# THE TECHNIQUE OF BLOOD EXAMINATION IN MALARIA

CINCHONA PRODUCTS INSTITUTE, INC.
NEW YORK
1948

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*The colored illustrations of malaria plasmodia, presented herewith, were reproduced from original drawings made under the direction of Dr. C. W. F. Winckel at the Institute of Tropical Hygiene, Amsterdam, Holland.*

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In the treatment of malaria cinchona bark and quinine have a history of over three hundred years. While modern opinions differ as to the correct dosage, the short quinine treatment advocated by the former League of Nations Malaria Commission is still followed by many.

## Quinine Dosage as recommended by the Malaria Commission of the former League of Nations:

20 grains of quinine sulfate for 5-7 days in *vivax* malaria. Take no more quinine until there is a recurrence or relapse, when the initial dosage is repeated. Slightly more quinine is needed for *falciparum* malaria.

# EXAMINATION OF THE BLOOD

## Description of Technique

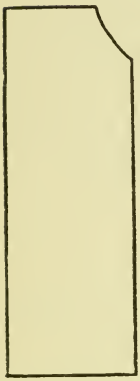


Fig. 1

### *EQUIPMENT*

1. A sharp edged needle for making the puncture.
2. Slides, carefully cleaned and put away in a mixture of equal parts of ether and alcohol.
3. A spreader, i.e. a slide with bevelled edge and a small corner cut off (see fig. 1). The film made with this spreader does not cover the whole width of the slide, but leaves the film edges free for investigation (see fig. 2).
4. Cotton-wool and alcohol.

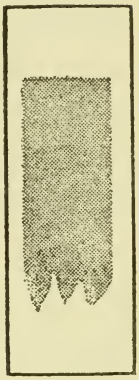


Fig. 2

### *TAKING A DROP OF BLOOD*

The lobe of the ear is a very suitable place to select for this purpose. It is more suitable than the finger-tip, as the latter is more sensitive and more exposed to infection. The ear-lobe should be cleansed with a little wad of cotton-wool damped with ether. As soon as the skin is dry a puncture or tiny incision is made. A drop of blood emerges, possibly only after slight pressure.

When sufficient blood has been taken for the desired films, a little wad of cotton-wool is put on the wound.

### *MAKING THE BLOOD FILMS*

#### *A. Thin smears*

A small drop of blood is put on the edge of the spreader. The slide is put in a horizontal position and the film is made after the manner indicated in fig. 3.

A little practice will show what size the drop of blood should be, at what angle the spreader should be held and at what rate it should be moved. The film dries within a few seconds and this process may be hastened by waving the slide to and fro. Fixation should follow immediately.

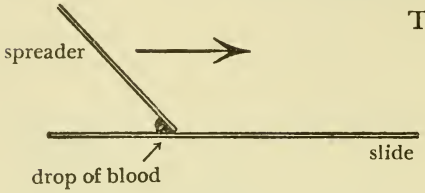


Fig. 3

## *B. Thick drops*

The slide is held against the drop of blood on the ear. The drop is thereby transferred to the glass and may have a diameter of about 5 mm. The slide is held in a vertical position so as to allow the drop to spread out. If the drop does not run of itself (as for example when it is not large enough) the lower edge of it may be touched by the needle with which the ear was pricked and the drop drawn out along the vertical slide to form a streak of blood about one inch in length. By this method a film that is neither too thick nor too thin is obtained, and one which after staining can be examined over its entire surface (fig. 4*a*), in contrast with the round drop which is too thick in the middle for staining and examination (fig. 4*b*).

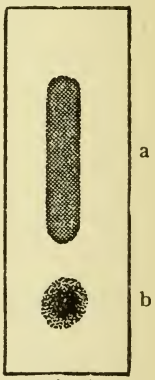


Fig. 4

The film should be laid horizontally to dry in the air but not in the sun. It dries in the course of a few minutes.

## *THIN SMEAR OR THICK DROP PREPARATIONS*

Each of these two methods of examination has its own advantages and disadvantages.

1. *Thin smears* are better suited for the examination of parasites and blood cells and hence make differential diagnosis easier.

If the blood specimen contains but few parasites it requires a long search to find them in a thin smear. Before the thick drop method came into vogue the accepted rule was that the film must be examined for a full hour before it could be pronounced negative. This was often necessary, because many patients had taken a dose of quinine on their own initiative before consulting the doctor, with the result that it took a very long time to discover the few remaining parasites.

Parasites are very scarce during the initial stages of the fever (*Korteweg*), and the same is true in the case of "healthy" carriers.

2. With a *thick drop* the same amount of blood may be examined in five minutes which requires a full hour with a thin smear. Hence the thick drop method is the only feasible one for large scale investigations, such as the examination of a great number of inhabitants of the same village, healthy parasite carriers or persons who have already had a dose of quinine.

The thick drop method increases the number of positive finds, but it requires a more accurate technique for staining than the thin smear method. If the following rules are observed, however, thick drop films will in many cases enable the investigator to use them also for the differential diagnosis of the various forms of malaria.

Hence our advice is as follows: For routine examination the thick drop is preferable to the thin smear; but the latter is needed when more delicate cytological examinations have to be made.

## *STAINING*

The same stain may be used for both the thin smear and the thick drop. Giemsa stain may be obtained ready made in small bottles from many dealers or it can be prepared from the following recipe.

Giemsa tablets, such as Burroughs and Wellcome's (London) Eosin-Azur Soloids, may be obtained from any dealer. Clean a small mortar thoroughly with methylalcohol and pound one tablet finely. Cover the powder thus obtained with 3 c.c. of glycerine. Rub the mixture again until it is entirely smooth. Then add 3 c.c. of methylalcohol, stir with pestle and allow to settle. Pour the solution into brown drop bottle with glass stopper. Rub sediment again, add 2 c.c. of methylalcohol, pour off once more. Rinse the pestle and mortar with another 2 c.c. of methylalcohol and pour this wash into the bottle as before. This should mean that all of the eosin-azure is now in the brown bottle. The result is 10 c.c. of Giemsa solution which must now be placed in an incubator at 37° C. for 24 hours and then kept at room temperature. This stain will not deteriorate for months if it is protected from heat and light. It goes through a ripening process and becomes most effective after standing for two to three weeks.*

### *A. Thick drops*

When the film is quite dry from exposure to the air, it is ready for staining. In order to remove the haemoglobin the slide is flooded with a solution of 0.1% of magnesium sulphate in distilled water. After five to ten minutes the fluid containing haemoglobin may be flushed off by pouring tap-

*Dr. Aimee Wilcox, in the National Institute of Health Bulletin, No. 180, page 21, 1943, writes that "The most dependable stain, particularly for thick films, is obtained with a good quality of Giemsa stain solution diluted with distilled water of a pH from 7.0 to 7.2. Grubler's dyes have long given complete satisfaction in this work. When Giemsa stains made from American dyes are used, they should be those stains which are certified by the Commission for the Standardization of Biological Stains. Recently certified samples of American Giemsa will be more likely to conform to the needs of the malariologist, as the staining of malaria parasites is now one of the tests used by the Commission prior to certification of Giemsa stains. If the Giemsa stain solution is made from powders, the best reagent methyl alcohol (neutral, acetone free) and glycerine C.P. (neutral) should be used. The glassware used should be chemically clean and dry. For thin films some Wright's and Leishman's stains made from standard formulae will also give satisfactory results."

water carefully over the slide from one end, out of a measuring glass. A white streak will be left, consisting of leucocytes, the stroma of the erythrocytes and the thrombocytes.

This tap-water is drained off and the slide is left to dry resting obliquely against some object. The film must be quite dry before staining is attempted, otherwise it may be washed away in the process.

The slide is now laid horizontally and flooded with a mixture of 2 drops of Giemsa stain in 2 c.c. of water. The water used for this purpose must not contain algae, water bacteria or the like; its pH must be about 7. Sometimes ordinary tap-water can be used, but often it is necessary to make use of a phosphate buffer solution (can be bought from stock) or of a 1/1000 magnesium sulphate solution. For this purpose a clean 10 c.c. measuring glass should be used after it has been rinsed with the buffer solution; after use it is again rinsed and put away filled with water of a neutral reaction. This glass should never be used for any purpose other than staining and never be cleaned except by rinsing with the buffer solution.

The staining fluid should be allowed to remain on the film for from 20 or 40 minutes to one hour, as indicated by previous experience in staining. The following factors affect the result:

*Temperature.* The best temperature is about 37° C. If the temperature is lower the process takes longer.

*Concentration of the stain.* The greater the concentration, the deeper and more rapid the staining, but it is accompanied by precipitation and unrecognizably over-stained leucocytes and plasmodia. In general it may be said that a long immersion in a highly diluted stain gives the best results, but of course there is a limit beyond which this is not the case. The slide should not be left in the stain for less than twenty minutes! If after this period the slide is too strongly colored, this may be taken as an indication for further dilution of the stain on the next occasion.

*Duration of coloring process.* This depends on the tem-

perature, the concentration of the stain, the age of the film, and the effectiveness of the stain used. A slide that is a day or two old does not stain as well as a freshly made one.

A thick drop is well stained if above a white ground it shows a purple color after flushing. Films are always bluer in tint after drying than before.

A film that has been stained for too short a period or too lightly is tinted pale to dark blue. This means that the red component has not had time enough to act and one notes among other things the absence of *Schüffner*'s dots.

Flushing should be done by pouring water carefully over the slide beginning at one end, care being taken not to allow the metallic looking film, floating on the stain, to come in contact with the bloodfilm as this would produce a troublesome precipitation. Never dry the slide by artificial heat or by putting it in the sun.

If a thick drop is properly stained, the microscope reveals a colorless or slightly yellowish background, not blue. The thrombocytes will be red-violet. The structure of the leucocytes should stand out clearly, even if they are rather strongly colored.

The stroma of the polychromatic erythrocytes, as well as basophile stippling, should be visible in the form of a blue "network" and easily distinguishable as forming fine or coarse nets.

The parasites have a red nucleus. The protoplasm is dark blue; the larger parasites show dark brown to black pieces of pigment. The erythrocytes with tertian parasites are stippled with orange red or purple red *Schüffner*'s dots; those with subtertian parasites show red spots (*Maurer*'s dots).

When the film is too lightly stained the *Schüffner*'s dots or *Maurer*'s dots do not show and the protoplasm of the parasite is tinted a too pale blue.

When the staining is too strong the parasite is shrivelled and unrecognizably covered by blue coloring matter and by red streaks due to colored threads of fibrin.

Films that are old, or that have been subjected to the action of light, are sometimes entirely or partly fixed and hence unusable. They haemolyse badly or not at all and staining is likewise not successful.

The above method is quite satisfactory for ordinary purposes, but occasionally more rapid work is required, when the object is not so much to obtain beautiful results and when one is content if only one can discover a plasmodium.

In such cases the following method is recommended:

*a.* Dry the film well.

*b.* Haemolyse for 10 minutes in a fluid consisting of 1 drop of stain in 2 c.c. of the 1:1000 solution of magnesium sulphate.

*c.* Flush carefully with water.

*d.* Dry again.

*e.* Stain for ten minutes with 3 drops of stain in 2 c.c. of the solution of 1:1000 magnesium sulphate.

*f.* Pour off stain, but do not flush.

*g.* Dry by leaning slide against the wall.

## *B. Thin film*

*a.* This is fixed by immersion in methylalcohol. Fresh films should be left in the bath for 2 minutes. In the case of older films drain off the fluid after 3 to 4 minutes.

*b.* Flush directly in plenty of running water.

*c.* Let it dry in the air until quite dry.

*d.* Pour over stain: 2 drops of Giemsa solution in 2 c.c. of water. The process of staining takes as long as in the case of the haemolysed thick drop.

*e.* Flush with plenty of running water. The metallic-looking film on the stain must not be allowed to touch the smear. Lean the slide against the wall to dry.

A well made and well stained thin film shows the following charactersitics:

*a.* The erythrocytes lie next to one another and are colored grey. If good stain is used and the staining has been

allowed to go on sufficiently long, some will show polychromasia.

*b.* The nucleus of the parasite is colored dark red and sometimes the structure of the nucleus is discernible too. The protoplasm is dark blue; brown pigment is present in tertian, darker, sometimes nearly black, pigment in subtertian and quartan parasites. Tertian parasites show coarse red *Schüffner*'s dots, except in the case of the youngest forms. Subtertian parasites show *Maurer*'s dots. The vacuole shows no color just as the achromatic zone round the nucleus.

*c.* The thrombocytes are colored purple-red.

*d.* The lymphocytes show a little too much color, although their structure is still clearly discernible.

*e.* The structure of the neutrophil cells comes out well and the eosinophils are especially characteristic. The leucocytes are a little too strongly colored.

The results obtained from staining depend on the following:

a. *The nature of the stain.* If the stain gives the erythrocytes a bluish tint and does not bring out *Schüffner*'s dots or only indistinctly in tertian parasites, matters may be improved by adding a little eosin to the buffer fluid (1 to 30.000).

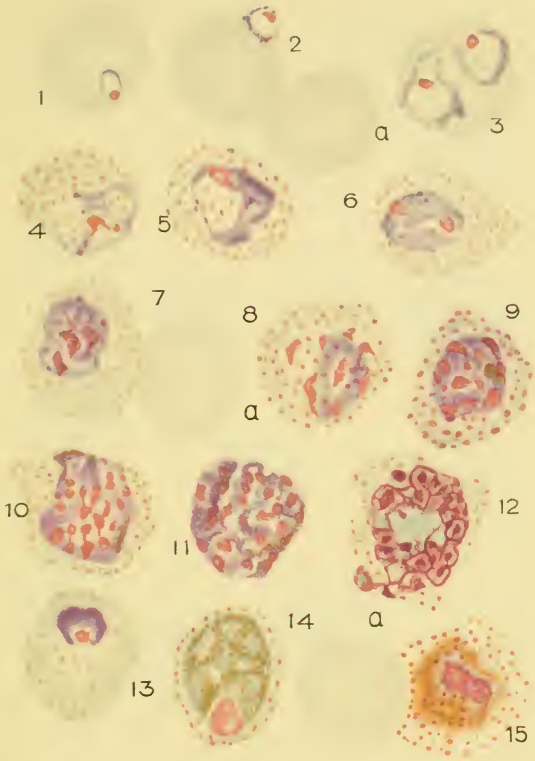
*b. The temperature.*

*c. The concentration of the stain.*

*d. Time allowed for staining.*

*e. Time allowed for fixation.* The longer the time allowed for fixation the longer the period needed for staining. If too little time is given to fixation haemolysis of the blood cells may occur.

*f. The age of the film.* The older the film, the longer the time needed for staining. Insufficiently stained films are immersed in Giemsa solution once more for re-staining.




*B. Bayens del.*

## I. PLASMODIUM VIVAX

*Benign Tertian Malaria (thin smear)*

1 young ring form; 2 young ring form, marginal; 3 double infection in erythrocyte with basophil dots; 4, 5 larger rings, amoeboid form, Schüffner's dots; 6—10 presporulating stages; 11 schizogony completed; 12 the same, with sharply differentiated merozoites; 13 young gametocyte; 14 female gametocyte; 15 male gametocyte. Schüffner's dots persist till the end. *a* normal erythrocyte.

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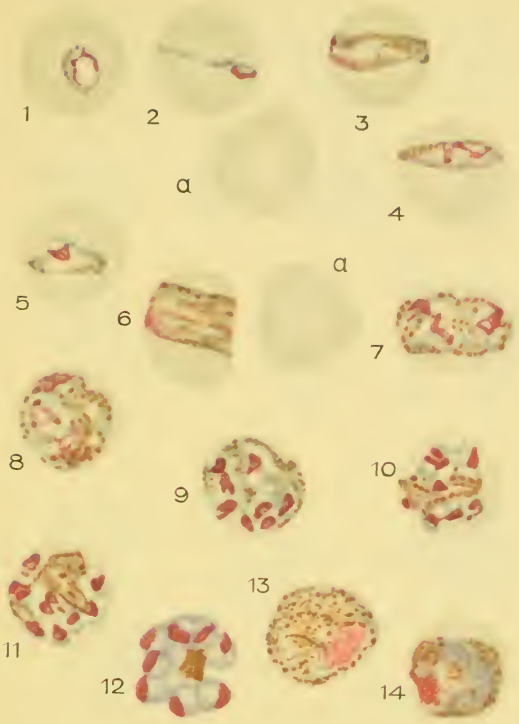


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## II. PLASMODIUM MALARIAE

*Quartan Malaria (thin smear)*

1 young ring form; 2 narrow band form; 3—6 older parasites in broad or irregular band form; 7—11 presporulating stages; 12 schizogony completed; 13 male gametocyte; 14 female gametocyte. *a.* normal erythrocyte.

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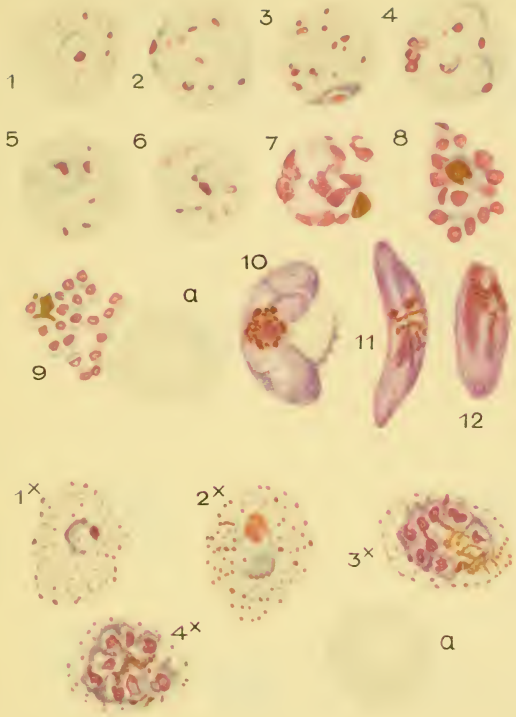


*B. Bayens del.*

## III. PLASMODIUM FALCIPARUM

*Subtertian Malaria (thin smear)*

1 young ring form; 2, 3 marginal forms; the Maurer's dots appearing in 1 are fully developed; 4 double infection and ring with double chromatin-dot; 5, 6 older rings; 7 presporulating stage; 8 schizogony completed; 9 liberated merozoites; 10 female gametocyte (crescent); 11, 12 male crescents. *a* normal erythrocyte.

## III A. PLASMODIUM OVALE

*Benign Tertian Malaria (thin smear)*

$1^x$ young ring form; $2^x$ older ring form; $3^x$, $4^x$ presporulating stages.

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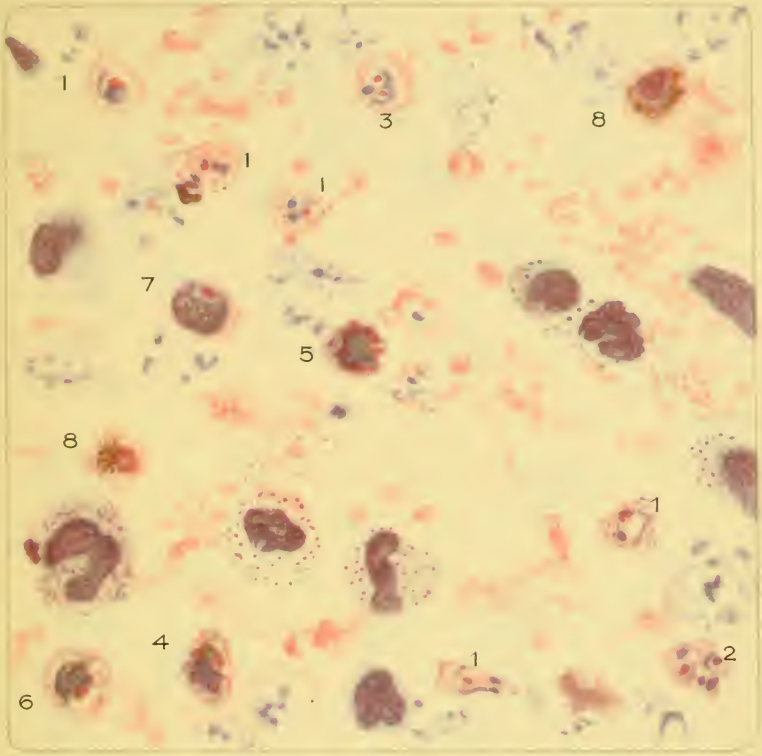


B. Bayens del.

## IV. PLASMODIUM VIVAX

*Benign Tertian Malaria (thick drop)*

1 ring forms; 2 double infection; 3, 4 presporulating stages; 5 complete schizogony; 6 young gametocyte; 7 female gametocyte; 8 male gametocyte.

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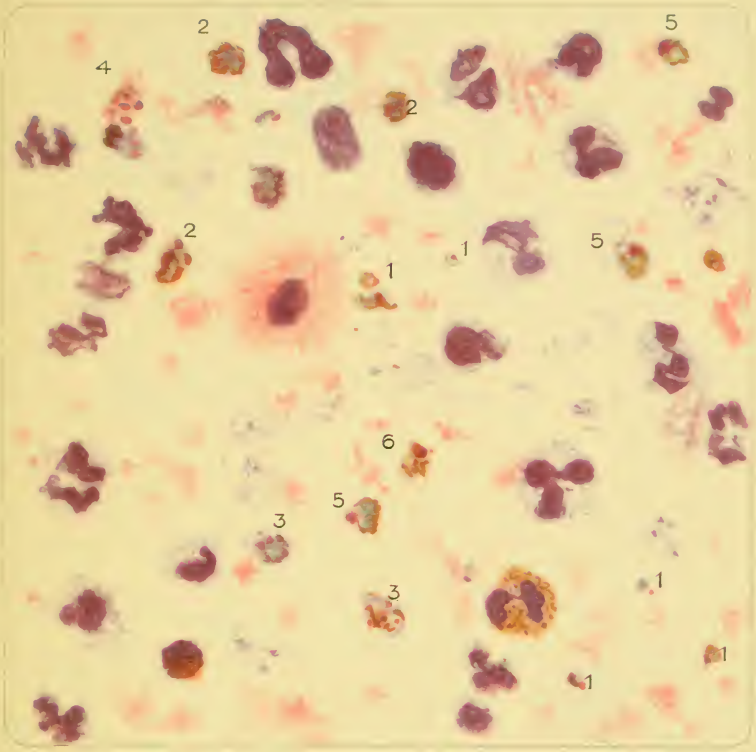


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## V. PLASMODIUM MALARIAE

*Quartan Malaria (thick drop)*

1 ring forms; 2 presporulating stages; 3 schizogony completed; 4 liberated merozoites; 5 female gametocyte; 6 male gametocyte.

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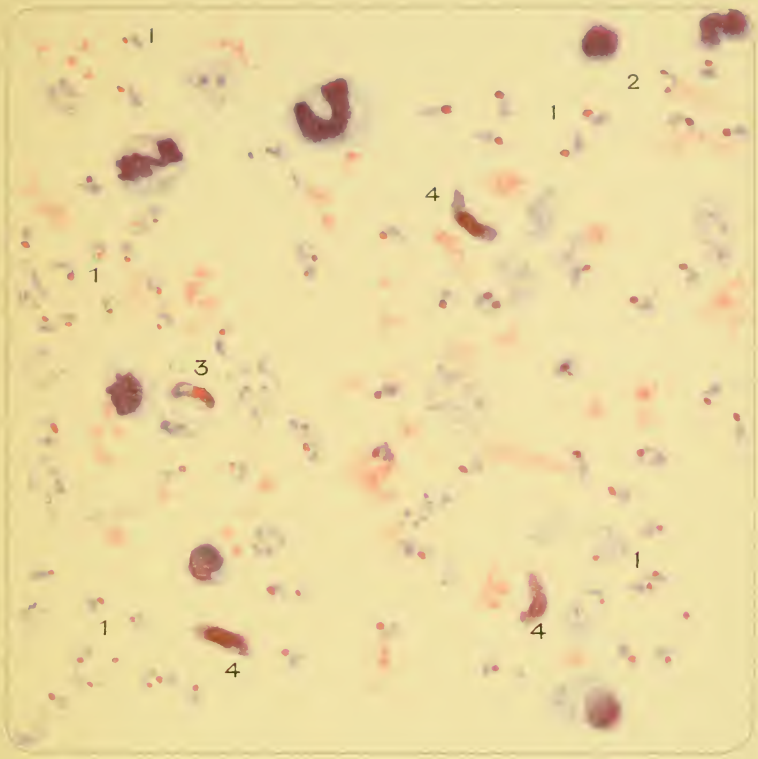


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## VI. PLASMODIUM FALCIPARUM

*Subtertian Malaria (thick drop)*

1 ring forms; 2 ring form with double chromatin-dot; 3 female gametocyte; 4 male gametocyte (3 and 4 crescents).

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## CHARACTERISTICS OF DIFFERENT MALARIA PARASITES IN STAINED FILMS

| Parasite | Duration of asexual development | Pigment | Changes in the red blood cells | Characteristics of asexual forms (Trophozoites) | Adult Schizonts | Merozoites | Adult Gametocytes |
|---|---|---|---|---|---|---|---|
| *Plasmodium vivax* (Malaria tertiana) | 48 hours | Fine granules; yellowish brown. Absent in young forms. | Enlarged, pale with fine red stippling (Schüffner's dots). | Rings, ⅓ to ½ diameter of red cell. Usually one nucleus only. The larger forms are irregular in shape on account of amoeboid movement. Vacuole present. Double infection of an erythrocyte rare. At most 2% of the erythrocytes are infected. | Larger than red cell. Rosette-shaped or irregular. | About 16 | Round or oval, larger than red cell.<br>♀ dark blue cytoplasm, small compact dark red nucleus near the periphery.<br>♂ cytoplasm much paler blue, sometimes reddish brown. Larger, more centrally located, paler nucleus than in ♀. |
| *Plasmodium malariae* (Malaria quartana) | 72 hours | Coarse, dark brown to almost black. The pigment is already present in young forms. | Not enlarged. No stippling with ordinary stain. | Uninuclear ring or band-like shape with only small vacuole that soon disappears. Compact, intensely colored cytoplasm. Segmentation begins after 48 hours. Double infection of an erythrocyte rare. At most ½% of the erythrocytes infected. | Same size as red cell. "Daisy head" form on account of regular arrangement of chromatin in form of wreath. Pigment clumped. | 8, very occasionally 10 or 12. | Round or slightly oval, almost or entirely filling the red cell.<br>♀ deep blue cytoplasm with small, dark red nucleus.<br>♂ pale blue or yellowish red cytoplasm, with large pink nucleus. |
| *Plasmodium falciparum* (Malaria tropica) | 48 hours | Darker than in the other forms. | Not enlarged. When intensely colored Maurer's dots appear. | Small rings about ⅛ diameter of red cell; often with 2 chromatin dots. Adult parasites seldom found in peripheral blood. Double infection of erythrocytes often occurs. In serious cases as many as 30% of the erythrocytes may be infected. | Irregular in shape. Smaller than red cell. Pigment black. Extremely rare in peripheral blood. | Number very variable, ranging between 8 and 24. | ♀ crescent shaped, deep blue, with compact red central nucleus and pigment aggregated round it.<br>♂ banana or crescent shaped, pale blue, with large pale nucleus. Pigment scattered. |
| *Plasmodium ovale* (Malaria tertiana) | 48 hours | Coarser than *P. vivax*. Dark yellowish brown. | Slightly enlarged; pale with fine pink stippling (Schüffner). Infected cells are often distorted or oval. | Rings, ⅓ diameter of red cell. No amoeboid movement, even in older forms. Compact, intensely colored cytoplasm, large nucleus. Band-like forms rare. | Slightly smaller than red cell. Often daisy-head shape. | 8 to 12. Chromatin may be crescent shaped. | Resembling those of *Pl. malariae*, but showing Schüffner's stippling in red cells. |

The following publications have been issued and will be sent free upon application:

Malaria Map of the United States (in color, mounted, 20x28 inches)

Drift or Mastery in the Treatment of Malaria *28 pages*

Quinine and Quinidine in General Practice *8 pages*

Quinine and Quinidine in the Treatment of Malaria *16 pages*

Malaria, Chills and Fever, Ague *8 pages* (for school children and teachers)

Treatment of Malaria *4 pages*

Quinine Formulary *48 pages*

Quinine: The Story of Cinchona *18 pages*

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