remains unknown. However, a single experiment done with *P. pitheci* (see p. 461) suggests that *P. pitheci* is probably not infective to man.

The opportunity of man acquiring orang-utan malaria seems even more remote than the zoonosis problem in West and Central Africa, where at least the animal reservoir is much larger than in Borneo and Sumatra, and we agree with the opinion of the World Health Organisation Scientific Group (W.H.O. 1969) that the available evidence suggests that the malaria parasites of non-human primates present little danger to human populations.

We examined blood films from forest workers and others in Sabah, suffering from malaria, and we compared the morphology of the parasites with that of *P. pitheci* and particularly *P. silvaticum*. The human infections all seemed to be typical *P. vivax*; the differences between these species are discussed on p. 469.

The level of transmission of malaria to man has been considerably reduced by the intensive antimalarial campaigns that have been conducted in Sabah over the past decade. In 1971, for example, 9652 blood films were examined from the 65000 population of the Sandakan area in the passive and active case detection operations of the Malaria Service of the Department of Health. Of the 209 films found positive, 158 contained P. *falciparum*, 44 *P. vivax*, 6 *P. malariae* and 1 a mixed infection. In the same year, 29512 blood films were examined from the 51000 population of the Tawau area which lies further south and adjoins the border districts of Kalimantan where malaria transmission continues at a high level. The infection rate was higher than in the Sandakan area; 783 blood films contained *P. falciparum*, 505 *P. vivax*, 1 *P. malariae* and 3 mixed infections (M. Colbourne, private communication). The blood films that we examined in 1972, in which we found typical *P. vivax*, came from both the Sandakan and Tawau areas. Figures supplied to us by Mr Peter Govind of the Malaria Service indicate that, while *P. falciparum* had virtually disappeared, *P. vivax* was relatively common during 1973. The cases were however classified as imported since, according to the records of the Malaria Service, every one of the 195 infections recorded was in a Philippino refugee of whom thousands had fled to eastern Sabah from the neighbouring islands of their homeland in recent months due to civil disturbances. The situation had changed by early 1974 when we were unable to find a single autochthonous infection of vivax malaria in the Sandakan area.

If one poses the hypothesis that either *P. vivax* in this area is in fact a zoonosis (i.e. *P. silvaticum* in man) or that, conversely, *P. silvaticum* is an anthroponosis (i.e. *P. vivax* in the orang-utan), it is evident that the quantum of infection in both man and apes would decrease in parallel, especially if the main vector is common to both primate hosts. The evidence we have presented, however, makes this hypothesis almost certainly untenable. The apparently higher level of suscepti-

bility of the chimpanzee to *P. vivax* than to *P. silvaticum*, the minor but consistent morphological differences between the blood and tissue stages of the two parasites, and the lack of infectivity of *P. silvaticum* to the gibbon which can be infected with *P. vivax*, together provide an accumulation of data confirming the separate identities of the two species. Unfortunately, biochemical comparison of the two parasites (e.g. characterization of the nuclear DNA and of isoenzymes) which would provide valuable confirmation may now never be forthcoming.

7. A DESCRIPTION OF THE MALARIA PARASITES OF *PONGO PYGMAEUS*

The following descriptions have been given in detail, because, with the strong possibility of the animal becoming extinct, there are unlikely to be many opportunities for the future study of the primate; in fact, material may never again be obtainable. The notation 'plate 3, 1–36' signifies drawings 1–36 on plate 3.

(*a*) *Plasmodium* (*Plasmodium*) *pitheci* (*plate* 3, 1–36)

(i) *Blood stages*

P. pitheci has been described by Halberstaedter & Prowazek (1907), Shibayama (1910), Dodd (1913), Wenyon (1926), and Coatney *et al.* (1971). Garnham (1966) attempted to amalgamate the earlier accounts into a new description, partly based on an examination of some of Wenyon's blood films of an orang-utan which had died in the London Zoological Gardens in 1936. It is clear today that some of these observations referred to infections of *P. pitheci* mixed with *P. silvaticum* and the following account of the former is now presented (see also the preliminary communication of Garnham, Rajapaksa, Peters & Killick-Kendrick 1972). Wenyon attached a note to his 1936 material which reads as follows: 'Deeper staining by Giemsa shows stippling of r.b.c. In many cases the infected cells show curious ring markings, schizonts up to 14–?16 merozoites, red cells not enlarged;' the note was accompanied by a sketch of a female gametocyte with a peripheral nucleus and a central nucleolus. These details clearly indicate that he was dealing with *P. pitheci* as described below.

P. pitheci is redescribed from natural infections as encountered in orang-utans in Borneo during the years 1969 to 1974. Concentrated work was carried out on four animals kept under close examination for about 5 weeks during our visit to Sabah in 1972. The heaviest infections were found in Barbara, Martin, Wallis and Ken Norton; in the first two animals, the infection was mixed with *P. silvaticum*. Fortunately the two species are easily differentiated from one another.

Ring forms (*plate* 3, 2–9). The youngest parasites are small solid bodies about 1.5 μm in diameter and consist of nucleus and cytoplasm. A vacuole is soon acquired. The nucleus remains spherical, is often inside the ring, and is darkly staining. After some hours about half the nuclei assume a curved shape, or appear to be split into two portions of equal or very unequal size. In other species of *Plasmodium* (e.g. *P. knowlesi* Sinton & Mulligan, 1932) when unequal dots are present, the tiny fragment is referred to as an 'accessory dot'. 'Accolé' parasites are occasionally seen (plate 3, 6). The parasite is now about 2.5 μm in diameter, The erythrocyte (a mature red blood corpuscle) is unchanged in size and in the first stages of growth of the parasite, no stippling is visible. Multiple infections of the host cell are uncommon.

Later, the parasite increases in size (up to 3.5 μm) and the cytoplasm becomes slightly

amoeboid; the surface may be crenulated or even long thin processes may project. The nucleus may now have a pale centre with two darkly staining dots at opposite poles.

Trophozoites (*plate* 3, 10–19). With continual growth, the nucleus enlarges but the parasite is still vacuolated; it may occupy two-thirds of the host cell and measure up to 5 μm in diameter, The staining quality of the nucleus continues to change and darker and lighter portions become very obvious. Stippling of the corpuscle begins now (or even earlier) and soon becomes highly characteristic; it is best described as comprising relatively few and easily countable dots of large but variable size and of a deep red colour. This stippling, even under a low magnification of the microscope, is easily distinguishable from Schüffner's dots as produced by *P. silvaticum* (and *P. vivax* (Grassi & Feletti, 1890) etc.). About the same time, approximately 20 h after invasion of erythrocytes by merozoites, fine inconspicuous pigment granules appear in the cytoplasm. The pigment is light brown in colour and is unstable, in that it begins to fade in blood films within 2 or 3 years.

Eventually the vacuole disappears and sometimes the parasite assumes the 'band form' (plate 3, 18) so well known in quartan malaria parasites (*P. malariae* (Grassi & Feletti, 1892), *P. brasilianum* Gonder & von Berenberg-Gossler, 1908, etc.). The developing parasite causes no hypertrophy of the host cell.

Schizonts (*plate* 3, 20–30). Nuclear division does not start until the parasite is about 36 h old or later, when it has come almost to fill the red blood cell. Interesting nuclear changes occur, but the finer details could not be discerned with accuracy in the conditions in which we were working. Nevertheless within the nuclear membranes of the binucleate parasite, we could sometimes distinguish 3 or 4 parallel bars of chromatin, and during the next division, one nucleus might be drawn out into a worm-like structure stretching almost from one end of the body to the other; all the nuclei had a heterogeneous composition, and had an irregular border, Such nuclei might measure 4 μm in length, in a schizont 6–8 μm in diameter. Sometimes comet-shaped nuclei were seen with round heads and light pink tails. Probably these changes were due to the rapidity of division which was also responsible for the frequent presence of 'nuclear threads' in the cytoplasm. Eventually, after a cycle of 48 h, 16 nuclei are formed and these give rise to 16 merozoites. Now the nuclei are in a resting stage, solid and dense, and like those of the early rings.

Pigment granules in the schizonts are as inconspicuous as in the trophozoites and are of a yellowish grey colour. They remain scattered in the cytoplasm and only agglomerate when the parasite is practically mature.

The stippling of the erythrocytes remains prominent, though as the schizont fills the cell, the dots become compressed between the margin of the parasite and the cell border and may bulge out from the membrane of the infected cell. The stroma of the erythrocyte is unchanged in colour, but it must be much damaged by the presence of the parasite, as the infected cell is distorted in outline or even assumes an oval and fimbriated shape, like that caused by *P. ovale* Stephens, 1922 (plate 3, 24 and 25). In the later stages of development the parasite may give rise to a very slight degree of enlargement of the corpuscle.

Schizogony of *P. pitheci* takes place commonly in the peripheral blood (though undoubtedly some schizonts pursue their course of maturation in the internal organs). The process is synchronous, but the presence of two broods of parasites maturing on alternate days may confuse the picture. Rupture of the schizonts takes place predominantly at midday every 48 h, and examinations of films of peripheral blood taken at 09.00, midday and 15.00 reveal a characteristic count. The morning film shows perhaps half the parasites as binucleate or quadrinucleate

schizonts; at midday mature schizonts are easily found; in the afternoon, the population is about 90 % in the form of young rings. *P. pitheci* thus has a tertian and not a quartan periodicity as was originally suggested by Halberstaedter & Prowazek (1907).

Gametocytes (*plate* 3, 31–34). Gametocytes were seen from time to time in nearly all *P. pitheci* infections in orang-utans, but they only occurred in large numbers during or shortly after recrudescences of the asexual parasitaemia. Usually macrogametocytes were more numerous than microgametocytes.

Owing to the late onset of nuclear division in the asexual cycle, often not until the parasite practically fills the erythrocyte, it is difficult to identify with certainty immature sexual stages, especially females. The pigment granules of gametocytes however tend to be slightly larger and darker than in asexual forms, an aspect best demonstrated in understained films.

The mature macrogrametocyte (plate 3, 32) is round or subspherical and measures from 7.5 to 8 μm in diameter, i.e. slightly larger than the host cells. The cytoplasm stains a blue colour. The nucleus is very often peripheral and elongated (up to 2.5 μm in length); it may contain a few, darkly staining dots or consist of lighter and denser portions. Some extranuclear chromatin is occasionally seen as a pinkish 'splash' in the cytoplasm. The brown pigment granules are not very conspicuous except in understained films, and are distributed regularly throughout the cytoplasm.

The microgametocytes (plate 3, 33 and 34) are the same size as the macrogametocytes, from which they are easily distinguished by the colour of the cytoplasm and the size of the nucleus. The cytoplasm stains feebly and has a light brown or pink colour; the nucleus is diffuse and occupies a large band across the parasite or takes up at least one third of its size. The nucleus may be homogeneous in apparent structure, but often streaks or lines of darker dots may be present. Brown pigment granules are more easily seen in the male than in the female parasite (probably because the cytoplasm stains less heavily in the former); they are numerous and distributed throughout the cytoplasm except over the nucleus.

Gametocytes cause pronounced stippling of the specialized character in the host cells, but when the parasites are mature the dots become condensed or agglomerated round the border.

(ii) *Sporogonic stages*

Exflagellation and ookinete formation. Exflagellation of the mature microgametocyte in blood under a coverslip occurs in $9\frac{3}{4}$ minutes, with the production of 8 microgametes (plate 3, 35).

Ookinetes were found in smears of the contents of the midgut of experimentally infected mosquitoes 18–20 h after feeding.

Oocysts and sporozoites. The growth curve is shown in figure 3 where it is seen that the oocyst becomes mature on the 10th day after the infected feed. The pigment is fine and the granules lie in two or more parallel lines or semicircles. On the maturity of the oocyst, the pigment becomes concentrated.

Sporozoite formation can be observed from the 9th day, and on the 10th day the oocyst (now about 45 μm in diameter) is packed with sporozoites which reach the salivary glands on that or the following day. The sporozoites are rather short (9–12 μm); in dried, stained specimens the mean length is 10 μm. They tend to be straight, and the nucleus may be fragmented (plate 3, 36). They are non-motile. An experiment to test their infectivity to man is described below.

These observations were made in Sandakan and relatively few mosquitoes were examined. Four out of 6 *A. maculatus* and 5 out of 5 *A. balabacensis* became infected in the positive batches.

Other attempts proved unsuccessful, although a hundred or more mosquitoes were fed on lightly infected orang-utans.

(iii) *Differential diagnosis*

The relationship between *P. pitheci* and other malaria parasites found in primates is discussed on p. 471. Only two species have been found in the blood of orang-utans and *P. pitheci* may be easily distinguished from *P. silvaticum* on the following grounds. *P. pitheci* is smaller and less amoeboid; it causes only slight if any enlargement of the host cell and the latter exhibits a highly characteristic type of stippling, differing entirely from the Schüffner's dots produced by *P. silvaticum*; schizogony in the peripheral blood is profuse in *P. pitheci*, rare in *P. silvaticum*; the sexual forms of the two species can easily be distinguished from each other by size.

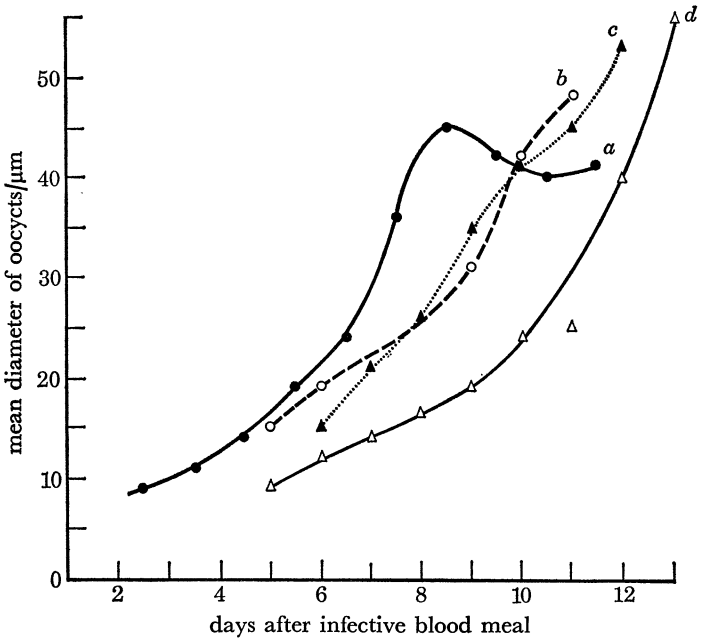

FIGURE 3. Growth curves of oocysts on the midguts of *Anopheles balabacensis*. *a*, *P. silvaticum* at 27–29 °C; *b*, *P. pitheci* at 27–30 °C; *c*, *P. hylobati* at 25 °C; *d*, *P. jefferyi* at 25 °C. (*c* and *d* based on data in Coatney *et al.* 1971.)

(iv) *Infectivity to primates other than* Pongo pygmaeus

On 21 February 1972, blood containing a moderate infection of *P. pitheci* was taken from Barbara (see p. 449) and was sent by air in a thermos flask with ice from Sandakan to Kuala Lumpur. The blood was immediately inoculated into a splenectomized baby gibbon (*Hylobates lar* (Linnaeus, 1771)) which had been born in captivity. Parasites were seen on a single day in early March and again in scanty numbers on 24 March 1972. Ring forms and schizonts

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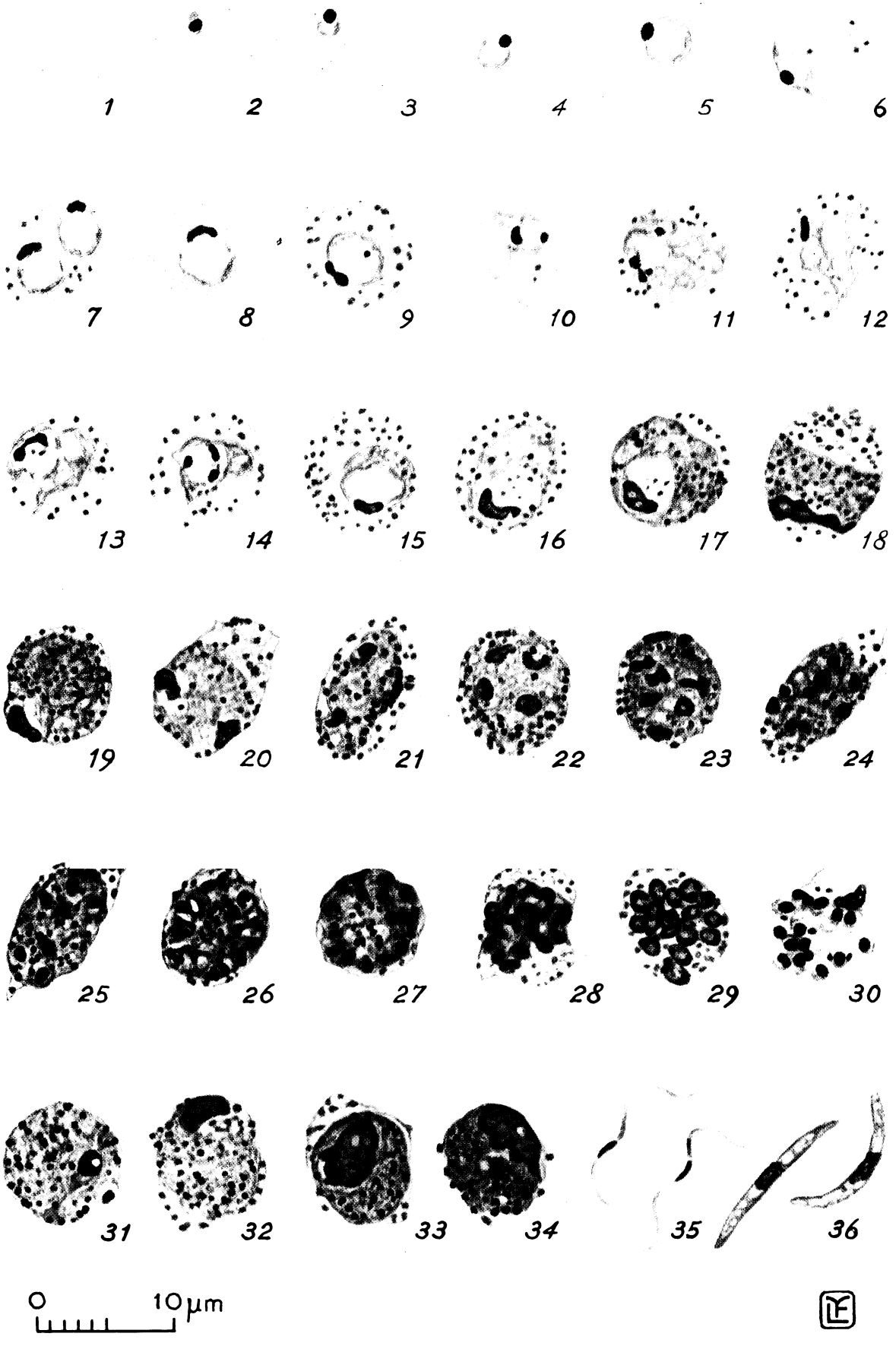
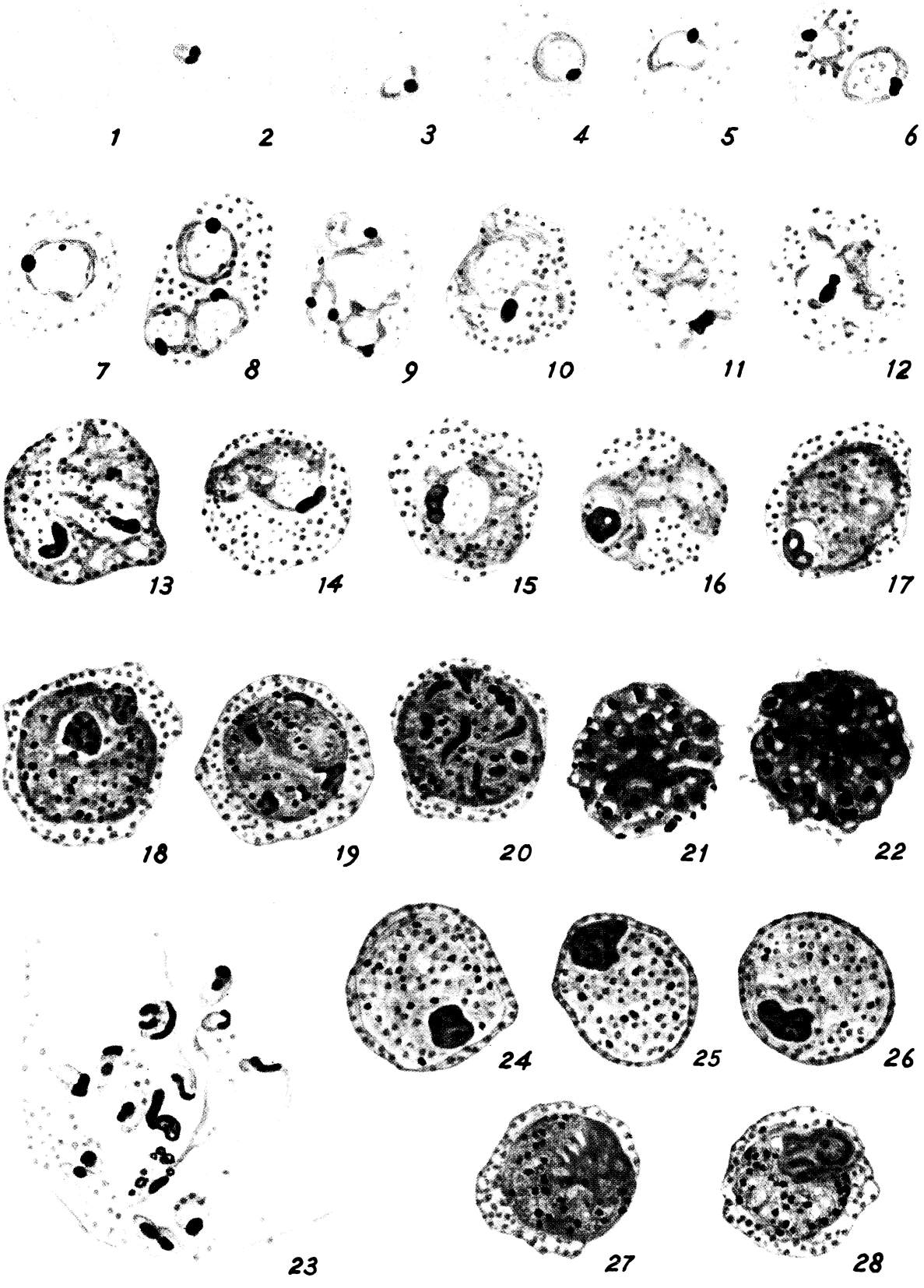
DESCRIPTION OF PLATE 3

Plasmodium pitheci in the blood of the orang-utan (*Pongo pygmaeus*); microgametes, and sporozoites in *Anopheles balabacensis*. 1, Normal erythrocyte; 2–9, ring forms; 10–17, developing trophozoites; 18, 'band-form' trophozoite; 19, mature trophozoite; 20–23, developing schizonts; 24, 25, schizonts in 'ovale-like' host cells; 26–28, maturing schizonts; 29, 30, mature schizonts containing 16 merozoites; 31, 32, macrogametocytes; 33, 34, microgametocytes; 35, free microgametes from *in vitro* preparation; 36, sporozoites from the salivary glands of *Anopheles balabacensis*.



For description see opposite.



For description see opposite.

were present (plate 5, 23–33). The rings had double dots of chromatin, and immature schizonts had 4 nuclei with fine brown pigment between the nuclei. The characteristic stippling of *P. pitheci* was present in erythrocytes infected by the younger and older parasites. No enlargement of the erythrocyte was produced. The blood remained negative on all subsequent examinations, until a year later, when the animal was inoculated with *P. youngi* Eyles *et al.* (1964) from another gibbon. A heavy infection of this parasite quickly ensued and the animal died a week later.

This single cross-infection experiment is important, because it illustrates (i) a difference in susceptibility of the gibbon between *P. pitheci* and *P. youngi* and (ii) the absence of any immunity conferred by *P. pitheci* on a subsequent infection with *P. youngi*. The close resemblance of these two species is discussed on p. 471.

A single experiment was carried out to test the susceptibility of man to *P. pitheci*. Two specimens of *A. balabacensis* infected with this parasite were allowed to bite one of us (R.K.-K.). After the feed the mosquitoes were dissected and were found to have heavily infected salivary glands. Blood films from the volunteer remained negative, and he experienced no fever. From this we conclude that *P. pitheci* is probably not infective to man.

(*b*) *Plasmodium* (*Plasmodium*) *silvaticum* (*plates* 4–6)

(i) *Blood stages in* Pongo pygmaeus (*plate* 4, 1–28).

The following description is based mainly on serial thin blood films taken from a young female, Barbara, with supplementary descriptions of parasites seen in the blood of a young male, Martin. Barbara's blood was studied from 5th to 10th February when the infection with *P. silvaticum* was replaced by *P. pitheci*. Martin showed a mixed infection throughout and was examined from 5th to 14th February. Both animals showed evidence of a double brood of *P. silvaticum*.

Ring forms (*plate* 4, 2–8). The smallest forms seen in the blood of *Pongo* were the youngest rings about 2 μm in diameter. They have a prominent, rounded, darkly staining nucleus that lies within the outline of the parasite and measures about 1.0 μm in diameter. In some mature schizonts the merozoites have a small vacuole in the cytoplasm; after the merozoites have invaded the red cell this becomes enlarged to produce the typical ring form. The parasites grow rapidly and after about 2 h measure just over 3 μm in diameter, but grow more slowly from then on, so that by about 6 h they measure only about 3.5 μm. During this period they show early evidence of amoeboid activity and stained films contain parasites of numerous shapes including some with filamentous, 'tenue-like' processes. A nucleus comprised of double chromatin dots of equal size is seen only rarely and, when seen, the total chromatin content appears to be about the same as that of the single chromatin dots of most ring forms. Very small accessory dots occur quite frequently and these are usually situated at the pole oppposite the main part of the nucleus. Multiple infections of the host cells are not uncommon; up to four parasites have been seen in a single cell. No pigment is visible in these young parasites. The host cells start to enlarge.

Description of plate 4

Plasmodium silvaticum in the blood of the orang-utan (*Pongo pygmaeus*). **1**, Normal erythrocyte; **2–8**, ring forms showing single and multiple infections of host cells; **9–17**, trophozoites in various stages of development (**13** showing multiple infection); **18–21**, schizont formation; **22**, mature schizont with merozoites; **23**, rupturing schizont liberating merozoites and residual body; **24–26**, macrogametocytes; **27, 28**, microgametocytes.

early and this is already obvious 3 or 4 h after they have been invaded. From an average initial diameter of 7 μm the erythrocytes reach about 8 μm after 6 h. Their colour in Giemsa-stained preparations becomes pinker than that of surrounding uninfected red cells; stippling resembling Schüffner's dots begins to appear at about 4 h and is distinct in most host cells after 5 to 6 h, the individual dots increasing in both number and size with age while the 'flushing' also increases.

Trophozoites (*plate* 4, 9–17). The developing trophozoites are highly amoeboid so that, as they grow, the parasites acquire a wide variety of bizarre shapes. Between 21 and 24 h of age trophozoites occupy a large proportion of the host cell, frequently taking up a peculiar dumb-bell shape with one half containing the nucleus and the other consisting only of cytoplasm. However, more solid forms are also common. The parasites measure roughly 5–6 μm across with a nucleus having a diameter up to 2 μm. The nuclear chromatin may form a single, solid bead, be linear or curvilinear, or appear to be fragmented. The total mass of the fragments is about equal to that of the single nuclei in other trophozoites, and fine chromatin connections can usually be detected joining the fragments. The nucleus generally stains an even dark colour but may appear granular or contain clear vacuoles. Doubly infected red cells are not common at this stage. Pigment is first visible in trophozoites of about 20 h and older; it appears as indistinct, straw coloured or slaty grey granules which are difficult to see in normal light. It tends to accumulate towards the periphery of the trophozoites away from the nucleus and is distinguished best with polarized light under which it is strongly birefringent. Stippling of the red cells is very intense and the cells enlarge considerably, reaching 9–10 μm in diameter within 24 h. The individual dots become deep pink in colour and the host cell stroma between the stipples is also markedly more red than neighbouring uninfected erythrocytes. Infected cells are thus very conspicuous and readily detected in a thin blood film, even under a low power of the microscope.

As late as 30 h the trophozoites continue to show marked amoeboid activity. Parasites of this age differ little from those of 24 h except that they are slightly larger, and their nuclei approach 3 μm diameter and become more granular. The pigment is more conspicuous, tending to accumulate in a band or in irregular masses in the cytoplasm, but it is still mainly a faint straw colour. The host cells continue to enlarge and the stippling is very prominent, often concealing the parasite in heavily stained preparations.

Schizonts (*plate* 4, 18–23). The trophozoites continue to grow without evidence of nuclear division until they are about 44–46 h old. They are then more solid and occupy most or all of the host cell. The preschizonts measure 9–10 μm and the host cells 10–11 μm in diameter. Some time before this stage many of the parasites appear to leave the peripheral circulation, and it is assumed that schizogony is completed largely in the capillaries of the deep circulation. The nucleus, which by 44 h is approaching 4 μm in diameter, undergoes a series of changes in preparation for division. A darker staining central mass forms within a rather homogeneous, lighter staining background of nuclear material. The central mass becomes angular in outline and appears to break up into several separate bands or dots. The nucleus then begins to divide, with material of both colours going into each division which starts at 44–46 h of age. Nuclear division may be accompanied by a shrinkage of the entire schizont which is most apparent from 47 to 48 h. The cytoplasm condenses around individual daughter nuclei without actual cytoplasmic division taking place until some 16–24 nuclei are present. The pigment in the early schizonts tends to be in rod-shaped or rounded granules, from light to dark brown in colour. When nuclear division is well advanced, the pigment begins to clump until it forms a well defined mass

containing 10 or more large grains. The red cell by this time is up to 11 μm in diameter. The Schüffner's dots are pushed out to occupy the residual stroma surrounding the schizont so that the remaining host cell sometimes takes on a dark red appearance due to the accumulation of dots. Individual mature merozoites measure about 2 μm and contain a nucleus half this diameter. Schizonts mature at about midday on alternate days. In both series studied (i.e. from Barbara and from Martin) double broods of the parasite were present so that some mature schizonts were present each midday, though it must be noted that such forms can be found only after a long search. Within each brood a marked degree of synchronicity was apparent.

Gametocytes (*plate* 4, 24–28). It was difficult to distinguish between immature macrogametocytes and mature trophozoites just before nuclear division. Macrogametocytes apparently about 24 h old are solid parasites about 5.5 μm in diameter with a uniformly dark blue cytoplasm and a single darkly staining nucleus nearly 2 μm in diameter. The pigment is very pale and scattered throughout the cytoplasm. The parasites grow to a diameter of about 7 μm with nuclei roughly a third of that size. They lie within red cells that have become enlarged up to about 9 μm in diameter and which contain heavy Schüffner's stippling. The pigment in mature macrogametocytes consists of numerous, scattered roundish granules, straw to light brown in colour or sometimes more bacilliform in shape. The compact nucleus is usually eccentrically situated and may be associated with a vacuole in the cytoplasm; it is sometimes oval or a convex lens-shape. The parasites continue to enlarge until they almost completely fill the red cell, compressing the Schüffner's dots together, and sometimes forming a dark red rim outside the macrogametocyte. At this stage the parasites reach a diameter between 9 and 12 μm, the host cell being slightly larger. The nucleus which may show an outer light pink zone and an inner, more densely staining core, is from 3 to 4 μm in diameter. In these mature macrogametocytes the pigment is often bacilliform, dark brown to black and may concentrate peripherally, or at least leave a clear area adjacent to the nucleus.

Microgametocytes are somewhat smaller than the macrogametocytes, stain a mauve colour and contain a large diffuse granular nucleus that occupies about half the diameter of the parasite. The microgametocytes reach a diameter of 7.5 to 9 μm or more, with a nucleus of about 4–5.5 μm. The host cells which have the usual heavy Schüffner's stippling enlarge to 9–10 μm. The pigment, which is variable in shape and size, and tends to accumulate in bands is darkish brown in colour and absent over the nucleus. The microgametocytes grow to 10 μm in diameter and the nuclei to 6 μm. At this stage the nuclear chromatin is usually coarsely granular. The diameter of the host cell may reach nearly 11 μm.

(ii) *Blood stages in* Pan troglodytes verus *Schwartz*, 1934 (*plate* 5, 2–21)

The morphology and cycle of the erythrocytic stages of *P. silvaticum* were observed in the blood of a young splenectomized chimpanzee, Khan, who was inoculated with blood taken from orang-utan Barbara. The infection first became patent 23 days after the inoculation. After a few days it was apparent that a double brood was present as in the donor.

The general morphology of the ring stages and trophozoites (plate 5, 2–11) is very similar to that seen in the blood of the orang-utan although the blood of Khan showed marked anisocytosis and poikilocytosis. Numerous unidentified objects (plate 5, 6–9) were seen in erythrocytes on most of the blood films made over a period of 14 days, some resembling *Eperythrozoon* but others appearing to occupy an intracellular position. The nature of these bodies is discussed on p. 452. Mature erythrocytes appeared to be slightly larger than those of the orang-utan, about 7 μm or

a little more in diameter. The youngest ring forms seen, estimated to be from 1 to 2 h old, were just over 3 μm in diameter with a nucleus about 1 μm. Schüffner's dots were already beginning to appear even in red cells occupied by such young parasites. These showed evidence of amoeboidicity when only about 2 h old.

By about 20 h the parasites become very amoeboid and grow to about 6 μm in diameter with a nucleus of 1.5 μm. The host cells at this time vary greatly in size, ranging from about 8.5 to 9.5 μm, reflecting the anisocytosis noted in uninfected red cells. Schüffner's dots are prominent. Forms with two nuclear masses are relatively common at this stage. Within the next 6 h many trophozoites become more solid in form and some light brown pigment granules are detectable, usually localized at the periphery. The nuclei may take on a more granular appearance. As in the orang-utan, schizogony appears to start very late in the cycle. As the nucleus starts to divide, (plate 5, 12–14), clefts appear in the cytoplasm which shows a foamy appearance (plate 5, 15–17). A curious gap between the margin of the parasite and the stroma of the erythrocyte was sometimes seen; such a split has not been observed by us in other malaria parasites. Clefts are also visible within the cytoplasm of some of the larger parasites (plate 5, 16). The straw coloured pigment gathers towards one part but only collects into the residual body when the merozoites are virtually mature, when the granules appear dark golden brown to black in colour. The mature schizonts (plate 5, 18 and 19) are about 8 μm in diameter within a cell, some 10–11 μm in diameter. This contains heavy Schüffner's stippling which remains evenly distributed throughout the pale-staining residual host cell stroma. Individual merozoites are similar to those in the orang-utan and 16–24 are normally found in each schizont; a maximum of 28 nuclei was seen.

In the later stages of the infection, multiple infection of red cells was common, with parasites of all ages present.

Macrogametocytes and microgametocytes (plate 5, 20 and 21) develop in a similar manner to those in the orang-utan. Pigment granules are bacilliform or rounded, variable in size and blackish in colour.

Immature macrogametocytes were provisionally identified on the 5th day of patency, but as mentioned above, it is difficult to be sure that such forms are not asexual trophozoites. A strange feature was noted in such parasites: in the centre of the reddened stroma of the erythrocyte, unoccupied by the parasite, was a darker rectangular mass, perhaps representing the accumulation of Schüffner's dots. Maturing gametocytes of both sexes (with the females predominating) first appeared on the 14th day of patency and became numerous two days later.

(iii) *Sporogonic stages* (*plate* 6, 1–3)

A more complete record of the sporogony of this species was obtained than that of *P. pitheci*, as the experiments were carried out on an experimentally infected chimpanzee at Kuala Lumpur,

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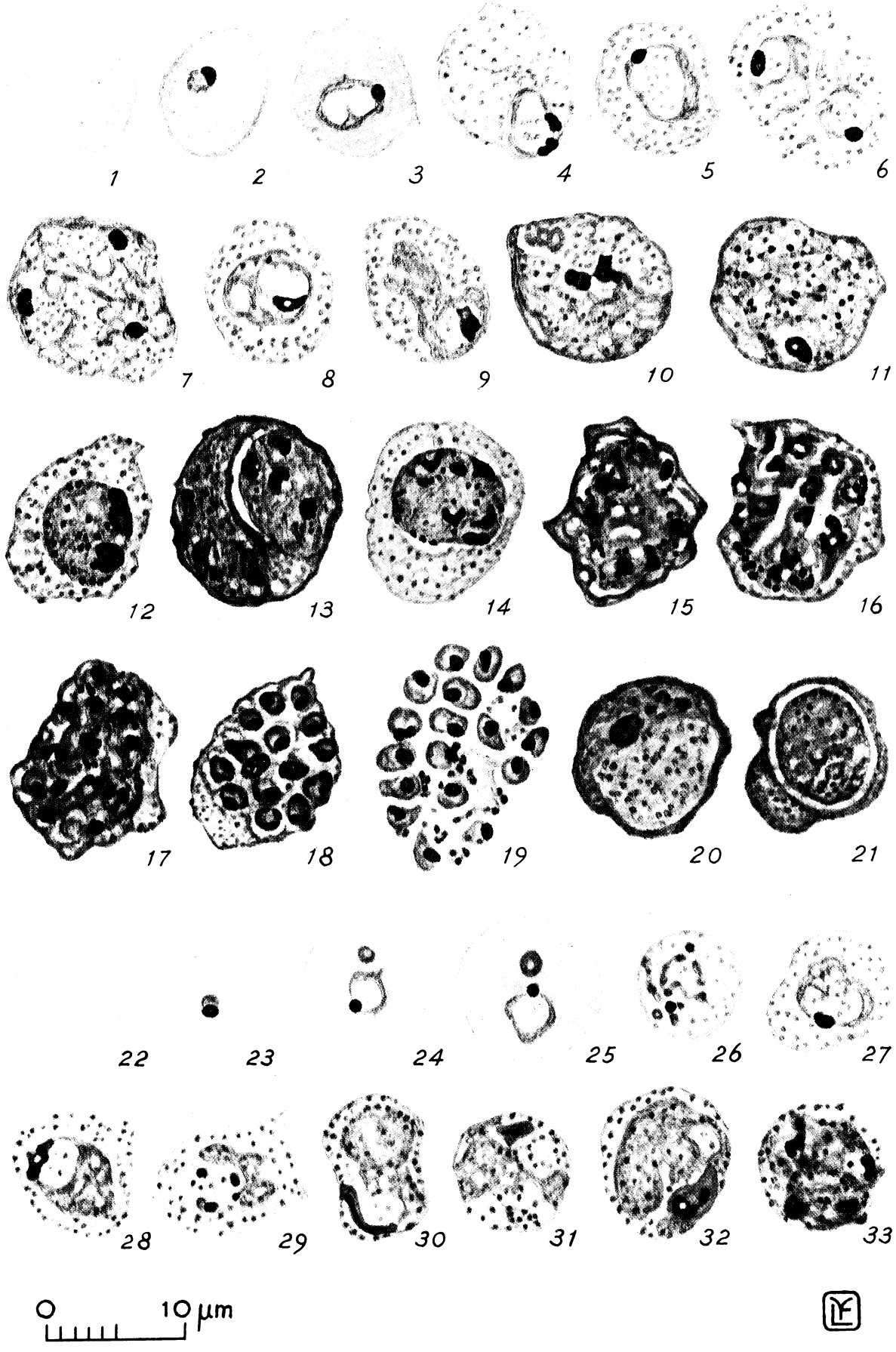
DESCRIPTION OF PLATE 5

Plasmodium silvaticum in the blood of the splenectomized chimpanzee (*Pan satyrus*); *Plasmodium pitheci* in the blood of the splenectomized gibbon (*Hylobates lar*). 1, Normal chimpanzee erythrocyte; 2–21, *P. silvaticum*; 2–5, ring forms; 6, 7, multiple infections with young trophozoites; 8, developing trophozoites; 12–14, young schizonts; 15–17, maturing schizonts with clefts in cytoplasm; 18, 19, mature schizonts with 12 or 16 merozoites; 20, macrogametocyte; 21, microgametocyte; 22, normal gibbon erythrocyte; 23–33, *P. pitheci*; 23, very young trophozoite; 24, 25, ring forms; 26–32, trophozoites in various stages of development; 33, immature schizont.



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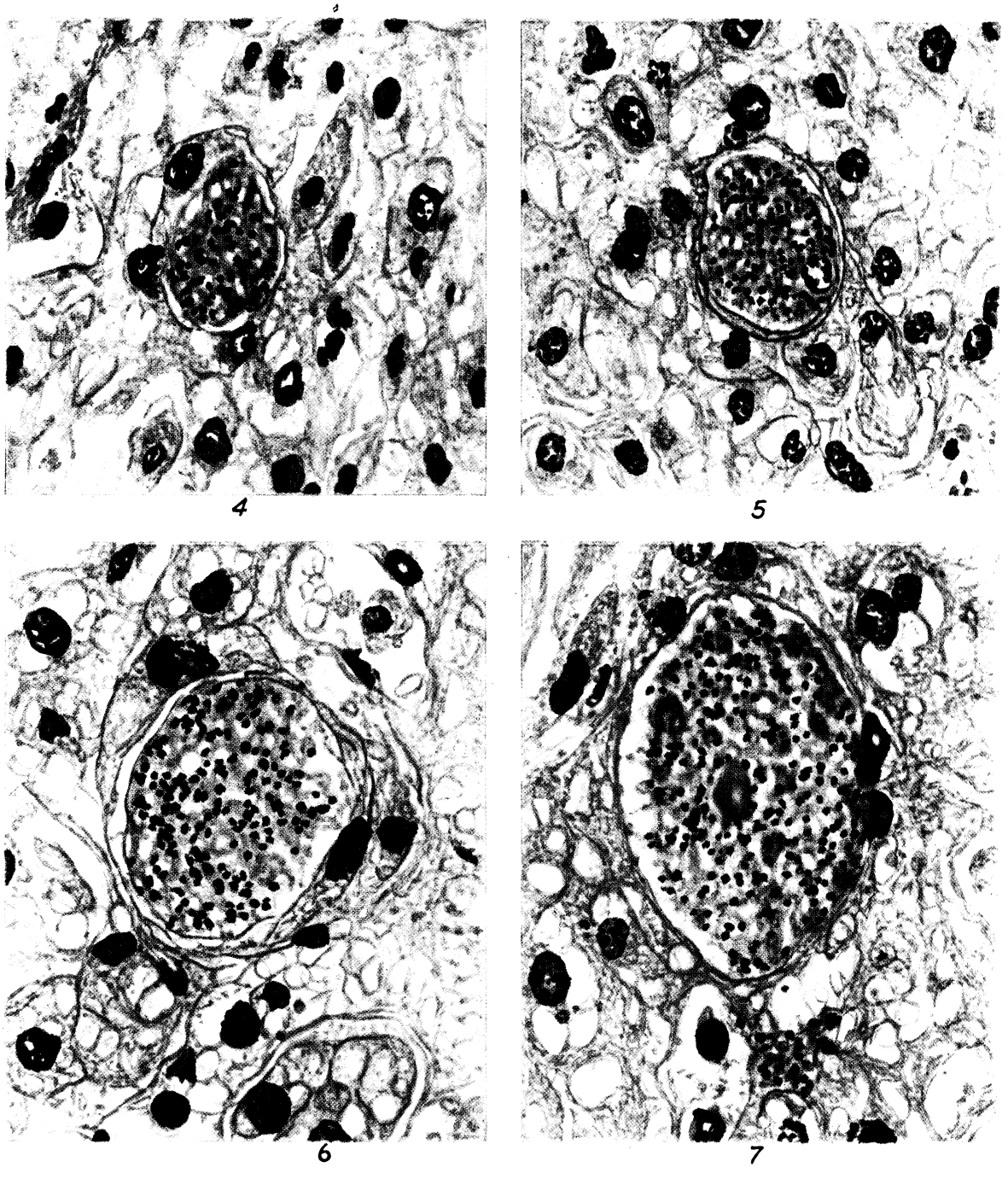


For description see opposite.

and conditions were easier than when mosquitoes were fed on orang-utans in the field. The same basic techniques were used as for the latter species (see p. 481).

Exflagellation and ookinetes. Exflagellation of the microgametocyte begins 9 minutes after taking a preparation of the blood and continues for 5–10 min, at a temperature of 26–27 °C. The free microgamete (plate 6, 1) is about 18 μm long, and contains a prominent oval nucleus.

Ookinetes (plate 6, 2) are numerous in smears of the contents of guts of mosquitoes which had fed on the infected chimpanzee. After 18 h they appear rather stumpy (14 × 4 μm) with one end broad and the other narrow. The broad end sometimes terminates in a small protuberance (possibly the retractile apex as demonstrated in other species by electron-microscopy by Garnham, Bird & Baker 1969). A deeply staining dot is present inside or close to the nucleus, and the nucleus itself is composed of 4 or 5 small dots set in a lighter staining background. A circular vacuole is sometimes present in the cytoplasm and at the broad end the azurophil cytoplasm is replaced by a zone of fine pigment or yellowish material.

TABLE 2. OOCYST INFECTION RATES OF *P. SILVATICUM* IN MALAYAN *ANOPHELES*

species	number dissected	infection rate (%)
A. balabacensis Perlis	298	51
A. balabacensis Sabah	8	25
A. kochi	199	41
A. maculatus	209	21
A. sundaicus	199	20

Oocysts and sporozoites. Figure 3 (p. 460) shows the diameter of oocysts of *P. silvaticum*, measured in fresh specimens of mosquito guts at different days after feeding. The mosquitoes were kept in an insectary at a temperature of between 27 and 29 °C. On the 4th day, the pigment in the oocyst is scattered, but on the following days it usually assumes a linear or semicircular pattern; the individual grains are small and brown in colour. Late in sporogony, the pigment is invisible. On about the 7th day, the contents of the oocyts become differentiated by sporoblastoid formation and actual sporoblasts can be seen on the 9th day. The latter are 4–8 μm in diameter and about 40 are present. When such oocysts are ruptured, immature sporozoites are seen to project from the surface of the sporoblasts and the object resembles a sea-urchin. Mature oocysts are first found on the 10th day. Most are 40–48 μm in diameter but some are outside this range.

Sporozoites first appear in the salivary glands 11 days after the infective feed, and are very numerous. They are non-motile and in fresh preparations are 11 μm in length (plate 6, 3).

The infectivity rates in different species of *Anopheles* varies, but the best host seems to be the Perlis strain of *A. balabacensis*, in that the greatest numbers of oocysts are produced and the oocysts in the mosquito show least variation in size; also the salivary glands are invaded more consistently. Even in this species, the individual number of oocysts in mosquitoes of the same batch varies greatly from 1 to 500. The infection rates are shown in table 2.

DESCRIPTION OF PLATE 6

Sporogonic stages of *P. silvaticum* in *Anopheles balabacensis* and exoerythrocytic schizonts in sections of liver of the chimpanzee (*Pan satyrus*). 1, Microgametes from *in vitro* preparation; 2, ookinetes; 3, sporozoites; 4, 5, five-day old exoerythrocytic schizonts; 6, 7, seven-day old exoerythrocytic schizonts.

(iv) *Tissue stages in* Pan troglodytes verus (*plate* 6, 4–7)

Orang-utans could not be subjected to dangerous experimental procedures, and we therefore planned to study the tissue schizogony of *P. pitheci* in other primates. For this purpose, we had taken two splenectomized chimpanzees and two night monkeys (*Aotus*) to Borneo (see p. 478). Observations on the tissue forms of many other malarial parasites have been made on similarly abnormal models ('surrogate hosts') when the use of the natural hosts was impossible or undesirable, e.g. the malaria parasites of man. With few exceptions, morphological differences in the schizonts are minimal in these circumstances.

When we found the second parasite (*P. silvaticum*), it was decided to use one chimpanzee and one *Aotus* for *P. pitheci*, and the other pair for *P. silvaticum*. Unfortunately, *P. pitheci* did not prove to be infective to these hosts, and we were unable to obtain sufficiently large numbers of infected mosquitoes (after feeding on orang-utans exhibiting this parasite) to attempt to demonstrate the tissue forms of this species. *P. silvaticum*, on the other hand, became successfully established in the blood of the chimpanzee Khan, and this infection eventually produced enough gametocytes to infect a good proportion of mosquitoes (see p. 465).

On 8 March 1972, an estimated 3000 hungry female mosquitoes comprising four species (*A. balabacensis*, *A. kochi*, *A. sundaicus* and *A. maculatus*) were permitted to engorge on Khan. By 20 March sporogony was complete and sporozoites were in the salivary glands of the mosquitoes; glands from 140 *A. balabacensis* were dissected, and a suspension of sporozoites was prepared and inoculated intravenously into the second chimpanzee, Sandy. A second suspension was prepared from the glands of 70 less heavily infected mosquitoes (*A. balabacensis*), and was inoculated two days later.

On 27 March, 7 days after the first inoculation and 5 days after the second, a biopsy of liver was taken from Sandy. A second biopsy was planned for 29 March, but the animal died under the anaesthetic just before the first operation.

In the liver, one schizont was found in about every 20 sections (80 mm^2); in some sections portions of three schizonts were seen. We anticipated that there would be schizonts of two different ages (7 and 5 days), with fewer younger than older schizonts. We based this opinion on two considerations: (1) small schizonts must be visible in fewer serial sections than are large ones, and therefore they always appear to be less abundant; (2) the second inoculum giving rise to 5-day-old schizonts was from a comparatively small number of mosquitoes which were less heavily infected than those used to prepare the first. From the size and relative numbers of the two types of schizonts our supposition appears to have been correct. The mean diameter of 11 schizonts considered to be 7 days old was 31 μm (28–35 μm); that of 3 schizonts thought to be only 5 days old was 20 μm (14–23 μm). (In a preliminary note (Killick-Kendrick *et al.* 1972) we gave the mean diameter as 37 μm; this was an over-estimate based on too few measurements.) Between these two sizes there was no overlap in the measurements, with the maximum size of the 5-day-old forms being 23 μm and the minimum size of the 7-day-old forms, 28 μm. The age of the many other schizonts examined could similarly be judged by their size.

No fully mature schizonts were seen, and no parasites were found after exhaustive searches of thick blood films prepared from Sandy at the time of his death. The precise time of development of the exoerythrocytic schizonts of *P. silvaticum* in the liver of the chimpanzee is, therefore, not known. However, the general appearance of the 7-day-old schizonts leads us to believe that they would probably have matured after one more day. It must be admitted that this is no more

than an estimate based on observations on many other species of malaria parasites of primates; there were no morphological features, such as aposchizogony, to confirm that the schizonts were in the last stages of development.

The general morphology of schizonts judged to be 5 or 7 days old differed little and except where otherwise stated the following description applies to both. The general shape of the schizonts was oval or, less commonly, circular. Nearly half the larger schizonts had shrunk at one side leaving a space in which strands of cytoplasm were seen connecting the mass of the parasite to the outer membrane (plate 6, 7). The limiting membrane was fine and often clearly visible; it looked thicker in shrunken schizonts.

The cytoplasm was characteristically granular and vacuolated. With one exception, the vacuoles were small and indistinct, and were often most numerous at the periphery. The exception was one small schizont (25 μm × 19 μm) which contained two vacuoles up to 4 μm in diameter, around the edges of which were red granules and streaks (plate 6, 5). Clumped masses of cytoplasm, up to 15 in number in one section, were common (plate 6, 7). In one schizont, curious small vacuoles lay in the cytoplasmic clumps. The cytoplasm of some schizonts, notably the smaller, was markedly granular and stained a deep blue, partially obscuring the nuclei. In others it formed a palely staining tenuous network against which the nuclei stood out boldly.

Most nuclei were round and stained heavily. In a few schizonts there were a number of elongate or angular nuclei scattered among the others. Nuclei usually varied in size from 0.5 to 1.0 μm in any given schizont. However, those of some were practically all the larger size and of strikingly uniform appearance. They were presumably in a synchronous state of development just prior to division. In some schizonts the nuclei tended to lie along the edges of strands of cytoplasm, whereas in others they looked as if they lay in vacuoles. There were no obvious nuclear patterns or cytoplasmic clefts, but in a few instances a small number of nuclei lay in short parallel lines.

A rare feature was the presence of red dots patchily distributed in the cytoplasm. The colour of the dots was the same as that of the nuclei and they may have been deposits of extranuclear DNA. They bore no obvious relationship to the nuclei, but sometimes lay in pairs resembling small diplococci. The dots were seen only in schizonts with palely staining cytoplasm; when the cytoplasm stained heavily they would have been obscured.

The nuclei of infected parenchymal cells were almost completely unaffected by the parasites. They were not enlarged and, in comparison with the nuclei of uninfected cells, the only difference was a slight change of staining reaction from the typical rich purple colour to a lighter reddish tinge. This was most noticeable when viewed under low power objectives (× 10 or × 20).

Our observations on exoerythrocytic schizogony of *P. silvaticum* were confined to a single, 'surrogate host' – a splenectomized chimpanzee. The susceptibility of this model is less than that of the natural host as shown by the behaviour of the parasite in the blood. We could also anticipate changes (including the size) in the appearance of the tissue schizonts of *P. silvaticum* in the abnormal host. It has been shown, for instance, by Sodeman, Contacos, Jumper & Smith (1972) that the 7-day schizonts of *P. hylobati* Rodhain, 1941 are larger in another abnormal host, *Aotus trivirgatus*, than in the natural host, *Hylobates moloch*. On the other hand, Sodeman, Contacos, Coatney & Jumper (1969) found that the schizonts of the human *P. falciparum* (Welch, 1897) are smaller and take longer to develop in the *Aotus* monkey than in man or the chimpanzee. Similarly, Baerg, Rossan & Young (1974) observed that the tissue stages of *P. vivax* grow more

slowly in the liver of *Ateles* E. Geoffroyi, 1806 than in that of man or chimpanzee. However, the exoerythrocytic stages of *P. falciparum* and *P. vivax* are practically identical in man and chimpanzee. It follows from this that some signs of abnormality, e.g. degeneration, could perhaps be expected in our material and indeed a few schizonts in the liver of the chimpanzee showed an apparent coagulation of the cytoplasm which was broken up into strands or pieces separated by large spaces; the nuclei in such schizonts were reduced in number. The size of these forms was somewhat less than the healthy schizonts. There were no signs, however, of phagocytosis of dead schizonts.

The characters differentiating the tissue stages of *P. silvaticum* from some other malaria parasites of primates are discussed on p. 471.

(v) *Differential diagnosis*

The differential diagnosis of the erythrocytic stages of *P. silvaticum* and *P. pitheci* is given on p. 460. *P. silvaticum* and *P. vivax* resemble each other closely in the blood of their respective hosts.

P. silvaticum develops easily in *Anopheles balabacensis* and less well in other Malaysian species. The details of sporogony are fairly similar to those of *P. pitheci*, although sporozoites of the former appear in the salivary glands a day later; the distribution of malaria pigment in the oocysts is the same in the two species. Exoerythrocytic schizogony has hitherto not been observed in *P. pitheci*, and the exact length of the process in *P. silvaticum* was not determined; however, the appearance of the 7-day schizont in the liver suggests that it would mature on the 8th day after inoculation of sporozoites. The schizonts at 7 days are smaller (31 μm) than those of other species in the '*vivax*' group and large vacuoles are absent; there is no enlargement of the host cell nucleus.

(vi) *Infectivity to primates other than* Pongo pygmaeus

P. silvaticum is infective to splenectomized chimpanzees, but the splenectomized gibbon, splenectomized *Aotus* and rhesus monkey are apparently insusceptible. The susceptibility of man is unknown.

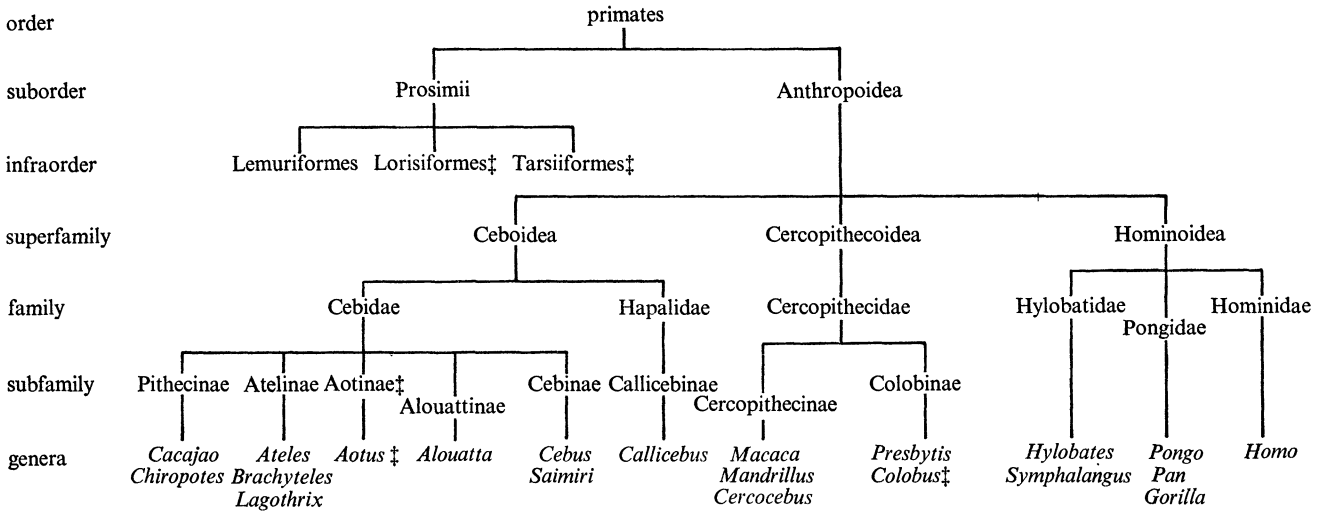
8. Phylogeny and affinities of the malaria parasites of the orang-utan and of other anthropoid primates

As a prelude to consideration of this topic it may be noted that all these primates share several species of intestinal protozoal parasites, e.g. *Entamoeba histolytica*, *Balantidium coli*, and various flagellates, though some other parasites are limited to special hosts. The curious ciliate *Troglodytella* Brumpt and Joyeux, 1912, for example, is found only in gorillas and chimpanzees.

More specificity is exhibited, on the other hand, by the blood protozoa of primates, and particularly by the intraerythrocytic malaria parasites (see p. 470). Susceptibility of a primate other than the normal host is greatly enhanced if its spleen is removed, though this is not invariable, e.g. the human malaria parasites will not develop in the Old World monkeys even if they have been splenectomized. In contrast to this, human parasites multiply readily in *Aotus trivirgatus griseimembra* Elliot, 1912 and some other New World monkeys, animals which are assumed to be less closely related to man than are the Old World monkeys (see table 3). This susceptibility is associated with the presence of 2 α-globulins in the serum of these monkeys (Collins *et al.* 1974). (The much less susceptible nominate subspecies *Aotus trivirgatus trivirgatus* possesses 3 α-globulins.)

The original undifferentiated primate stock presumably possessed the genes for susceptibility to the malaria parasites of higher primates. Such genes are present in the ceboid line which became isolated in the New World; they are also present in the hominoid line but they never entered the Cercopithecoidea.

TABLE 3. CLASSIFICATION OF THE PRIMATES† SHOWING THOSE WHICH ARE NATURAL HOSTS OF *PLASMODIUM* SPECIES



† After Napier & Napier (1967).
‡ Taxa in which there are no known natural hosts of *Plasmodium*.

The orang-utan, chimpanzee, gorilla, and man, possess the minor haemoglobin-A_2 component, which is also present in *Aotus* and other New World monkeys that are susceptible to human species of *Plasmodium*, but which is absent from most Old World monkeys (Barnicot, Wade & Cohen 1970; Barnicot & Jolly 1966; Huser 1970). The latter are insusceptible to the human *Plasmodium* spp.

Kingdon (1971) points out that the African apes and man differ markedly from *Pongo* in the patterns of their serum proteins and in their chromosome karyotypes, although the chromosome diploid number of *Pongo* (48) is the same as that of *Pan* and *Gorilla* (Napier & Napier 1967). The number in *Homo* is 46, and in *Hylobates* it is 44 (Chiarelli 1962).

One of the chief objects of the work in Borneo was to see how *Plasmodium pitheci* (and, as it turned out, the new species, *P. silvaticum*) fitted into the evolutionary pattern. We think that the following criteria are likely to be most significant for this purpose: (*a*) morphology of the parasite – in *Anopheles*, liver and blood; (*b*) changes produced in the host's erythrocytes; (*c*) periodicity, i.e. length of developmental cycle (in the three sites); (*d*) susceptibility of other primates, and (*e*) cross immunity to related parasites.

(*a*) *Relationship between* P. silvaticum *and other* vivax*-like parasites of the higher primates*

P. silvaticum bears similarities to *P. vivax* of man, *P. schwetzi* Brumpt, 1939 of the chimpanzee and gorilla and *P. eylesi* Warren, Bennett, Sandosham & Coatney, 1965 of the gibbon. This newly found species has a tertian periodicity in the blood, where it enlarges the erythrocyte and produces the highly distinctive Schüffner's dots. Such characters show that *P. silvaticum* belongs

to the *vivax-cynomolgi* group, though there are a few features (e.g. blood stages) which distinguish it. It most closely resembles *P. eylesi*, but its behaviour in other primates is slightly suggestive that *P. silvaticum* is nearer to the parasites of the higher hominoids than to those of the gibbon. The parasite proved to be non-infective to the gibbon, rhesus or *Aotus* even when the recipient animals had been splenectomized. It produced a good infection in a splenectomized chimpanzee although the incubation period was longer than expected (23 days), and the course of parasitaemia was rather short. In other words, the splenectomized chimpanzee is not quite as good a host for *P. silvaticum* as it is for *P. vivax*. However, the affinities of these parasites are best established by observing their respective behaviour in the surrogate host – a splenectomized chimpanzee – and we made a direct comparison of *P. silvaticum* and *P. vivax* in the blood of such animals. Malaria species are commonly observed in hosts other than the natural one and comparisons of morphology are frequently not made in the natural host. True affinities will only be appreciated if the parasites are studied (*a*) in their natural hosts, (*b*) in each other's hosts and (*c*) in a surrogate host susceptible to both. Unfortunately all three procedures cannot often be followed.

TABLE 4. SUSCEPTIBILITY OF BLOOD OF MAN, APES, AND SOME LOWER MONKEYS TO MALARIA PARASITES OF THE *VIVAX* GROUP (SEE GARNHAM 1966)

	Homo	*Pan*	*Gorilla*	*Pongo*	*Hylobates*	*Macaca*	*Alouatta*	*Aotus*
P. vivax	N	+++S	.	−†	±‡	−S	.	+++S¶
P. schwetzi	±	N	N	.	.	−§	.	.
P. silvaticum	.	++S	.	N	−S	−S	.	−S
P. eylesi	±	.	.	.	N	−	.	.
P. cynomologi	+	.	.	.	.	N	.	.
P. simium	+	.	.	.	.	−S	N	.

N, natural host; S, splenectomized; +++, high parasitaemia; ++, moderate parasitaemia; +, low parasitaemia; ±, transient infection; −, no infection.

† Koch (1900). ‡ Cadigan, Ward & Puhomchareon (1968).
§ Rodhain, Van Hoof & Muylle (1938). ¶ Young, Baerg & Rossan (1975).

The two strains of *P. vivax* that we have studied in *Pan* are from Madagascar and Korea, although the ideal comparison would have been one between *P. vivax* of Bornean origin and *P. silvaticum*, but such material was not available.

When the host has been splenectomized all stages of the blood forms may be seen in advanced infections and synchronicity tends to become lost, both in *P. vivax* and *P. silvaticum*. The following points of difference were observed between these two species in the blood of *Pan*:

Red cells containing ring forms of *P. silvaticum* show an earlier, light pink staining, even before Schüffner's dots are numerous, and trophozoites of *P. silvaticum* are more amoeboid than those of *P. vivax*. The pigment remains very pale and inconspicuous in *P. silvaticum* but darker and more obvious in *P. vivax*. Even half grown trophozoites of the latter have very conspicuous pigment. The Schüffner's dots are fine in *P. vivax* and coarser in *P. silvaticum* but the most obvious differences in the host cells are the marked pink flush of those infected with *P. silvaticum* and their large size. These distinctions are accentuated as schizogony progresses, and stippling becomes condensed as a rim around the schizonts of *P. silvaticum*, but not of *P. vivax*. The host cells are much larger in *P. silvaticum* than in *P. vivax* infections (up to 15 μm as compared with 10–11 μm in the latter). Clefts in the cytoplasm of *P. silvaticum* and their absence in *P. vivax* are a notable distinction.

Nuclear division of *P. silvaticum* begins later and proceeds more rapidly than that of *P. vivax*. There is no difference in the number of merozoites formed in the two species.

Perhaps the most important difference is the readiness with which *Pan* is infected with *P. vivax* following splenectomy, whereas in our single experiment with *P. silvaticum* there was a long prepatent period after blood inoculation. However, it must be remembered that Khan's blood contained an unidentified structure (p. 452) and that this may have interfered with the establishment of the malaria infection in this animal. Once established, *P. silvaticum* proliferated and produced gametocytes that proved infective to anopheline mosquitoes.

The relative susceptibility of the blood of primates to homologous and heterologous malaria parasites of the *vivax-cynomolgi* group is shown in table 4.

No direct comparison has been made between the African species *P. schwetzi* and *P. silvaticum*; it does appear that, like those of *P. vivax*, the schizonts of *P. schwetzi* show less tendency to retreat to the deep capillary circulation in their natural hosts than do those of *P. silvaticum*. Moreover schizogony starts earlier in the cycle in *P. schwetzi* than in *P. silvaticum* where it begins exceptionally late.

From the blood stages of the gibbon parasite, *P. eylesi*, the main distinguishing features from *P. silvaticum* are the following:

(1) the trophozoites of *P. eylesi* are only slightly, instead of highly amoeboid;

(2) extreme multiple infections of erythrocytes are seen only in *P. eylesi*, though double and occasionally triple or quadruple infections are not rare in *P. silvaticum*;

(3) the peak rupture of schizonts is 5 h earlier in *P. eylesi*;

(4) probably the character of the stippling is slightly different in the two species.

The size and rate of growth of exoerythrocytic schizonts of primate malaria parasites are useful taxonomic characters, and it is interesting to compare the figures relating to *P. silvaticum* with those of *P. vivax* and *P. cynomolgi*. The last two parasites mature at about 8 days after the inoculation of sporozoites, the estimated day of maturation of *P. silvaticum*. At 7 days, the diameter of schizonts of all three is approximately the same, between 25 and 30 μm. Morphological characters are also similar, e.g. vacuoles on the 7th day, solid type of nucleus, absence of plaques and pseudocytomere formation. This confirms the close relationships of these parasites as exhibited by the blood stages. Summing up the available information, it seems likely that *P. silvaticum* stemmed from the *vivax* : *eylesi* : *cynomolgi* stock (fig. 4), but at an early date, since when it has become well isolated and its characters fixed.

(*b*) *Relationship between* P. pitheci *and similar parasites of gibbons*

Malaria parasites of the *pitheci* group (*P. pitheci*, *P. youngi* and *P. hylobati*) are readily differentiated from other tertian parasites of primates by the characteristic appearance of infected erythrocytes. They cause little or no enlargement of the cell, and give rise to stippling which is markedly larger than the Schüffner's dots of the *vivax* group and which does not normally coalesce to form plaques as in *P. fieldi* Eyles, Laing & Yap, 1962. *P. jeffreyi* Warren, Coatney & Skinner, 1966, one of four species described from gibbons, is separable from all other tertian parasites of primates by an absence of stippling of any kind.

Within the *pitheci* group, identification by characters of blood stages is difficult, and well stained films with numerous parasites are essential. Diagnosis of poorly stained material is generally not possible. The principal morphological differences of the blood stages of *P. pitheci* of the orang-utan and *P. youngi* and *P. hylobati* of gibbons are summarized in table 5. Schizonts of

the parasites of the gibbons produce a wider range of merozoites than *P. pitheci*. *P. hylobati* is separable by the character of the stippling and the compactness of the trophozoites. *P. youngi* is more difficult to differentiate from *P. pitheci*. In similarly stained films, however, differences in the stippling are recognizable. Stippling of young trophozoites of *P. youngi* is more numerous and somewhat finer than that of *P. pitheci* at a similar point of growth. Stippling of older forms is superficially similar, but the stippling of *P. pitheci* is notable by always consisting of discrete, countable dots. Of the three species, *P. pitheci* and *P. hylobati* have lighter pigment whereas that of *P. youngi* is prominent and obvious. All these characters are subtle and some would be inapplicable if the parasites were in an abnormal host.

TABLE 5. MORPHOLOGICAL DIFFERENCES IN THE BLOOD STAGES OF MALARIA PARASITES OF THE *PITHECI* GROUP IN NATURAL HOSTS

	P. pitheci (*Pongo*)	*P. youngi* (*Hylobates*)	*P. hylobati* (*Hylobates*)
trophozoites	amoeboid or compact	amoeboid	rarely amoeboid
schizonts	14–16 merozoites	12–20 merozoites, usually 14	12–20 merozoites, usually 14
pigment	inconspicuous	conspicuous	inconspicuous
host cell			
(i) shape	'ovale' shape common	'ovale' shape uncommon	'ovale' shape absent
(ii) stippling of young forms	few large stipples	many fine stipples	absent?
(iii) stippling of old forms	large and discrete	large and abundant	tends to condense around rim

Perhaps the strongest evidence for the difference between *P. pitheci* and the two gibbon parasites of this group is the insusceptibility of a splenectomized laboratory-bred gibbon (see p. 460). *P. pitheci* is not as easy as *P. silvaticum* to classify within a group. Both species have a tertian periodicity in the blood, but the absence of the characteristic stigmata in erythrocytes infected with *P. pitheci* and the lack of enlargement of the host cell indicate that this species does not belong to the *vivax-cynomolgi* group. The stippling however is very striking and differs markedly from that produced by any other species occurring in man or African apes; it is also different from the stippling of parasites of the Asian monkeys and, as stated above, resembles most that of *P. youngi* or *P. hylobati* of the gibbon.

Sepilok Forest in common with other forests in Borneo and Sumatra has a large population of gibbons and monkeys, most of which are hosts of malaria parasites. We examined a few specimens from Sepilok and found *Hepatocystis semnopitheci* (Knowles, 1919) in two specimens of *Macaca nemestrina*, and *Plasmodium hylobati* in one out of three gibbons (*Hylobates moloch*). The latter parasite was of some interest owing to its resemblance to *P. pitheci* and, as both gibbons and orang-utans occupy the same environment, it would be easy for *A. balabacensis* to convey the infection from one to another, and for the morphology of the respective parasites to become altered in the other host. Two hypotheses can be suggested. Either, *P. pitheci* represents *P. hylobati* (or *P. youngi*) in orang-utans in present-day conditions, or *P. pitheci* arose in the past from *P. hylobati*, gradually became adapted to and finally speciated in the new host (the orang-utan).

A strong argument against the former theory is that a splenectomized gibbon (laboratory bred) was either insusceptible to, or could only very feebly support, an infection of *P. pitheci*. Moreover, the gibbon, when challenged later with *P. youngi*, died of a fulminating infection, i.e.

there was no cross-immunity between the two species. Incidentally, there is no cross-immunity between the two parasites of the orang-utan – *P. silvaticum* and *P. pitheci*, which readily co-exist in the same animal in nature. The affinities of *P. pitheci* seem to be closer to the gibbon parasites (*P. youngi* and *P. hylobati*) than to those of the higher hominoids (see table 5).

Halberstaedter & Prowazek (1907) failed to demonstrate parasites in the blood of gibbons, *Macaca nemestrina* or *M. fascicularis* Raffles, 1821 inoculated with orang-utan blood containing *P. pitheci*. In our experience, man, splenectomized chimpanzee, *Aotus* and *Macaca mulatta* Zimmerman, 1780 are all insusceptible to *P. pitheci*, although man was not inoculated with infected blood. However, Coatney *et al.* (1971) refer to successful (and successive) inoculations of *P. pitheci* into two splenectomized chimpanzees; parasitaemia was not apparent until 72 and 18 days respectively after the inoculations and the parasites remained at a low density. They did not succeed in infecting *Aotus trivirgatus*, *Macaca nemestrina*, *Macaca mulatta* or gibbon (*H. lar*), but their report does not specify if these animals were intact or splenectomized.

(*c*) *Evolutionary origins of the malaria parasites of the orang-utan*

Opinions differ as to the site of origin and course of evolution of *Plasmodium* Marchiafava & Celli, 1885 species in primates. According to Hill (1972) simians first made their appearance on the African continent (for example in the Faiyûm area), rather than in Asia. Today malaria parasites are common in Asian monkeys in which speciation is clearly still very active, while *Plasmodium* is extremely uncommon in African species. The single malaria parasite, *P. gonderi* Sinton & Mulligan, 1933, is limited to mangabeys (*Cercocebus* spp.) and drills (*Mandrillus leucophaeus* (F. Cuvier, 1807)) living on the West African littoral between Liberia and Zaire. For this reason Asia has been called the cradle of primate *Plasmodium* (Garnham 1973*b*) or its nidus (Coatney *et al.* 1971). Two strong lines developed: (1) the tertian-Schüffner type, and (2) the quartan, both of which have persisted into the Hominoidea (figure 4), although only the former line entered the gibbon–orang-utan branch. A third line exists which is less definable as the periodicity may be quotidian (in one species. *P. knowlesi*), the stippling is variable in character and, although the species affect many Old World monkeys, they do not reach the Hominoidea. Some species easily infect man, either accidentally or experimentally (e.g. *P. cynomolgi* Mayer, 1907 and subspecies, *P. brasilianum* and *P. knowlesi*). The splenectomized chimpanzee was shown to be highly susceptible by Draper (private communication) to *P. knowlesi*.

No quartan parasites have yet been found in the gibbons or orang-utan, nor has any example of the crescent-bearing subgenus, *Laverania* which is confined to the higher African apes and man (as *P.* (*Laverania*) *reichenowi* (Sluiter, Swellengrebel & Ihle, 1922) and *P.* (*L.*) *falciparum* respectively).

The distribution of the quartan parasites is interesting. There are only two species-complexes in the Anthropoidea: (1) *P. inui* Halberstaedter & Prowazek, 1907 (with one or more subspecies, e.g. *P. i. shortti* Bray, 1963) of the Old World monkeys, and (2) *P. malariae* (in three adapted strains – the human *P. malariae*, the so-called *P. rodhaini* Brumpt, 1939 of the chimpanzee and the so-called *P. brasilianum* of New World monkeys). These parasites (*P. inui* and the *P. malariae* complex) have a different morphology and biological behaviour in all stages and the only important character they share is the quartan periodicity of erythrocytic schizogony. *P. malariae* possibly evolved from *P. rodhaini* in chimpanzees in Africa but the source of the latter is unknown for there are no simian homologues in that continent. *P. malariae* was perhaps taken by man in recent centuries to the New World where it infected, as a zoonosis in reverse, the local monkeys

(see p. 456). The *P. inui* complex (which is not included in figure 4) on the other hand appears to have emerged and speciated in Asia.

As to the origin of malaria parasites in monkeys and apes, one suggestion is that they arose in Asia in spite of the possibility that their hosts originated in Africa, and that this accounts for the paucity of species on the African continent. An alternative explanation is that simian *Plasmodium* did indeed originate on the African continent. After the emergence of the tertian-Schüffner line (but before the emergence of the quartan line) primates migrated into the Asian continent, accompanied by their tertian parasites. The subgenus *Laverania* almost certainly developed still more recently and was thus restricted, like the original *malariae* line, to the African continent. The tertian-Schüffner line was able to flower in the actively speciating simians of

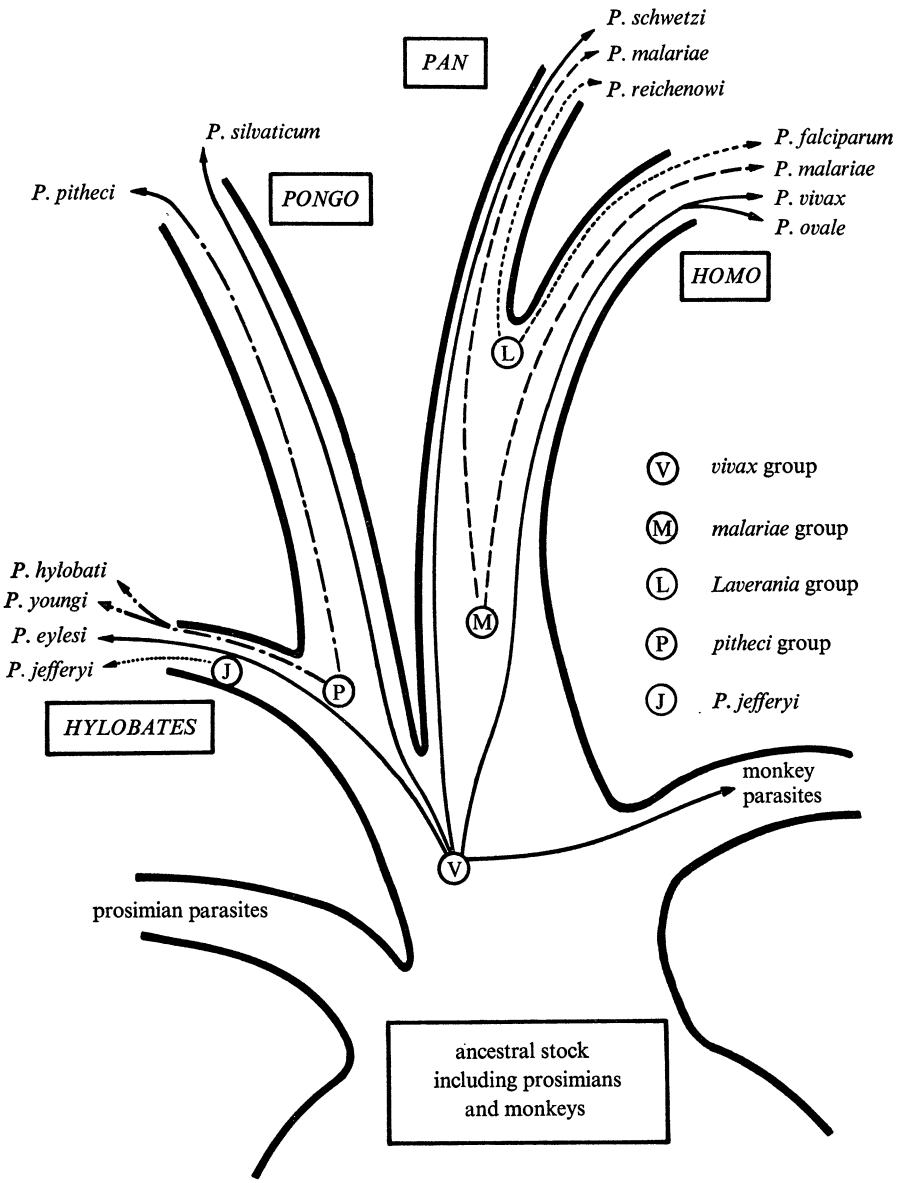


FIGURE 4. A figure to show the suggested evolution of *Plasmodium* (*Plasmodium*) and *Plasmodium* (*Laverania*) of apes and man.

Asia, including the gibbons and orang-utans in which, at a later date the tertian *pitheci* line also evolved. The scarcity of simian malaria species today on the African continent may perhaps be considered in terms of a relict fauna in contrast to that of Asia. The problem remains unsolved, but the ubiquity of the *Culicoides*-transmitted *Hepatocystis kochi* (Laveran, 1899) in nearly all species of monkeys throughout tropical Africa represents a striking contrast to the rarity and limited distribution of the mosquito-transmitted *Plasmodium* in only two genera of monkeys. The reverse picture is found in southeast Asia where nearly all monkeys are infected with *Plasmodium* but fewer are infected with *Hepatocystis*.

Hepatocystis is likely to have evolved at an earlier date than *Plasmodium* (as did the vectors of *Hepatocystis*), and this perhaps suggests that the origin of *Hepatocystis* (and its successor, *Plasmodium*) was African, where species of *Hepatocystis* are widely and intensively prevalent today. The explanation of the paradoxical scarcity of *Plasmodium* in Africa may be found in the present infrequency in that continent of suitable anopheline vectors of non-human primate malaria (none in fact has been incriminated in Africa). In contrast, anopheline vectors (the *leucosphyrus* complex) abound in southeast Asia and their rôle in the transmission of simian malaria is well known.

The study of malaria parasites of the orang-utan tends to support the idea that this animal has closer affinities to the gibbon than to other anthropoid apes.

We illustrate this point in figure 4 on which the *Pongo-Hylobates* branch is shown splitting off the main trunk lower than the *Pan-Homo* division. Sarich (1970) suggests in his diagram that the gibbons and orang-utan branched off separately. Our ideas of the evolution of the malaria parasites of primates are based on the assumption that the parasites arose in a primitive stock from which the modern species of *Plasmodium* of primates have arisen (see table 3). Speciation of the parasite is presumed to have accompanied this process. Figure 4 deals in detail only with the malaria parasites of the higher anthropoids. It has often been stated that *P. malariae* is the oldest of these parasites, because of the relative mildness and chronicity of the infections which it provokes in man, in contrast to *P.* (*Laverania*) *falciparum* which is generally thought to be the most recently evolved species because of its high pathogenicity. We preserve the latter hypothesis but disagree with the former and suggest that *P. malariae* is a relative late-comer in contrast to *P. vivax* which is shown here as the oldest parasite. This new hypothesis is based on two observations, (1) the absence of *P. malariae* (and of any quartan parasite) in the older *Pongo-Hylobates* branch and (2) the absence of *P. malariae* in the monkey parasites. In the latter there is present a malaria parasite (*P. inui*) which, although quartan in periodicity in its blood stages, in other respects is unlike *P. malariae* of the higher apes (see p. 473). Although the evolution of the monkey and lemur parasites is outside the scope of this paper, it may be noted that the *P. vivax* line appears to have emerged almost unchanged from *P. cynomolgi* – the common malaria parasite of oriental monkeys.

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Professor M. Colbourne sent us information on the prevalence of malaria in the human population of eastern Sabah, and Dr S. Ramalingam and Dr F. Y. Cheng data on mosquito collections. Dr R. E. Kuntz and Dr S. S. Kalter of the World Health Organisation Regional Reference Centre for Simian Viruses, San Antonio, Texas, U.S.A. examined chimpanzee serum for evidence of virus and *Mycoplasma* infection.

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Appendix

Studies on the full life cycle of malaria parasites of primates require a series of highly sophisticated techniques. Such work is normally carried out in an established laboratory where the necessary apparatus is in regular use and assistants trained in the specialized procedures are available. The present project, much of which was to be attempted far from any research centre, posed exceptional logistical and technical problems. Because of the unusual nature of the project, an account of the logistics and methods is presented in this appendix.

(*a*) *Logistics*

(i) *Experimental animals*

From the outset, the possibility of carrying out hazardous experimental procedures on orang-utans was dismissed. Plans were made accordingly to obtain potentially useful surrogate hosts, i.e. chimpanzees and night monkeys, and to transport them with us to East Malaysia. As the

animals were to be splenectomized before the experiments, time had to be allowed for convalescence.

In England two young chimpanzees (*Pan troglodytes*) were bought from a reputable animal dealer who had obtained them from Sierra Leone. The same dealer supplied two night monkeys (*Aotus trivirgatus griseimembra*) which originated from Colombia; they were the subspecies known to be susceptible to the malaria parasites of man (Collins *et al.* 1974). On arrival in England, the animals were placed in quarantine in accordance with current British regulations.

Chimpanzees are natural hosts of three malaria parasites, infection of which may be subpatent. In order to ensure that the animals were free from malaria, they were splenectomized in England 3 months before departure. If they had been naturally infected, a latent infection would have been rendered patent and there would have been time to give radical curative treatment before the experiments began. Neither of the chimpanzees was found to be harbouring parasites and the appearance of the spleens, which were unpigmented, suggested that they had never had the disease. The animals made an uneventful recovery from the operation except that one developed a localized infection at the operation site, which was successfully treated by the topical application of an antibiotic.

As night monkeys are less robust than chimpanzees, it was decided to delay splenectomizing them until after their arrival in Borneo in order to avoid submitting them to stress before the journey. Unlike the chimpanzee, *Aotus* is not a natural host of malaria parasites, and post-operative surveillance of the blood was not expected to reveal any natural infections. These animals were splenectomized at the Government Hospital in Kota Kinabalu in Western Sabah.

There were two staging posts *en route* to Sandakan, namely Kuala Lumpur in Peninsular Malaysia and Kota Kinabalu. In both places accommodation was available for the animals so that the accumulation of stress due to the long journey could be avoided by providing two periods of rest. Thus on arrival in Sandakan all the animals were in excellent condition.

(ii) *Operational bases*

Members of the expedition took all the equipment required for the field work to Borneo. At Sandakan a laboratory was established in the Veterinary Department where the chimpanzees were transferred from a travelling box to two spacious wooden cages.

Before the expedition began, arrangements had been made to move to the Institute of Medical Research, Kuala Lumpur, at a later stage. It was hoped that either the chimpanzee or *Aotus* monkeys would prove to be susceptible to *P. pitheci*, and anopheline mosquitoes of as many species as possible would then be needed in order to study the sporogony of this parasite. If experimentally infected mosquitoes were obtained, an attempt to demonstrate exoerythrocytic schizogony was planned. Although Sandakan was ideal as a field base, the facilities there were unsuitable for the difficult procedures necessary in the demonstration of the full life cycle. In the event, *P. pitheci* failed to infect the chimpanzees and night monkeys and investigations in Kuala Lumpur were made on the previously unknown parasite, *P. silvaticum*.

The establishment of two bases proved essential to the progress of the work. Chilled blood from an orang-utan with *P. pitheci* was transported successfully by air from the field base in Sandakan to Kuala Lumpur where it was inoculated into a gibbon and a rhesus monkey. The former animal developed a transitory infection (see p. 460) whereas the latter proved to be refractory. However, attempts to send mosquitoes from Kuala Lumpur by air to Sandakan in

order to feed them on an orang-utan while we were in the field met with only limited success. In the absence of an established insectary, such work is more likely to succeed if the gametocyte carrier is taken to the mosquitoes, as with *P. silvaticum* in the chimpanzee. Unfortunately *P. pitheci* never became established in the chimpanzee, night monkey, gibbon or rhesus monkey, and observations on the sporogony of this parasite were in the end limited to infections in mosquitoes which had been sent from Kuala Lumpur to the field base in Sabah.

(*b*) *Techniques*

(i) *Splenectomies*

Splenectomies were performed under general anaesthesia. Classical left lateral incisions were employed. Vessels were tied, and the incisions were closed in layers with catgut and silk sutures by using conventional surgical techniques. The anaesthetic for the chimpanzees and the night monkeys was phencyclidine followed by halothane.

(ii) *Care and handling of chimpanzees and* Aotus

Experimental primates are normally handled in cages with a moveable section which immobilizes the animal for the preparation of blood films or administration of injections. This facility was not available in the field, and the handling of the chimpanzees initially caused difficulty. During the journey from England to Borneo, the chimpanzees were caged together. On arrival in Sandakan they were separated and two members of the expedition handled and exercised the animals daily. In this way the animals, denied each other's company, became dependent upon their handlers. Blood films were at first prepared daily from blood of fingers or toes, the skin of which had been cleaned with alcohol but this procedure caused stress to the animals and they refused food. From previous experience of working with chimpanzees without crush cages (Godfrey & Killick-Kendrick 1967), it was known that it was possible to train them to permit blood films to be prepared and injections to be given without restriction. We therefore adopted this approach and trained the animals so that further blood films could be made from the edge of the ear. Any intramuscular injections that were required were made in the animals' thigh muscles.

The night monkeys which were housed together were taken from their cage with gloves. Since they were known to thrive best when left as undisturbed as possible, they were kept in a room away from all activity. Blood films were prepared from the edge of the ear.

Both chimpanzees and night monkeys were fed a mixed diet of fruit including bananas, oranges, rambutan and soursop, and a proprietary infant protein concentrate (Complan®). Water was freely provided.

(iii) *Preparation and examination of blood films*

Blood films from orang-utans were made with blood obtained by pricking a finger. Unlike the chimpanzees they rapidly learned to hold out one hand for this purpose.

Thick and thin blood films were stained in Giemsa's stain at pH 7.2. The thick films, which were not fixed, were stained for 30 minutes in a 5 % solution of stain. The thin films were fixed momentarily in absolute methanol and stained for 45 min in a 10 % solution. Exflagellation of male gametocytes was studied in two ways. Blood was examined first in the fresh state under a coverslip, and then in a series of stained smears. The fresh blood was kept moist in a damp chamber for intervals for 3 to 15 min, after which the slides were removed from the chamber,

dried, fixed in methanol and stained in Giemsa's stain as for thin blood films. Films were examined with a × 50 oil immersion objective and the morphology of the parasites was studied with a × 100 objective. Pigment was examined under polarized light (Shute & Maryon 1966; Garnham 1966).

(iv) *Inoculation of blood into experimental animals*

In attempts to infect experimental animals, blood taken from orang-utans with natural infections was inoculated into chimpanzees, night monkeys, gibbons and rhesus monkeys. The chimpanzees were taken to the Sepilok Rehabilitation Centre where they and the donor orang-utans were first tranquilized with phencyclidine hydrochloride (1 mg/kg). With heparin as an anticoagulant, blood was drawn from the saphenous vein of the orang-utans and inoculated at once into the same vein of the chimpanzees. Blood given to the *Aotus* was taken in crushed ice from Sepilok to Sandakan and given intravenously without delay. In the attempt to infect a gibbon and a rhesus monkey with *P. pitheci*, infected blood of the orang-utan was flown in crushed ice to Kuala Lumpur. Since a transitory infection arose in the gibbon, it is known that the blood remained infective.

Observations on the susceptibility of a gibbon and rhesus monkey to *P. silvaticum* were carried out in Kuala Lumpur. Infected blood from chimpanzee Khan was inoculated intravenously into the other animals, neither of which became infected.

(v) *Breeding mosquitoes*

Four species of *Anopheles* (see p. 447) (*A. balabacensis*, *A. kochi*, *A. maculatus* and *A. sundaicus*) were bred in the insectaries of the Entomology Department of the Institute of Medical Research, Kuala Lumpur. The larvae were fed on a special mixture including yeast, vitamins and liver powder, and the adults on a 10 % sugar-vitamin solution and the blood of guinea pigs or macaques. Mating of mosquitoes was accomplished artificially (Esah & Scanlon 1966).

(vi) *Infection, maintenance and examination of mosquitoes*

The sporogony of *P. pitheci* was observed in *A. balabacensis* flown from Kuala Lumpur to Sandakan. The adult mosquitoes were put into waxed paper cups, the mouths of which were covered with gauze. Sugar solution was provided on dental wicks. The cups were packed in metal boxes for transit. A high humidity was maintained in the boxes by lining them with wet lint. About 70 % of the mosquitoes survived the journey.

On arrival in Sandakan, cups which had become soaked in water collapsed on handling. The mosquitoes were therefore transferred to containers improvised from sections of bamboo, about 3 in in diameter and 5 in long, the ends of which were covered by gauze held in place with strips of plaster. Access was by a hole in the side of the cup closed with a plug of cotton wool. Each bamboo container was kept in a closed plastic bag containing a swab of wet cotton wool; this not only ensured the maintenance of a high humidity, but also guarded against attack by ants.

Mosquitoes were permitted to engorge on the abdomen of an orang-utan (Barbara) in the blood of which mature gametocytes were seen. The animal was first sedated by the intramuscular inoculation of phencyclidine hydrochloride. (This tranquilizer was well tolerated by orang-utans and proved valuable when, for example, they needed to be immobilized for the dressing of wounds or other veterinary treatment.)

Without an insectary in the field it was difficult to select a suitable place to keep the mosquitoes. The principal problem was the widespread domestic use of insecticides. An initial attempt to establish a temporary insectary in a large animal cage in the forest at Sepilok was abandoned when orang-utan Joan entered the cage and destroyed the cups containing the mosquitoes. Finally, they were kept successfully in a hotel bathroom where the temperature remained fairly constant at approximately 27–30 °C.

Conditions were more controllable at Kuala Lumpur where the studies on the sporogony of *P. silvaticum* were performed. Many hundreds of hungry females were available and they were easily permitted to engorge on chimpanzee Khan after first tranquillizing the animal with phencyclidine hydrochloride, and lying him on top of cages containing the mosquitoes. Infected mosquitoes were maintained in an insectary at 27–29 °C and 80 % relative humidity.

Midguts and salivary glands of infected mosquitoes were dissected in saline by conventional methods (Shute & Maryon 1966) and examined under a coverslip. Permanent preparations of infected midguts were made by fixing the midgut by drawing 10 % formalin under the coverslip and staining the gut as a whole mount in Ehrlich's haematoxylin. Smears of infected salivary glands were permitted to dry, and were stained in Giemsa's stain as thin blood films. Oocysts and sporozoites were measured with a calibrated eyepiece.

(vii) *The demonstration of tissue schizonts*

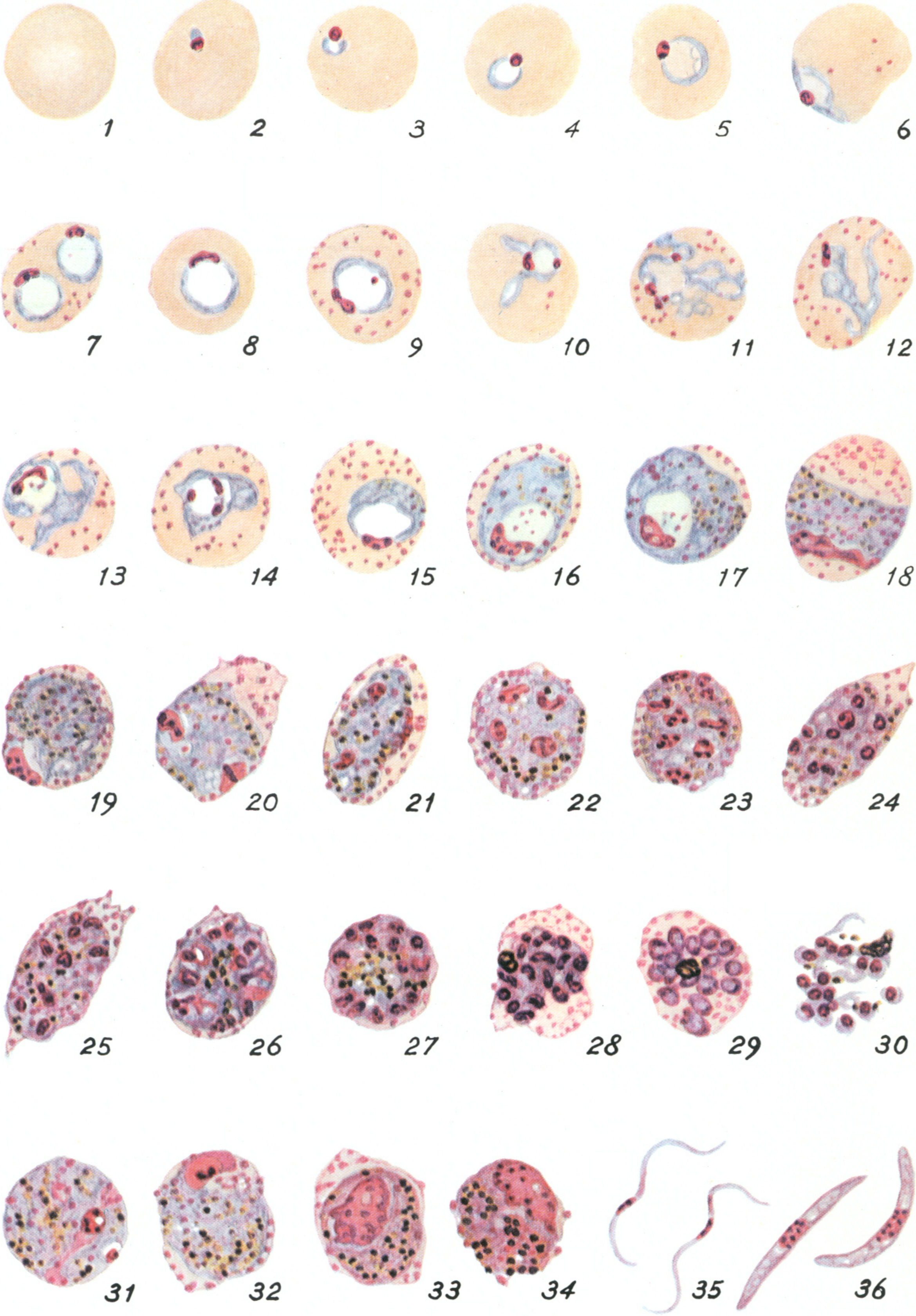
Suspensions of sporozoites of *P. silvaticum* were prepared in Kuala Lumpur from the glands of batches of *A. balabacensis* infected by engorging on chimpanzee Khan. Mass dissections were performed by first stunning the mosquitoes in a test tube, removing the legs and wings and dissecting the glands on a perspex slide in a cold solution of 10 % human serum in sterile Locke's fluid. On each of two occasions, the procedures were limited to 1 h from the first dissection. Sporozoites were released from the glands by gently grinding with a Teflon pestle in a glass tube. The suspensions were inoculated into the saphenous vein of chimpanzee Sandy who was previously tranquilized by the intramuscular injection of phencyclidine hydrochloride.

Liver was later to be taken from Sandy at laparotomy. The animal was denied food for 12 h, and phencyclidine hydrochloride was given as a premedication. Anaesthesia was by open ether (halothane not being available), but the animal responded badly and respiration stopped as the operation began. Resuscitation was attempted but after 40 min the animal had failed to revive and liver was taken *post mortem*. It was fixed for 4 h in Carnoy's fluid in slices about 5 mm thick. After dehydration in an ascending series of ethanol, the liver was cleared in Supercedrol, embedded in paraffin wax and sectioned at 5 μm. The sections were mounted in series and stained by the Giemsa-colophonium method (Bray & Garnham 1962). A few sections were differentiated in dilute acetic acid which was found preferable for demonstrating details of the nuclei of some tissue schizonts.

Top. Accommodation for sick animals and veterinary laboratory at the entrance to the Sepilok Forest Reserve; the main footpath into the reserve lies immediately behind these buildings. (Photograph by courtesy of Mr Stanley de Silva.) *Bottom left.* Typical tropical rain forest at Sepilok containing numerous species of dipterocarps, the crowns of which form the forest canopy at a height of 30–40 m (photograph by courtesy of the Sabah Forestry Department). *Bottom right.* The female orang-utan Joan aged 15 years (photograph by courtesy of Mr Stanley de Silva).

Top and centre. Unidentified structures (arrowed) in erythrocytes of the splenectomized chimpanzee Khan. The blood films which were stained with Giemsa's stain were photographed with phase-contrast optics (bar = 5 μm).

Bottom. Electronmicrographs of erythrocytes of the splenectomized chimpanzee Khan showing unidentified structures corresponding to those seen in top and centre figures (bar = 1.0 μm). (Blood prefixed in glutaraldehyde and post-fixed in osmic acid – electronmicrographs by courtesy of Professor V. Zaman.)



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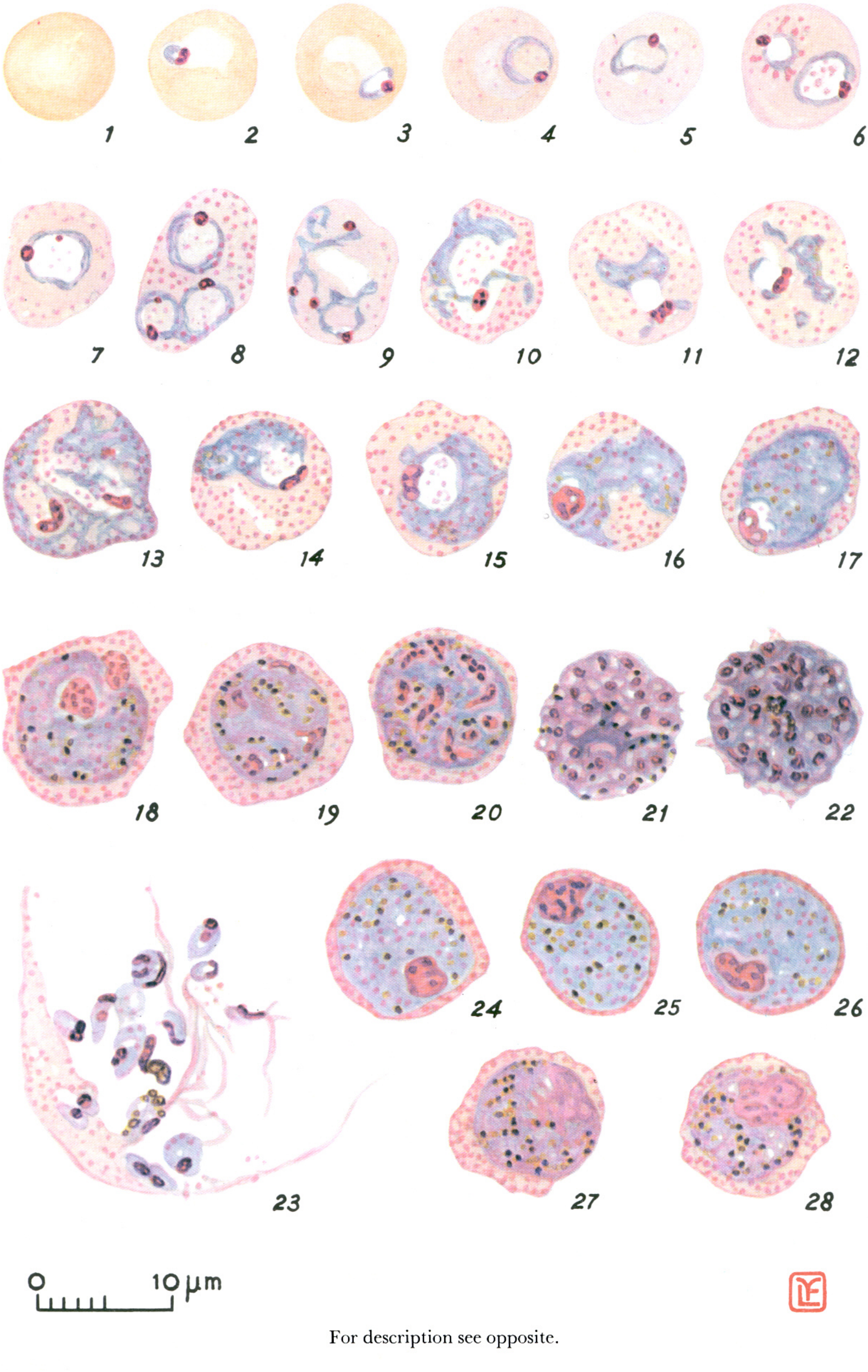

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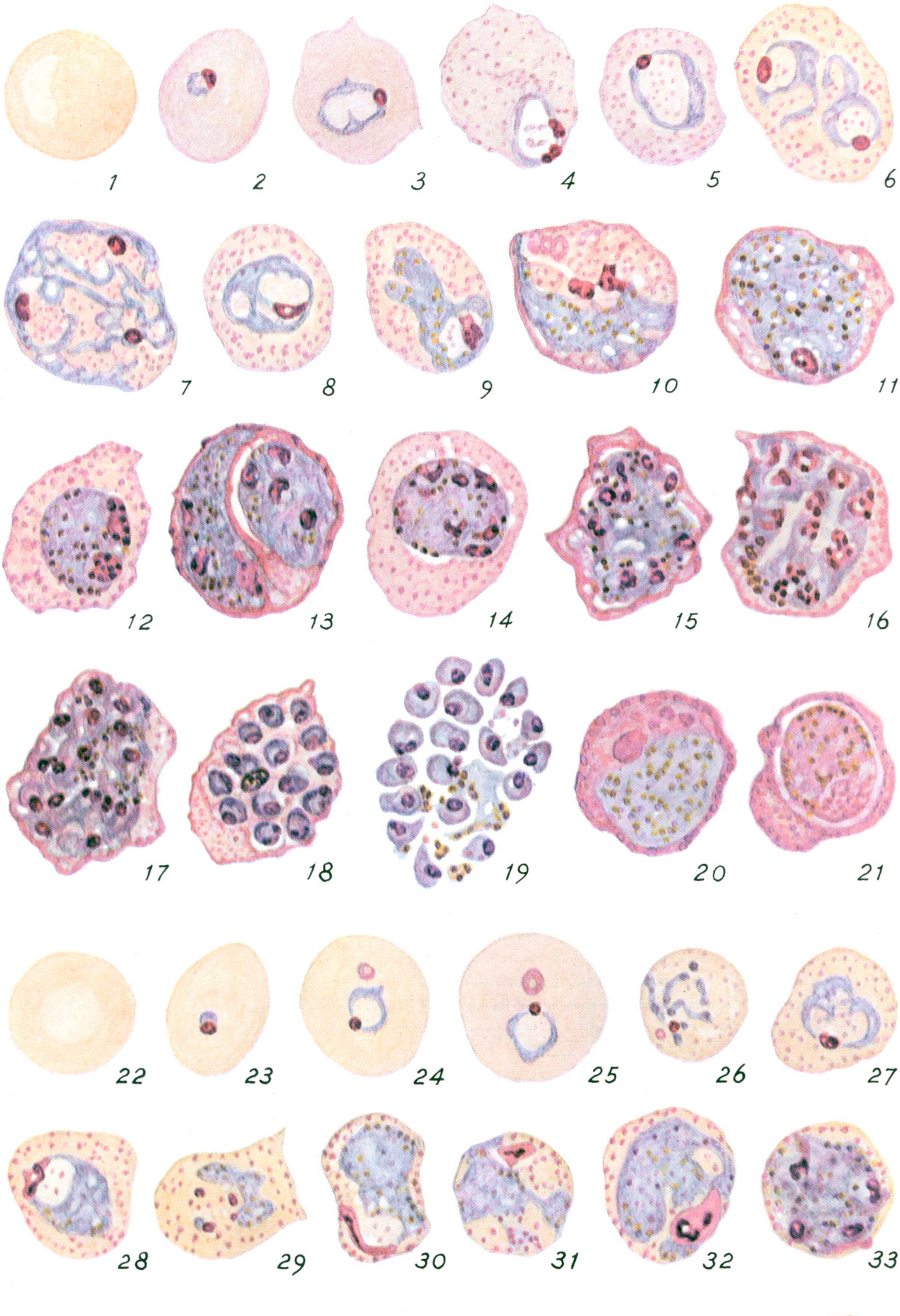

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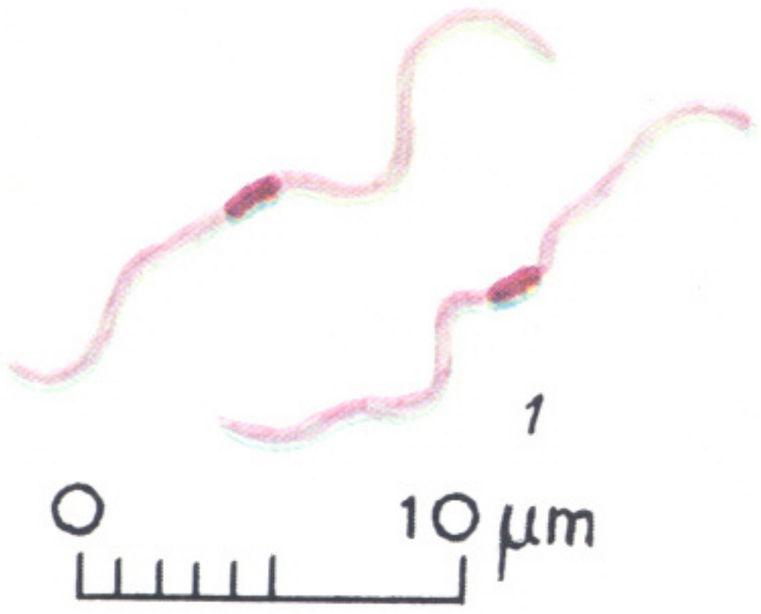

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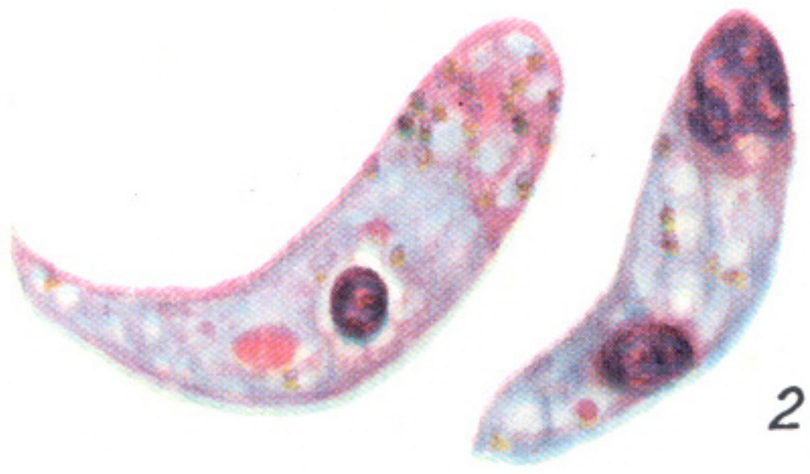

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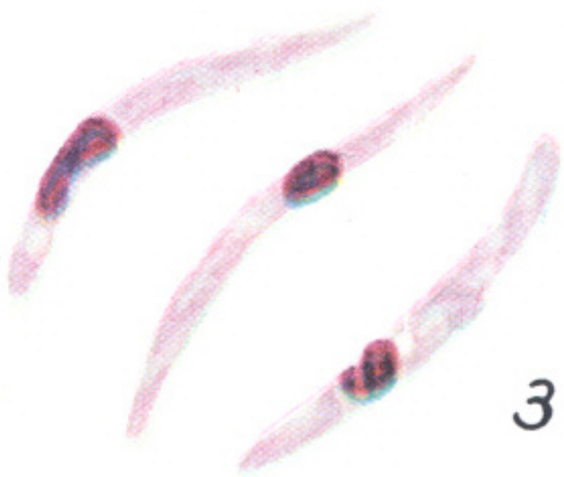

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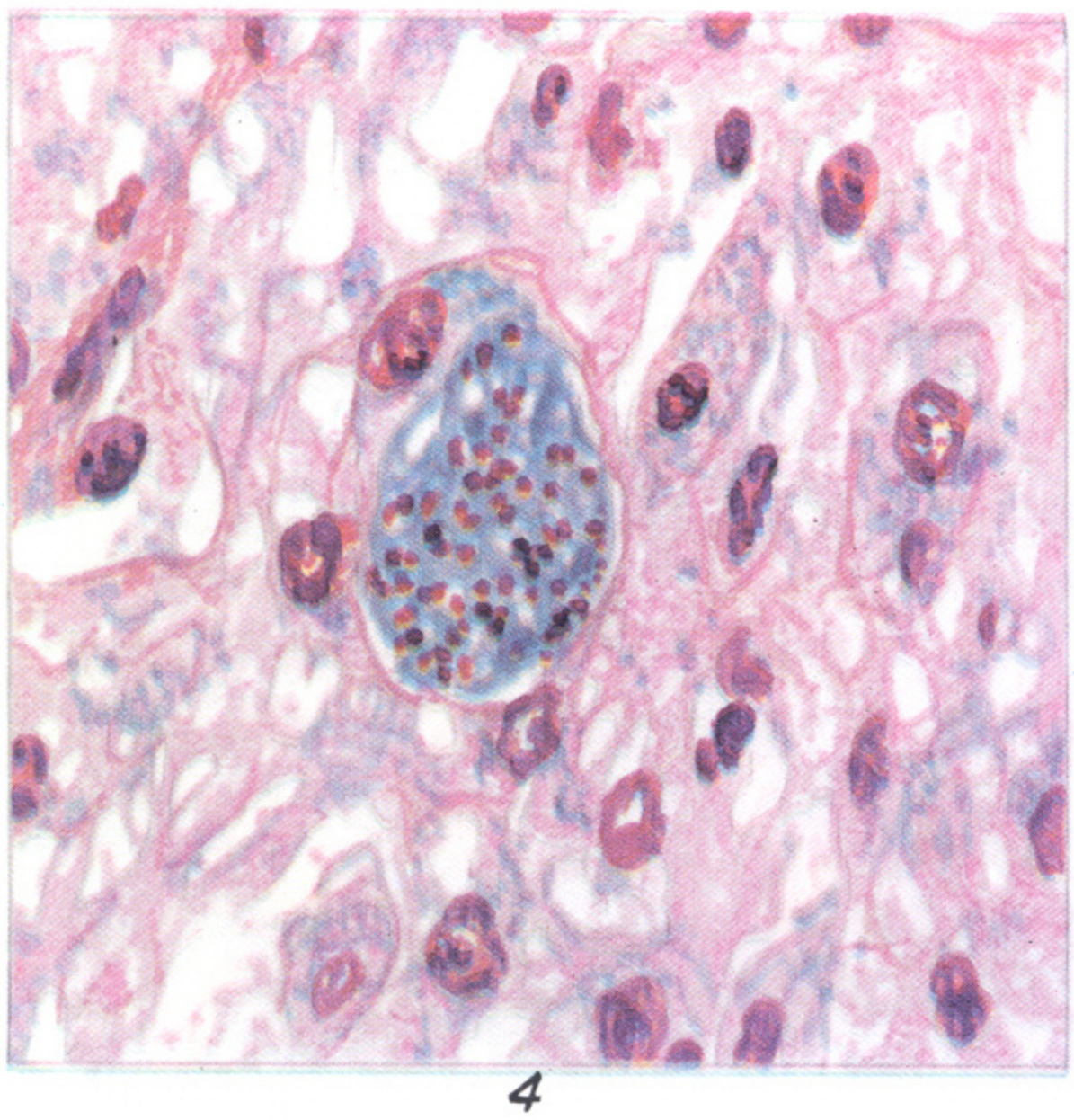
10 µm



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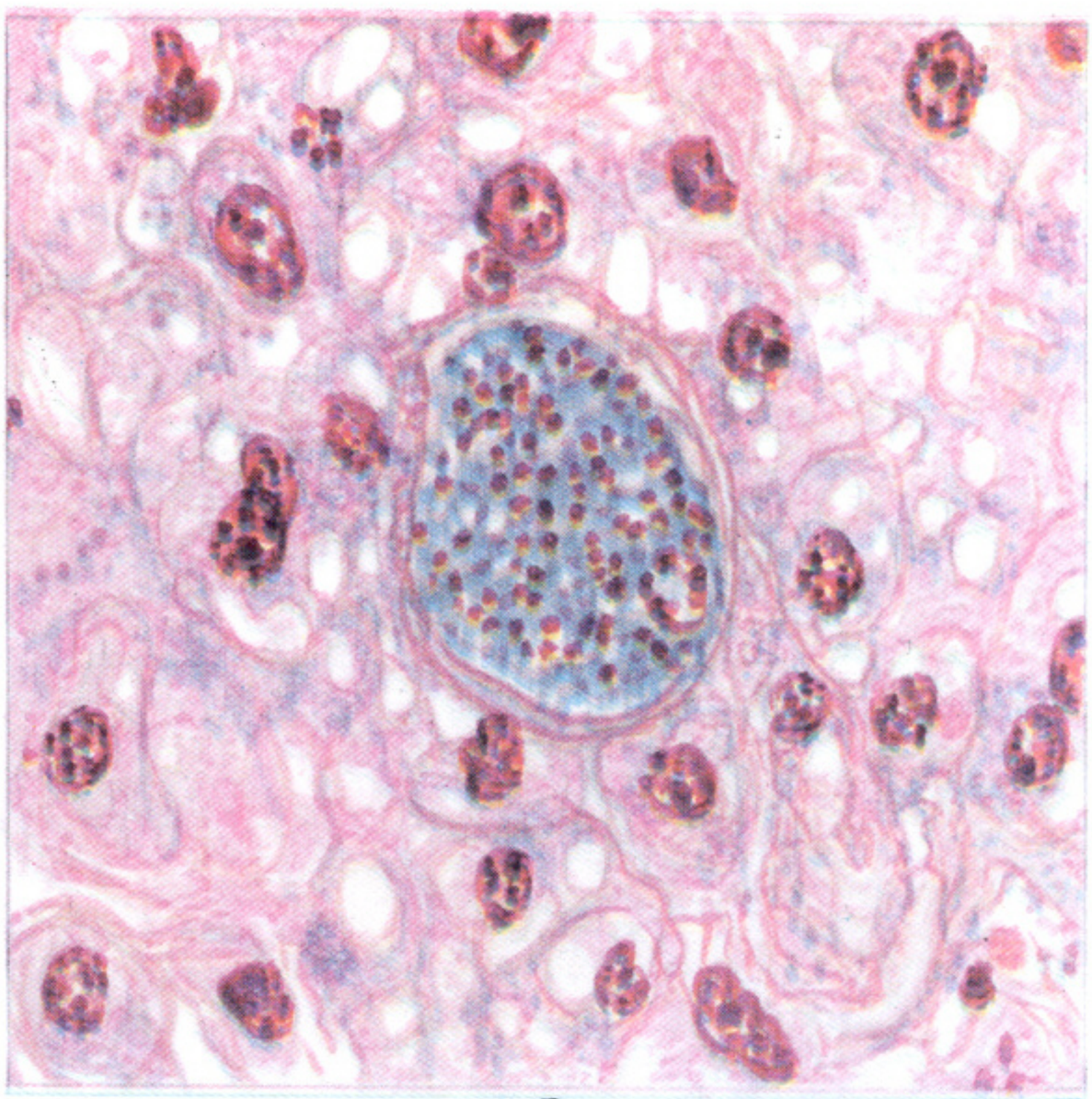

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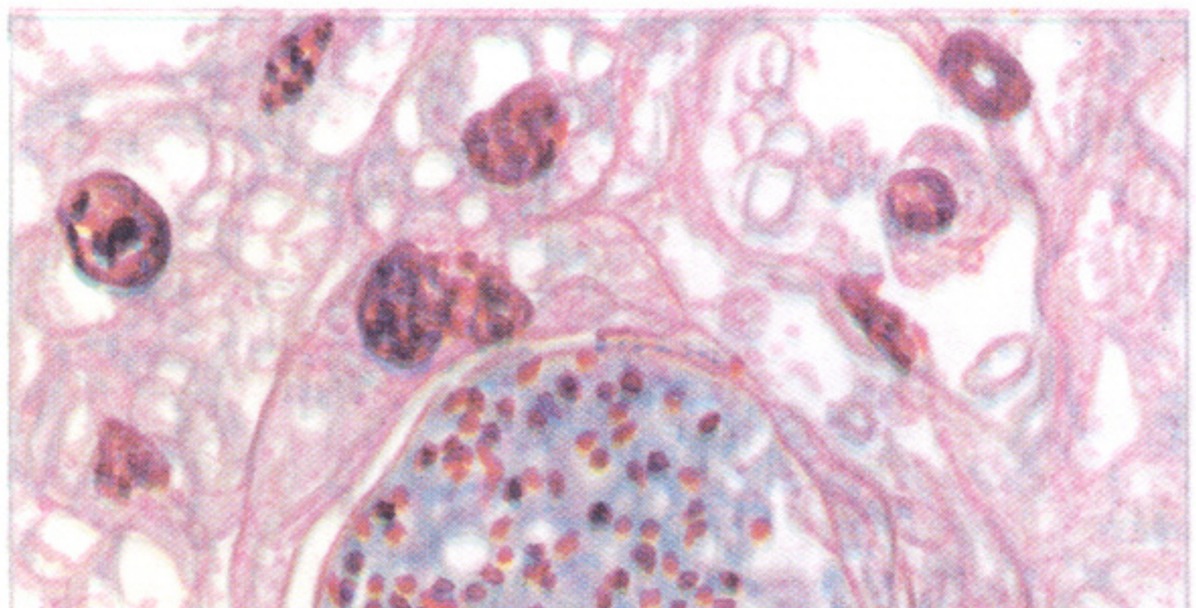

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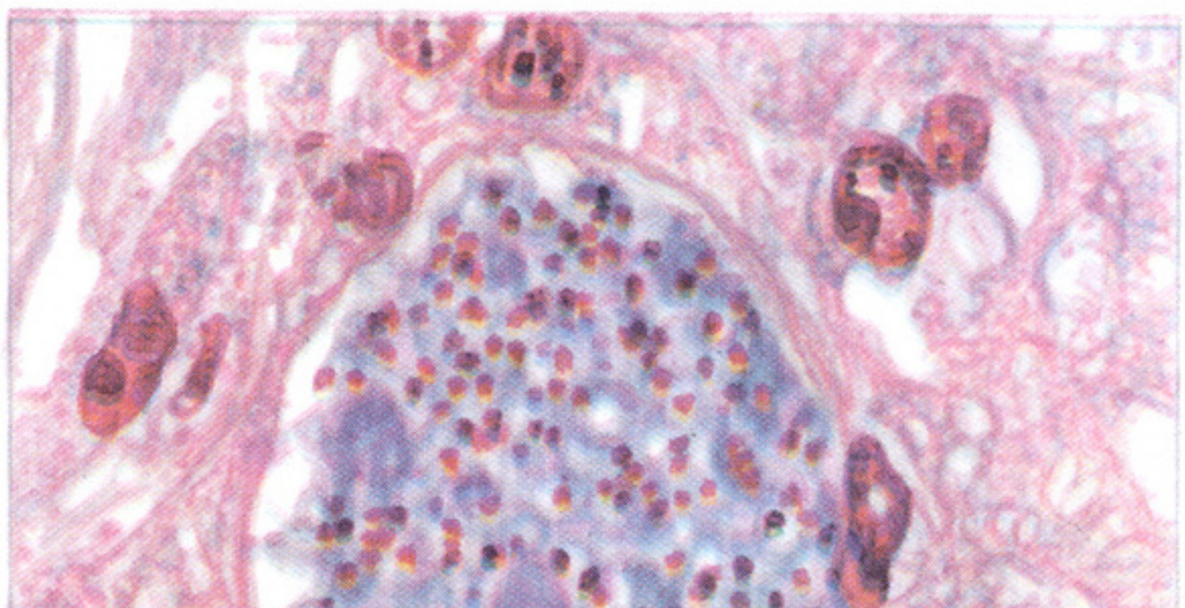

7

20 µm

For description see opposite.