CEYLON J. MED. SCI. (D) Vol. XIV No. 1, (August 1965)

Two New Malaria Parasites, *Plasmodium cynomolgi ceylonensis* subsp. nov. and *Plasmodium fragile* sp. nov., from Monkeys in Ceylon *

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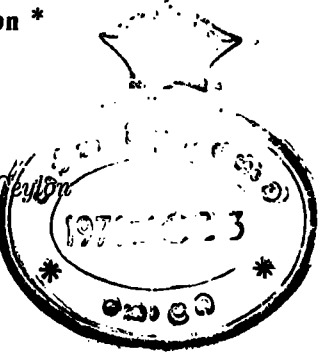


INTRODUCTION

In a recent communication a brief account was given of three malaria parasities isolated from Ceylon monkeys (Dissanaike, 1965). These parasites were provisionally identified as *P.cynomolgi*, *P.shortti* and a new species. The *P.shortti* like parasite (Plate III) is morphologically indistinguishable from the same parasite isolated by Shortt, Rao, Qadri and Abraham (1961) from South India. It was recently described in detail by Jayewardene (1963). The other two are considered to be different to hitherto known species and are described in the present paper as *P.cynomolgi ceylonensis* subsp. nov. and *P.fragile* sp. nov.

These parasites are fairly widely distributed in Ceylon, in Puttalam and Maho in the North Western Province, Passara and Buthala in the Uva Province and Lahugala in the Eastern Province. Infected monkeys occur in jungles bordering the main roads and have often been shot by the roadside. They abound in the not very dense jungles that are close to human habitations and often do a great deal of damage to various plantations. The majority of positive animals have been shot in dry zone areas at very low elevations, except in the foothills of Uva (Passara) where the geography is perhaps similar to the Nilgiris and the forest is a little denser.

Of the sixteen positive monkeys recorded so far, the host distribution of the three parasites is summarised in Table I below, which also indicates the numbers found positive for each species.

It is not certain whether the parasite resembling *P.cynomolgi* in the grey langur (*Presbytis entellus*) is the same as that isolated from *M.sinica* as the only attempt made to transmit the parasite to a laboratory toque monkey was unsuccessful.

* This investigation received partial assistance from the World Health Organization.

TABLE I

(Host distribution of *P.c.ceylonensis*, *P.shortti* and *P.fragile*).

Species of Monkey	*P.c.ceylonensis*	*P.shortti*	*P. fragile*
Macaca sinica sinica	8	3	3
M. sinica aurifrons	5	4	1
Presbytis entellus thersites	2	0	0

The first strains of *P.c.ceylonensis* and *P.shortti* were isolated from *M.sinica* (M 5) shot in Puttalam and were transferred by blood inoculation into a rhesus (M 1) and a laboratory toque (M 2). Blood from M 1 was subsequently sent to the London School of Hygiene and Tropical Medicine where a mixed infection with *P.shortti* was noted in a subinoculated rhesus monkey and subsequent separation of the two parasites was made by mosquito infection. *P.fragile* was isolated from three different monkeys shot in Maho (M 50, M 52 and M 53) and maintained in three laboratory toques (M 3, M 4 and M18). All these monkeys had mixed infections with *P.c.ceylonensis*. Blood from M 3 was sent to the London School of Hygiene and Tropical Medicine and a pure strain of *P.fragile* was isolated by serial passage of minute amounts of infected blood. Table II summarises the isolations of the two parasites. The descriptions that follow are based on studies of the parasites that have been made in laboratory infected rhesus and toque monkeys both in Ceylon and in London.

Plasmodium cynomolgi ceylonensis

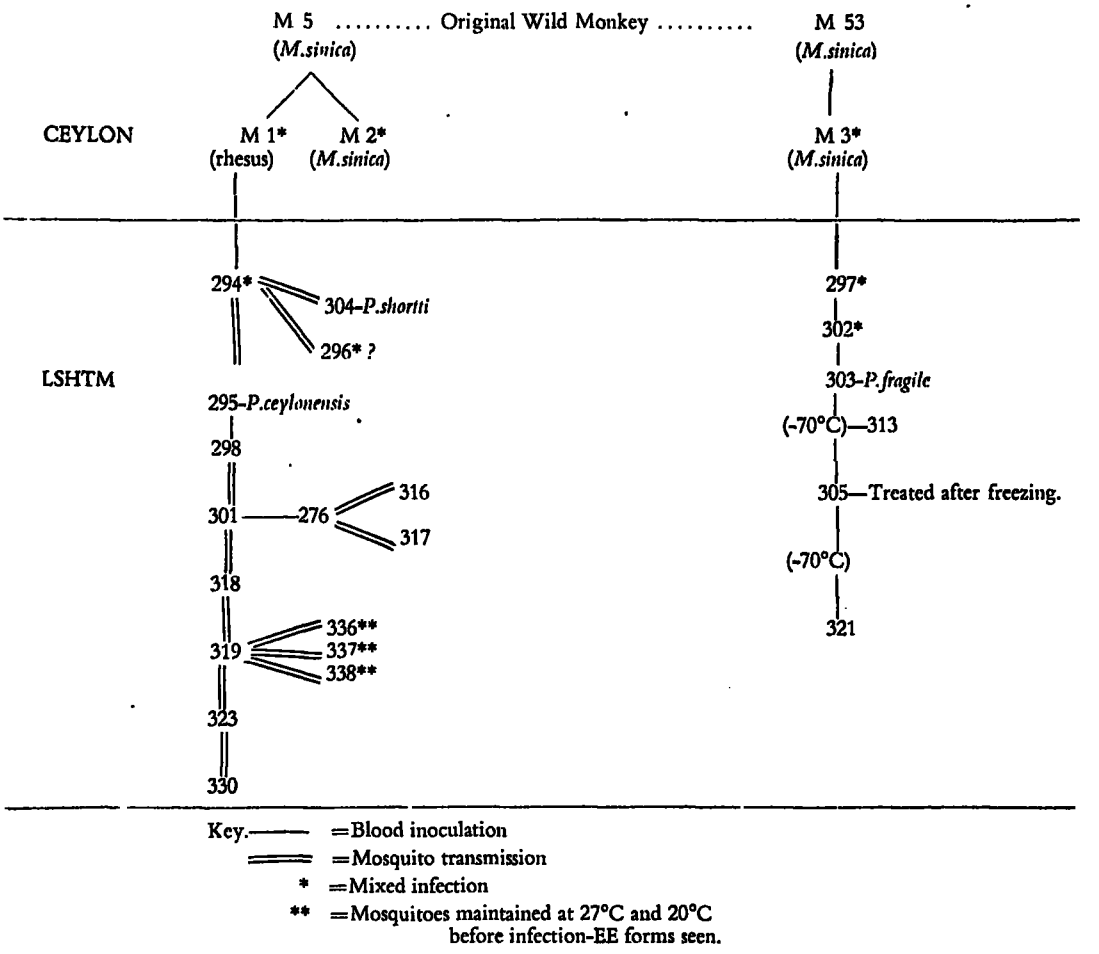
Exoerythrocytic stages (Plate IV).

Tissue forms of *P.c.ceylonensis* were found after the intravenous inoculation of sporozoites into rhesus monkeys. Their maturation takes place towards the end of the 8th day or at the beginning of the 9th day. Two stages were seen. The 7th day schizont has a diameter varying from about 20 to 26 μ. The substance of the parasite is characterised by the presence of numerous large spheres of dense cytoplasm ; occasionally one or two large vacuoles may be present, though these are very much less common than in the type strain of *P.cynomolgi*. The nuclei are irregular in shape, though sometimes in the form of little rods.

The 8th day exoerythrocytic schizont varies in size from 30 to 40 μ. It usually has an oval shape with a smooth contour formed by the thin membrane. The cytoplasm may still show a few flocculated masses, or even an occasional vacuole ; the nuclei have now divided further, so that they are closely packed together. The nuclei are darkly staining and at this stage are apparently still some way off the stage of merozoite formation.

TABLE II

(Isolations of *P.c.ceylonensis* and *P.fragile* in laboratory monkeys).



Asexual cycle in the Blood (Plate I).

The periodicity of schizogony in the blood is tertian, the majority of the schizonts rupturing between 9·0 and 11·0 a.m. on alternate days. Reticulocytes and mature corpuscles are invaded indiscriminately. The younger stages are typical rings. The chromatin becomes curved and very often divides into two dots. At this stage the parasite is not very amoeboid, but later, pseudopodia project from the surface of the trophozoite. The parasite continues to grow in size, and the vacuole disappears. The nucleus has still not divided and it is not until the parasite almost fills the corpuscle that binucleate schizonts are formed. Then, after further division, the nuclei often take up a marginal position, leaving the rest of the cytoplasm free of chromatin. The pigment is light brown in colour and not very conspicuous.

Finally, 12 to 18 merozoites are formed, the average number being 16. The infected corpuscle becomes enlarged and often oval in shape. The degree of enlargement is less, however, than in *P.c.bastianellii* (and probably the type) as the following average measurements indicate :— (i) uninfected corpuscle –7·35 μ; corpuscle infected with rings of *P.c.ceylonensis*– 7·44 μ; corpuscle infected with trophozoites of *P.c.ceylonensis*–8·34 μ.

(ii) uninfected corpuscle– 7·48 μ; corpuscle infected with rings of *P.c.bastianellii*– 8·36 μ; corpuscle infected with trophozoites of *P.c.bastianellii*– 9·53 μ.

Schüffner's dots appear early and show an intense staining reaction in the later stages though remaining pin-point in size. A particular feature of the subspecies is that the substance of the corpuscle itself darkens in colour in contrast to the pallor induced by the type.

Gametocytes

Gametocytes usually appear in the peripheral blood at the end of the first week of parasitaemia after a sporozoite infection, and usually persist in large numbers for two weeks. The microgametocyte contains numerous brown pigment granules of the usual type. The macrogametocyte is larger in size, often reaching 9 μ in diameter, and is nearly always oval in form. Its nucleus consists of two portions—a lighter staining outer zone, and an inner zone comprised of darkly staining threads or granules.

Sporogony

Laboratory bred *Anopheles maculipennis atroparvus*, *Anopheles aztecus* and *Anopheles stephensi* were used in the studies. All three species are easily infected with the parasite and show an infectivity rate around 75 % when maintained at 27°C. This rate remained the same when in later experiments the mosquitoes were maintained at 20°C.

At 27°C sporogony proceeds as follows :— On the third day the oocysts are 12 to 14 μ in diameter with unequal rod like pigment granules, dark brown in colour, arranged in short rows. By the 5th day the oocysts are 30 μ in diameter with the pigment granules arranged in short and long rows. Clumping of the pigment granules is seen in some of the five day old oocysts, and by the 6th day most of the pigment is obscured. The oocyst is now 50 μ in diameter and sporozoite formation begins. Mature oocysts and rupturing ones are seen on the 7th day. Invasion of the salivary glands by the sporozoites is complete on the 8th day. The sporozoites measure 13 to 14 μ in length.

Due to the failure to obtain exoerythrocytic stages in the liver of monkeys inoculated with these sporozoites, in later experiments the mosquitoes, after being maintained at 27°C for the first twenty four hours following the infective feed, were then kept at 20°C. The rate of growth of the oocysts was now slower but the oocysts grew to a much larger size before maturity. In a batch of *A.m.atroparvus* maintained in this manner sporogony was as follows :— on the 9th day the oocyst was 24 μ in diameter, on the 10th day 27 μ and on the 14th day 56 μ. Sporozoites were first seen developing on the 15th day when the oocyst was 72 μ in diameter, and on the 17th day mature oocysts measuring up to 96 μ were seen. The glands were invaded by the sporozoites on the 18th day at a temperature of 20°C and this batch gave rise to exoerythrocytic schizonts.

Relationships

The type form of *P.cynomolgi* was described in detail by Mulligan (1935), and the subspecies *bastianellii* by Garnham (1959); the new subspecies *ceylonensis* differs in many minor respects from these two parasites in all stages of its development. Exoerythrocytic schizogony occupies a longer duration than either of the other two, taking 8½ to 9 days instead of the 8 days of the type and 7 days of *bastianellii*; the schizonts have no prominent vacuoles like the type and no pallisade distribution of nuclei like *bastianellii*. The oocysts of *P.c.ceylonensis* reach greater dimensions than those of the other two parasites, while the sporozoites reach the salivary glands in a shorter time. The blood forms also differ in that the schizonts attain maturity between 9 and 11 a.m. instead of 5 and 7 a.m. (type) and noon (*bastianellii*); the stippling of the infected erythrocyte is more intense and is accompanied by a deep reddening of the corpuscle; but there is less enlargement of the erythrocyte. There should be little difficulty in recognising this subspecies, if the above criteria are applied. A further point of distinction may be that *P.c.ceylonensis* is apparently unable to infect man. Infective blood was inoculated into four patients with Buerger's disease either subcutaneously or intramuscularly in amounts of approximately 2 ml., but none of the recipients developed an infection. Two volunteers were bitten by *A. atroparvus* heavily infected with sporozoites, and again the infection failed to take.

Ramakrishnan and Mohan (1961) and Satya Prakash and Chakrabarty (1962) recently described *P.cynomolgi* from the closely related monkey, *Macaca radiata*, in South India; this form of the parasite in the erythrocytic phase resembles in many respects the new subspecies from Ceylon with which it is probably identical.

On the basis of the above points, the parasite is named *Plasmodium cynomolgi ceylonensis* subsp. nov. with the type locality Ceylon and the type vertebrate host *Macaca sinica*.

Plasmodium fragile

Asexual stages in the Blood (Plate II).

The duration of the erythrocytic cycle of schizogony is about 48 hours. There is a strong tendency for the parasite to retreat to the internal organs to undergo schizogony, although later in the infection many advanced forms will be found in the peripheral blood. Maturation of schizonts appears to occur about mid day, and young rings flood into the circulation in the afternoon.

The youngest parasites are hair-like rings, and occasionally multiple invasion of the corpuscle occurs. An accessory dot, staining rather feebly, is often present, as well as the main nucleus. The ring becomes amoeboid, but the most striking feature is the accumulation of heavy and numerous pigment granules scattered throughout the cytoplasm. The colour of the pigment is black, with a pronounced yellow sheen, and the shape of the granules is more spherical than the rice grain shape of the pigment in *P.coatneyi*.

The schizonts do not usually fill the erythrocyte, and often lie on one side. The merozoites appear to bud off a residual mass of cytoplasm. Finally about 10 to 14 merozoites are produced; rarely a schizont with 16 merozoites may be seen.

The pigment remains bulky, but it is obviously unstable in composition, because it tends to fragment into smaller particles or even disappear completely. In fresh pre-

parations the pigment can be seen in large golden black masses. The corpuscle itself shows very marked changes from the trophozoite stage onwards. At first a faint flush appears in the substance of the corpuscle, or it may lose its colour entirely. This is followed by a faint stippling and very soon the erythrocyte itself becomes distorted in various ways, It is obviously fragile; it is sticky and may be pulled out into an oval or fimbriated shape, but the corpuscle never becomes enlarged.

Gametocytes

Gametocytes are abundant and appear in the peripheral blood after 3 or 4 days of parasitaemia. The microgametocyte may be irregular in shape with amoeboid projections. It has the usually diffuse red nucleus with a prominent karyosome. The pigment is found outside the nuclear area in the cytoplasm, either as a few large granules or in smaller particles. The macrogametocyte is spherical or oval with a concentrated nucleus, and again with pigment which may be found to be undergoing disintegration.

Sporogony

Two attempts were made to study the development of this parasite in mosquitoes without success. In both instances laboratory bred *Anopheles maculipennis atroparvus* and *A.aztecus* were used. In the first experiment the mosquitoes were maintained at 27°C after the infective feed. In the second experiment the mosquitoes were maintained at 27°C for the first twenty four hours, and then at 20°C. No developmental stages were seen in any of the mosquitoes.

Relationships

Plasmodium fragile bears a close resemblance to *P.knowlesi edesoni* and to *P.coatneyi*; it may be distinguished from the former by its tertian instead of quotidian periodicity in the blood, and from the latter, by the smaller number of merozoites, the more abundant and different type of pigment, and by the different reaction of the infected host cell which moreover is usually a mature erythrocyte instead of the reticulocyte preferentially invaded by *P.coatneyi*.

The new species however seems to be identical with the parasite described by Eyles (1963) as the "New Nilgiri Parasite", and originally isolated by Ramakrishnan and Mohan (1961) in South India, the latter observers provisionally identifying it as *P.inui*. Eyles fully intended to name this parasite, and in fact was preparing a coloured plate; but his premature and much regretted death prevented this, and the parasite has remained unnamed. We now formally name it, from the Ceylon strain, *Plasmodium fragile* sp. nov., with the type locality Ceylon and the type host *Macaca sinica*. The name is derived from the Latin *fragilis* = fragile, and was suggested to us by Dr. Charles Wilcocks; as in the case of *P.ovale*, the descriptive name applies more to the effect of the parasite on the erythrocyte than to the organism itself. The marked tendency of the parasite to deform or even to destroy the corpuscle is one of its most striking features.

P.fragile is also characterised by the confinement of erythrocytic schizogony largely to the internal organs. It exhibits a close antigenic relationship with *P.coatneyi* and *P.knowlesi*; and like these two parasites, it often kills rhesus monkeys by fulminating infections. The sporogonic development of the parasite in ordinary laboratory bred mosquitoes is obviously difficult; Eyles (1963) had a little more success with the strain from the Nilgiris in that he managed to obtain a few oocysts, but no sporozoites, in *Anopheles maculatus*.

Three patients with Buerger's disease were inoculated intramuscularly with blood containing *P. fragile;* their blood was examined daily for a month, but no infection was detected. On this limited evidence, it is probable that *P. fragile* is non-infective to man.

DISCUSSION

It is of considerable interest to note both that true malaria parasites of monkeys have only recently been isolated from the Indian subcontinent, and that the three species from South India have their representatives in Ceylon. These parasites appear to be peculiar to India and Ceylon, and it may be stated that, so far, no quotidian parasite has been found in this region. One wonders therefore whether the original records of Donovan (1920) and Mulligan and Swaminath (1940) of *P.cynomolgi* and *P.inui* like parasites in the Nilgiris might not really have referred to *P.c.ceylonensis* and *P.shortti* respectively. It is also possible that these earlier workers had seen *P.fragile* and identified it as *P.inui*, just as Ramakrishnan and Mohan (1962) and Satya Prakash and Chakrabarty (1962) did with the parasite isolated in the Nilgiri hills. On the other hand, the report of *P.cynomolgi* and *P.inui* in *M.mulatta* in East Pakistan by Schmidt et al. (1961) may well be correct, for the origin of these infections, as suggested by the authors, may have been from *M.irus* and *M.nemestrina* on the Burma Assam border, the latter monkeys being well known hosts of *P.cynomolgi* and *P.inui*.

Recently, Choudhury, Wattal and Ramakrishnan (1963) have incriminated *Anopheles elegans*, a member of the "leucosphyrus" group, as the vector of the simian malaria parasites of South India, and it would be interesting to know what the vector in Ceylon is.

The differential characters of the subspecies of *P.cynomolgi* are shown in Table III.

SUMMARY

1. Of the three malaria parasites recently isolated from Ceylon monkeys, two are described as new—*P.cynomolgi ceylonensis* subsp. nov. and *P.fragile* sp. nov. The former differs from the type and from *P.c.bastianellii* in the E. E. cycle and in its behaviour in experimentally infected mosquitoes, and is probably the same as the strain of *P.cynomolgi* recently isolated in South India.

2. *P.fragile* sp. nov. is a tertian parasite characterised by its marked tendency to deform and distort the erythrocyte and form more abundant pigment than any other known *Plasmodium*. It simulates *P.k.edesoni* and *P.coatneyi* in many respects though it is quite distinct from them, but it is considered to be identical with the "New Nilgiri Parasite" of Eyles. The E. E. stages and the complete sporogonic cycle are unknown.

ACKNOWLEDGEMENTS

We are deeply grateful to Dr. J. R. Baker, Dr. R. S. Bray, Mr. P. G. Shute, Mr. R. Killick Kendrick and Mr. P. E. Nesbitt for help in various ways and particularly in the mass dissections of infected mosquitoes. We should also like to thank Dr. M. Agnes Fernando, Mr. M. P. A. Jayetilleke and Mr. S. Kumaran Rajan for their assistance.

TABLE III

(Differential Characters of Subspecies of *Plasmodium cynomolgi*)

	P.c.cynomolgi	*P.c.bastianelli*	*P. c. ceylonensis*
1. *P.E.Cycle*			
6th day	18μ	24μ	—
7th day	26—34μ	30μ	20—26μ
8th day	over 35μ	under 35μ	30—40μ (oval)
Maturation	8th day	7th day	8-9th day
Large vacuoles	Present	Rare	Few or none
Peripheral patterning	Absent	Present	Absent
2. *Erythrocytic cycle*			
Schizonts	Late division, fill red cell.	Early division	Late division, fill red cell.
Time of maturation	Early morning	Before noon	9—11 am.
Stippling	Schüffner's dots	Schüffner's dots finer.	Schüffner's dots finer, more abundant with reddening of corpuscle.
3. *Sporogonic stages* at 27°C			
Size of oocyst on 7th day	27μ	50μ	50μ
Duration	9—10 days	8 days	8 days
4. *Transmissibility to man*	Positive	Positive	Negative

EXPLANATION OF PLATES

PLATE I—Erythrocytic stages of *P.c.ceylonensis*

Fig. 1. Merozoites.
2. Normal red cell.
3—11. Ring stages.
12—16. Amoeboid trophozoites.
17—20. Schizonts.
21. Female gametocyte.
22. Male gametocyte.

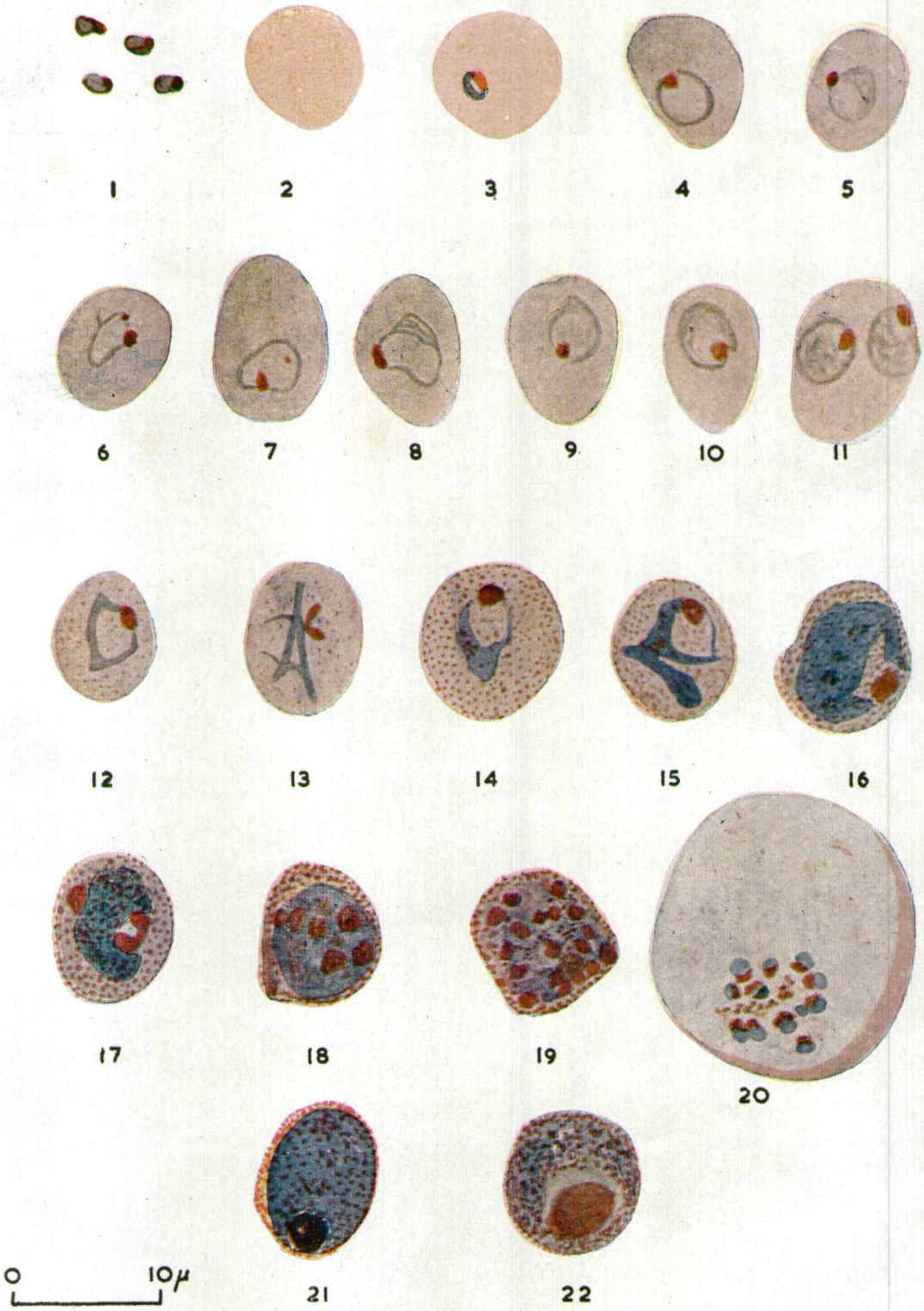
PLATE II—Erythrocytic stages of *P.fragile*.

Fig. 1—11. Ring stages.
12—15. Later trophozoites with fine pigment.
16—24. Trophozoites with abundant pigment.
25—30. Schizonts.
31—33. Female gametocytes.
34—35. Male gametocytes.

PLATE III—Erythrocytic stages of *P.shortti*.

Fig. 1—13. Ring stages.
14—17. Amoeboid trophozoites.
18—20. Schizonts.
21—22. Male gametocytes.
23—25. Female gametocytes.

PLATE IV—7 day E.E. Schizont of *P.c.ceylonensis* (X 2000).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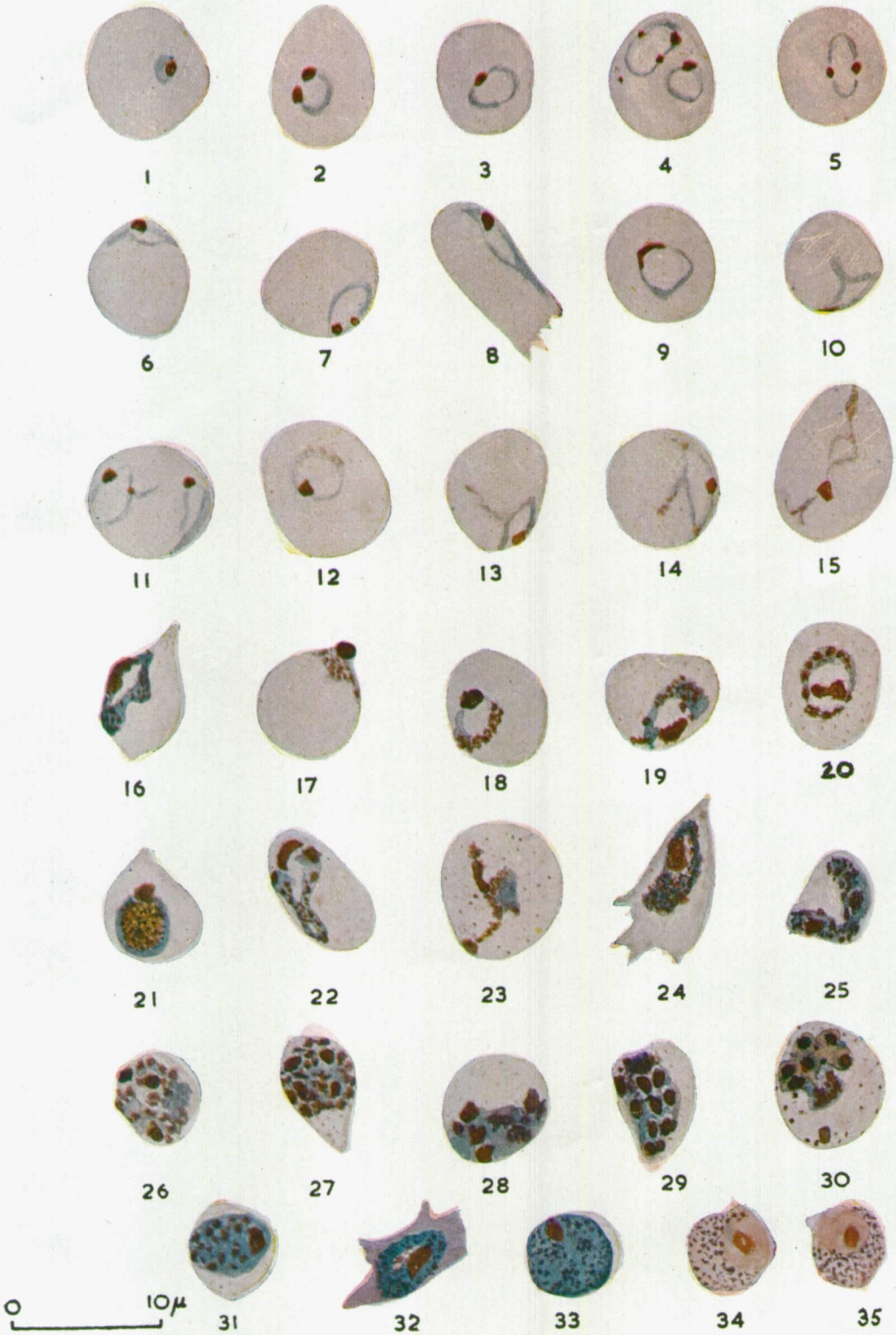
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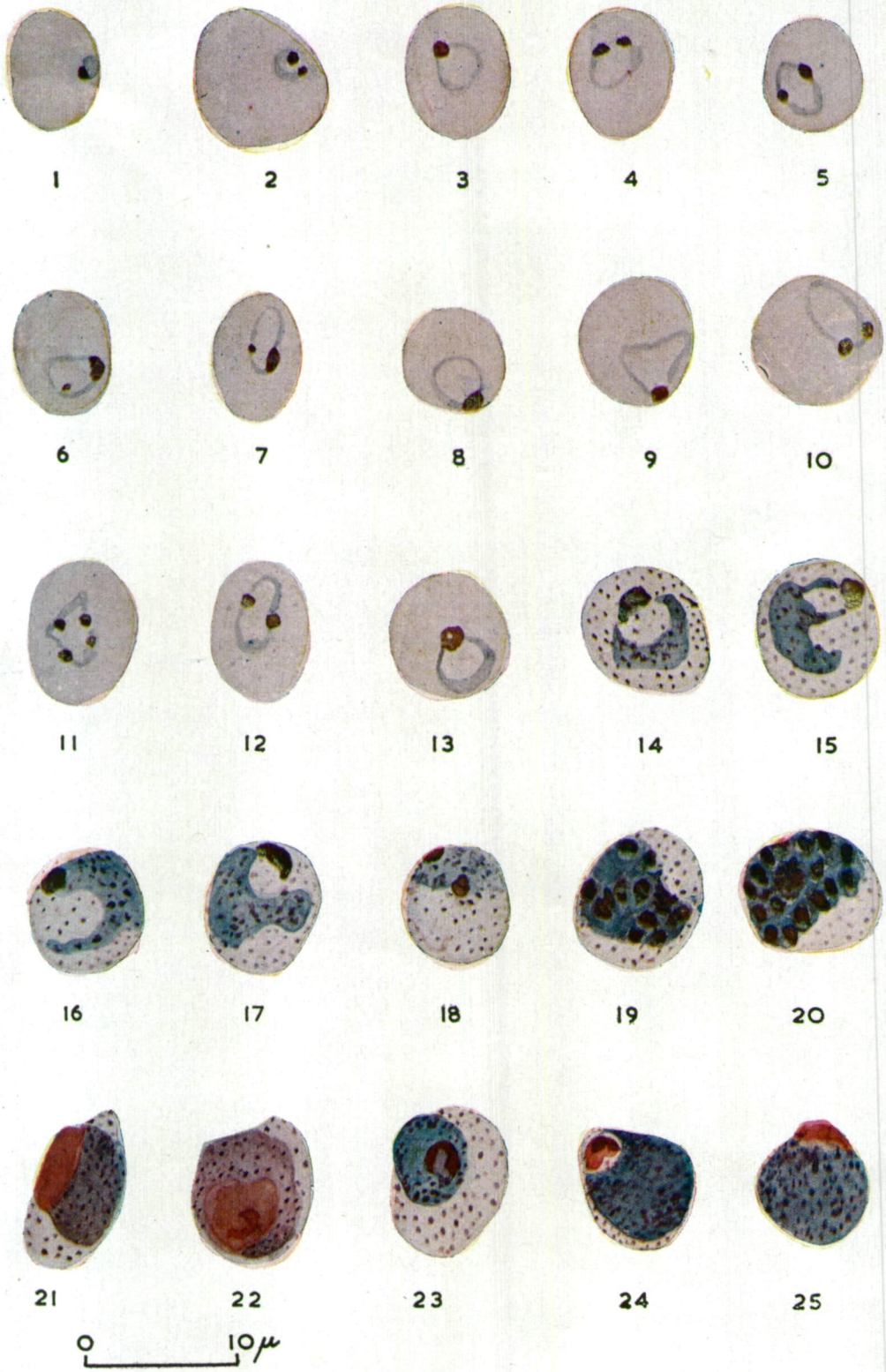
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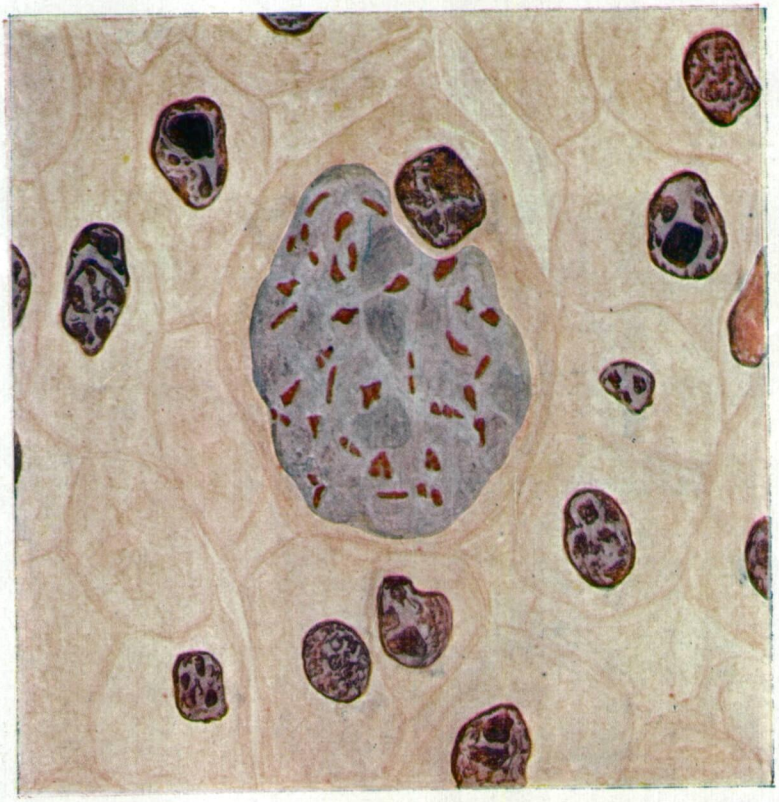

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