MANUAL FOR

THE MICROSCOPIC DIAGNOSIS

OF

MALARIA

THIRD EDITION

PAN AMERICAN HEALTH ORGANIZATION
Pan American Sanitary Bureau, Regional Office of the
WORLD HEALTH ORGANIZATION

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INTRODUCTION

This manual has been prepared principally for the purpose of establishing uniformity in the laboratory techniques used in the microscopic diagnosis of malaria in programs for eradication of this disease. It is also intended as an aid in the teaching of parasitology in malaria eradication training centers sponsored by PASB/WHO or operated with its collaboration. Moreover, the manual is designed to facilitate the work of laboratory technicians making regular examinations of blood specimens, particularly negative specimens, which will account for the majority once the first stages of eradication are completed.

In this third edition, the contents have been arranged under the four major headings: The Malaria Parasite; Preparation of Blood Slides; Microscopic Examination; and Laboratory Services. In 22 Appendices, supplementary information on basic equipment and techniques has been added to provide a ready reference for the student.

The recommended techniques are simple and are developed from the thick-blood-film point of view. The use of these techniques should result not only in rapid and high-quality diagnosis, but also in an increase in the microscopist's daily output.

The principles and recommendations in the manual, presented in the simplest terms, make this handbook applicable to any malaria eradication program.

PART I

The Malaria Parasite

1. THE LIFE CYCLE OF THE MALARIA PARASITE

A practical training aide for use in conjunction with this manual is the sound movie in color—*The Life Cycle of the Malaria Parasite*—edited by Prof. H. E. Shortt and distributed by the Imperial Chemical Industries in both English and Spanish. It depicts clearly and schematically the life cycle of the *Plasmodium* in the mosquito and in man. The final part, showing the thin smear diagnosis of *P. vivax* malaria, may be deleted as it predates the basic principles used in this manual.

Another useful aide is the short, black-and-white sound movie in English (USPHS, No. M-138B, CDC, Atlanta, Georgia), presenting a cinematographic record of a mosquito in the actual act of biting the skin of a hamster's cheek. A final portion of this film, taken with phase microscopy, shows the exflagellation of the male gametocyte and the fertilization of a female by one of the microgametes. Thick blood films drying very slowly under suitable temperature and humidity conditions can demonstrate partial and even complete exflagellation. This is the major point of diagnostic significance contained in this film.

The female *Anopheles* mosquito is driven, by the urge to secure blood for the maturing of her already fertilized eggs, to bite a warm-blooded animal. If that animal is a man in whom an infection with one of the species of the malaria parasite is well established, the blood will contain sexual forms of the parasite as well as the asexual forms which are responsible for the symptoms of the disease.

These asexual forms, on reaching the mosquito's stomach, quickly die and are digested, but the sexual forms—gametocytes —escape from the human red blood cells ingested by the mosquito. The smaller male gametocyte soon develops spermatozoa-like motile elements called microgametes (exflagellation), which detach themselves from its residual body and actively move away in the surrounding liquid of the mosquito's stomach. The larger female gametocyte undergoes some maturation process which enables the motile microgamete to enter and move actively within the cytoplasm of the female macrogamete.

Within a few hours, a fertilized ookinete has developed and has moved between the cells lining the mosquito's stomach, coming to rest beneath the membrane separating the lining cells from the body cavity of the mosquito. Here, the united nuclear material begins to divide in the large, highly refractile cell, which still contains the original pigment of the female gametocyte. The encysted ookinete, now a spherical oocyst, becomes larger and larger, penetrating farther into the cavity around the intestinal tract of the mosquito. After two to three weeks the oocyst, distended by thousands of tiny hair-like structures, called sporozoites, each containing a tiny piece of chromatin, bursts and releases the mature sporozoites into the fluid-filled cavity of the mosquito. The current of the body fluid carries the sporozoites forward into the thorax of the mosquito. Once in contact with the salivary gland cells, they enter them and ultimately reach the salivary ducts with the saliva.

The USPHS film, which shows the mosquito in feeding action, indicates that the proboscis probes the skin of its victim repeatedly until it reaches the lumen of a capillary or venule. Saliva is released by the mosquito during the biting and sucking operations, and the malaria sporozoites are injected into the victim intravascularly, as if by a needle in a vein. By subinoculation they can be shown to circulate up to 10 minutes, but rarely after 30 minutes. Since the entire amount of blood in the human body passes through the liver every three minutes, in man it is relatively simple for numbers of sporozoites to enter liver cells immediately adjacent to blood-filled liver sinusoids. Probably many are lost to phagocytes, but those which are successful begin their liver multiplication immediately and this process lasts either 6, 8, 9, or 11 full days, depending on the malaria species. The growing form in the cytoplasm of a liver parenchymal cell expands and its nucleus divides repeatedly until the large cyst-like, irregular-shaped, mature schizont ruptures. Numbers of the liver merozoites that result from this rupture find their way between neighboring liver cells to the nearest sinusoid that contains the human red blood cells. Thus, the pre-erythrocytic stage is over and the disease-producing erythrocytic stage begins. At the end of each 48- or 72-hour period, many mature erythrocytic schizonts rupture and numbers of tiny merozoites hasten to enter other red blood cells before they are captured by the phagocytic monocytes and other cells.

2. BEHAVIOR OF THE INFECTIONS WITH P. FALCIPARUM AND P. VIVAX IN THE LIVER OF MAN

After the exo-erythrocytic stages of the *Plasmodium* were first discovered in the endothelium of birds, it was long presumed that these stages would be found in the endothelium of man. This has not been the case. Several years before the discovery of the liver stages, schematically pictured in the movie *The Life Cycle of the Malaria Parasite* (see p. 3), experiments had been carried out in Australia during World War II to determine the exact effects of the then well-known drug atabrine in malaria therapy. The results of these experiments dramatically demonstrated everything about the exo-erythrocytic stages of *P. falciparum* and *P. vivax* in man.

So complete and exhaustive were the experiments that only the location of the stages remained to be discovered. Unlimited numbers of non-immune volunteers were used in an area where malaria did not exist. Mosquitoes and strains of *vivax* and *falciparum* malaria from hyperendemic areas of the southwest Pacific region were brought in. The subinoculation of 300 to 500 cc of blood was used to demonstrate the presence or absence of infective forms in the peripheral circulation. This was the most important feature of the tests, since up to that time only 10 to 50 cc had been inoculated into a very small number of subjects.

As briefly as possible, the results obtained may be summarized as follows:

P. falciparum. Mosquitoes with demonstrable sporozoites of *falciparum* were allowed to feed on the right arm of one volunteer while 300-800 cc of blood were withdrawn simultaneously from a vein of the left arm and immediately injected in another volunteer. Both subjects developed parasitemia and symptoms at the end of the customary incubation period of 11 days. But a similar sample of blood withdrawn one-half hour or more after the mosquitoes had stopped feeding consistently failed to infect a second recipient. Thereafter, blood withdrawn and injected daily for 5 days did not produce any sign of disease in the recipients. In fact, not until after 144 hours had passed was any subinoculation reported as

successful. This means that a period of 6* full days elapsed before the blood of the person bitten could induce a demonstrable infection in another individual.

Daily subinoculations continued to be positive in the second individual until a curative amount of mepacrine had been taken or the patient had developed sufficient immunity for a spontaneous cure. Once any of these cases became negative they remained so, but all were followed for one year.

On the other hand, the weekly subinoculations were always positive from those cases that had received insufficient medication, although they remained symptom-free for several weeks or months before relapsing.

This demonstrated conclusively that there was a sharply defined period of 6 full days during which some type of development of the *falciparum* infection continued outside the peripheral circulation.

It was repeatedly demonstrated that an individual who received 100 mg of mepacrine daily for 7 days previous to bites by *falciparum*-infected mosquitoes, and for a minimum of 3 weeks thereafter, never developed patent parasitemia or symptoms, although occasionally subinoculations made on days 9 to 13 after treatment were positive. This was the first experimental proof that a drug could completely eliminate the *falciparum* infection. It also demonstrated that 3x100 mg mepacrine tablets administered daily for 8 days could produce a radical cure of this infection.

Although there were some cures of individual cases treated with quinine, this drug could not be depended upon to produce a cure of *falciparum* malaria in more than 50 per cent of the cases. Its reported successes could well have been assisted by the development of a high degree of immunity. Proof of the correctness of this observation is the virtual disappearance of blackwater fever from those areas where mepacrine replaced quinine, either for suppressive or radical treatment.

P. vivax. Similar experiments were repeated for *vivax* malaria with equal success but with markedly different results. *Vivax* subinoculations on the 7th and 8th days were negative. Only when 8 full days had elapsed did the recipients become infected, even when simultaneously bitten by *vivax*-infected mosquitoes and

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* *falciparum*—6 days; *vivax*—8 days; *ovale*—9 days; *malariae*—11 days.

falciparum-infected mosquitoes. *Falciparum* appeared on day 7 and day 8; day 9 produced both.

The blood of the *vivax*-infected person continued to be positive throughout the duration of the primary attack of 3 to 5 weeks, or until curative treatment was given. It then became negative and weekly subinoculations for as long as 2 to 5 months completely failed to infect recipients. Not until near the time when a relapse might normally occur did the first positive subinoculations appear. When a relapse, with its patent parasitemia and symptoms, subsided, subinoculations continued to be positive for a number of days and then became negative. They remained so until within a few days of the next relapse, if such occurred. With the daily administration of mepacrine, as indicated above, transient parasitemias were not encountered as with *falciparum,* but even if the administration was continued for 3 weeks after the biting, parasites and symptoms usually appeared in the patient shortly after the drug was discontinued. The curative treatment with mepacrine, which had proved so effective in *falciparum,* always controlled the primary attack and eliminated the parasitemia, but the same treatment did not prevent the subsequent development of a relapse in *vivax* infections.

The situation in patients infected by blood inoculation rather than by mosquito bites was quite different; these individuals received only the asexual erythrocytic forms. As long as these persisted in the blood, subinoculations were positive even though the number of parasites in the inoculum was submicroscopic. The patients with either *falciparum* or *vivax* were readily cured with smaller amounts of drugs; once they became negative to subinoculation they continued to remain so. Such experiments showed beyond all possibility of doubt that there was a pre-erythrocytic stage in the *falciparum* infection of 6 days' duration, after which time it was apparently completely exhausted, and that once subsequent asexual infection was eliminated the patient was completely cured.

In sporozoite-induced *vivax,* the pre-erythrocytic stage lasted a full 8 days. However, evidently it did not exhaust itself entirely because after a period when no circulating forms could be detected in the blood, one or more renewals of parasitemia and clinical activity were frequently, though not constantly, encountered. This never occurred when the infections were induced by injections of blood. The deductions made from these experiments suggested the existence of a *hidden developmental* stage which produced, in

falciparum, merozoites which infected only red blood cells and nothing else, whereas in *vivax* it was postulated that this hidden stage produced merozoites infective not only to the red blood cells but also to some cells capable of maintaining them in a site then unknown.

The marked difference in exo-erythrocytic behavior between *falciparum* and *vivax* justifies the creation of a new genus: *Laverania.* Therefore, *P. falciparum* may ultimately be called *L. falcipara.*

The following points are based on a full understanding of the above-described experiments:

1. *Falciparum* malaria is entirely curable by an adequate dose of blood schizontocides.
2. All *vivax* infections do not necessarily relapse; up to 30 per cent do not.
3. Medication that will eradicate *falciparum* infection will not eliminate *vivax* relapses, which, if they occur, rarely if ever continue to do so for more than 3 years.
4. Prolonged suppressive medication may also eradicate *falciparum,* but *vivax* is prompt to appear clinically when medication is discontinued.

Apparently, the suggestion to explore the liver for these hidden stages originated in an observation made by Garnham in East Africa, where he had noted tiny vesicles on the surface of the liver of monkeys whose blood contained the gametocytes of *Hepatocystis kochi.* These cysts, which were later shown to contain numerous merozoites, were never found in uninfected animals.

The liver stages were first demonstrated by Shortt and Garnham in a monkey infected with *P. cynomolgi* and shortly thereafter in an induced infection of *P. vivax* in man. The liver stage of *P. falciparum* in man was demonstrated in England and in America some time afterward. In 1953 *P. ovale* was shown to have an hepatic stage of 9 days' duration. In 1959 Bray found that the hepatic stage of *P. malariae* can last 11 days. In all four species the actual liver stages are similar in appearance. The youngest forms are found in the cytoplasm of a single liver cell; the chromatin divides by binary fission. At maturity, *vivax, malariae,* and *ovale* contain hundreds of merozoites, whereas the *falciparum* liver schizont may contain several thousand. The only damage to the liver is apparently a compression of a few adjacent cells, as no reactionary cellular infiltration is seen. When the schizont ruptures, naked

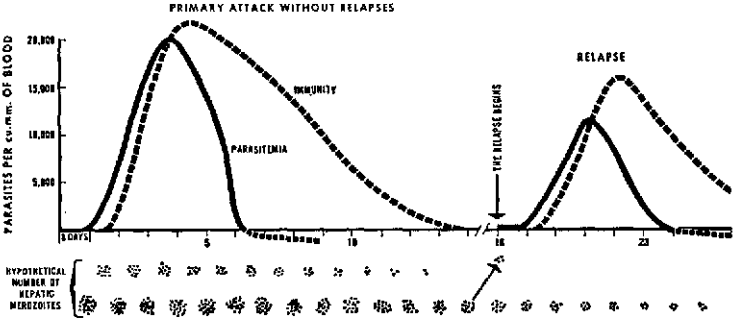
merozoites are extruded between adjacent liver cells until a liver sinusoid containing red blood cells is encountered. At this point the erythrocytic stage begins.

Another monkey plasmodium, *P. knowlesi,* which has a 24-hour erythrocytic cycle and a pre-erythrocytic stage lasting 5½ days, has been used repeatedly to infect man artificially.

More than 100 successful infections of man with *P. cynomolgi* and other simian species of malaria have been studied in 1960-62.

DIAGRAM 1

SCHEMATIC DIAGRAM OF THE ROLE OF THE EXO-ERYTHROCYTIC STAGE IN THE PRIMARY ATTACK AND RELAPSE OF VIVAX MALARIA



The wholly imaginary schematic diagram shown above (Diagram 1) is an attempt to explain the role of the exo-erythrocytic stage of *vivax* malaria in:

1. A solitary primary attack.
2. One relapse following a primary attack.

The solid line shows the parasitemia as measured by the scale on the left. The parasite level is submicroscopic where the line becomes interrupted. The dotted line suggests the degree of immunity; the bottom scale marks the duration of each complete exo-erythrocytic cycle, or 8 days.

Beneath the diagram, the upper row of diminishing clusters of small dots suggests the number of liver merozoites reaching the circulation after successive liver schizogonies. This number decreases with each hepatic cycle until the formation of liver schizonts ceases.

The lower row of clusters of dots represents a much larger number of liver merozoites released every 8 days. Only a fraction of

these are required to initiate the parasitemia of a relapse (as at the end of the 18th liver cycle), provided the immunity is so low that the progeny of the first erythrocytic schizogony escapes destruction and successfully invades new red blood cells. One must perhaps assume that the merozoites coming directly from the liver are unaffected by the immunity of the host; however, those produced by the first and subsequent erythrocytic schizogonies undoubtedly are susceptible to immunity already developed.

It may well be that the relapses derive from latent or very slowly growing liver schizonts. Latent development would explain also the delayed primary attacks of the so-called temperate climate *vivax*.

3. BLOOD IN RELATION TO THE DIAGNOSIS OF MALARIA

Blood is the medium in which malaria parasites are found. Since it is the vehicle which brings the parasite into the microscopic field of vision of the person who is searching for it, it is highly desirable to know something about the blood itself.

Blood consists of a liquid called plasma, wherein are suspended the cellular elements (erythrocytes, leukocytes, blood platelets), which are developed in the bone marrow and released to the peripheral circulation as required.

Erythrocytes, or red blood cells, are biconcave discs[1] which appear singly or in groups, or like rolls of coins called "rouleaux." When slight pressure is applied to fresh blood under a coverslip, they can be compressed or greatly distorted without injury. They are tawny yellow in color because of their hemoglobin-filled contents, in which parasites, if present, can be recognized only if they are large enough to contain pigment. The examination of fresh blood, therefore, is not practical. Red blood cells have a maximum life of 120 days.

Erythrocytes are also derived from a type of bone marrow cell and the early stages contain a nucleus. Just before these developing erythrocytes are released into the circulation, the nucleus is lost and these young red blood cells of varying size can contain

[1] See Diagram, Appendix 3, p. 94.

blue-staining elements (seen only with special staining in the thin smear), variously described as reticulum (reticulocytes), polychromasia, and punctate basophilia, all of which disappear in 1-3 days after the cells are released into the circulation. In thick blood films[1] not only blood platelets but bluish masses ranging from the size of a small lymphocyte to a large spread-out polymorphonuclear leucocyte can be seen in the otherwise clear spaces between the leukocytes. These bluish masses are the remains of young erythrocytes (reticulocytes). They vary in appearance from a fine haze, through mottled cloudiness, to dense blue dots of variable size. Their numbers also vary from 1-3 per microscopic field in normal blood, to a countless number where a malaria or other infection has resulted in a severe anemia which has forced the bone marrow to put out large numbers of cells before the red-cell maturation is complete.

Under changing conditions of their surroundings, the fresh red blood cells may alter their appearance greatly. In serum or in 0.85 per cent solution of sodium chloride the smooth, round outline of the cell is preserved. If the amount of salt is increased to 1.5 per cent, the cell shows little protuberances of its outer surface, resembling those of a submarine contact mine. Such cells are termed "crenated" and may even retain this appearance after staining. Too much or too little salt in the solution causes the cells to rupture and to release their hemoglobin, and the solution water becomes and remains red.

Exposure to strong alcohols, application of heat, or the passage of time will "fix" the hemoglobin in the cells and dehemoglobinization will not occur. Hence the need to stain thick films as soon as possible after they are taken.

Leukocytes, or white blood cells, are transparent, highly refractile, and may show movement of their cytoplasm. In general, their nuclei should be rich blue-violet in color. They vary in size and shape, and the cytoplasm may be clear or granular, depending on the type of cell. The life of a leukocyte is short—3-5 days only—so that the appearance of some of the polymorphonuclear types varies from compact, clear-cut, well-stained individuals to large, pale, irregular, and often distorted cells. Diagram 2 gives some examples of the common leukocytes observed in the examination of a thick blood film.

[1] See Section 6, pp. 33-35.

DIAGRAM 2

LEUKOCYTES

POLYMORPHONUCLEAR LEUKOCYTES

EOSINOPHIL

SMALL LYMPHOCYTE

MONOCYTE

LARGE LYMPHOCYTE

PLATELETS

CELL REMAINS

ERYTHROCYTES

Blood platelets (Diagram 2) do not have a nucleus, originating as they do from the cytoplasm of an enormous cell of the bone marrow called a *megakaryocyte*. They should be recognized as separate individuals and in groups. They are of different densities on the same microscope slide but the color can range from pink to violet (never blue) in different specimens. They vary in size and shape; in blood which has dried slowly or has been defibrinated, they may resemble almost anything. If slides have been long delayed before staining, platelets may stain so deeply as to hide small parasites.

Detection of malaria parasites in the blood. If malaria parasites cannot be found on microscopic examination, this does not necessarily mean that they were not present at the moment the blood was taken; possibly their numbers were too small to be detected in ordinary examination. For example, in one instance in Ghana, women in a prenatal clinic were carefully followed by means of regular thick-blood-film examinations of 100 microscopic fields each. Gametocytes of *P. falciparum* were found in 7 per cent. When 10 thick films were made simultaneously and examined for 100 fields each, 20 per cent showed gametocytes. Interestingly enough, 6 examinations were sufficient to encounter 18 per cent positive cases and 4 further examinations produced only 2 per cent more cases.

The only certain way to prove that a person has no malaria parasites in his blood is to inoculate 2-400 cc of blood into a human volunteer. If no parasitemia develops, the blood was negative; if it does, the original infection was submicroscopic when the blood was drawn.

It should be appreciated as well that the blood of a normal, healthy, well-nourished individual can be quite different from that of a person who has had malaria or some other debilitating illness over a considerable period of time. Changes in the usual appearance of parasites may be produced by alterations in the shape of the red blood cells themselves, quite independent of the species of malaria infection.

Despite the way parasites appear in the dehemoglobinized thick blood film, malaria parasites are incapable of an independent existence. Except for the briefest period when they are moving from one cell to another, they are *intracellular* in red blood cells.

When whole blood is allowed to stand in a test tube, it coagulates as a result of the interaction of the platelets and elements of

the blood plasma which produce fibrin. When the sponge-like mass of fibrin, which entangles most of the red blood cells, contracts to form a clot, clear yellow *serum* separates out after standing. Unless spread promptly, a drop of blood on a microscope slide will clot in exactly the same fashion as in the test tube but with considerable change in the plasma; this affects the manner in which the preparation sticks to the slide. If the blood is stirred after clotting begins, areas of different thickness and different concentrations of cells are produced, which can easily be recognized by inspection with the low-power microscope objective. Streaks or clumps of leukocytes are seen instead of the even, regular distribution of same in the promptly-spread preparation.

The liquid part of the blood contains such antibodies as are produced by the body and provides liquid medium to keep cells and parasites moist. Drying destroys both. The cellular portion provides a variety of elements in the blood preparations which not only give information about the patient but also aid in the appraisal of the quality of the preparation and its staining.

A drop of *fresh blood* placed on a slide, covered with a coverslip, and illuminated with light greatly reduced by closure of the iris diaphragm of the microscope, may be examined under both low-power objective (10x) and the high power (43x). The oil immersion objective[1] may also be used if the cells are not moved by the focusing action.

Appearance of the Blood Elements in a Well-Stained Thick Blood Film

Leukocytes. In general, the many-shaped nuclei of leukocytes should be of a rich, blue-violet color, while the cytoplasm varies with the type of cell. *Neutrophiles* have granules of different colors which are irregular in size, shape, and distribution. Staining of inferior quality may make the cytoplasm so red that the uninitiated may make the error of classifying these cells as eosinophiles. By contrast, the cytoplasmic granules of the *eosinophiles* are so large and regular in size, shape, and compactness that they can be recognized easily in the unstained state in which the nuclei cannot be distinguished. The compact granules themselves are dark copper-red in color, inconspicuous rather than prominent and not bright-

[1] See Appendix 16, p. 109.

red to pink, which is the familiar color of eosin-stained tissue sections. If one were to select a single feature which suggests inferior quality of the staining, it could well be such an appearance of these granules.

Lymphocytes and monocytes (see Diagram 2, p. 12) have only a single mass of nuclear material. The *small* lymphocytes are extremely important to the microscopist because they serve as the unit for measuring size in the thick blood film, just as the red blood cell is similarly used in the thin blood smear. The small lymphocyte is the most uniform cell in the blood and it measures 8-10 microns. It probably survives in the blood stream about 100 days. The cytoplasm of lymphocytes is pale blue, somewhat transparent, and sometimes it contains a few bright red granules.

Monocytes are, in the clinical sense, the most important cells for the patient and their numbers increase during the malaria infection. As active phagocytes, they are capable not only of taking up malaria pigment but even red blood cells containing mature malaria schizonts. Their cytoplasm shows a fine blue-grey stroma and the nuclei have a more or less prominent cleft (Diagram 2).

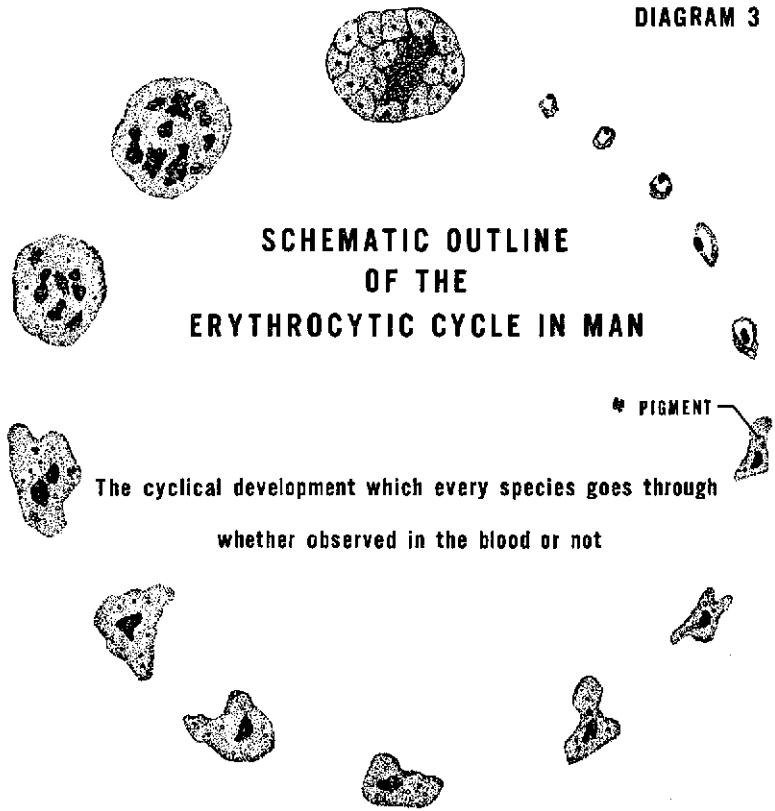
4. GENERAL CHARACTERISTICS OF PLASMODIUM SPECIES IN THE PERIPHERAL BLOOD

Individual parasites vary in size, shape, and appearance exactly as any multicelled animal.

Some individual forms of one species, namely, small gametocyte-like stages of *P. vivax,* may appear identical with larger stages of *P. malariae.* In fact, the only form of the parasite that may be considered *unique* or *typical* is the *P. falciparum* gametocyte.

To achieve an accurate species diagnosis under microscopic examination, it is necessary to have only a sufficient number of different examples of the parasite present to show the *pattern of variation* that is constant for each species. It will not suffice to memorize a list of appearances which have been commonly observed in the thin blood smears of various species to which have been attributed specific characteristics that are not always valid. It is necessary that the examiner know the variations of forms

DIAGRAM 3



that are possible, not only in each of the different species but with different conditions of the blood.

The examiner should always ask himself two questions with each new parasitic form that comes into focus: (a) Is this truly a parasite? and (b) If so, does its appearance fit into the *pattern of variation* that can be expected for the species suspected? (Diagram 3).

The answer to (a) is usually obtained by searching in the immediate vicinity for a parasite about which there is no doubt whatsoever. The color and density of the chromatin is appraised, as is the appearance of the cytoplasm. If these are not similar in the newly-found object, it is unlikely that it is a genuine organism.

Once it has been decided to consider the object a true parasite, the first question is: How many are present? The second: Does its developmental stage fall within the range of development which, as learned by observation and experience, can be present at a cer-

tain point in the cycle of "X" species, or is this a stage that is never present in the cycle of "X" species?

Owing to the fact that, for purposes of species diagnosis, the checking of slides is mandatory, it has become important to know, once parasites are recognized, whether they are numerous or scanty. If they are numerous, it is likely that the diagnosis of the species is correct; when there are very few parasites confirmation may be difficult. A system of symbols can give an approximation of the numbers present.[1]

A minimum of 50 microscopic fields of a positive blood film should be covered before a definite diagnosis is made. If it is evident that not all the forms pertain to "X" species, further examination is necessary to confirm a suspicion that some specimens of a second species are also present.

Broods.[2] All species may have more than one brood of parasites at the same time, as is demonstrated schematically in the film by Shortt referred to on page 3. Although *falciparum, vivax,* and *ovale* require 48 hours for maturation of the asexual erythrocytic forms, it is also possible, and not uncommon, for each to show a daily paroxysm. This daily paroxysm indicates the existence of two broods of one of the foregoing species, or may reflect the presence of three broods for *malariae*. Once the dominant brood is established, all others are suppressed.

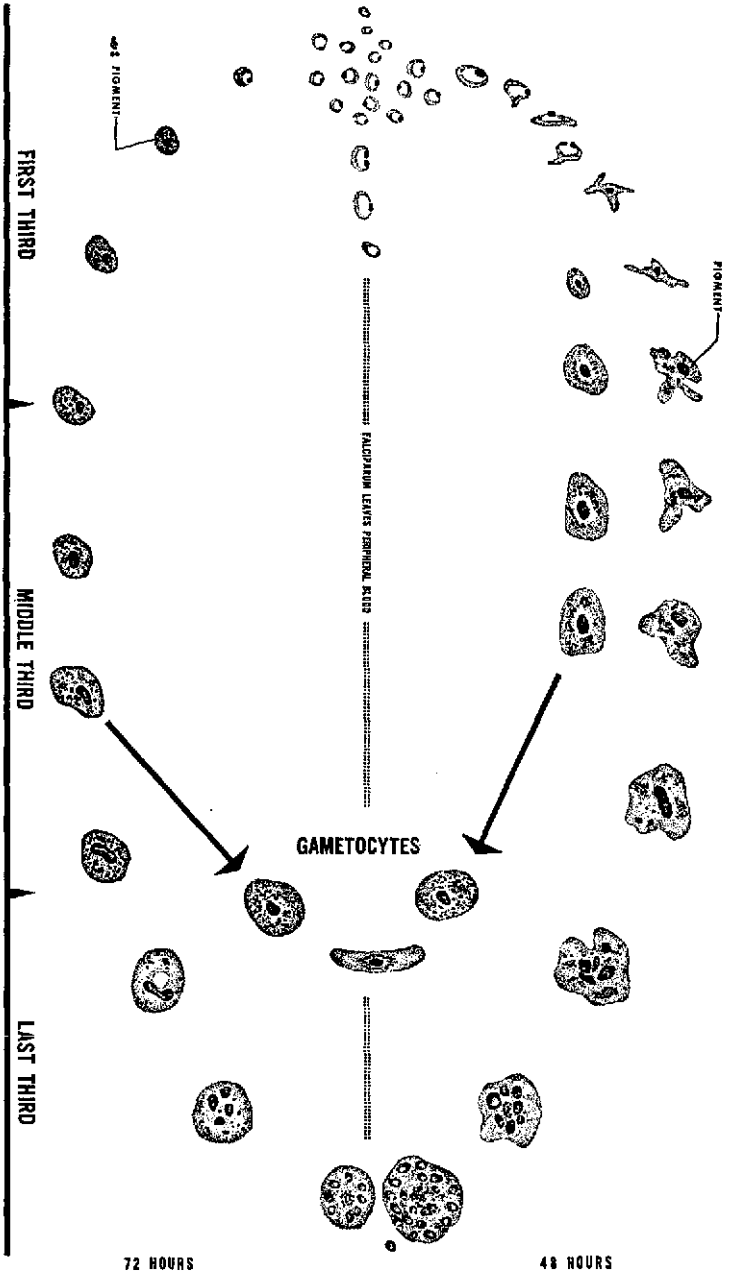
Different broods arise from the liberation of liver merozoites at different times in the primary attack of a mosquito-induced infection. They may also appear spontaneously in a continuing infection which has shown a regular single-brood periodicity for some time. The infection need *not* be of sporozoite origin for the development of a second brood. Apparently, at the schizogony of a single-brood infection (end of each 48-hour period), the majority of the parasitized cells rupture within the same hour, and the remainder complete their division at various intervals before or after the majority. If this takes place over a 5-hour period, instead of one hour, and the majority rupture during the 3rd hour, some

[1] See Section 14, Recording and Reporting of Results, pp. 63-65.

[2] *Definition* — Broods are unsynchronized population densities of the parasites found in the peripheral blood which may result in abnormal (out-of-step) clinical symptoms of the patient. They are formed from variation in the time of release of merozoites into the blood from the liver or from a natural difference in development which is exaggerated within few cycles. They presumably could also result from a new infection by biting infectious mosquito (when the immunity is not already established).

DIAGRAM 4

THE ERYTHROCYTIC CYCLE IN MAN



do not divide until 2 hours later. Those which began the schizogonic process did so 2 hours before the majority.

One cycle later, the offspring group which began schizogony is now 4 hours ahead of the majority (the last groups complete the process 4 hours later). After enough cycles, there will be a considerable number of parasites matured 24 hours before and 24 hours after the majority. Together, these may make up a sufficient number to produce another schizogony 24 hours in advance of the one caused by the majority, i.e., the dominant brood. Thus a new brood is developed quite independently of the exo-erythrocytic stage.

Diagram 4 presents, in a condensed schematic manner, the erythrocytic cycle, as seen in the peripheral blood, of the three common species of malaria in man. It illustrates the three stages of schizogony. At the top, merozoites are shown just after rupture of the mature schizont. These merozoites quickly enter the red cells (*vivax* on the right). Although stretching from the top to the bottom of the diagram, the cycle is a continuous one, and the smallest form (shown at the top) is encountered immediately after the largest schizont ruptures (shown at the bottom). The diagnosis of species will be greatly simplified if each parasite found is mentally transferred to its appropriate position in this diagrammatic cycle. Gametocytes of the three species have a completely different development, so their origin and growth is considered apart from that of the asexual forms.

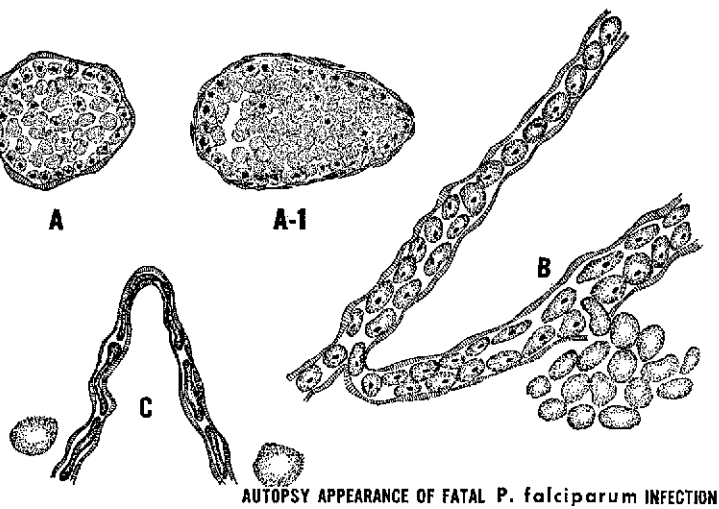
5. CHARACTERISTICS OF INDIVIDUAL SPECIES IN THE PERIPHERAL BLOOD

There are differences in the behavior of the individual species that are responsible not only for the symptoms produced in the patient but for the forms that are present in the blood as well.

P. falciparum. Apparently, when the *falciparum* merozoite enters a red blood cell, its very presence produces some change in the red blood cell itself, which might be described as a stickiness of its external layer or membrane. It is certainly not due to any specific property of the cell, since *falciparum* merozoites enter *young and old* red blood cells indiscriminately, while *vivax* shows a preference for younger cells and *malariae* has an equally definite preference for old cells.

Diagram 5 shows the autopsy appearance of a fatal *falciparum* infection. Perhaps it should first be explained that the well-known appearance of parasites and parasitized cells, as seen in a well-stained blood smear, cannot be expected in tissue sections. With the customary hematoxylin and eosin staining the actual parasites are rarely seen, but their presence is indicated by an amount of pigment proportional to the size of the parasite during life.

DIAGRAM 5



AUTOPSY APPEARANCE OF FATAL P. falciparum INFECTION

Chromatin and cytoplasm can be distinguished in material that has been placed in the appropriate fixative promptly and stained with Tomlinson's stain.[1]

A survey of any tissue, particularly the brain, will demonstrate the frequency with which parasitized red blood cells are found along the walls of small blood vessels where both flow and pressure are low. In smaller vessels where there is little or no turbulence, these lightly-stuck parasitized red blood cells are not dislodged. Thus, a small venule may have its border completely lined with parasitized red blood cells, while few if any red blood cells show the presence of parasites in the lumen of the vessel crowded with red blood cells (A of Diagram 5). Although A was first drawn in 1946 to help explain the phenomenon of stickiness, 8 serial

[1] Boyd, Mark F., ed.: *Malariology*. Philadelphia and London: W. B. Saunders Company, 1949, pp. 899-900.

sections from the brain of a fatal case of *falciparum* malaria which occurred in Costa Rica in 1959 showed the cross section of a small venule virtually identical with A, as shown in the reproduction of photomicrograph A-1.

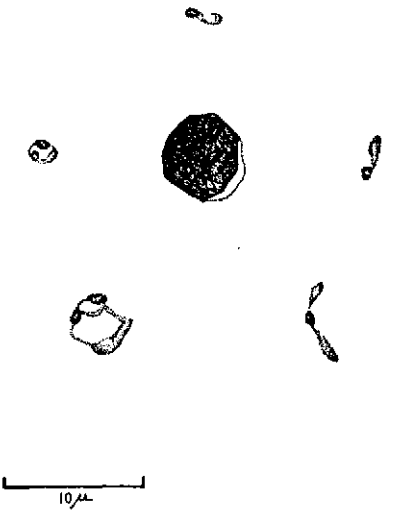
More dramatically, the capillaries almost always show every single red blood cell having a spot of pigment which represents all that is left of what was a parasite during life. What has taken place may be understood if petechial hemorrhages from a ruptured capillary are found. Not one of the red blood cells making up the hemorrhage will contain a parasite, because the parasitized cells in these confined spaces are stuck to the endothelial lining (B). With their well-known ability to accommodate themselves to a variety of constrictions and obstructions, the *non-parasitized* cells were able to worm their way around the fixed cells (C) as long as some pressure existed and to escape through the opening which followed too severe damage to some of the lining cells. As long as the endothelium remains intact, hemorrhages are not encountered.

Death occurs not as the result of the existence of some toxic substance given off by the parasites, but rather from the interference with the normal function of the vascular endothelium. It is obvious that death occurs when the number of parasitized cells has become so great that tissues depending on this vascular supply for their existence are deprived of essential oxygen, electrolytes, etc., especially if this condition exists in close relation to vital centers. It is unlikely that death takes place at the time of schizogony, but rather just after a maximum number of newly-parasitized cells have removed themselves from the circulating blood and arranged themselves over the surface of the vascular endothelium. It is probable that the dramatic apparent improvement often noted in severe cerebral infections coincides with a schizogony which temporarily frees the endothelium of the cells which are clogging it. The increased number of infected cells resulting from this schizogony occupy so much more of the endothelium that the effect cannot be supported. Death therefore occurs probably 15 to 24 hours after that final schizogony.

In pathology it is most probable that the difference between a fatal case of *falciparum* and a severe one which recovers is only one of degree. Although severe *falciparum* infections with high parasitemias are increasingly difficult to find, it is to be hoped that if one does occur in a center equipped with the appropriate facilities, needle biopsies of the liver may be attempted for the purpose of demonstrating the pathological picture during life.

DIAGRAM 6

P. falciparum



From the foregoing, it is easy to understand why only the young forms of the growing parasite are found in the peripheral blood. Only when shock develops can embolic phenomena be seen. Evidently, lack of muscle tone of the vessel walls enables the slightly stuck cells to move from their sites.

It is supposed that the growth and development of the *falciparum* gametocytes take place in red blood cells similarly adherent to the endothelium lining, but with the maturation of the gametocytes and their elongation, the containing cell is liberated into the circulation.

The characteristic high parasitemia is possible since these blood vessels, whose lining is occupied by parasitized cells, are also full of uninfected cells. Schizonts rupturing in their fixed position liberate merozoites, which are literally pressed against hundreds of cells. One or more merozoites quickly enter the nearest red blood cell regardless of whether the cell is young, old, or middle-aged.

In a primary attack of *falciparum* malaria, the parasitemia can be seen about the 10th to 12th day, followed within 24 hours by the appearance of symptoms. If the individual is ready for the development of gametocytes, these will not begin to appear until

8 to 10 days later. If untreated, ring forms and gametocytes may persist together for several days or weeks until sufficient immunity is acquired to eliminate the asexual forms. No more gametocytes are produced when the asexual forms have disappeared, but those already present may continue to circulate from 2 to 4 weeks. (See Appendix 2, The Behavior of *P. falciparum* Gametocytes, p. 93.)

The symbols F for rings only, F + g for rings with gametocytes, and Fg for gametocytes only,[1] accurately describe the blood findings of the three phases through which *falciparum* infection passes.

The student who begins to study species diagnosis should have access to at least one dozen different bloods containing F and F + g. Each parasite should be studied carefully until the student has seen as many *falciparum* rings as possible. He should recognize the degree of variation to be encountered and should try to learn the maximum size that can be attained by the growing *falciparum* parasite that can be seen in the peripheral blood.

This alerts him to the fact that when larger than the maximum forms are found at any time, the infection is more likely to be *vivax* or *malariae*. The student will also know what are the smallest rings that can be seen. *Falciparum* rings are described as small, medium, and large. The nearer to the last schizogony that the blood is taken, the larger will be the proportion of tiny rings. If the proportion of large rings is the greatest, it may be assumed that the total number of parasites will diminish remarkably within 6 hours. It should also be clear that where the infection involves a single brood, parasites may be entirely absent from the blood for several hours, as can be seen in Diagram 4 (see p. 18). On the other hand, single-brood infections are unusual in semi-immune populations.

It is, of course, possible to have more than one *falciparum* brood at the same time, with the result that parasites are never absent from the peripheral blood, although their numbers may vary considerably.

P. vivax. The word *vivax* means lively and it accurately describes the almost frenzied activity frequently exhibited by this species. The *vivax* merozoite shows a distinct preference for younger red blood cells, which are more elastic than the mature ones; as a result they are able to enlarge to better accommodate the growing parasite. However, since there is no stickiness of the

[1] See Section 14, Recording and Reporting of Results, p. 63.

DIAGRAM 7

P. vivax

host red cells as in the case of *falciparum*, the parasitized red blood cells circulate freely at all times throughout the entire cycle or series of erythrocytic cycles as schizogony takes place in the circulation. The period during which the liberated merozoites are seeking a new host cell is much longer than with *falciparum*. Even then, it probably does not exceed 5 minutes, and the number of parasites lost by phagocytosis is much greater. Heavy parasitemias are never as high as in *falciparum*. In their haste to find new homes, the *vivax* merozoites may sometimes rush into a new cell until one, two, three, or more have entered. As each divides, it is not uncommon to find more than one piece of chromatin in the new parasites; the second chromatin piece is often smaller than the first. Although these relatively early parasites already appear to be undergoing division, such is not the case. The *vivax* parasite moves throughout the entire newly-entered red blood cell, putting out cytoplasmic pseudopodia that reach all parts of the cell. This results in the appearance of bizarre forms so numerous and so variable that it is hopeless to attempt to draw more than a fraction of them. Consequently, a large, irregular parasite may not be recognized completely in the thick blood film. The tiny threads of cytoplasm connecting the individual fragments are frequently

too fine to be seen (Diagram 8). Frequently, a fragment of the parasite is compact and may perhaps contain all the pigment from the rest of the cytoplasm, with the result that nearby smaller masses of cytoplasm are ignored. In fact, to estimate what constitutes the entire parasite in a thick blood film often requires including everything within an area similar to the size of the largest polymorphonuclear leukocyte currently seen in the same blood. The confusion so frequently encountered in the diagnosis of *vivax* and *malariae* is due to the fact that these large dense portions are considered as the entire parasite.

Instead of becoming sticky the red blood cell membrane undergoes some change which, after staining in the thin smear, produces a series of reddish granules in the cell envelope which are regular in size, shape, and distribution. Fine at first, they become larger and more prominent. They are known as Schüffner's dots.[1] In the thick blood film, especially at the periphery, this staining phenomenon is seen as a pinkish halo around the parasites, of the size and shape of the host red blood cell. Schüffner's staining occurs only

DIAGRAM 8

APPEARANCE OF P. vivax PARASITES IN THICK BLOOD FILM AT 12-HOUR INTERVALS

[1] See Appendix 6, The Staining Phenomenon of Schüffner, p. 98.

in *vivax* and *ovale* infections. Advantage should be taken only of its presence, *not of its absence,* since a certain degree of excellence of staining is necessary to demonstrate this phenomenon. Only some of the routinely stained slides show these changes.

As the *vivax* parasite matures it becomes somewhat round and regular, and the full-grown adult pre-schizont looks so much like the gametocyte that it is not easy to distinguish between them. The gametocytes appear very promptly as the parasitemia becomes established. Unlike *falciparum,* the *vivax* gametocytes appear in the circulation when they are not fully developed and grow up in the peripheral blood. These growing gametocytes, with compact, dense cytoplasm and frequently with considerable pigment, are sometimes confused with growing *malariae* parasites in the first half of the cycle.

Also unlike *falciparum,* they do not continue to circulate for a long period after a schizontocidal drug has been taken. They are as susceptible to it as are the schizonts. *Vivax* gametocytes need never be confused with *malariae* gametocytes, because of the scarcity of the latter.

The proportion of the entire cycle found in a single drop of blood may be as much as two thirds or as little as one fourth of the cycle. The number of merozoites in mature schizonts just before rupture usually falls between 14 and 24. When two broods exist, all stages of the parasite may be encountered.

Since all stages of the parasite are commonly encountered in a single blood specimen, it is superfluous to specify as trophozoites, schizonts, and gametocytes the parasite forms encountered in each.

The simple designation V (V=*vivax*) implies that all such forms can be present. Although it is sometimes claimed that only *vivax* ring forms may be found, this is rarely if ever the case, and careful examination will reveal numerous small irregular stages never encountered in *falciparum.* If a careful search is made, it is most likely that advanced dividing forms will be encountered as well.

It should be stressed that *falciparum* dominates *vivax* and *vivax* dominates *malariae,* but it is unusual to find any combination other than *vivax* accompanied by *falciparum* gametocytes. The so-called mixed infections do not exist clinically, except for 24 to 48 hours when the two species present are fighting. Again—*falciparum* consistently dominates *vivax* and *vivax* regularly dominates *malariae,* i.e., F> V> M. In the clinical sense, at least, a mixed infection almost never occurs.

Some indication of the proportionate numbers would give a truer picture. The subordinate species may be indicated by enclosing its symbol in parentheses, e.g., +++F (17M),[1] which means that there are over 20 *falciparum* rings per microscopic field with 17 *malariae* parasites per 100 fields.

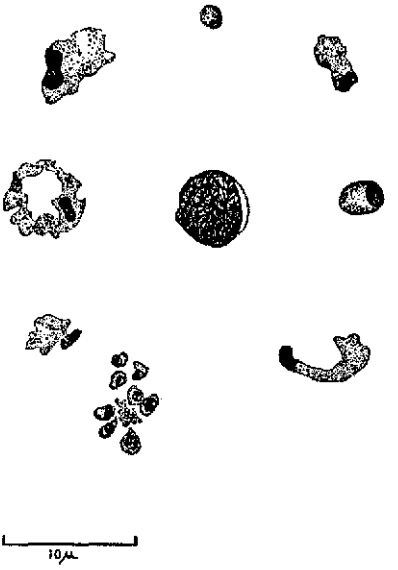
P. malariae. The quartan infection as exemplified by *P. malariae* is usually present in regions where the other two species are found; ordinarily it subordinates itself to them and is therefore sometimes called a dry-season species. Because gametocytes are unusually scanty, transmission by mosquitoes in the laboratory is rare. The pre-erythrocytic stage lasts 11 days and parasites can appear in the blood any time between the 19th and 30th days. The persistence of *malariae* infections is well known. One case is believed to have continued for 52 years. Since modern schizontocidal drugs are very effective, it is unlikely that as many such long-term infections will be encountered in the future, as there have been in the past.

P. malariae infections differ from those of the two more important species in having side effects not encountered in the others, namely, some effect on the kidney and a loss of hemoglobin in excess of that due solely to the rupture of parasitized cells. In contrast to *vivax,* the *malariae* merozoite prefers older red blood cells. This, coupled with its average schizogony of 8 to 12 divisions, results in a lower total parasitemia than in the case of *vivax.* The 50-per-cent increase in the time for its complete development, and the distinctly slower activity shown in comparison to *vivax,* result in early and increased production of pigment. Once within the cell, the quartan parasite remains relatively motionless. There are no extruding pseudopodia. Advanced pre-segmenting and segmenting forms occasionally show considerable irregularity as the result of the parasite's having entered a doughnut-shaped red blood cell.[2] The entire parasite is contained in the tubular peripheral portion of the biconcave discs of red blood cells containing less than normal amounts of hemoglobin. Similar forms are encountered in *vivax* infections which have persisted long enough to decrease the hematocrit and hemoglobin values. A lesser segment of the cycle is seen in a single drop of quartan blood than is the case in *vivax.* Three broods exist when all stages of the cycle of *malariae* are present in the same specimen of blood (Diagram 9).

[1] See Section 14, pp. 63-65.
[2] See Appendix 3, p. 94.

DIAGRAM 9

P. malariae



From shortly after the termination of ring stages to actual mature schizonts, the general characteristic of the *malariae* parasite is compactness, marked pigmentation, and uniformity and regularity of shape. A type of stippling also occurs, but its detection requires a degree of technical excellence seldom achieved in routine preparations.

It is not too uncommon to find 1 to 4 per cent of undoubted quartan parasites persisting in an infection which is otherwise patently *falciparum* or *vivax*. A common error resulting in an incorrect diagnosis of *malariae* is that which occurs when a blood containing only gametocytes of *falciparum* dries so slowly that the parasite has time to round up. If these forms are somewhat numerous, they will be miscalled quartan parasites.

However, true mixed infections do not occur frequently enough to justify a separate column for them in report forms. The existence of such a column implies a frequency which does not exist, suggests an importance which in fact is very minor, and favors careless diagnosis and poorer staining standards. It is hoped that

the reports and the records thereof will reflect accurately the infections in the population rather than a stilted statement of parasitological findings whose value is more academic than practical.

P. ovale. True *ovale* infections are exceedingly rare and a suspicion of *ovale* should not take up too much time of busy microscopists in the Western Hemisphere. Prolonged *vivax* infections, when the blood is sufficiently sub-standard, may go through a phase in which the containing red cells are deficient in hemoglobin. The resulting changes in the parasitized cells produce appearances of *vivax* morphologically indistinguishable from true *ovale*. It is therefore hazardous to attempt a diagnosis of *ovale* on morphological grounds alone. Only if the supposed *ovale* is transferred, either by needle or by mosquito, to another person, and the resulting new infection follows the *ovale* pattern, may an *ovale* diagnosis be considered. If by chance the blood specimen is taken near the time of schizogony, repeated specimens may demonstrate non-*vivax* behavior.

P. ovale is found most frequently on the west coast of Africa but also occurs in East Africa, the Middle East, Malaya, Indonesia, and the Philippines. It resembles a quartan infection with a 48-hour cycle and Schüffner's dots are visible in the uninfected portion of the red blood cell containing the parasite. In fact, if the staining is not good enough to show Schüffner's reddish coloration around the parasites in a thick film, as is frequently seen in *vivax* infections, the species may not be recognized for what it is. Previously thought to be somewhat innocuous, some *ovale* strains have shown a degree of severity equal to *vivax*.

The oval or fimbriated red blood cells containing the parasites responsible for the name given to the species, are not seen in the thin smear except where there is high humidity and when the smear is promptly dried. Accessory chromatin dots are as frequent as in *vivax*.

PART II

Preparation of Blood Slides

6. THE MAKING OF THICK BLOOD FILMS

Clean slides are packaged in lots of 5, 10, or 15. The package of 5 is probably more appropriate for field work (see Section 16, Microscopic Slides, p. 66). The end of the package is torn across, exposing the ends of the slides (Diagram 10, fig. 1). Two slides are withdrawn and placed on the outside of the package. A clean, clear flat working area is sought. If no table, bench, or chair is available, a sturdy piece of cardboard may be used and should be carried for this purpose. If the floor or other available surface is not flat, the cardboard may be held flat by a volunteer assistant from among the ever-present onlookers. It is useful to place a smooth piece of white paper under the slide when spreading the blood. Beginners should be provided with a *template or guide*[1] to the position on the slide of single or multiple thick films.

Blood is taken from the lobe of the ear, if the latter is fleshy enough, from the forefinger of the left hand, or, in the case of infants, from the large toe. The side, rather than the ball, of the finger is punctured with a sharp, single-use or multiple-use sticker. Of the latter, the most commonly used are ordinary steel pen-nibs, of varying shapes, with half of the writing end broken off. Special spring-type needles are seldom sharp enough and cause a discomfort that is out of proportion to the magnitude of the operation. For continuous daily work, the Bard-Parker No. 11 blade merits consideration. The blunt top of the blade is stuck through the under side of a small cork, which serves as a holder or handle. When not in use, the point should be inserted in the upper surface of the larger cork of a moderately wide-mouthed alcohol bottle of 30 cc capacity, since the blade rusts excessively if left permanently in the alcohol. It can be kept completely clean and sharp if rubbed on fine (No. 0 or 00) emery paper before every day's work or when required (see Diagram 10, figs. 2, 3, and 4).

A piece of this emery paper 4 x 6 cm is placed around the bottle with the emery surface to the glass, and held by string or rubber band, where it is constantly available (fig. 3). Ninety-five per cent alcohol is kept in the bottle. When the blade is to be cleaned, the emery paper is laid over a package of slides or other firm, smooth surface and the end of the blade is rubbed against the

[1] See Appendix 5, pp. 97-98.

paper until all traces of dried blood, rust, etc., are removed and the sharpness of the edge and point cannot be seen readily without a hand lens (fig. 4).

The skin to be punctured is wiped firmly with cotton or gauze moistened with the alcohol to remove gross dirt and dried sweat, and is then *wiped with dry cotton or gauze* (fig. 7). Long-fibered cotton is more desirable than the cheaper grades, which have much more lint. Gauze or loose-woven bandage cut in small pieces is by far the most satisfactory. The blade or pen is moistened with alcohol, wiped (fig. 6), and stuck lightly and quickly through the skin; the first drops are wiped away with dry gauze. When the finger is being punctured, the operator grasps it with the fingers of his left hand in a constricting manner. After puncture, the tip of the finger is squeezed gently until the blood wells up in a spherical drop on the dry skin (figs. 8, 9, and 10).

The edge of a clean slide is immediately pressed firmly against the forefinger of the operator (fig. 11) and the surface of the slide is lowered to the donor's finger until it touches the upper surface of the drop. The amount of blood taken will determine if another drop is needed; if so, it is placed beside the first. The slide is quickly laid flat on any smooth, flat surface, preferably with a white background, and the blood is spread (fig. 12) with the corner and first five millimeters of the long edge of a second slide until a square or rectangular patch of approved thickness is obtained.

If more than one thick film is to be taken from the same person, when the skin has been wiped perfectly dry a fresh drop may be picked up and spread directly from the corner of another clean slide. To avoid transfer of bloods from drop to drop, the corner used for spreading must be wiped clean immediately after use.

A thinner streak of blood or partial thin smear is made from the blood remaining at the site of puncture and is used for the identification (figs. 13 and 14) which is written later with a soft No. 1 graphite or "film mark" pencil[1] when the blood has dried.

Thick blood films are not made as thick as formerly, nor are they stirred or defibrinated. Adequate density of a thick film is, of course, the maximum thickness after staining that can be seen through when examined under the oil immersion. This can be estimated by first making one film that is definitely too thick; it is spread insufficiently and the slide is turned perpendicularly or on its edge; a drop is immediately formed and runs to the dependent border. With the next slide, a similar amount of blood is spread

[1] See Appendix 12, Pencils Used for Marking in Blood, p. 104.

HOW TO MAKE A THICK BLOOD FILM

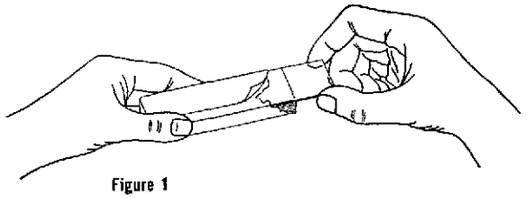


Figure 1

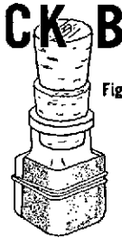


Figure 3

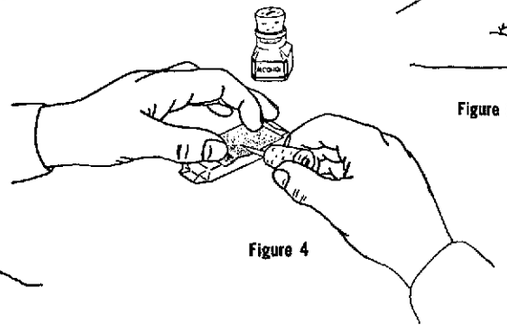


Figure 4

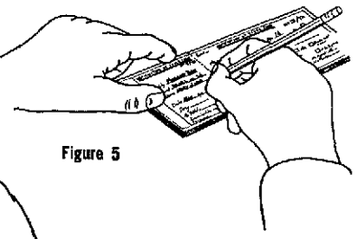


Figure 5

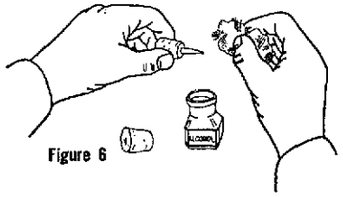


Figure 6

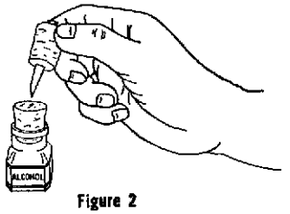


Figure 2

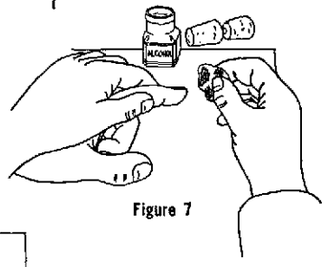


Figure 7

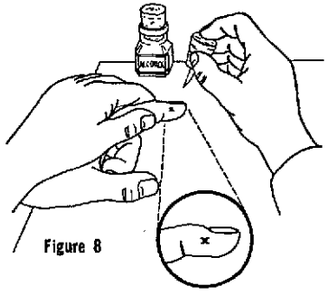


Figure 8

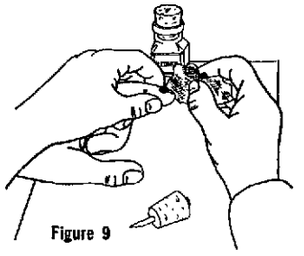


Figure 9

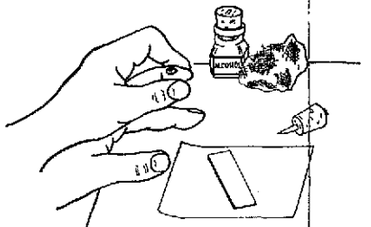


Figure 10

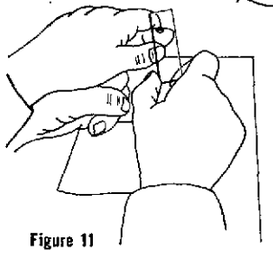


Figure 11

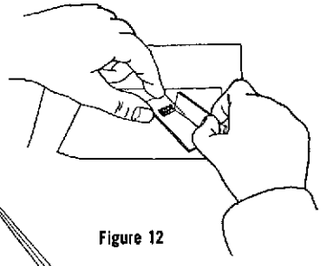


Figure 12

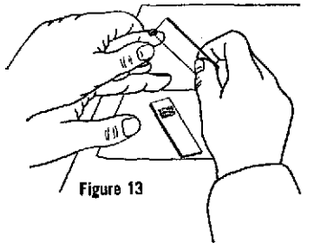


Figure 13

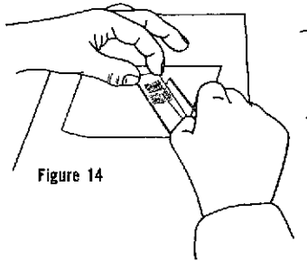


Figure 14

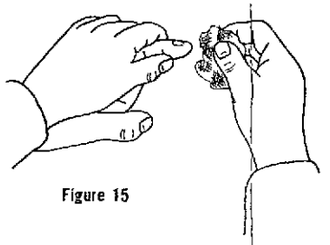


Figure 15

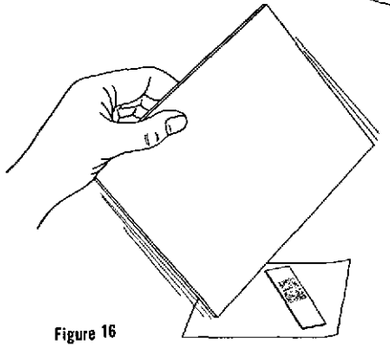


Figure 16

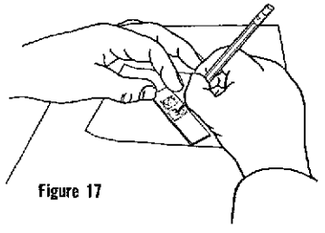


Figure 17

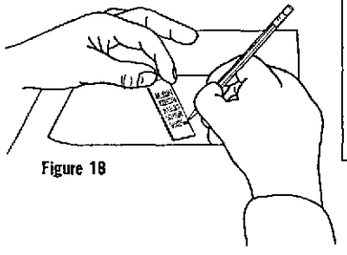
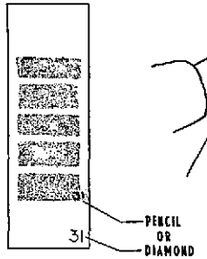


Figure 18



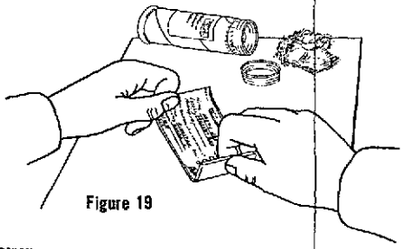


Figure 19

INSTRUCTION

Figure 1 The package of new slides is opened by tearing off one end of the wrapping paper. Slides should always be held at the edges, or the ends, between the thumb and forefinger, to avoid touching the surfaces.

Figure 2 Any clean, sharp-pointed blade can be used as a sticker. The sticker should be inserted in the under side of a small cork, and when not in use the point should be stuck in the upper surface of the larger cork of a moderately wide-mouthed alcohol bottle of 30 cc capacity, since the blade rusts excessively if left permanently in the alcohol.

Figure 3 A piece of emery paper is attached to the sticker bottle by means of a rubber band; this paper is used to clean and sharpen the sticker as needed.

Figure 4 When sharpening the sticker, hold the emery paper over a package of slides or other solid, flat surface.

Figure 5 Fill in all the required information on the "Notification of Fever Case" forms, taking care to indicate the collaborator's post number and the serial number of the slide.

Figure 6 Wipe the blade of the sticker clean with a wad of gauze or absorbent cotton moistened with alcohol. Place the cork holding the clean sticker on the table, on its side, in such a manner as to prevent the sticker's point from touching any surface or object.

Figure 7 Before puncture, clean the skin around the area selected with a wad of gauze or absorbent cotton saturated with alcohol, after squeezing the wad lightly to remove excess alcohol.

Figure 8 Puncture the finger with a sharp stab of the sticker at the point marked "X".

Figure 9 The first drop of blood should be carefully wiped off with a piece of *dry* gauze or cotton.

Figure 10 With a squeezing motion, another drop of blood is forced on to the surface of the finger.

Figure 11 A clean slide is held carefully at one end and the edge is steadied against the forefinger of the operator's left hand, which is holding the donor's punctured finger; the slide is then gently lowered to the donor's finger until its upper half contacts the upper portion of the blood drop and a part of the drop adheres to the slide. Care should be taken to prevent the slide from coming in contact with the skin. If sufficient blood remains on the finger, a second, smaller drop is placed on the slide ¼" below the first one, thus preparing for the operation shown in fig. 13.

Figure 12 Place the slide face up on a sheet of paper and, using about ¼" of the lower edge of a second slide, spread the blood to form a square or rectangular smear, and immediately wipe blood from spreader slide.

Figure 13 With the same corner of the second slide, pick up some of the blood remaining on the finger.

Figure 14 Spread this blood, or the second drop mentioned in fig. 11, to be used to write on.

Figure 15 Wipe the donor's finger with a wad of cotton or gauze saturated with alcohol; if bleeding continues, hold a piece of dry cotton over the puncture point until bleeding stops.

Figure 16 To dry the blood smears, fan the slide with a piece of cardboard until the blood smear loses luster.

Figure 17 Using a No. 1 or a Dixon #2225, "Film Mark" pencil, write on the second of the two smears the collaborator's post number, the serial number of the slide, and the date on which it was prepared.

Figure 18 When a large number of smears from different donors are to be taken at the same time, five transverse, narrow thick films may be placed on the same slide. The drop of blood taken first may be streaked to the edge of the slide for identification. The number of the donor from whom this first drop was taken will serve to identify all smears on the same slide, and that number is marked at the right edge of the first drop, or better still and if possible, is written with a diamond-point pencil on the lower right corner of the slide. Such numbers will always end in 1 or 6.

Figure 19 One or two slides can be wrapped in the same identification slip so long as the smears they contain were taken from the same person. Also, three or more slides from different persons may be placed together and packed tightly in a slide wrapper. The identification slips are then wrapped around the compact package.

wider and the slide is turned up again. If the drop forms quickly, it is spread still wider. This is repeated until the freshly-spread blood no longer runs to the inferior border but merely appears to crawl.

The finished thick film should occupy the inner half of the distal two thirds of the slide, and a free space of 1.5 cm should be left at each end of the slide for easy manipulation while wet.

The place for writing the identification may be spread with blood that has remained slightly longer on the surface of the skin. If the malaria service has no particular key or symbol to identify the donor, the latter's initials and the date may be printed plainly with a soft pencil, using Roman numerals to indicate the month, *viz.*: P M B 17 XII 3 (for 17 December 1963). Only slides that are made for permanent specimens require a digit for the decade.

Where the humidity is high, drying may be accelerated by brisk fanning with a piece of cardboard (fig. 16). The inexpensive Humigraph[1] is useful to show humidity changes which affect the drying of slides.

Five transverse, narrow thick films from five different donors may be placed on the same slide if desired (fig. 18) and identified by symbol or number written with a No. 1 lead pencil at the lower edge of the first drop, or written with a diamond-point pencil in the lower right corner of the slide. Also, to show which drop was taken first, that blood may be streaked to the edge of the slide.

7. THEORY OF BLOOD STAINS

Blood staining has always been complicated by the inherent variability of methylene blue. Virtually all blood stains are derived originally from methylene blue processed in one of several ways. When it is understood that widely varying results can be obtained even when the same lots of methylene blue are used in identical procedures, it becomes obvious that the same variation occurs with Giemsa, Wright's, or Leishman's stains. In some instances the difference may be so great that certain lots of stain may be useless.

In 1925 the Biological Stain Commission was created in the

[1] A. Daigger and Co., 159 West Kinzie Street, Chicago 10, Illinois (see Appendix 13, p. 105).

U.S.A. to test each lot of stain for the purpose of eliminating those that were unsatisfactory. When acquiring U.S.-manufactured blood stains, then, it is most desirable that they bear a certification number from the Stain Commission, which assures that at least at the time they were tested the results were satisfactory.

With regard to the Giemsa stain, which is of maximum utility in malaria work, a number of laboratory workers still prepare their stains from the original dye components used, often with surprisingly good results. However, except under exceptional circumstances, the beginner is advised against attempting this not-always-successful method. Although there are many good-to-excellent Giemsa stains available in the world markets, it is not possible to recommend any given one with certainty. The safest procedure would be to obtain small (1 to 5 grams) samples from at least three different sources. These should be extensively and repeatedly tested with the techniques and under the conditions of the locality. Only then should a large supply be requisitioned.

The foregoing may explain the almost total dependence of some of the early workers on individual brands or types of stains. It may also explain how some Giemsa stains may be satisfactory if dissolved in one brand of pure methyl alcohol and distinctly less so in another. The dissolved elements are evidently in such delicate chemical equilibrium that very slight changes of reaction may produce surprising results.

The basic constituent of a blood stain is some type of eosinate of methylene blue dissolved in either pure methyl alcohol or an equal proportion by weight of that alcohol and pure glycerin. The methyl alcohol is ordinarily acetone-free, though not all acetone-free alcohols give satisfactory results. This alcoholic solution of Giemsa stain is the only convenient form from which may be prepared the aqueous solution that actually does the simultaneous staining of red, blue, and violet elements in blood. It is unlikely that the dissolved elements stay in solution longer than 45 to 90 minutes, by which time all the active staining elements are completely precipitated out of solution. This is significant in two ways: (1) all Giemsa solutions should be prepared immediately before use; and (2) if water contaminates the alcoholic stock solution, then valuable portions of the staining elements will be precipitated out of this stock solution in proportion to the amount of water present. The results following the repeated introduction of a wet pipette into the stock bottle may be disastrous. Watery contamination of the stock alcoholic stain occurs most frequently and more

subtly because of the inherent ability of pure alcohol to take up moisture. This may occur quite rapidly in the tropics, where the humidity is high. This fact gave rise to the old general belief that blood stains deteriorate in the tropics. Therefore, the screw caps on bottles should be tightened at intervals and cork stoppers renewed when their elasticity is lost. Ground-glass stoppers, if used at all, should be cleaned every time they are put in the bottle, since the accumulation of dried stain may prevent the stoppers from fitting properly.

To prevent the occurrence of this undetectable and continuous damage to the Giemsa stain, it is advisable to use small working bottles whose content will be sufficient for only one or two weeks. When no plastic dropping bottles are available, the old small test tube strapped to the work bottle may be used, but it is a poor substitute. Small plastic bottles with a dropper opening and closed tightly by a screw cap, used for perfumes and some medicines, make ideal Giemsa work bottles.[1] Stock bottles are opened only when it is necessary to replenish the working bottles.

The same precautions described for Giemsa stain should be taken with all other Romanowsky-type stains such as Wright's and Leishman's, although glycerin is not ordinarily used. *They are dissolved in pure methyl alcohol in the proportion of 0.15-0.18 gm per 100 cc pure methyl alcohol,* whereas Giemsa is mixed as follows:

Giemsa powdered stain (certified)	0.75 gm
Pure methyl alcohol	65.0 cc
Pure glycerin	35.0 cc

In the absence of Giemsa, excellent results may sometimes be obtained by using the Wright's or Leishman's powders *in the same concentration as Giemsa,* and of course with the same technique.

To prepare the stains, it has long been recommended that they be mixed in a mortar. Prolonged grinding with glycerin or methyl alcohol, or both, is still routine procedure. In dry climates this can probably be done without risk, but exposure to moist atmosphere is unduly prolonged with this method. Moreover, lumps of moistened stain powder invariably adhere to the sides of the mortar and the face of the pestle. In place of that method, the procedure that has been used successfully for years is the one whereby the dry powder is added directly to a bottle of convenient size,

[1] See Appendix 11, Plastic Dropping Bottle, p. 104.

containing the proper amount of alcohol-glycerin mixture. A minimum of 50 scrupulously clean, solid glass beads of varying sizes, not to exceed 5 mm in diameter, are also added to the bottle. This bottle is shaken thoroughly at intervals, 6 to 10 times per day, for a minimum of three days. Small samples are then removed daily, filtered through medium-coarse filter paper, and tested with fresh thick blood films. When all elements of the blood are seen in their appropriate colors, enough stain is filtered into one or two working bottles and the stain is then ready for use. The balance of the stain is stored, without filtering, until required. Because of the possible variation of the individual ingredients, the stock bottle should bear a large label carefully listing the name, lot number, and the amount of each of the ingredients, as well as the date of preparation.

It is repeated, for emphasis, that *all liquid stain containers must be kept tightly stoppered at all times.* If the foregoing directions are carried out meticulously, it is usually found that instead of deteriorating with time, these stains improve, whether "in the tropics" or elsewhere.

Diluents. As only freshly prepared aqueous solutions of blood stains give the well-known colors to the preparations, the diluent used assumes considerable importance. The following have been used as diluents for blood stains at one time or another: water from wells, springs, streams, rivers, and piped water supplies, also distilled water, rain water, and even double and triple distilled water; and finally, the two most frequently used, buffered water and neutralized water. When distilled water is temporarily in short supply, the buffer salts may be added to tap water. This combination should never replace the use of distilled water. Rain water collected in a clean enamel or other smooth-surface container raised two feet above the ground *is* distilled water.

It should be noted that it is not possible to stipulate a reaction which will suit every stain. *The ultimate test of the suitability or reaction of the diluent is the appearance of the blood seen through the microscope.* Therefore, any combination which gives consistently good results should be used no matter how unorthodox it may appear. In some areas where the ground water soaks down through forest and grassland, sand, hard clay, or rock, the tap water may be highly satisfactory. On the other hand, where the water soaks through porous, chalky rock or limestone, it may be quite useless. Water which has acquired aerogenic bacteria, yeasts, or algae may

no longer be suitable, unless active boiling for 5 minutes and filtering or sedimentation restores its clarity. All waters that are no longer crystal-clear should be discarded.

When buffer salts were first added to the diluting water, a dramatic improvement in the quality of stained blood preparations was noted at once. Generally speaking, the reaction of diluents which gave the improved results was close to the point of neutrality (pH 7.0). Experience has shown that no standard pH can be depended upon with all types of stains and that the most suitable reaction for the stain in hand should be sought. In practice, the range is usually between pH 6.6 and 7.4, which, coincidentally, is the range of the indicator phenol red.

Sodium phosphate (Na_2HPO_4) and potassium phosphate (KH_2PO_4) are the buffer salts used generally. Because the crystalline sodium phosphate contains 12 molecules of water of crystallization, it soon becomes covered with white powder on exposure to the air; exact weighing is no longer possible. A moist mass results when mixed with other crystals. It is therefore imperative to specify that the sodium monohydrogen phosphate be *anhydrous;* the monopotassium dihydrogen phosphate can thus be mixed with the anhydrous sodium salt in any proportion and still remain in a dry state. In practice, useful buffer solutions may be prepared rapidly by adding to each liter of distilled water one gram of a mixture of the sodium and potassium salts in the proportion of 6 to 5 *or any other proportion that has been found satisfactory.* Correct amounts of these salts are thoroughly mixed and ground in a mortar and the homogeneous powder is weighed out in lots of one gram (or more) and placed into small well-stoppered tubes, or if for immediate use it may be folded in glassine papers or dissolved in small amounts of water.

For the competent staining of thin blood smears, distilled water appropriately neutralized for the occasion may be superior to buffered waters. This is prepared by neutralizing it to an indicator phenol red with a weak alkali such as 0.2 per cent lithium carbonate. Repeatedly shaken (a minimum of 50 times) until the desired color remains for at least 20 minutes, it may then be used with Wright's or Leishman's stains. This neutralized water is used not only to dilute the alcoholic stain but also for the final washing of the slide. Buffers act as a kind of elastic chemical material to inactivate (within a limited range) varying amounts of acid and alkali. Neutralization, on the other hand, is a fixed chemical reaction allowing for no variations.

8. STAINING TECHNIQUES—GENERAL

Thin blood smears are prepared by spreading a very small drop of freshly exuded blood with the smooth, even, undamaged edge of a new microscopic slide. The optimum thickness is a layer of blood cells one cell thick; in contrast, the thick blood film may contain 6 to 20 times as much blood spread over a roughly rectangular area 1.5 by 1.2 cm.

In the thin blood smear, a single layer of cells lies horizontally on the surface of the glass. In the thick blood film there are many layers of cells in their customary "rouleau" formation and the axis of an individual cell may be in any direction. The staining of the flat layer of cells in the thin blood smear is intended to demonstrate the maximum detail of the blood cells and their contents. These preparations are therefore "fixed" by the application of pure methyl alcohol in order that the hemoglobin may be retained in the cells and so be affected by the stain. It is relatively easy to see through a single layer of stained red blood cells.

If the red blood cells in the thick blood film were so "fixed" it would be quite impossible to see anything, except perhaps at the extreme edges. It is therefore necessary to remove the hemoglobin from the red blood cells by one of various methods, separately or during the staining process. In the past, weak solutions of hydrochloric acid, distilled water, or various other mixtures were used to remove the hemoglobin before the blood stain was added. This frequently resulted not only in complete lysis of the red blood cells but also lysis and distortion of the parasites, leukocytes, and other elements of the blood. Later the practice was developed of having the dehemoglobinization take place in the stain solution.

The first step in the staining of thin blood smears is fixation, which is directly opposed to the principle of thick film staining.

Both time and heat tend to "fix" the hemoglobin in the red blood cells, whether in thin or thick preparations. Therefore, it is apparent that the more promptly the thick films are stained, the more complete the dehemoglobinization will be. The longer they are allowed to remain without staining, the less clear the preparations will be. Seven to 10 days in a moist, warm climate may render a thick blood film unsuitable for examination after staining. Thin blood smears, on the other hand, may present quite a good appearance.

To show what would happen if thick films were "fixed," simply drop pure methyl alcohol on the lower half of the thick blood film held perpendicularly and expose to the usual staining technique for thick films.

Thick Films. The original thick film staining consisted of flooding a rather large thick drop with diluted methylene blue. As visibility in the central portion was virtually nil, it was thought that examination would be easier if the blood were thoroughly stirred. There followed a long period when defibrinization was considered imperative, although a little less blood in the drop would have obtained the desired result. Since that time, *Giemsa stain has been virtually the only one used and the dilution of one drop to 1 cc of distilled water has become customary.* Slides with thick films were laid across glass rods and the diluted Giemsa stain was poured on them and allowed to act for approximately one hour, after which they were ready for examination. Larger numbers were stained on the edge or ends in rectangular or upright staining dishes.

In 1929 Barber and Komp placed the thick film at one extremity of the slide and separated the slides by a rather thick 1″ square piece of cardboard at the other extremity. Groups of 25 and even 50 slides could be stained simultaneously using these compact packets placed upright in a dish of stain. The practice of placing the thick blood film at the extreme end of the slide arose from this method. If a single, very heavy *falciparum* infection was present on one of the slides in those packages, parasites might be transferred occasionally to adjacent slides because of the debilitated character of the blood. Careful rinsing or use of a detergent may prevent this.

Following Pampana's discovery of the dehemoglobinizing action of isotonic solutions of methylene blue, Field originated his rapid staining method for thick blood films used so extensively in World War II. It consisted of a one- to three-second dip in Solution A (a mixture of methylene blue and phosphates), followed by brief washing in distilled water, and a similar dip in Solution B (a mixture of eosin and phosphates). The rapidity of the method did not allow time for thorough dehemoglobinization, but showed both leukocytes and parasites brightly and clearly colored. A variation of this same stain was later developed in India and is known as the J.S.B. stain.

During the 1920's a London manufacturer made a staining plate

slightly curved in such a manner that when a slide was inverted across the curvature of this plate, a space not to exceed 3 mm in depth remained below the slide into which the stain was directed. Because of the particularly heavy weight of the hemoglobin molecule, this inverted position of the thick blood film permitted total dehemoglobinization as compared to the other positions in which the slides had previously been stained. A one-second dip in a methylene blue phosphate preserved the cellular elements of the blood to a great extent without interfering with dehemoglobinization. When diluted Giemsa was run under the slide inverted on the curved plate or on the reverse of a rectangular enamel pan, such as shown in Diagram 12-E (p. 44), it was found that this blue step permitted excellent staining after exposure of only 6 to 10 minutes to the Giemsa solution.

The staining of the combined thick film-thin smear requires that the thin smear be separately "fixed" with methyl alcohol before the whole slide is stained with some variation of the aforementioned techniques.

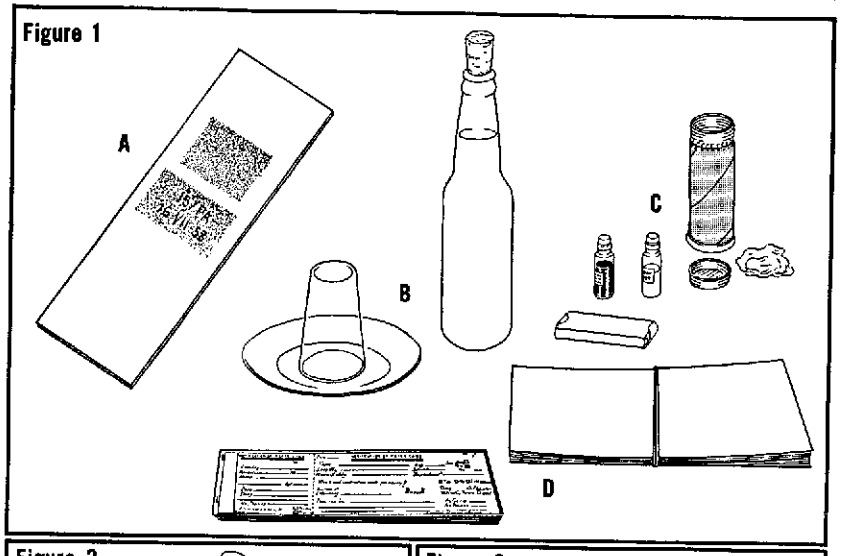
Thin blood smears alone may be stained with Giemsa stain following previous fixation, or by any of the May-Grünwald, Wright's, or Leishman's stains whereby fixation is obtained by the application of the undiluted stain followed by the addition of the diluent.[1]

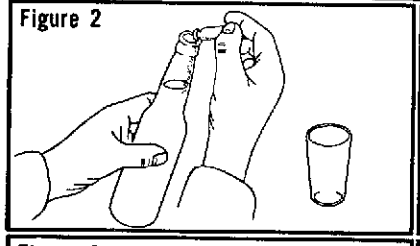
9. PRE-TREATMENT OF THICK BLOOD FILMS

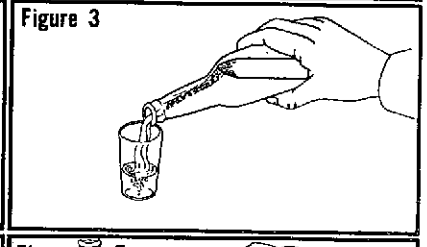
Whenever it is expected that thick blood films will be long delayed in reaching the persons capable of using Giemsa stain properly, they may be "pre-treated." First, dip them for one second in the blue phosphate solution or simply pour the blue over the blood (Diagram 11, fig. 4). Second, prolong the rinse in buffered water longer than in the case of the complete staining technique, but only until the margin of the thick film is faintly blue-gray. Do not attempt to wash until the red color disappears from every part of the drop. Third, drain the slides and dry with gentle heat, or in the sun, to avoid fungus growth. Fourth, wrap in packets of 15 slides or less and on the outside identify the contents with a soft

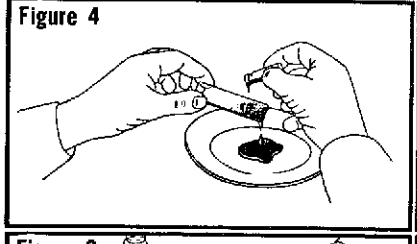
[1] Further details of the staining of thin blood smears are given in Appendix 8, pp. 100-101.

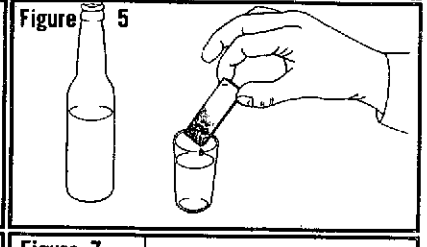
DIAGRAM 11

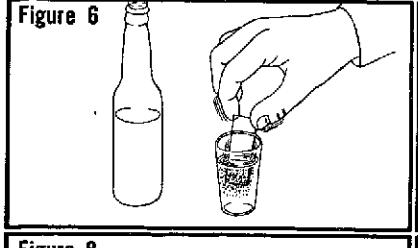


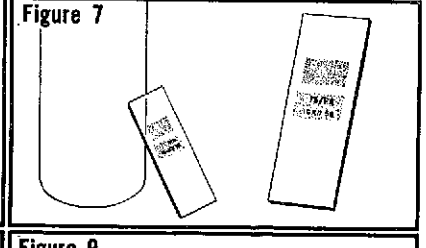


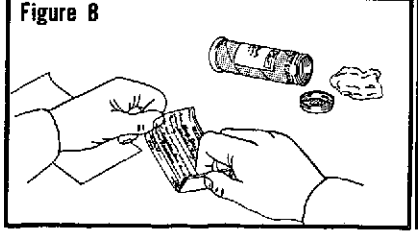


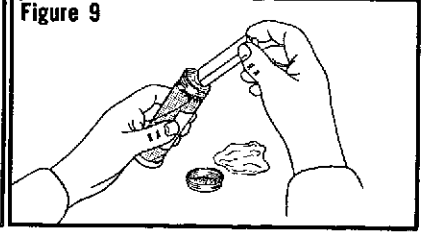












PRE-TREATMENT OF THICK FILM BY VOLUNTARY COLLABORATORS

Figure 1

A. Appearance of the thick film before pre-treatment.

B. Simple equipment used by collaborators to pre-treat thick films with methylene blue phosphate solution. It consists of a well-rinsed bottle containing 300 cc of distilled water or rain water, a small drinking glass, and a small saucer.

C. Cylindrical cardboard mailing tube which, when sent to the collaborator, contains small vials of methylene blue phosphate solution, and dry buffer salts, and a package of three slides.

D. Pad of notification forms and a package of slide wrapping papers.

Figure 2

Pour the buffer salts (white powder) into the bottle of water.

Figure 3

Once the salts are dissolved, fill the drinking glass three-quarters full with this solution. The buffer solution can be used until it becomes turbid.

Figure 4

Holding the slide at a 20-degree angle over the saucer, quickly pour sufficient methylene blue phosphate solution (blue solution) over the slide to cover the blood. One second is sufficient for the action of the blue solution.

Figure 5

Dip the slide immediately into the buffer solution in the glass.

Figure 6

Move the slide gently to-and-fro in the buffer solution until only the margin of the thick film loses its red color. Whenever the solution becomes markedly blue, replace with fresh solution from the bottle.

Figure 7

To drain dry, incline the slide against a suitable object. The figure shows the slide as it appears after treatment (quite transparent).

Figure 8

Wrap the slide in its notification form.

Figure 9

Place the wrapped slides in the cylindrical mailing tube for shipment to the laboratory, using packing as needed.

pencil, always on the edges rather than on the flat side of the slides. Fifth, store in a dry place until they can be finally stained with freshly prepared Giemsa solution. Since no further dehemoglobinization is possible, it serves no useful purpose to invert them. Five to 10 minutes with Giemsa is usually sufficient. Determine minimum time required by trial.

Diagram 11 shows how a small number of slides can be treated in the home of a collaborator, and outlines each of the steps taken. For large numbers of slides, the steps can be reduced to those shown in Diagram 12 (A and B); the slides are gently moved to-and-fro in the second buffer water until only a trace of red color remains.

Slides which have been poorly stained may be restained in a variety of ways, sometimes with considerable success. However, no single technique has yet been devised which gives generally satisfactory results.

Plastic dropping bottles ranging from 30 cc to 150 cc capacity (see Appendix 11, p. 104) are available. Giemsa stain, when placed in them, is more completely protected from contamination with moisture than in any container requiring a separate pipette for delivering the appropriate number of drops. Therefore, *evaluation personnel can be equipped to stain slides in the field,* and pretreatment of slides need be done only by the collaborators. All that is needed to equip evaluation personnel, beyond what has already been recommended, is a small additional supply of buffered water, and a clean surface with a depression, as shown in Diagram 12-E. All slides taken by evaluation personnel may thus be stained in the field when a convenient number have been accumulated on a daily basis or every other day. Such a step should provide the microscopists with the *best possible preparations for examination.*

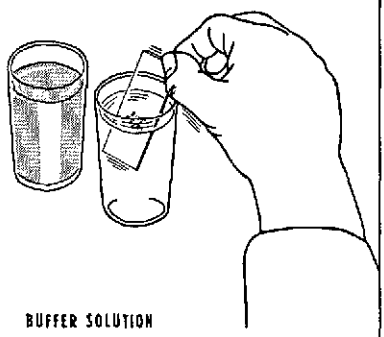
Commentary. The amount of stain powder required is only an average and the amount can be varied according to the results desired. For example, if one Giemsa gives good results when dissolved in a certain proportion with glycerin and alcohol, these quantities can be used. When the alcoholic solution is too strong and the leukocytes are overstained, 5 to 6 drops in 10 cc of the diluent should be sufficient. However, it is better to dilute the stain with more alcohol-glycerin mixture in order to maintain the universal proportion of one drop of alcoholic solution to 1 cc of buffer solution.

Similarly, with the buffer solutions, attempts should be made to

A

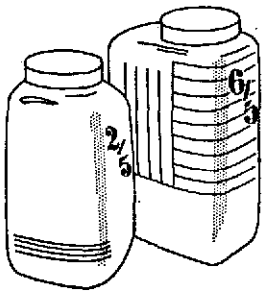
METHYLENE BLUE-PHOSPHATE SOLUTION

B



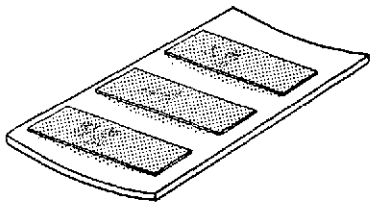
BUFFER SOLUTION

C



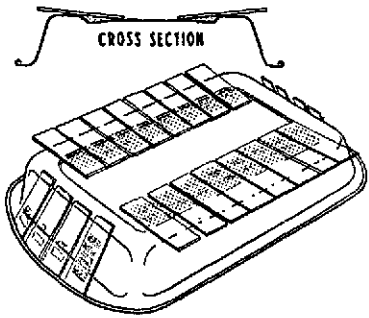
BOTTLES FOR SOLUTIONS

D



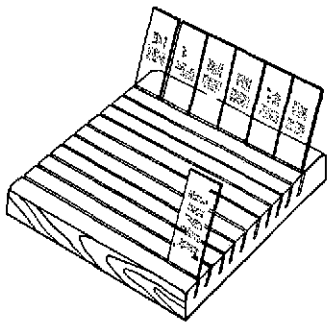
CURVED PLATE FOR STAINING

E



ENAMEL TRAY

F



RACK FOR DRYING SLIDES

find that proportion of salts which will give optimum results. A series of different amounts of the sodium and potassium phosphates is made in the following proportions: 2:5, 4:5, 6:5, 8:5, and 10:5.[1] A number of preparations of the same blood are stained with the same Giemsa using the buffer made with 1 gm of each of these proportions to one liter of distilled water. *Those proportions giving the best results are used.* The proportion 6:5 is probably the most frequently selected.

10. STAINING TECHNIQUES—DETAIL

Thick films were often stained with Giemsa solutions alone. The slides were placed face upward across glass rods 5 cm apart and carefully levelled; fresh Giemsa solution, one drop to 1 cc of distilled or buffered water, was poured on the slides and allowed to act for 30 to 60 minutes. After being rinsed with the same diluent, the slides were drained and dried with heat.

Coplin jars and rectangular staining dishes held slides for staining with Giemsa and the afore-mentioned blocks of slides were stained in the same manner.

Staining times may vary with each lot of stain and should be tried out carefully. Dehemoglobinization is not as complete with the Field method (see p. 47) as with the methylene blue-Giemsa technique, but the colors at the margin of the thick film are approximately the same with the two methods.

Staining of Thick Blood Films (Walker) (Diagram 12)

1. Check carefully the identification of the specimen and be sure it is adequate. Use soft No. 1 graphite pencil for any additions or corrections. Avoid grease pencils.

[1] The pH for mixtures as measured by Beckman Potentiometer:

pH	proportion of buffer salts
6.25	1/5
6.50	2/5
6.90	4/5
7.05	6/5
7.10	8/5
7.20	10/5

2. Dip for one second—no more—(count aloud "one-one-thousand") in the methylene blue phosphate solution. To lessen the number of changes of buffer water after the dip in methylene blue phosphate solution, the dependent end of the slide should be touched momentarily to a moist pad of plastic sponge that has been thoroughly wet and then squeezed out so as to remove the excess blue quickly.
3. Dip five times in buffer solution (made by adding one gram of the 6:5 phosphate mixture to one liter of distilled water), the same as is used for diluting the Giemsa. Use 2 wide-mouth glasses for more than 10 slides and change solution whenever it becomes markedly blue. (Buffer salts may be added to tap water when distilled water is temporarily unavailable.)
4. Place the slides upside down over the 2-3 mm depression of a curved stain plate or enamel pan.
5. Allow freshly prepared Giemsa solution (1 drop-1 cc buffer solution) to run under the slide until depression is filled. Remove any bubbles which collect on or near the drop of blood.
6. Allow stain to act 6-10 minutes.
7. Dip briefly in buffer solution to remove excess Giemsa.
8. Drain and dry with heat.
9. Examine under oil immersion.

Preparation of Various Solutions for Diagnosis of Malaria

1. *Methylene blue phosphate*

Methylene blue, medicinal	1.0 gm
Disodium monohydrogen phosphate anhydrous (Na_2HPO_4)	3.0 gm
Monopotassium dihydrogen phosphate (KH_2PO_4)	1.0 gm

These are thoroughly mixed in a dry mortar and one gram quantities are placed in small well-stoppered vials. The contents of one vial are dissolved in 250-350 cc distilled water or filtered if necessary.

2. *Giemsa stain, certified, liquid or*

Giemsa stain powdered, certified	0.75 gm
Pure methyl alcohol	65.0 cc
Pure glycerin	35.0 cc

Shake well in bottle with glass beads 6-10 times daily until it is thoroughly mixed. Keep tightly stoppered at all times. If Giemsa powder is not available, Wright's powder can be used in the same proportion. No heating is required; use approximately on the third day or as soon as daily trials with normal blood show it to be satisfactory. Filter only when necessary, each time plastic bottle is refilled.

3. *Buffer water*

Disodium monohydrogen phosphate anhydrous[1] (Na_2HPO_4)	6.0 gm
Monopotassium dihydrogen phosphate (KH_2PO_4)	5.0 gm

Mix thoroughly in mortar. 1 gm mixture to 1,000 ml distilled water

4. *Field's stain consists of two aqueous solutions:*

Solution A:

Methylene blue	0.8 gm
Azure I=Azure A	0.5 gm

dissolve in 500 cc 4/5 buffer

Solution B:

Eosin, yellow w.s.	1.0 gm

dissolve in 500 cc 4/5 buffer

To stain, dip for one second (1-3 secs.) in Solution A; wash gently in distilled or buffer water; dip for two seconds (1-4 secs.) in Solution B; wash gently, drain, and dry.

5. *Wright's stain, certified, liquid, or*

Wright's stain powdered, certified	0.15-0.18 gm
Pure methyl alcohol	100 ml

Shake well in bottle with glass beads and keep tightly stoppered in small bottle. Filter only if necessary.

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[1] *Primary Sodium Phosphate:*
1.25g of $Na_2HPO_4 + 2H_2O$ = 1g of Na_2HPO_4 anhydrous.

Sodium Phosphate Crystals:
12.6g of $Na_2HPO_4 + 12H_2O$ = 5g of Na_2HPO_4 anhydrous.
2.5g of $Na_2HPO_4 + 12H_2O$ = 1g of Na_2HPO_4 anhydrous.

PART III

Microscopic Examination

11. MICROSCOPES

Three kinds of microscopes are used in malaria eradication programs:

1. The compound microscope (monocular or binocular)
2. The stereoscopic microscope
3. The hand lens or loupe, with magnification of 2x to 10x

The loupe, considered primarily a tool of entomology, is extremely useful for the examination of the various parts of the compound microscope, such as worn screw threads, damaged rack and pinions, or hardened specks of oil and dirt on smooth or glass surfaces. The stereoscopic microscope, standard equipment for every malaria program, may also be used where greater magnification is desired. A 5x loupe is indispensable for the examination of objectives whose clarity of detail is impaired.

Compound microscopes are supplied with either monocular or binocular bodies. Some makers also supply a monocular tube with the latter.

Diagram 13 shows that the monocular body consists of a single tube. It has long been customary, for reasons of comfort, to incline or tilt the monocular microscope on its base so as to give an inclination to the ocular, similar to that of the binoculars. In cases where only monoculars are available, it is desirable to supply inclined monocular tubes. If these have a 1.5x magnification, a proportionately lower ocular is indicated.

An inclined binocular body (Diagram 14) enables the observer to use the microscope for prolonged periods with much less fatigue than with a

DIAGRAM 13

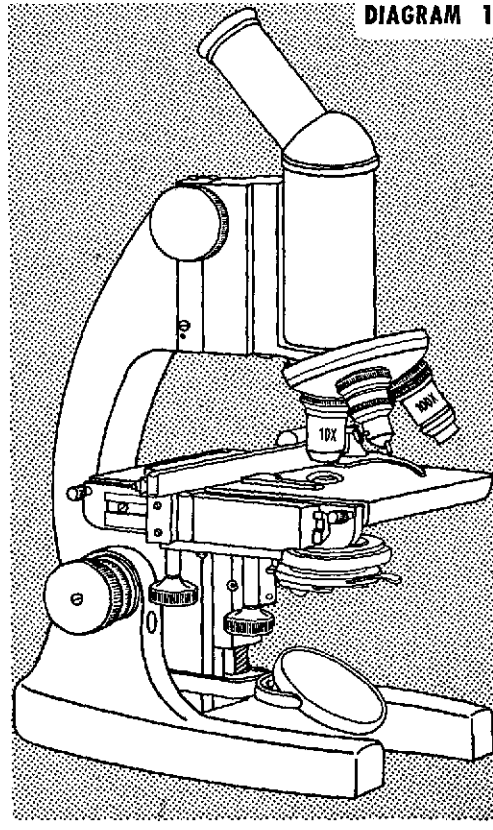
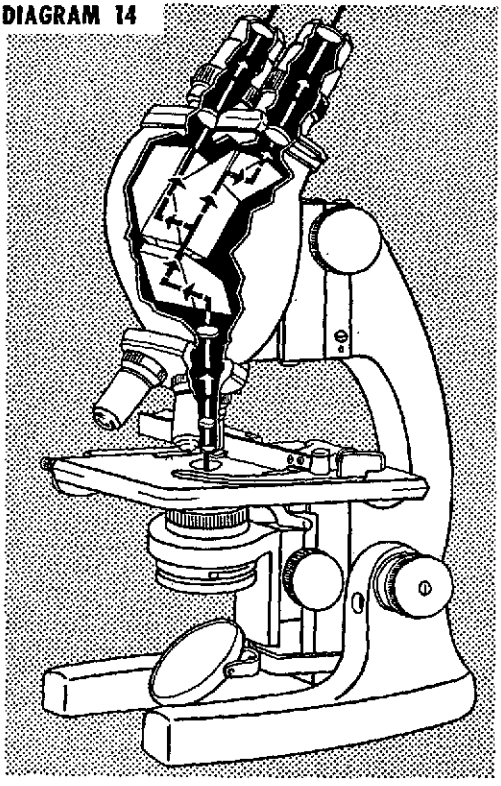


DIAGRAM 14



monocular. The same diagram shows that a considerable amount of additional optical equipment is required to bring the same image into both eyes. Not only is the total light reaching the slide via mirror and condenser divided in two, but every lens or prism in the binocular system reduces the amount reaching each eye. It should be clearly understood, then, that the amount of light which gives adequate results in the monocular microscope may be hopelessly inadequate for the binocular.

Again, where focusing of the fine adjustment is sufficient to obtain maximum resolution in the monocular eyepiece, in the binocular instrument an additional focusing of the adjustable ocular tube to match the resolution of the ocular in the fixed tube must be carried out before use. When these adjustments are properly made, a degree of resolution can be obtained which is not possible with a monocular instrument. Moreover, eyestrain is greatly reduced. The use of the binocular microscope is complicated by the extra weight of the binocular body, which has a tendency to slip down and get out of focus after some time.

Some binocular bodies, often of European manufacture, may increase the magnification of the image because of their complex prism-lens system, and they are accordingly marked 1.25x, 1.5x, 1.6x, or even 2.5x. Unless they are so marked, the body magnification may be taken as 1x.

There is no distinction between the remaining features of monocular and binocular microscopes. Each has a revolving nosepiece containing 3 or 4 openings into which the customary objectives are inserted. The platform should have upon it, or incorporated in it, some type of slide holder (mechanical stage) which can be moved mechanically and accurately backward and

forward and from side to side. Since virtually all malaria work involves methodical searching, one cannot be sure that the same fields have not been inspected repeatedly *unless a mechanical stage is used.*

The upper surface of the top lens in the Abbe condenser can be seen through the opening in the platform. When it is in position, it should be as near as possible to the under surface of the slide. The function of the condenser is to concentrate to a maximum, at the upper surface of the slide, all the light reflected by the plane mirror through the large lower component of the condenser. The mirror which is supported in position directly below the condenser is used to reflect comparatively parallel rays of light from the light source to the condenser. To obtain the maximum effect of the condenser, *only the plane mirror is used;* the curved mirror is provided only for operations in which the condenser is removed. The parts of the microscope located below the slide are related to illumination and those above, to magnification and resolution.

The total magnification obtained in the compound microscope when the oil immersion objective (usually 90x to 100x) is used may vary from 450x to 1,500x, depending on the eyepieces (oculars) available. Experience has shown that increased magnification by the ocular beyond an optimum point decreases the clarity of detail or *resolution* obtained.

The usual objectives available on microscopes are the low power, or 10x, the "high dry," or 43x, and the oil immersion, usually 100x. The low power may be used to inspect the blood preparation for its position on the slide, general appearance and staining, as well as the distribution of leukocytes. Little use has been found for the 43x objective in malaria diagnosis and it may be eliminated. It may be substituted in the revolving nosepiece by some type of object marker for the purpose of circling interesting microscopic objects for further examination. The total magnification is determined by combining the magnifications of the optical elements involved—namely, ocular, body (if any), and objective. Technicians in training will appreciate more detail with an *intermediate magnification of between 600x and 800x.* After they have acquired considerable experience, they may find that 500x magnification increases the speed of their work, usually because the increased amount of light more than compensates for the loss of detail due to decreased size. Also, 5x oculars may be used conveniently with body magnification over 1x. Other ocular magnifications are 4.3x, 6x, 6.3x, 6.4x, 7x, 7.5x, 8x, and 10x. The latter is

too high for ordinary use with the oil immersion objective.

In recent years it has been customary to treat all air-exposed surfaces of lenses and prisms with a "coating,"[1] which compensates for the unequal wave length of the colors of the spectrum, and which results in a substantial reduction in the dissemination of light. Lenses and prisms so treated may then function adequately with considerably less light than those that are uncoated. When viewed with incident light, coated surfaces may be distinguished by the appearance of a bluish-violet luster. This treatment on modern microscopes is becoming increasingly frequent. (See Section 12, Illumination.)

In warm, moist climates it is imperative that the microscopes be stored at night in a cabinet or cupboard fitted with light bulbs or other heating units so as to maintain the temperature between 29° and 35° C. If the temperature is allowed to drop beyond this point, though it be higher than room temperature, it may actually aid the growth of fungus. This simple storage procedure, therefore, will prevent the development of fungal mycelium on the surface of lenses and prisms. It is preferable that the microscopes used daily not be stored in their respective carrying cases. Wood dust is commonly deposited on the prisms and upper surfaces of oil immersion objectives, causing impaired resolution. This dust, however, can often be removed by a few blasts of air from an ordinary 4-oz ear syringe.[2]

Sometimes, when the mass of the binocular body cools considerably below body temperature during the night, or when the instrument has been out of use, the resolution, though good at first, may suddenly become impaired. When the warmth of the examiner's face heats the air around the prisms in the binocular body, there may be a sudden deposition of moisture on the cold surfaces, which disappears as soon as the air and surface temperatures are equalized.

A disadvantage of inclined oculars in the tropics and elsewhere is that the eyelashes soon become laden with sweat and the grease thereon is transferred to the upper surface of the ocular. Frequent washing of the face with soap and water will reduce this difficulty. Small pieces of Kleenex-type tissues should be used frequently to clean the exposed surface of the eyepiece lens.

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[1] Anti-reflexion film.

[2] See Appendix 18, p. 112.

12. ILLUMINATION

Microscopic objects on slides are found and examined with light directed through the condenser by the mirror. This light passes through the preparation until it reaches the oculars, and these convert the rays of light into a recognizable image. Such light is called "transmitted" light, whereas light such as that which permits the recognition of white bands on the palps of a mosquito with the simple, stereoscopic microscope is "incident" light. Solid pigments of various colors of the spectrum appear in those colors in incident light, but since they are solid, in transmitted light they merely obstruct the passage of light and they appear as gray or black.

"White" light consists of all the colors of the spectrum as can be demonstrated with a prism. Each color has a different wave length. When white light meets a glass surface, a certain proportion is reflected from the smooth surface. But it is not reflected as white light; each color is reflected at a different angle according to its individual wave length. The effect could be compared to a layer of fine bubbles or foam on the surface of water through which one might wish to look. Light passing into the water is obstructed by this foam and much more light is required to show what is below the surface than if this foam did not exist. The coating of lenses with an anti-reflexion film reduces to a minimum the confused reflexion of different wave lengths and permits a smaller light source to work than is required when lenses are not so coated. An ultimate saving of electricity can also be effected through the use of coated lenses, even at an initial extra cost.

Binocular microscopes with their prisms, and often with additional lenses, require an enormous amount of light as compared to that required for the monocular microscope. The total available light is divided between the two eyepieces. At least twice the amount of light which is adequate for the monocular should be available to the binocular. A coated system in the binocular body is therefore a distinct advantage.

It is doubtful whether any other aspect of medicine demands such a high standard of microscopy as does the recognition of the small malaria parasites in a dehemoglobinized and stained thick blood film. Usually, tiny bacteria or other organisms are stained one color and are easily distinguished in the contrasting color of the background. For malaria it is obligatory that the background

be as clear and white as possible so as to show up tiny objects 0.5 to 2.0 microns in diameter which are stained both red *and* blue.

By comparison, the thin blood smear, with its single layer of red blood cells lying flat on the surface of the slide—larger than normal because it has been spread on a very smooth surface—can be viewed with minimal light and considerable detail can be recognized. For this reason the *thin smear should never be used in evaluating* the adequacy of the light and the quality of the image. A well-made and well-stained thick film should be kept at hand for this particular purpose.

The field—the oil immersion field—should be evenly illuminated with a blue-white light at all times. The microscope, lamp, and filters should be arranged in such a way as to obtain the maximum amount of light possible, after which the light may be reduced to suit the individual worker by using the iris diaphragm of the condenser.

At one time it was thought that only daylight was the ideal light for the microscope of the period: a monocular. Benches were located at a window with northern exposure and the mirror was set to reflect the light from white clouds. Under such conditions, daylight is still a good, though inconstant, source of illumination for a monocular microscope. For the binocular microscope, only electricity provides a constant source of uniform light; when it is used, however, an additional factor must be taken into account: electric light is frequently quite yellow.

There are many different microscope lamps that are excellent for malaria work, as well as some that are not satisfactory. It should be recognized that many of the high-quality microscope lamps are specifically designed for microphotography. There is no need for such in malaria eradication programs and the utilization of simpler apparatus is entirely adequate.

Therefore, illuminators which depend on special types of light bulbs should not even be considered, whereas those that can be used with bulbs obtainable in the local market should be. Also, lamps with special sockets should be converted whenever possible; "built-in" illuminators should not be purchased, but the usual mirrors should be ordered for those microscopes with built-in illumination already on hand.

It is not possible to utilize direct sunlight with the microscope because of the glare; it is equally impossible to use bulbs with transparent glass. If the latter are provided, a ground-glass filter is obligatory, either incorporated in the lamp or placed directly be-

neath the condenser of the microscope. A thin, fine-ground glass is preferable for a low light source, whereas a thick, coarse-ground glass is required for a stronger source. Frequently the ground glass supplied is so thick that it cuts off a great deal of light, to the point where the light reaching the microscope is insufficient. Too often, the ground-glass filter is combined with a blue filter and together they lower the available light below working level.

Some type of blue filter is essential in order to obtain the blue-white background for the competent inspection of leukocytes, parasites, and platelets. Often the blue glass circles to be used as substage filters are too blue for the light source; when combined with a ground-glass filter, the combination reduces light excessively.

One hundred-and-ten-volt light bulbs give off heat proportionate to their wattage, hence the use of transformers and special low voltage lamps is recommended. However, the heat from bulbs of 100 watts or less can be tolerated with proper ventilation. Open or unshaded bulbs cannot be used because of the glare in the operator's eyes or in the eyes of those around him.

The foregoing explanation refers only to the use of the oil immersion objectives, since many illuminators are quite satisfactory for pathology, stool, and urine examinations, etc., but do not produce enough light for malaria work.

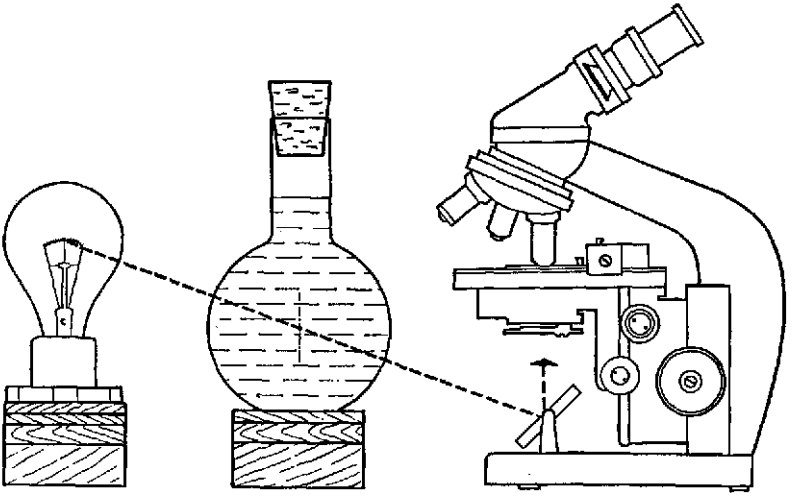
An example of good, adequate illumination *for a monocular microscope* is the ordinary blue, inside-frosted, "daylight" 60-watt bulb. If voltage is good, the 40-watt is preferable. This bulb, in a plain porcelain base and suitably screened on three sides, gives an abundant amount of blue-white light. No filters are required.

Approximately 150 watts are needed for a plain binocular microscope, but since the blue color and the degree of "pearling" are the same as with the 60-watt bulb, the blue is insufficient for so much light and a further blue filter is required. Any 150-watt light gives off too much heat to be of practical use. A lamp on which a series of filters are used usually has a special transparent bulb as a light source and should not be considered. The model A.H.T. Cat. No. 6958, an old German design intended for dark-field illumination, is excellent for the purpose. The 200-watt transparent bulb and the adjustable internal bracket are not required in malaria work, but the No. 6958-E, with 100-watt inside-frosted bulb is satisfactory. A regular 100-watt pearl bulb behind a water-filled 250-300 cc Florence flask is quite adequate if the water is tinged with blue. A sufficient number of drops of "artificial daylight" solution (20 per cent copper sulphate solution 9 cc + aniline blue 0.6 per cent—

1 cc) is added to the water in the flask to give the basic blue-white background. Excess of blue is worse than insufficient blue because it reduces the light enormously. The water is changed as required. Slight turbidity can often be removed by the addition of a few drops of strong acetic acid or ammonia.

The light source should be surrounded with some opaque material, except where it shines on the microscope's mirror. The Chalet type of illuminator (American Optical No. 361 *without* blue or frosted glass, $16.00) will control the glare and hold either the 60-watt or the 100-watt bulb suspended from above. The blue flask may be supported on blocks in an appropriate position between the lamp and the mirror.

DIAGRAM 15



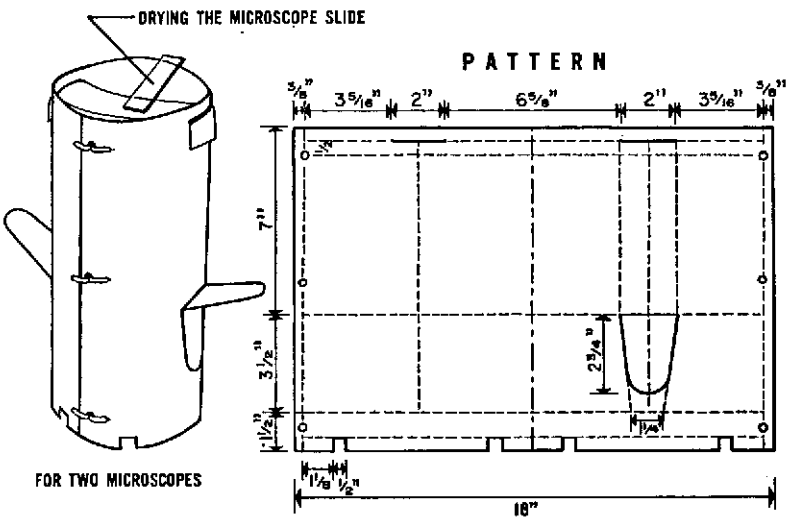
The above installations may be improvised readily by using a strong dark cardboard (insulating paper), a plain porcelain base or socket, and 10 feet of wire. As can be seen from Diagram 15, the relation of the brightest part of the bulb, the center of the spherical flask which acts as a lens, the mirror, and the relative heights of each in relation to each other, are of the utmost importance if optimum results are to be obtained. A number of wood blocks 1-2 cm thick may be used to obtain the proper height of lamp and flask.

An electronic light meter which measures the number of foot-candles of light reaching the eye is most useful in helping the stu-

dent obtain the maximum light. A valuable piece of equipment to aid both in the teaching of proper microscope illumination and in demonstrating accurately the actual amount of light reaching the eye, is the photoelectric exposure meter "Photovolt" (see Appendix 20, p. 114).

The same principle as seen in Diagram 15 can be applied to a homemade lamp, as represented on the left of Diagram 16, which shows a scale drawing of a lamp shade which is held together by brass paper clips. Two openings are used when two operators are seated opposite one another.

DIAGRAM 16



One-hundred-watt inside-frosted bulbs vary considerably not only in size and shape (especially length), but also in the amount of light they give out and the degree of deterioration after use. Some inferior grades darken the interior surface of the pearl glass to such an extent that a loss of 30 per cent may occur. When the line voltage is lower than the specified voltage of the bulb by 10 or more volts, a line voltage regulator may be required to obtain the maximum light.

Degrees of color blindness impair the work of microscopists. Prospective candidates should be given one of the color vision tests, for which American Optical H-R-R Pseudoisochromatic plates are excellent.

13. TECHNIQUE OF MICROSCOPIC EXAMINATION

In order to avoid loss of time and prevent errors, the following steps, which may appear to be insignificant, should be learned thoroughly before attempting to search for parasites:

1. Place a small drop of immersion oil near the edge of the thick blood film.
2. Check and record on the work sheet the identification of the slide, as well as any unusual appearance that may be present.
3. Set the slide between the open arms of the mechanical stage of the microscope and make certain that it is firmly placed against the movable bar of the stage. If this is not done, doubtful or suspicious objects may be lost before they can be permanently located by a circle made with a suitable object marker.[1]
4. Survey briefly the entire thick film with the 10x objective until a suitable area is found where the leukocytes are numerous and apparently well stained. If it proves difficult to locate such an area, spread oil thinly over the entire stained area and survey again systematically with the 10x objective.
5. When an acceptable area is located, place another drop of oil in the center of the illuminated area. Swing the 100x objective into position. Holding one's head well to one side, and using the coarse-adjustment screw, lower the objective into the oil until the tip barely touches the glass.
6. Place the eyes at the aperture of the ocular and continue to raise the body with the *coarse adjustment* until leukocytes come into view. Bring to the center of the field and into optimum focus with the *fine adjustment* a well-stained polymorphonuclear leukocyte, preferably one with some platelets near it. Rotate the fine adjustment back and forth almost violently and rapidly through 50 divisions on the shaft while the eyes view the field. If, during these movements, the leukocytes appear to move radially, even slightly, in any direction, *stop* and adjust the mirror or its height, or the distance from the light source, until the leukocytes appear to hover over the same spot as the result of a wide movement.

The clear areas or the field between the leukocytes should be white with a slightly bluish tinge. It is likely that the light will be

[1] See Appendix 15, pp. 107-108.

less yellow and more abundant than before the adjustments were made. If not, further alterations are in order until maximum illumination is obtained.

7. Next, clean the oculars thoroughly on their upper surfaces with a small piece of Kleenex-type or equally fine tissue. Dust that is on the *outside* of the lower lenses of the oculars, however, should be removed with a blast of air. Dust on the inner surface of the lower lens collects more slowly. Then adjust the oculars to the exact, previously determined interpupillary distance on the scale, such as 68. The single adjustable ocular tube is moved up or down until the resolution is equal in each eye.

8. Review the elements of normal blood described in Section 3, pp. 10-13. Make note of any deviation from the usual color and appearance.

9. Only when the normal blood elements appear in their proper colors can it be expected that any parasites present will appear in their appropriate colors. Therefore, if the nuclei of leukocytes are too red in some area of the specimen, it is unlikely that any blue will be detected in the cytoplasm of parasites. Or, if the leukocytes are too blue it is unlikely that the particles of chromatin will show much red color. It should not be overlooked, however, that occasionally where the staining of the cellular elements is pale or poor, parasites may continue to stand out clearly and distinctly for several weeks.

10. Acquire ability to recognize the appearance and color of malaria pigment as early as possible, because many suspicious objects, up to the size of a small lymphocyte or larger, may immediately be dismissed as not being parasites if no pigment can be recognized within them.

11. During all these operations, note automatically whether the cells and other elements inspected for color are also seen as good, clear-cut, and distinct images, i.e., whether the resolution ranges from excellent to good. If it does not, the cause should be corrected before continuing.

Once the above steps have been learned thoroughly, it is time to examine the specimen. Since it is customary to search a minimum of 100 fields of the thick film before calling the blood negative, it would be advisable to learn precisely what constitutes one practical microscopic field.

Since few microscopic fields appear entirely flat in the oil objective, it is costly in terms of time to examine in detail every object in the illuminated area. Any such object which requires careful exam-

ination should be moved to the center of the field where resolution is maximum. Therefore, for *searching purposes,* the microscopic field may be defined as that part of the illuminated area which is in as clear focus as something located in the exact center. This may sometimes include no more than two thirds of the illuminated area.

Once the usefulness of the exact center of the field is appreciated, any object under discussion may be moved there, so that crosslines or pointers in the ocular are superfluous.

Beginning at one edge of the thick film, the slide is moved in a zigzag fashion under the objective as rapidly as this central area can be scanned. Each time a new area of the same size has been observed it is considered to be one microscopic field.

To compensate for the variable performance of individuals, the examination of a single specimen should be for a specific number of fields rather than for a certain number of minutes. Supervised novice workers have recorded from 39 to 215 fields examined during the same observation period. A thick blood film of approximately 1.0 to 1.5 cm^2 may contain 500 to 800 satisfactory microscopic fields. It is obvious that an extension of examination to include the entire preparation can be done only under very special circumstances, such as in infection and drug test experiments. Where symptoms of a febrile patient are due to malaria, the parasites are usually readily found, that is, several per field. When they are very infrequent they may be recorded as the number found in 100 microscopic fields, e.g., 37/100, or simply 37 V (when referring to *vivax*).

If the observer has been examining slides with similar preparations almost daily, the recognition of what is chromatin will be automatic. But if the microscope has not been used for some days it is well worth while to find several undoubted parasites in order to be oriented as to the specific and characteristic density that chromatin uniformly shows. A single parasite in a preparation should be diagnosed with caution; at least three parasites should be found. The search is no longer made for specific forms of the parasite; instead, as many definite parasites as can be seen in the examination are checked as to the size of the smallest, the largest, and the majority (see Appendix 4, p. 95). It is suggested that a good positive slide be examined for at least 50 fields so that evidence of a second infection will not be overlooked.

When it is desired to investigate any *unusual* parasitic appearances in the thick blood film, if the patient is available a thin smear

may be taken and examined in the hope of finding forms which will explain such unusual appearances—for example, the doughnut-like appearance of early schizonts and pre-schizonts of both *P. vivax* and *P. malariae*. Attempts to diagnose species with only two or three parasites in the thin smear can be unwise.

14. RECORDING AND REPORTING OF RESULTS

In services devoted exclusively to malaria it can be a waste of effort to write *Plasmodium, Plas.*, or *P.* every time reference is made to any of the species. Adequate symbols or abbreviations take up less space and constitute less work, *viz.*, F, Fg, V, M.

If these are coupled with the intelligent use of approximate numbers distributed under only four headings, it is possible to record the exact picture of the blood findings.

It is therefore recommended that the abbreviation *P.* and the word "positive," which by itself means very little, be eliminated. The word "negative" has now been accepted to signify that no parasites have been encountered in 100 fields of the thick blood film. When the report form has a space for showing the total number of slides examined and the positives are recorded by means of numerical reference, it is not necessary to write "neg." after each negative. A dot or check is sufficient to show that the slide has been examined.

The *falciparum* infection is divided into three phases:

1. Rings alone = F
2. Rings and gametocytes = F + g
3. Gametocytes alone = Fg

No other explanation is needed.

On the other hand, the gametocytes of the other three species do not require any special mention except to remind the observer that they may persist through several cycles and that old, faded ones may be encountered. Unlike *falciparum*, they require no special drug to remove them from the circulation. All the different forms of the developmental cycle may be seen in the peripheral circulation at some time during the 48 to 72 hours they require for the cycle, but they all disappear after a schizontocidal drug is taken.

V is the only record required for all forms of *vivax;* M for all forms of *malariae;* Ov for *ovale* (not yet proven to exist in the Western Hemisphere).

The *important* thing is some approximation of number of parasites present. Not that high numbers can be interpreted as suggesting new or recent infections and low numbers as older, but solely because species diagnosis is more likely to be correct when made from large numbers than when there are very few. Review of the slide becomes important in the case of scanty parasites.

The numbers can be recorded roughly as follows (the numerical values assigned to ++ and +++ are pure approximations):

Where parasites average 1 per field = +
2-20 per field = ++
21-200 per field = +++
more than 200 = ++++
When the number of parasites actually counted in 100 fields lies between 40-60 = $\frac{+}{2}$

Any number less than 40 per 100 fields should be written in full, i.e.: 33

When recorded under only four column headings, these approximations or actual numbers can be used for the exact diagnosis of any possible combination of species, e.g.:

F	FG	V	M
++	14	•	•
•	•	+++	•
•	37	++	•
•	29	•	++
•	•	$\frac{+}{2}$	9

Three parasites per 100 fields is the largest permissible number of parasites which can be found later by a second examiner (reviewer) in a slide already reported as "negative." This may be taken as a baseline for errors. If no more than three are found, a simple notice of the finding is returned to the original examiner instead of the "error in diagnosis" slip sent when the diagnosis has been wrong.

15. GENERAL PROCEDURES SUGGESTED FOR THE ORDERLY EXAMINATION OF STAINED SLIDES

1. Adjust the seat to the proper height for the individual examiner.
2. Remove all finger marks, dust, or oil from the entire microscope with a soft cloth or fine soft-type tissue paper. Make sure that the mechanical stage moves freely in both directions. If it does not, remove and clean both the underside of the stage and the platform. DO NOT OIL. Use vaseline on all dry movable surfaces.
3. Check the solution in the flask, which should be transparent and not too blue.
4. Close iris diaphragm slightly to use the 10x objective; always open when 100x is turned into position.
5. Use the left hand for the constant focusing of the fine adjustment screw, and the right hand for changing fields with the knobs of the mechanical stage. Constant focusing is a *must* with thick films.
6. Review the features of the blood (see p. 10) to facilitate the evaluation of the quality of the stain and the recognition of parasites if they are present.
7. When the examination of each slide has been completed, remove all oil and grease-pencil marks, if used, by wiping gently with tissue moistened with toluene. For large numbers of slides it is preferable to use a 2-oz wide-mouth bottle filled with toluene. First blot up the excess oil on the slide with soft-type tissue paper; dip the slide in the toluene bottle; then wipe lightly with a small piece of dry tissue.

8. At the end of the work period, remove the last slide and turn the 10x objective until it is directly over the condenser. Lower this objective until checked by the automatic stop. Remove oil from the 100x objective.
9. Move mechanical stage to its center position.
10. Cover the instrument with dust-protection cover and return it to its numbered position in the warm cabinet.

16. MICROSCOPIC SLIDES

These commonplace objects are so well known that most people seem to believe that they are pieces of glass of a standard length, breadth, thickness, and quality. This is not so. Slides from different manufacturers as well as from different countries vary slightly in thickness,[1] length, and width.[2] Before placing an order for microscopic slides, a review should be made of what will be expected of them. They will be exposed to washing and strenuous wiping; they will be carried distances under varying conditions, stored, and then distributed. Before receiving their specimens of blood they may have been transported long distances by jeep, horse, boat, or afoot. They then bear blood samples which cost from a few cents to several dollars each to obtain. They will go through many hands before the specimen is finally examined, checked, and then stored for further reference. They must remain intact throughout all this process. If these points are taken into account, it is unlikely that considerations of cheapness will outweigh the advantages of a really good slide.

Should a large supply of previously used slides be the only ones available, they should be inspected one by one. Those showing even a beginning of corrosion should be discarded and the remainder separated into groups of identical color, thickness, length, and width. Individual groups may then be washed and packaged in accordance with the directions appearing below.

One of the best available microscopic slides are Micro slides, noncorrosive, Red Label, Special, A.H.T. No. 7030. These are very uniform and sturdy. Their thickness is 1.10-1.30 mm. They have polished edges and slightly rounded corners; the long edge is

[1] 0.8-1.3 mm. Measure 10 slides in order to obtain average thickness.
[2] 77 x 27, 76 x 26, 76 x 25, 75 x 25, and 74 x 24 mm.

slightly bevelled (appreciably reducing the number of finger cuts during wiping). The list price is $3.35 per gross. The No. 7030-C "clinical" slides are identical, lacking only the bevelled edge. The list price is $1.90-$2.85 per gross.

Several brands of "pre-cleaned" slides are on the market. If they come near to the above specifications, prove to be clean enough for use with Giemsa stain, and are completely grease-free, they may be tried. Possibly they could be wiped out of 90 per cent alcohol and could then become usable, but they are expensive.

The so-called "safety-grip" rough-edged slide is also thin, less than 1 mm. It is doubtful whether this slide could be cleaned as completely as a smooth-edged slide.

New slides which have never been used but which have been stored for months under favorable conditions may show varying degrees of corrosion. If at all possible, these should not be used for blood work, but rather diverted to some other activity.

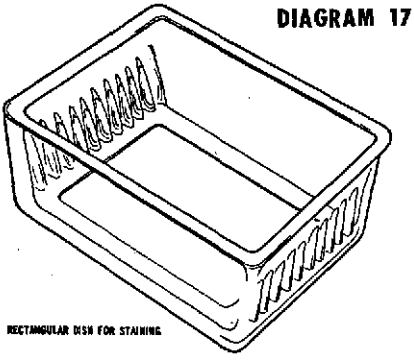
New slides are usually received in cardboard boxes which have a small space remaining after 72 slides have been placed in them. When this space is filled with an adequate number of clean slides—usually 3 to 8—the full box becomes a convenient and satisfactory unit for shipping or storage. For mailing, these boxes may be reinforced with a single layer of corrugated cardboard and covered with a tough paper wrapping. All such cardboard boxes should be saved for this use.

The Cleaning of Microscope Slides

The staining of malaria and other parasites in blood is in reality a very delicate chemical reaction which is easily upset by contact with dilute acids or alkalies, soaps, disinfectants, and absorbent materials such as dried serum or sweat. A trace of fat or oil hinders the penetration of the stain and is the most common cause of blood washing off the slide in small flakes during staining.

For this reason there has not been as yet any "short cut" to the slide-cleaning routine. It is not enough that their surfaces be highly polished; they should also always be firmly wiped with a clean cloth until nothing is adhering to the glass. In fact, so much pressure is exerted to achieve this end that novices frequently break many slides when they are learning to wipe them properly. Well-cleaned slides also reduce the number of confusing artifacts that may appear in any blood preparation.

DIAGRAM 17



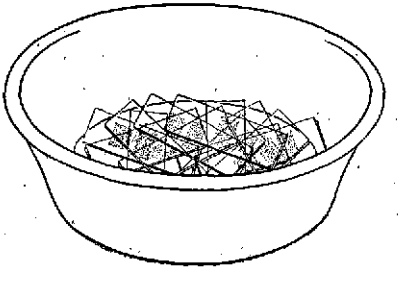
RECTANGULAR DISH FOR STAINING

The assumption that new slides from a freshly opened box are the best that can be used is almost universal. This is by no means true because new slides are often contaminated by chemicals during the polishing process and these are seldom removed by washing or prolonged soaking in clear water before being squeezed together to remove the excess water from large quantities of slides.

Staining dishes which will keep slides separated one from the other during the washing, soaking, and rinsing process are indispensable (Diagram 17). They have a loose-fitting cover and are grooved to keep slides separated (A.H.T. No. 9194). Each worker should have a minimum of four available. One cover can serve for four dishes piled one on top of the other.

Where the number of slides to be washed is so large that the staining dishes are no longer practical, the slides should be distributed in an empty basin in such a way that the water reaches all surfaces (see Diagram 18).

DIAGRAM 18



New slides. New slides are treated by placing them in glass dishes which keep them separated one from the other, and immersing them for at least 12 to 24 hours in glass-cleaning fluid prepared as follows:

Potassium dichromate	60 gm
Sulphuric acid (concentrated, 95-98% H_2SO_4)	300 cc
Water	400 cc

(Add acid to water *very* slowly, preferably holding the flask in flowing water to cool.)

The glass-cleaning fluid should be kept in a glass-stoppered bottle conspicuously marked "DANGEROUS—Keep away from Hands and Clothes."

This solution is then poured through a wide funnel back into the bottle, and the slides, still lying on their edges, are thoroughly rinsed with running water from a faucet, or, if not available, in repeated changes of water until all traces of the acid have gone. If distilled water is plentiful the slides should be given a final rinse in it before they are wiped dry, using firm pressure of the fingers in a longitudinal direction. These slides are laid out individually on a clean piece of cardboard, placed on a table or bench, to permit complete drying of the edges. Recently, two shipments of new slides appeared to have a thin waxy film which survived passage through the rather weak acid. Stronger acid had no effect. After rinsing, they were placed overnight in a detergent solution which resulted in the complete disappearance of the waxy coating. If two or three trials using detergent alone demonstrate that the slides are really clean, then the acid step may be omitted.

If this cleaning operation has been done properly, there is no need for further immersion in good-quality alcohol to render the slides completely grease-free. If alcohol is used, the slides must be dried before being placed in it.

The cleaned and dried slides are packaged in tightly wrapped blocks of 10, 15, or 20 (if the slides are thin) and are then ready for use. It is advisable to mark the date on which the package was made since, depending on the humidity and contamination of the air by such substances as motor exhaust gases, slides kept in packages for several months may require washing again before use.

Used slides. All slides have some amount of immersion oil on them at the end of their first examination. If it is desired to pre-

serve the slide for further examination, a few drops of good-quality toluene are allowed to drop on the slide, which is placed in a wood drain block until dry. A better procedure is to dip them in a 2-oz wide-mouth bottle full of toluene to remove any oil that could not be taken off by a brisk blotting with dry soft-type tissue. Slides are then wrapped in onion skin paper 4½ x 8½", and their identification is written on the outside with a lead pencil. Stained thick films covered with oil are soon spoiled if left scattered about on the bench or exposed to sunlight.

For washing, provide a bowl of glass, enamel, or plastic (Diagram 18) at least 4" deep and one half filled with a strong solution (5 per cent) of soap or (½ per cent) detergent into which the discarded slides are placed pending washing. Any of the laboratory cleaning compounds similar to A.H.T. No. 3298 are less trying on the hands than commercial detergents of usual grades. Slides should never be kept soaking in shallow vessels such as trays or flat pans. These are almost always left to one side and forgotten and the slowly evaporating water or solution leaves corroded streaks across the surface of the slide which may be permanent. Slides should never remain more than three days in plain water, which eventually becomes slimy with an unpleasant scum on the surface. The slides in detergent or soap solution may be transferred every two or three days to a larger and deeper storage vessel. If, while wet, they are passed between the thumb and forefinger, most of the stained blood and oil will be left behind in the old container. Such slides may be massed together for storage in weak detergent solution, but for successful cleaning they should be separated individually or handled in the above-mentioned glass staining dishes or jars.

In such jars they are placed under a stream of running water for one half hour, or 20 changes of water. New slides may be placed in running water, gently rubbed between the fingers, and rinsed in running water for a further half hour.

Since used slides have been exposed to oil, it is often more difficult to make them grease-free. After being wiped out of water and allowed *to dry thoroughly,* they are placed in 90-95 per cent alcohol; they are then wiped out of the alcohol with a fresh cloth and packaged. Such alcohol may be used repeatedly if filtered each time it is returned to the stock bottle.

Commentary. This cleaning process may seem to represent a great deal of work, but to be convinced of the need for it, one has only to see a group of several slides prepared by a careless attendant and observe that only a ring of blood remains on the slide after staining, by which time the person from whom the smear was taken may be 100 miles away.

The bacteriological practice of flaming slides before use is not essential and may indeed make slides more brittle and hasten corrosion. Boiling is also unnecessary.

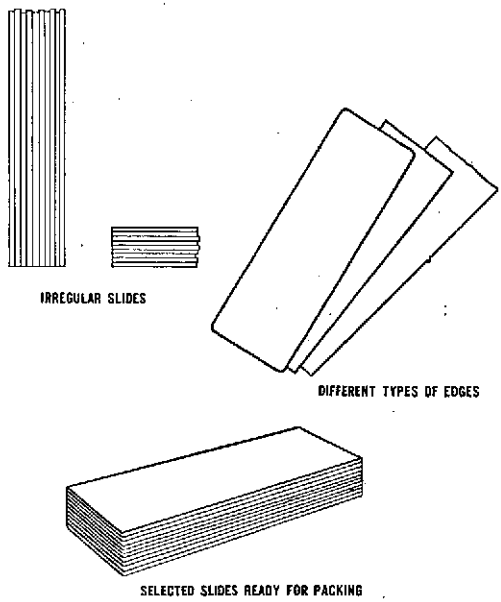
Cleaned slides may be stored, until needed, in wide-mouth jars 5 cm wide and 10 cm high, where they are submerged in 90-95 per cent alcohol. They are wiped with special clean cloths used for glassware and then packaged for later use.

Importance of cleanliness of towels used for glassware. To obtain the condition of scrupulous cleanliness required for glassware, a generous supply of towels of suitable cotton material with as little lint as possible should be provided. The material should not be too thick or too thin and its initial content of dressing must be removed by repeated washing. Material such as Indianhead is cut in pieces of 40 x 60 cm and the cut ends are hemmed. These towels should be used for no purpose other than drying the glassware—never for the hands or face or for wiping the bench or sink.

It should be understood that these cloths are rarely soiled when used only for glassware. However, they should be washed frequently since the hands perspire constantly, to a greater or lesser degree depending on the climate, and the perspiration is absorbed by the cloths.

Soiled towels are soaked in water and a good soap is thoroughly rubbed over the whole surface and into the material, or the towels may be immersed in a strong solution of detergent and allowed to soak for 15 to 30 minutes or overnight. After thorough agitation and strong rubbing on spots where dirt is visible, the towels are rinsed in repeated changes of water until all traces of soap or detergent are gone. Where distilled water is abundant, a final rinse in such water is advisable. Starch should never be used, and towels washed by commercial laundries should always be well rinsed before use. Ironing is superfluous except when done to accelerate drying.

DIAGRAM 19



Packaging of clean slides. When used slides are washed and wiped dry, they should be separated into groups of equal size (length, width, thickness) and color before being made up into packages of 5, 10, or 15 slides tightly wrapped in paper. If these packages contain some slides which are not uniform, the paper wrapper will soon be damaged. Diagram 19 shows the difference between a block of selected and unselected slides.

It is not recommended that clean slides be stored in the conventional wooden slide boxes with 25, 50, or 100 slots. In these, each slide is exposed to dust every time the container is opened. Moreover, constant shaking produces wood dust and even glass dust. The larger boxes require extra reinforcement, particularly for transport or mailing and are bulky as well. One to three such boxes should be kept in each central laboratory to hold reference and teaching slides[1] for ready reference.

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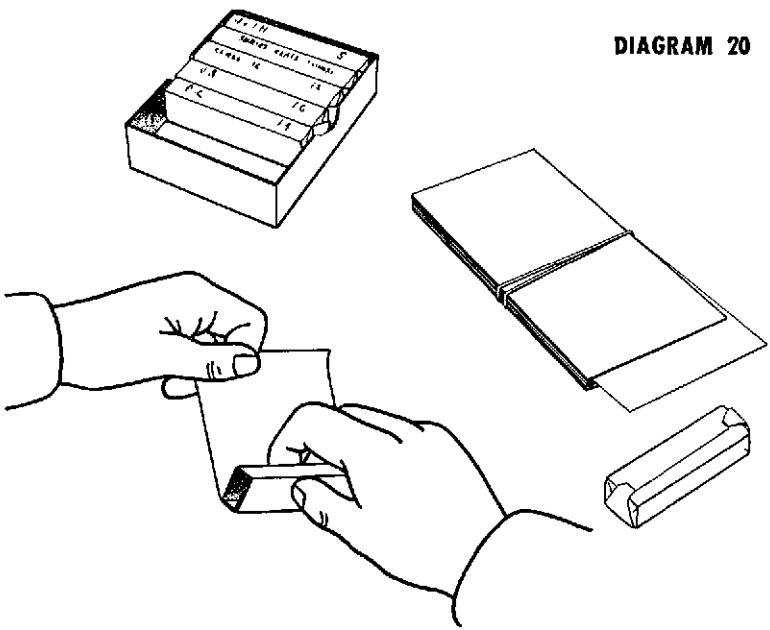
[1] See Appendix 10, p. 103.

Handling, Storing, and Transportation of Microscopic Slides Containing Blood Specimens

One advantage of modern packaging of merchandise is that the contents are arranged in such a way as to reinforce the container, that is, comparatively thin cardboard cartons are firmly supported by their compact contents. Except for the packaging of pieces of extremely delicate apparatus, strong wooden or metal cases with an abundance of soft packing have been entirely replaced by weight-saving and space-saving "skin-tight" cardboard containers.

As soon as completely dry, freshly prepared slides are made up into taut, tight packages of 8 to 15 slides, but no more, as shown in Diagram 20.

DIAGRAM 20



Any tough, thin copy paper such as onionskin is cut, preferably in a printer's cutter, in sheets measuring 4½ x 8½″, together with pieces of firm cardboard of the same dimensions. Fifty such sheets are placed between two pieces of the cardboard and are held together by a rubber band to keep the sheets flat and the edges even. The length should be 5″ if individual packs of five slides or less are made.

The predetermined number of uniform slides are placed across the narrow portion of the paper, allowing an equal amount of paper

to project beyond each end. The paper is turned back over three edges of the block of slides and the distal portion of the paper is held firmly so that the wrapping is completed by rolling the block of slides as a unit toward the free end of the paper. The ends are then neatly folded and compressed against the end of the pack. The importance of even edges will now be apparent, for the firm tight package can then stand upright.

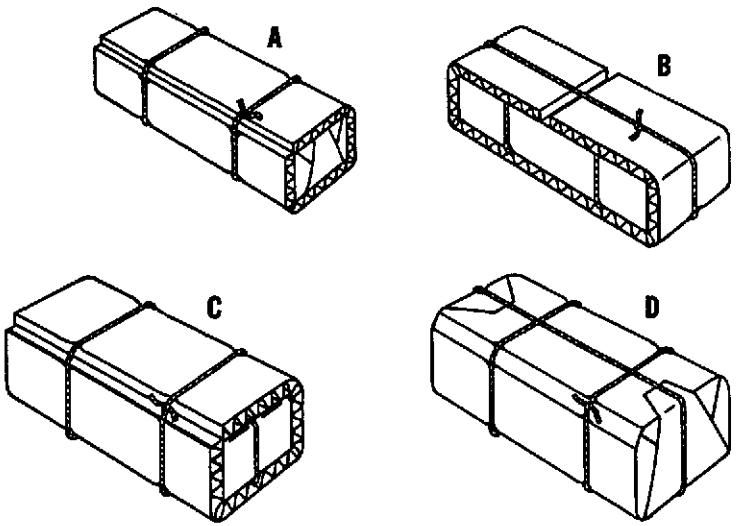
Any attempt to introduce paper between the individual slides defeats the principle of packing. The method of rolling slides in toilet paper results in a loose package as compared to the above and is, in addition, tedious to roll and unroll. Slides containing blood smears will withstand transportation better if packaged in the above-described manner because movement between slides is reduced to a minimum.

All necessary identification may be written with a soft pencil on the "edge" side of these packages so that they need not be opened for identification. (Packages of newly cleaned slides should also bear the date when they were cleaned.) Such blocks or packages of slides are stored in the empty cardboard boxes in which they were received, either in the open box or in the lower half and the lid of the box. Small metal cooking pans 18 x 28 x 3.5 cm can be used to hold as many packages of slides as can be conveniently handled, taking weight into account.

A slide carton full of such packages is the most compact, convenient unit for handling and shipping slides. Only neatly-cut corrugated cardboard is needed to cover all sides, which are tightly tied with string before the carton is wrapped in tough wrapping paper.

Single packages or less than a carton of slides may be packaged separately, using appropriate widths of cardboard. Strips of corrugated cardboard are cut in varying widths with a Gem-type razor blade in a razor-blade holder, or preferably with a heavy paper cutter. There are two sizes, approximatey 2.5 cm to 7.5 cm wide, with successive pieces each 0.5 cm wider than the previous ones. The cardboard grooves are to be transverse and the length not to exceed 60 cm. The pieces are then cut according to the individual block or blocks of slides. Strips are applied alternately in each longitudinal axis and tied separately until sufficient covering for the mass of glass has been applied (Diagram 21). The whole is finally covered with wrapping paper. Such packages can resist a single deliberate throw from a great height on metal, stone, or cement.

DIAGRAM 21



A traditional container used for mailing one or two slides only is the *wooden* Micro slide mailing case, reversible, A.H.T. 7056. Whether used in pairs or in series, they require only an outside paper wrapper. However, it is safer to apply a single layer of cardboard to each side of these mailers, held in place by tough wrapping paper, than to rely solely on the wrapper.

It should always be remembered that the specimen of blood on a slide represents a considerable expenditure of time and effort as well as travel on the part of the collector. Everything possible should be done to ensure that this is not wasted through loss or breakage of slides.

Adams two- or four-slide cardboard mailers, A-1615, can be very useful to field workers for the purpose of protecting slides from flies, etc., until dry, at which time the slides are incorporated into temporary packages.

Student or training slides may be quickly distributed and collected with Adams map-form slide trays for 20 slides A-1605.

An excellent 25-slide box made of polystyrene by the LaPine Co. (A. H. Thomas No. 7059-B) is suitable for the rapid handling of teaching slides. There is more space between the slides than in the common bakelite boxes and the polystyrene is less brittle.

Conventional slide boxes with slots for 25, 50, or 100 slides are more useful for the storage of demonstration and special material slides than for use in the field, where slides are exposed to dust, moisture, and flies.

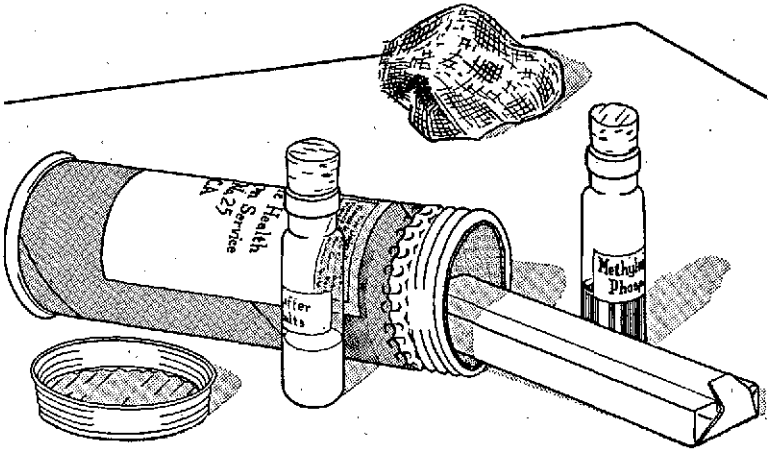
Positive slides and other exceptional material of special interest should be stored in a uniform manner and plainly labelled as pertaining to individuals and groups, so that any given slide may be located without an exhaustive search.

Frequently available are tubular cardboard containers with metal screw caps and base which measure 4¾ x 1¼". These can hold one package of 15 slides or two of 7 each, with the accompanying slips compactly folded.

This same container may be used for slides and tubes destined for collaborators who are located at such a distance that the slides with blood cannot reach a laboratory in less than five days. It contains a packet of three clean slides and three folded slips together with two small well-corked vials 1¾ x ½" (Diagram 22). One vial contains 5 cc of methylene blue phosphate solution and the other sufficient dry buffer salts (0.2 to 0.6 g) to dissolve in a soft drink or beer bottle containing distilled or rain water.

Wrapping for storage in freezer. Unstained thick blood films are first dried well and are then wrapped neatly in onionskin

DIAGRAM 22



paper in packages not to exceed 15 per package. The folded ends are stuck down with a narrow ½″ band of plastic tape 1″ long.

Next, the package is wrapped in aluminum foil 9 x 4½″. The ends are smoothly moulded in place.

A concise description of the contents is typed on stiff white paper and placed over the edges of the slides for the final wrapping in wax paper 11 x 5″, in such a way that the identification will show through. The folded ends are again held in place by short strips of plastic tape.

PART IV

Laboratory Services

17. LABORATORY SERVICES

The purpose of a malaria diagnosis laboratory is to examine competently the maximum number of blood specimens as rapidly as possible. In addition, the laboratory should be responsible for everything that pertains to this specialized work.

It should always be borne in mind that the examination of a blood specimen for the presence of parasites is really a very poor test when the parasites are present in very low numbers. Therefore, a type of minimum examination should be adopted, such as 100 microscopic fields per specimen, so that no undue amount of time is lost in the useless scanning of negative bloods.

For a laboratory service to function satisfactorily, it is important that all supervisory personnel know exactly what is expected of the microscopists and what their limitations are. The immediate "heads" of the laboratory will be obliged to undergo sufficient technical instruction in procedures so that each will be able to detect small deviations from the prescribed laboratory methods for the purpose of correcting them before the over-all quality of the work is affected. One example of this is when a worker calls for or uses something from an unlabelled bottle, perhaps handed to him by the cleaner or some other unskilled person.

Principles and practices must be determined in advance and all procedures standardized whenever possible. Modifications which give better results may be thoroughly tested at the central laboratory and adopted immediately after they have been proven satisfactory. Such rigid uniformity permits the rapid inspection of equipment and practices and allows easy interchange of personnel for temporary periods of observation, study, or relief.

Staff

Head of the laboratory service. An M.D. should be given preference for this post. He should be selected for aptitude and energy, rather than for degrees and formal training.

He should devote his first month to actual practice of all laboratory procedures; he should inspect in detail the quarters, fixtures, microscopes, lenses, and all other equipment in each laboratory under his supervision. He should appreciate the limitations of

this equipment. He should insist on a high degree of cleanliness and should understand and remedy any difficulties that may be involved in attempting to maintain it. He should personally investigate any failure of methods or materials and correct it himself. He should be the key person in recruiting microscopists, laboratory aides, and collaborators.

Chief technician. Here again, aptitude and energy are more desirable than diplomas and titles. This officer is responsible for the central laboratory and for all diagnoses; he confirms all positive slides and an occasional negative one; he supervises all records and reports, and also supervises the field laboratories.

Microscopists. The number of microscopists depends on the monthly work volume, based on a minimum of 75 thick film examinations per microscopist per day, plus additional staff to relieve them during vacations and increased workload periods. Each should have his color vision tested before the training period.

Cleaners. This personnel should be well instructed in the special precautions to be taken in this type of work. Those showing spontaneous interest should be given the opportunity to do microscope work as soon as they are familiar with the routine.

Clerical staff. This staff includes clerks, record keepers, and secretaries, depending on the methods used in the particular service and on the work volume.

Training of Personnel

The training of personnel should not be limited to a formal introductory course; it should be a continuing process. The student requires at least six months of bench work, examining routine slides, if he is to become acquainted with the variable appearances of blood. The central laboratory can function as a training ground or as an internship where technicians from outlying districts and private laboratories may rotate after learning the current procedures and modern viewpoints.

The approach should emphasize the thick blood film and ignore the thin smear. The student should be encouraged to discontinue all mention of the old-time classical terms such as *accolé,* band, "mixed infections," and the like. He should learn current usage and precise diagnosis. The latter includes three separate phases of *P. falciparum.* Instead of reports of "mixed infections," the dominant and subordinate species may be indicated clearly with

abbreviations (see Section 14, p. 63). Since the non-*falciparum* species show all the various stages of development in the peripheral blood no matter when the blood is taken, and since their gametocytes do not persist after the asexual forms are gone, it is pointless and pedantic to mention the presence of trophozoites, schizonts, and gametocytes when these are understood solely by the letters V and M (for *vivax* and *malariae*).

The beginner should be exposed to only the best possible study material until species diagnosis is thoroughly understood. The quality of specimen slides may then gradually be reduced to the level of the poorest specimen sent in from the field.

Any attempt to combine thin blood smear appearances with thick film diagnosis results in confusion and should not be permitted. Instruction should be continuous until the worker has complete confidence in the thick film alone. For many people, the detailed examination of several infections is most helpful. It provides a firm basis for understanding that the individual parasites are dynamic, growing, individual animals which react to their environment as do any other animals and can vary greatly in appearance, but that the behavior of the infection as a whole has very definite patterns.

18. EQUIPMENT

Minimum Requirements, Quantity and Quality, Operation and Maintenance

For the establishment of a satisfactory laboratory, use is naturally made of buildings, rooms, or other quarters that are available. In practice, space is often set aside for entomological laboratory work. Whenever possible, the diagnostic laboratory should be maintained apart from all other activities because of the nature of the work and because a quiet environment is most desirable.

When the prevalence of malaria declines, the work becomes progressively more tedious, because of the diminution in number of positive bloods which stimulate the interest of the workers. Within limits, everything possible should be done for the comfort of those engaged in this rather tiring work.

In order to facilitate the identification of slides, it is essential to have a large, well-ventilated and well-illuminated room. In warm, moist localities an electric fan, preferably in the ceiling, contributes to the success of the technical procedures as much as it does to

the physical comfort of the occupants. It goes without saying that the whole installation should be adequately screened. To protect material before screening, fly control measures of all kinds should be rigorously instituted. In humid climates, a "warm closet" should be provided from the beginning, of a size adequate to store at night the number of microscopes in use. It should also be equipped with electric bulbs or some other heating device to maintain a constant temperature not to exceed 35°C. This will effectively prevent the growth of fungus on lenses or prisms.

Solidly constructed tables or benches should be ample in size and number. The edges of the tables should be well rounded to protect the forearms of persons using microscopes. The surface should be painted dull black. The height should be between 30″ and 34″, and a minimum width of 32″ will permit the utilization of most types of illuminators.

Specific laboratory furniture is neither essential nor desirable. A stable, solid, homemade table with a crossbar suitable for a footrest is often preferable to types of desks or benches designed specifically for laboratory use.

In addition to furnishing a footrest and *ample knee space,* care should be taken to provide chairs, stools, or other type of seats that can be adjusted to the user's height, such as block or swivel chairs; cushions can also be used. The purpose is to aid the postural position so that fatigue will not occur earlier than need be. The provision of stools of different heights is usually satisfactory.

One or more sinks with running water are absolutely mandatory, since the cleanliness of slides and all glassware used in relation to staining procedures is of the utmost importance. A practical dust-proof cabinet for glassware, bottles of stain, and other reagents is most desirable, as are filing cabinets and a desk for the secretarial and record-keeping work.

A separate small, firm table may be required if a good-quality balance or scale is used, in order to avoid the damage resulting from constant movement.

Provision should be made for a constant and adequate supply of distilled water. It may be necessary to collect rain water in an appropriate manner and store it in a number of large glass containers. The modern electric still provides abundant distilled water of excellent quality. Demineralizers (cartridge de-ionizers) provide water entirely suitable for blood staining in a much simpler manner than stills.

The following list shows the basic equipment required for a

single examiner. It may be increased according to the number of microscopists contemplated.

List of Equipment for a Single Examiner

(Multiply by the number of examiners and students expected.)

1 clean binocular microscope with appropriate oculars for the particular multiplication factor of its binocular body, *viz.*

Factor	*Eyepiece*
1.6x	4.5x
1.5x	5x
1.25x	6x
1x = no factor	7x, 7.5x, 8x (maximum)

1 solid, smooth-working mechanical stage.

1 plastic dust cover.

1 light source to give abundant blue-white light.

Built-in illuminators with transparent bulbs (special) and bulbs of less than 25-watt capacity are inadequate. Thin ground-glass and pale blue filters for microscope and illuminator should be supplied. The American Optical Chalet-type Micro lamp, without the ground-glass and blue filters, may be combined with a blue water-filled Florence flask to give an excellent light if an ordinary 100-watt pearl bulb is used in the Chalet lamp. The flat top of the lamp provides a warm plate for drying slides (A.H.T. No. 6958-E). A homemade lamp gives the same results (Diagram 16, p. 59).

1 warming unit adequate to give a temperature up to 35°C in a storage cupboard, to protect prisms and enclosed microscope lenses from fungus in hot moist climates. A maximum-minimum thermometer (see Appendix 19, p. 113) is essential for this purpose.

1 small bottle for Cargille's Type A, lower viscosity immersion oil or Crown oil (ordinary 20-cc applicator bottles, with glass rod applicator, $0.75 to $1.00 per dozen, are better than those supplied by optical companies).

2 towels for glassware, lint-free, 16 x 24″.

4 rectangular staining dishes 2¾ x 3½ x 2″ (Diagram 17, p. 68).

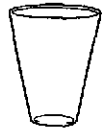
2 soft (No. 1) lead pencils.

1 package of 50 sheets best quality *onionskin* copy paper, 4½ x 8½″ (held between cardboard of same size.)

1 pointed Bard-Parker blade in cork of square 2-oz bottle with ¾″ mouth.
1 sheet 00 or 000 emery paper to maintain sticker bright, clean, and sharp.
1 package gauze wipes, small roll gauze or bandage, or, if unavailable, a small roll of good-quality (long-fiber) absorbent cotton.

For Staining Purposes:

1 interval-timer, 1 min. to 2 hrs., with alarm continuous until stopped.
1 120-cc wide-mouth bottle with methylene blue phosphate mixture.
2 small plastic glasses, with

perpendicular  rather than inclined  sides,

for buffer wash water.
1 30-cc bottle (or plastic dropping bottle) filled with *good* Giemsa stain.
1 10-25 cc graduated (preferably plastic) test tube. (Test tubes marked at 5, 10, and 15 cc work just as well.)
1 curved staining plate or equivalent with 2-3 mm depression, or 1 white enamel rectangular basin (see Diagram 12-D and E, p. 44).
1 glass or plastic bottle to hold 500, 750, or 1,000 cc.
1 bottle mixed phosphate salts of some previously selected proportion such as ⅘; and 6 small tubes with corks to contain 0.5, 0.75, or 1.0 gm of that mixture.
Blocks for the draining of slides are made in various sizes from good-quality, well-dried hardwood, ¾-1″ thick. Transverse saw cuts ¼″ deep and inclined at 110° are placed ½″ apart. The cuts should be sufficiently wide—$\frac{1}{20}$″ minimum—to receive thicker slides. One 1 x 8″ block will take a few slides, while one 6 x 9″ can hold 100.
1 package of paper towels.
1 plastic box for 25 slides to hold demonstration thick blood films.
1 Gem-type razor blade.

2 pieces ordinary package cardboard (corrugated). Sundry items for carrying, drying, and packaging slides (string, brown wrapping paper).

Supplementary Items for a Zone or Central Laboratory

1 spare oil immersion objective and 10 pairs of 7x or 7.5x oculars if microscopes which lack intermediate oculars are to be used.
1 adjustable object marker to circle microscopic objects, for each microscope (see Appendix 15, p. 107).
12-24 250-300 cc Florence flasks.
1 AO Pseudoisochromatic color vision test.
Wood blocks 1 cm and 2 cm thick—6-8 cm square (25 of each thickness).
Spare corks for all bottles.
36 plastic dropping bottles (see Appendix 11, p. 104).
1 30-60 cc dropping bottle for pure toluene for each three students.
1 oblong enamel pan for staining, 14 x 10 x 3″ for each three students, or 12 curved plastic plates.
1 pint Cargille or Crown oil (thinner grade).
Giemsa—either 30 cc stock solution of a proven high grade Giemsa or 4 x 5 gm bottles of a tested lot of Giemsa powder such as National Aniline NGe 18.
3 x 1 lb bottles best quality acetone-free pure methyl alcohol, $2.00 each.
2 lbs best quality pure glycerin.
½ lb solid glass beads, 5 or 6 mm in diameter.

Buffer Salts:

8 x ¼ lb bottles *anhydrous* Na_2HPO_4.
1 lb fine powder or crystals KH_2PO_4.
10 grams methylene blue, medicinal or best available.
1 2-liter bottle, polyethylene if possible, containing sulpho-chromic mixture (potassium dichromate and sulfuric acid).
1 7.5 cm enamel or glass funnel for pouring back sulpho-chromic mixture from rectangular staining dishes.
3 liters 90-95 per cent alcohol for wiping clean slides.
Enamel or plastic basins for detergent solution to receive used slides.
1-2 enamel or plastic buckets.

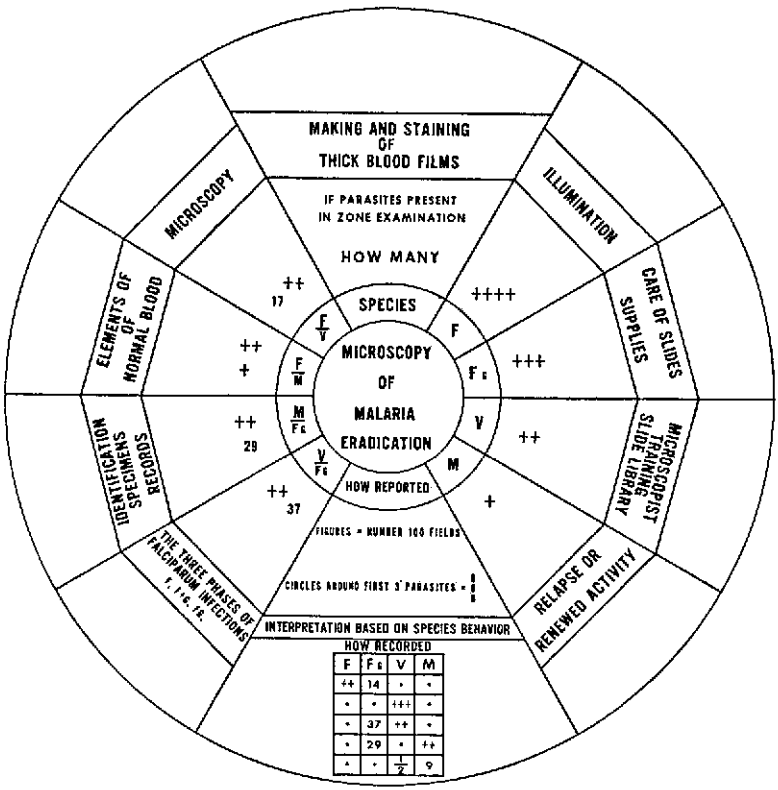
APPENDICES

The Ten "Deadly Sins" in Thick Film Microscopy

1. POOR RESOLUTION
2. INADEQUATE ILLUMINATION
3. FAILURE TO MOVE THE FINE ADJUSTMENT CONSTANTLY
4. PERMITTING ANY ADJUSTMENT OF THE ILLUMINATING SYSTEM WHICH ALLOWS RADIAL MOVEMENT OF THE LEUKOCYTES
5. FAILURE TO FAMILIARIZE ONE'S SELF WITH THE APPEARANCE OF LEUKOCYTES AND OTHER BLOOD ELEMENTS WHICH ARE FOUND IN EACH PREPARATION TO BE EXAMINED
6. WASTING TIME OBSERVING DOUBTFUL IMAGES OR POORLY STAINED FIELDS
7. USING CONCAVE MIRROR
8. USING SLIDES, FLASKS, OR BOTTLES WITHOUT LABEL OR IDENTIFICATION
9. FAILURE TO PROTECT ALCOHOLIC STAIN SOLUTIONS FROM ACQUIRING WATER IN ANY MANNER
10. FAILURE TO LEAVE THE 10X OBJECTIVE IN THE WORKING POSITION WHEN CARRYING OR STORING THE MICROSCOPE

Appendix 1

MICROSCOPY OF MALARIA ERADICATION



The above diagram contains the essential points described throughout this manual for the modern diagnosis of malaria, based wholly on a well-made, well-stained thick blood film.

In an eradication program it is mandatory to re-examine a portion of the total number of slides taken, *viz.*, all the positive slides and variable numbers of the negative slides. This involves the removal of oil from slides and the storing of all slides after the first examination.

Formerly, when a blood was declared "positive" the first ques-

tion asked was "What species is present?" Today that question is "How many parasites?" For if they are numerous the diagnosis of species is much more likely to be correct, whereas if the number is scanty the diagnosis may be uncertain and usually the re-examination is prolonged. More and more attention is being paid to scanty infections, for the discovery of a single case in a particular area, today, can be more important than was the finding of a dozen or a hundred cases 20 years ago.

The diagram also attempts to show, by the use of abbreviations, how all blood findings may be fully but simply recorded.

Appendix 2

THE BEHAVIOR OF P. FALCIPARUM GAMETOCYTES

CASE NO.	DAYS														WEEKS						
1	154 x	117 x	7	1	0	0	-	-	-	-	-	0	-	-	1	128	43	8	2	0	-
2	137 x	67 x	34	0	0	0	-	-	0	-	-	-	-	-	0	14	25	7	2	0	0
3	59 x	39 x	0	0	0	-	-	-	-	-	-	-	-	-	2	98	228	48	4	30	0
4	62 x	17 x	24	5	1	-	-	-	0	-	-	-	-	-	82	372	84	12	2	15	0

FIGURES = NUMBER OF GAMETOCYTES PER 100 THICK FILM FIELDS

Three to five weeks before the above observations were made in 1949, four small boys took unknown amounts of proguanil and quinine, probably only until their fever ceased. The first boy was found in a routine school survey and the others were later located in the same family.

Each of the children received 10 mg of iso-pentaquine on the first two days after the blood specimen was taken (indicated by the small x's seen in the first two columns).

On day 12, Case No. 1 complained of symptoms and there were scanty rings in his blood. Case No. 4 had + F. On day 13, they all received adequate amounts of amodiaquin as a single dose.

No further rings were seen in the subsequent 7 weekly examinations.

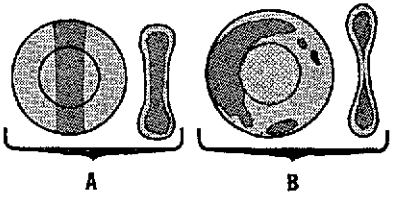
From this chart it may be deduced that:

1. Gametocytes rarely appear until the second week of asexual parasitemia;
2. They disappear spontaneously after 5-7 weeks; or
3. They disappear within 72 hours following the ingestion of primaquine-type drugs.

It would seem from the foregoing that, in areas of reduced transmission, it is more important to give adequate amounts of schizontocidal drugs on the first day of treatment, than a special drug against gametocytes later.

Appendix 3

DOUGHNUT-SHAPED RED BLOOD CELLS



A)-Normal red blood cell, face on and edgewise

B)-Enlarged younger red blood cell in which the central portion is exceedingly thin. The parasite is represented as the central portion of the cell in A) and occupying only the peripheral portion in B).

The distorted appearance of well-grown parasites of *P. vivax* and *P. malariae,* due to the type of cell in which they are contained warrants a word of explanation.

The above schematic diagram endeavors to show the size and shape, in both front and side views, of normal red blood cells (A). The destruction of many red blood cells by repeated schizogonies causes anemia which, as it becomes more marked, results in incompletely developed red blood cells reaching the circulation in increasing numbers. Not infrequently, red cells, larger in size than usual (B) show a marked constriction in their middle portion in contrast to normal cells. Instead of looking like thick biconcave discs, they resemble inflated inner tire-tubes or the cake-like article of U.S. diet known as the doughnut. Bluish cell-remains alone are also encountered in similar shapes.

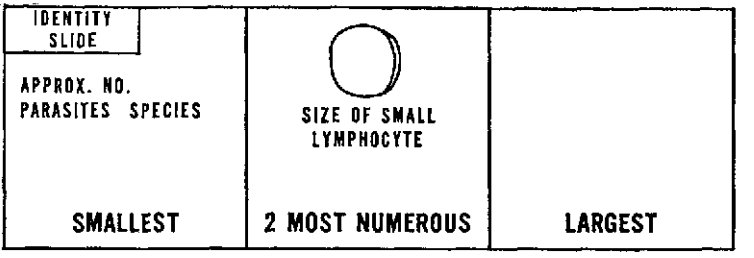
Evidently, the opposing cell membranes are so close together that the hemoglobin and other contents are forced toward the periphery. The resulting appearance, depending on the quantity of parasite material present, will look as represented by the shading in (B).

The student should understand that the distortion of the parasite by the shape of the cell containing it, does not signify any characteristic behavior of either *P. vivax* or *P. malariae.*

Appendix 4

DIAGRAMMATIC OUTLINES OF PARASITES OBSERVED

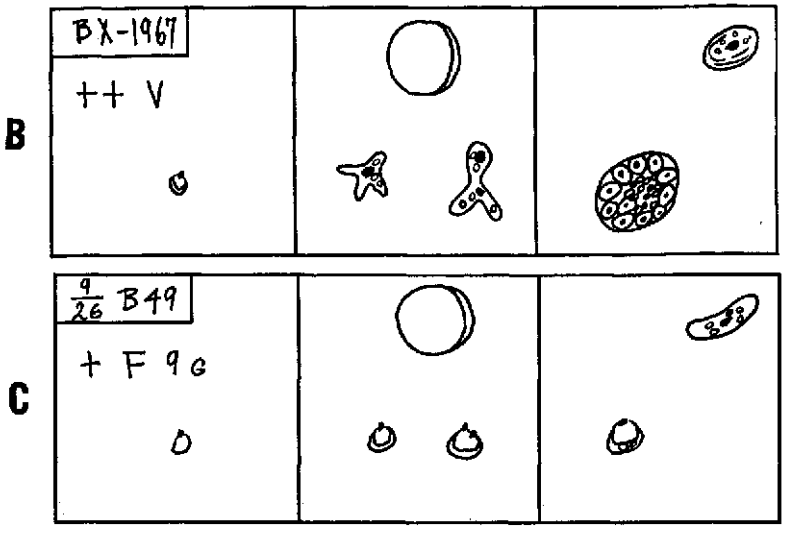
A



The above diagram is used to help the student learn the various developmental forms of the parasite which can be present in the same drop of blood. For use by the student, individual mimeographed forms similar to Diagram A are supplied and one is completed for each unknown blood examined until the student becomes proficient in representing the growth spread of each particular study slide examined.

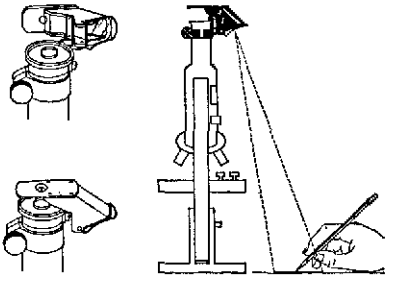
The mimeograph form, measuring 7 x 2" and divided in three equal parts, has a circle $\frac{5}{16}$" in diameter near the top of the middle square to represent the size of a small lymphocyte. The identification of the specimen that is being observed is written in the small rectangle at the top of the first square. When the examination is completed an approximation of the number of parasites present and the identification of the species diagnosed are placed just below the rectangle.

To complete the examination, the student moves slowly over the thick blood film until (depending on the number of parasites present) 30 to 300 parasites have been inspected with care. Then, after turning out the microscope lamp, he draws *from memory,* in a crudely schematic manner, four outlines to represent: (a) the smallest parasite encountered (drawn in the first square); (b) two examples of the stage which is the most numerous (in the second square); (c) the largest of the growing forms seen (in the third square). Only growing asexual forms are recorded. If it is desired to note the presence of mature gametocytes, a miniature drawing of one of these may be placed in the top right-hand corner of the third square.



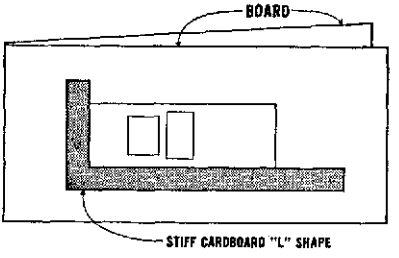
Artistic drawings of individual parasites are definitely NOT wanted. The important point is to make the drawings carefully in proportion to the size of the circle representing a small lymphocyte. After the size has been indicated, the position of the piece or pieces of chromatin and their shape are represented. Two or three small tiny circles may be used to signify the presence of pigment.

To enable the student to acquire quickly some concept of size in the microscope, he should be given the opportunity to see at least one blood specimen containing parasites with the camera lucida. When properly adjusted, this apparatus, by means of the prism, projects to the paper beside the microscope whatever objects are to be seen in the microscopic field. It is relatively simple to trace the outlines of a small lymphocyte and parasites nearby. Diagrams 6-8 (see pages 22, 24, 25) were made in this manner.



Appendix 5

TEMPLATE



Models, templates, or patterns—by whatever name they are known—are indispensable for teaching people how to make good, uniform thick blood films with a streak of blood in which the identification may be written with soft pencil.

An ordinary file folder is cut to a width of 6″ and re-cut in three to make three small folders each 3¾ x 6″. From another piece of stiff, firm, hard cardboard (such as a cardboard loose-leaf folder) an "L" is cut to measure 1¾″ on the vertical arm and 3½″ on the horizontal arm, with each arm measuring ⅜″ wide. The "L" is then pasted on one of the cardboard folders 1″ from the fold, as shown in the diagram, and is allowed to dry thoroughly.

A glass slide is then fitted into the "L" and the outlines of its top border and end are drawn with a fine line on the cardboard folder. At ¾″ from the left end of the slide a vertical line ⅝″ is drawn on the folder, equidistant from the top and bottom edges of the slide. Depending on the size of the thick film desired, a rectangle or square is then formed, as shown in the diagram. One eighth of an inch to the right, a larger rectangle is drawn, consistent in size for the number of identification letters or numbers to be used or large enough for the initials of the subject and the date.

With these two outlines clearly marked on the folder, the beginner lays a clean slide in the angle of the "L" and notes where on the slide the blood must touch to be to the left of the center of the first square or rectangle, which denotes the limits of the thick film. As far as he can, with the blood he has taken, he then quickly

spreads the blood on the slide within the limits of that drawing. The identification label is then written in the slide, in the space indicated by the rectangle on the right.

The outlines drawn on this template should be clean and sharp; this is not a drawing of what the finished slide should look like, but a device to ensure that uniform specimens are placed consistently in the same position on the slide.

Appendix 6

THE STAINING PHENOMENON OF SCHÜFFNER

In well-stained thin blood smears of *P. vivax* and *P. ovale,* the red blood cells containing parasites show reddish granules which have come to be known as Schüffner's dots, or stippling. In thick blood films which are equally well stained, a pinkish halo in the form of the containing red cell is frequently seen around the parasites. This is called Schüffner's staining, as definite dots are rarely distinguishable unless stained with Shute's saline technique.

No similar appearances are known to accompany the *P. malariae* parasites in the thick film.

A pinkish halo or shadow of the containing red cell sometimes is seen in bloods containing *P. falciparum* rings. These thick films are usually overstained or show some variation of staining or even fading.

In any case, the diagnosis is *always* based on the aggregation of developmental forms appearing simultaneously, not on the appearance of the stained red cell.

Appendix 7

SHUTE'S SALINE STAIN TECHNIQUE

Pampana (1938) was the first to show that Giemsa diluted with saline gave better results than Giemsa diluted with distilled water for thick films.

In 1955, Shute accidentally diluted some Giemsa stain with normal (0.85%) saline solution for the staining of thin smears and was surprised at the excellence of the staining of the parasite, as well as of the stippling, when present. He soon found that similar results were obtained with the thick film.

The dehemoglobinizing effect is apparently much more drastic than when the usual buffer solutions are used, and seems to give better results with slides that have been left unstained for a long period. The remains of young red blood cells are not so prominent; the field is unusually clear and parasites are more easily located. Staining 30 minutes or more brings out individual Schüffner's granules clearly and distinctly.

It would appear that anyone unfamiliar with this type of staining should immediately investigate the possibilities of using it under local conditions. Perhaps minor modifications could be found to reduce the staining time and the amount of Giemsa used. Obviously, the principle has great advantages.

The directions as given in Shute's book* are as follows:

The stain is prepared in the following proportions:

5-7 cc Giemsa stain to each 100 cc 0.85% NaCl [pH 7.0].
Stain in the inverted position, in blocks of 25-50; or in staining dishes—30-60 minutes.
Rinse with tap water, drain, and dry.
Use stain repeatedly as long as results warrant.

It is not practical to prepare dry proportions of salt and the buffer salts because of the tiny amount of the latter required. The following pH readings were obtained by adding 1% disodium monohydrogen phosphate to 500 cc normal saline:

0.85 cc—7.2
0.65 cc—7.1
0.4 cc—7.0

* *Laboratory Technique for the Study of Malaria.* Percy G. Shute and Marjorie E. Maryon. J. and A. Churchill Ltd., London, 2nd Ed. 1966.

Appendix 8

STAINING OF THIN BLOOD SMEARS

Thin blood smears are useful to demonstrate special features of malaria parasites and their containing red blood cells. Also, to illustrate unusual hematology, parasites in bone marrow, or skin scrapings, thin smears are made from the cut surface of the placenta and of the brain, spleen, or liver at autopsy. Either of the following two methods may be found suitable for the staining of these smears.

A. Fixation with alcohol and staining with Giemsa stain in buffer water.
B. Staining with Wright's or Leishman's stain diluted with suitably neutralized water.

The former is more common but the latter may give better hematological results.

It may be necessary to use a special buffer 3:5 to 5:5 proportions to obtain the staining of Schüffner's dots, in place of the usual 6:5 ratio.

The neutralized water is prepared by adding 20 to 40 drops of phenol red solution to 100-300 ml distilled water. Lithium carbonate (0.2%) is added drop by drop until a pink-violet color is obtained and remains after thorough shaking; the color should remain constant for about 20 minutes, and if it fades quickly more lithium carbonate should be added. The pH varies between 6.6 and 7.4 and that color which gives good results with the particular lot of Wright's stain is the one required, irrespective of its pH.

Method A

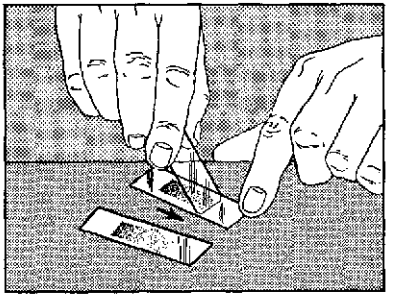
1. Fix thin smears with pure methyl alcohol.
2. Place slides across horizontal glass rods 2" apart.
3. Flood with fresh Giemsa stain prepared by using 1 drop Giemsa to each cc of 4:5 buffer water.
4. Stain 30-60 minutes.
5. Flood off stain with copious amounts of 4:5 buffer water.
6. Drain or blot.
7. Dry with gentle heat.

Method B

1. Place slides across horizontal glass rods.
2. Flood with undiluted Wright's or Leishman's stain.
3. Add 1-2 volumes of neutralized water.
4. Mix by gentle movement of rods. Do not blow.
5. Flood off stain after 20-30 minutes with copious amounts of the same diluent.
6. Drain or blot.
7. Dry with gentle heat.

Stained by either method, red blood cells are buff.

Where thin blood smears are indicated, Clay-Adams "margin-free" blood smear slides No. A-1463 are desirable as spreader slides (see diagram).



"MARGIN-FREE" SLIDE

Appendix 9

RESTAINING OF THICK BLOOD FILMS

Some thick blood films fade more quickly than others. Even in freshly stained preparations, the examiner is advised to look in the margin of the thick film for the best color contrast. Failure of the central part to stain may well be caused by the slower drying of that portion because it is thick or because the humidity is high. Absorption of CO_2 from the air continues as long as the blood is moist and may be a possible cause of the lack of red color in the center of many thick films. Other factors, such as the cleanliness of the slides, quality of the stain, suitability of the buffer water and, especially, the reaction of the mounting medium, must of course be considered.

Individual slides or groups of slides, and even whole sets of teaching slides, have become useless in three months or less. Evidently, only Shute can prepare thick films which sometimes last for decades.

Hitherto, restaining rarely has been satisfactory. Only when the preparations were excellently stained in the beginning are good results to be expected. Generally speaking, thick films which are most carefully protected from light are less likely to fade.

The principle of restaining is to use dilute stains, stain for a relatively long time, and use buffers containing less of the dibasic salt. It may be necessary to try buffer proportions of 3:5, 2:5, 1:5, and perhaps 0.1% potassium phosphate before a satisfactory result is obtained. Considerable success has been obtained with a good Leishman powder made up in the same way as Giemsa stain (see p. 46), diluted to 1 drop to 3 cc or 4 cc of the selected buffer and allowed to act as many 10-minute periods as necessary until no further improvement in the microscopic appearance can be noted. If the slides are placed face upwards across staining rods and flooded with the dilute stain, it will soon be apparent that those parts of the film which are more faded than others or are too red will decolorize the liquid stain lying directly over them, in the same pattern as on the thick film. Fresh stain should be added and staining continued until no further decolorization can be detected.

The fading of thin smears is seldom a problem, as it is well known that many retain their colors for years. Some method is needed, however, to restain those areas with special fields that have faded from hours of use as demonstrations.

Appendix 10

LIBRARY OF TEACHING SLIDES

While malaria infections are still fairly numerous, arrangements should be made to store sets of stained thick blood films. These can be used for the training of the present staff of microscopists and, with the aid of restaining, can continue to be used for the training of microscopists who will be required during the three-year surveillance period.

A good collection should consist of sets of 10-25 thick films, together with two thin smears of the same blood specimens taken at the same time. They should be carefully identified in a manner which does not reveal the species present, and should be promptly stained, packaged, and stored in the dark until needed. One week before they are to be used, clean cover-slips (No. 1) are applied with one of the newer resins dissolved in pure toluene. Euparal Vert delays fading, but it is much harder to remove when the slides are to be restained.

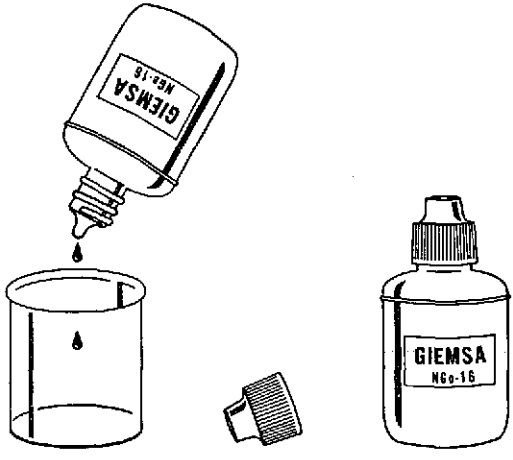
Sets of blood slides containing both numerous and scanty parasites should be made of each of the three phases of *P. falciparum* and of bloods taken at three different times in the cycle of *P. vivax* and of *P. malariae*. In instances where medicine has not already been given to the patient, it can often be postponed for 24 hours to allow a second drop of blood to be taken and placed alongside the first 24 hours later. One set of normal blood specimens and one taken after all parasites have disappeared, to demonstrate cell-remains, should be included.

Arrangements may be made with hospitals and active outpatient clinics, as well as with interested epidemiology inspectors and collaborators, to prepare sets of slides from patients in whom the immediate examination of the blood shows a pattern of infection not already included in the teaching collection. It is most advisable to supply packets of uniform, new, properly cleaned slides for this purpose.

Some results of current investigations on the fading of teaching slides suggest that thick blood films which have been well stained by Field's method may perhaps be used for many months before having to be discarded because of fading. A proportion, at least, of each lot of slides destined for class instruction should be stained by Field's method, in addition to those prepared with the usual methylene blue-Giemsa method.

Appendix 11

PLASTIC DROPPING BOTTLE



With the plastic dropping bottles now available (ranging from 30 cc to 150 cc capacity), the Giemsa stain is more completely protected from contamination with moisture than in any other container which requires a separate pipette for delivering the appropriate number of drops.

Appendix 12

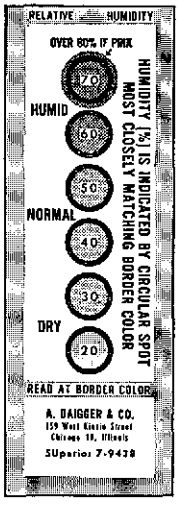
PENCILS USED FOR MARKING IN BLOOD

Although No. 1 or other soft graphite pencils are recommended for writing the label as soon as the blood is dry, a U.S. pencil, Dixon 2225 Film Mark, is excellent for writing in blood, as it has no loose graphite dust that can spread through the adjacent parts of the thick film.

Grease-pencils are to be avoided, since much of the grease mark may float off when the slides are immersed during the staining process. Moreover, if red or blue grease-pencils are used, some red or blue pigment may remain on the slide even after the oil is removed.

Appendix 13

HUMIGRAPH



The Humigraph is a card with six black circles containing material capable of changing color from blue to pink and vice versa. The border of the card is a neutral grayish color which changes as the humidity changes. The color of the rings is compared to the border color for the purpose of gauging humidity.

The humidity percentage is read from the figure in the circle whose color matches the border. Should one circle be more blue and the adjacent one more pink, the reading would be the lower figure plus 5.

The outside of the 70 circle can change to pink. When this occurs the humidity is 80% +, which will interfere with the prompt drying of the thick blood films.

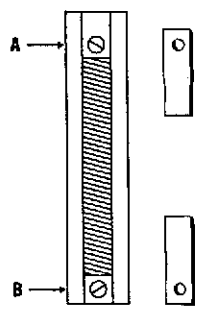
Appendix 14

HOW TO TIGHTEN COARSE ADJUSTMENT OF MICROSCOPE

When the coarse adjustment of a microscope begins to slip because of the weight of the binocular body, the oil immersion objective will not remain in focus when the hand is removed from the knob.

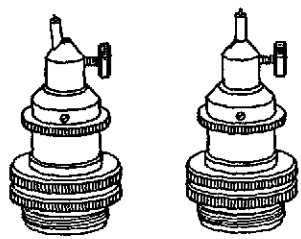
In some models of microscopes this factor can be adjusted immediately simply by turning the knurled handles of the coarse adjustment in opposite directions. In other models, there are two small screws in the stand, directly above the coarse adjustment, which can be tightened with a suitable small screwdriver until the tension is satisfactory.

When none of these adjusters are present, it will be necessary to remove the ratchet (see diagram) by loosening the screws at points A and B and to insert sufficient layers of tin or aluminum foil to control sliding.



Appendix 15

USE OF MICRO OBJECT MARKERS



A micro object marker, capable of making a circle at least as small as the oil immersion field, should be available to each microscopist or, as a minimum, one should be provided for each group of three working together. When properly used, the object markers can save enormous amounts of reviewing time. Doubtful objects on the slide may be circled for referral to the central laboratory, and that laboratory may circle objects to be sent back to the original examiner. The markers are used routinely in the case of scanty infections.

Once the search for parasites begins, the microscopist should keep a record of the number of fields examined. If the examiner does not find a parasite in the first 25 fields, he realizes that if infection is present it will be very scanty. It is not always easy to determine the species—every parasite located helps—when the parasites are few in number.

The number of the field in which the first parasite is found is recorded. The examiner, with eyes fixed on the parasite in the center of the field, first tests the firmness with which the slide fits in the mechanical stage. If it is not firm, the correction of the slide's position is made without losing sight of the object to be circled. Finally, when the slide is firmly set, the examiner uses the marker to circle the object—first with the tiny circle, and then with a much larger one to permit easy recognition.

If, for example, the first parasite was found in the 37th field, and two more are then found—one in the 61st and one in the 93rd field—the findings are recorded as 37, 61, 93 $\overset{o}{o}$. The three small o's, placed vertically after these numbers, indicate that three

parasites have been circled and the circles can be located with the 10x objective.

Once three bona fide parasites have been seen, the examiner should find enough parasites to make certain of the species, irrespective of the number of fields. If unable to make a diagnosis after 300 fields, because only seven parasites were found, he should be allowed to place an interrogation after the most probable diagnosis, e.g., F?—or merely write 7p/300 ♂♀ F, thereby giving the reviewer a definite idea of the frequency of the parasites. On one occasion, where a well-grown parasite was circled, the examination of 2,000 more fields failed to show another parasite.

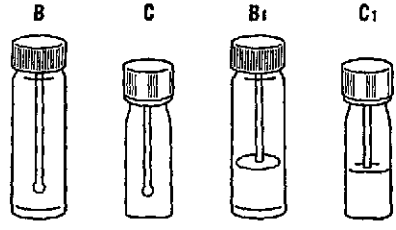
Appendix 16

IMMERSION OIL

A

The small drawing A represents a cross section of an oil immersion objective arranged to show that it has six different optical elements. The one which fills the pin-head sized aperture is the one that is most frequently and easily injured by external violence. It will be seen that this tiny lens is spherical and has a very small focal distance. The 90-100x magnification is so great that proper resolution cannot be obtained unless the upper surface of the slide bearing the specimen is connected to the objective by an adequate layer of oil which has the same index of refraction as glass.

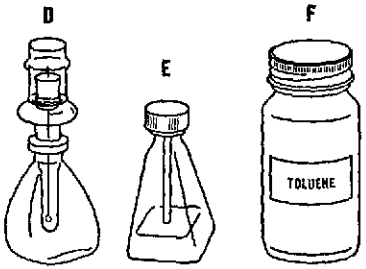
Drawings B and C show two types of working bottles for immersion oil, with bulbous tips on the glass applicators for easier application of the desired amount of oil. Since in these drawings the tips are surrounded by air, they can be seen clearly. Drawings B1 and C1 show how the tips disappear if immersed in an oil of suitable refractive index.



Since a number of modern synthetic immersion oils are available, the traditional cedarwood oil need never be used. That oil becomes tacky very quickly and dries like varnish, holding each particle of sand and grit which comes in contact with it. In that way it was responsible for much of the wear of the underside of mechanical stages. Because of the tackiness, it was also difficult to remove cedarwood oil from the slide after examination.

Drawing D illustrates an old type of oil-xylene combination bottle which is still used (the pyramidal bottle, shown in Drawing

E, is better). The narrow inner tube contained cedarwood oil, which was removed with a fine wire looped at the end; a drop or two of xylene was picked up on the end of the inner tube and touched to the objective to aid in removing the sticky oil. This bottle hinders the rapid handling of slides and is therefore replaced as a work bottle by types B and C, with a simple wide-mouthed screw cap, and type F, containing the more volatile toluene, into which the slide may be dipped after first removing the greater part of the oil by daubing with soft-type tissue or other absorbent paper.



There are a number of modern synthetic oils such as Crown, Mersol, some synthetic cedarwood oils, and Cargille or Shillaber's oil, which comes in two densities. Usually, the thicker oils slow down the work of examination. Anisole or methyl benzoate, a fat solvent, has been used because its refractive index is adequate and it is volatile. If it does not decolorize the thick films and there is no objection to its odor, it may be tried but is not recommended.

A satisfactory thin immersion oil may be prepared by mixing:

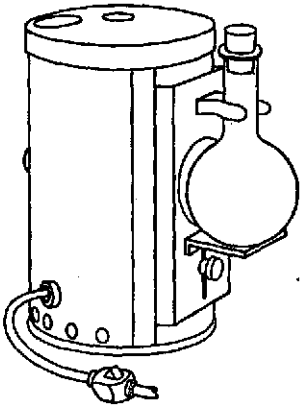
Liquid petrolatum USP or BP (heavy) or (Nujol)	82 parts
1-bromonaphthalene	18 parts

The 1-bromonaphthalene can be obtained from Matheson, Coleman and Bell, East Rutherford, New Jersey, USA.

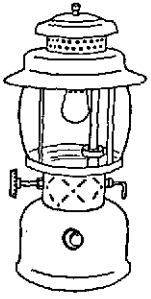
To see that no oil remains after the dip in toluene, a piece of lightly held Kleenex-type tissue is drawn across the thick film while it is still wet with toluene.

Appendix 17

TYPES OF MICROSCOPE LAMPS



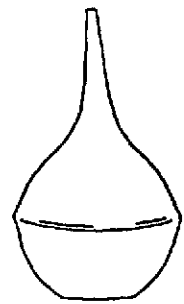
Microscope lamp described under A.H.T. catalogue No. 6958-E



Type of pressure lamp which can be used in localities lacking electricity, provided they are of *250 candle power or less.* The heat is intolerable with higher candle powers.

Appendix 18

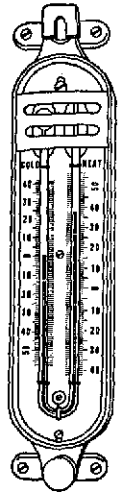
RUBBER EAR SYRINGE



This rubber ear syringe, of only 4-oz capacity, can produce a considerable blast of air. It is effective in removing lint, threads of paper, and dry dust which has settled on the surfaces of microscope lenses and prisms.

Appendix 19

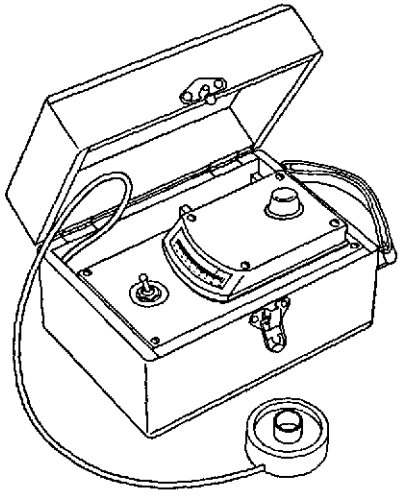
MAXIMUM-MINIMUM THERMOMETER



The reading of the maximum-minimum thermometer is self-evident. The indicators which show the highest point reached by the columns of mercury may be brought in contact with the mercury by means of the small magnet seen at the top.

Appendix 20

"PHOTOVOLT" PHOTOELECTRIC EXPOSUREMETER



Model M-200 of Photovolt Corporation, 95 Madison Ave., New York 16, N. Y., has a delicate photoelectric cell which, with the switch set at HI, registers fractions of a foot-candle on a scale on which 100 divisions equals 2 foot-candles. Readings may be recorded in foot-candles or, more simply, as the number of divisions the needle moves. It is advantageous to "zero" at 20 or 30, where the needle moves more freely than when set at 0. A special adapter to fit any eyepiece allows the light to be measured at the level of the eye. Such readings average one division lower than when the adapter is not used.

The amount of light in a binocular microscope set up by the average microscopist varies between 1.5 and 4 divisions. It is usually possible to improve the reading by 4 divisions through proper adjustment of all factors involved.

Invariably, students strive to obtain a higher numerical reading than their neighbor. In achieving that, they improve their own illumination.

Appendix 21

CONTINUING REVIEW OF MICROSCOPIC EQUIPMENT

Experience has shown that the only way to maintain a high degree of cleanliness of the microscopes of the entire malaria program of any country is to have continuous inspection of them every two to three months by a *competent* person.

This person should have, above all, an innate interest in both microscopy and the thick blood film. In addition, he should be equipped with the following:

A spare oil immersion objective in perfect condition. It is used for comparison with the oil immersion lens that is under inspection.

A selection of well-made and well-stained thick blood films to be used as test and demonstration slides.

Several pairs of eyepieces 5x, 6x, etc., to give a total magnification of 6-800x for the different makes of microscopes in use in that area.

1 hand lens (lupa) 5x, 2″ focal distance.
1 pear-shaped rubber ear syringe, 4-oz capacity.
1 tube vaseline.
2 sheets emery paper No. 00 or 000.
1 set of screwdrivers, different sizes.
1 set of tiny screwdrivers—dental or watchmaker.
1 small pair regular pliers; possibly a pair of needle-point pliers.
1 oz very fine absorbent cotton.
24 wood applicators.

1 "Photovolt" lightmeter (useful but not mandatory).

Appendix 22

DEFINITIONS

Trophozoites	All undivided parasites. The youngest trophozoites are called "rings."
Schizonts	All *adult* asexual forms with two or more divisions of nucleus.
Mature schizonts	Schizonts in which merozoites are completely formed.
Merozoites	Result of segmentation of either a liver schizont or an erythrocytic schizont. They may be separated from or contained in the original schizont.
Erythrocytic	Any stage of the parasite within red blood cells.
Exo-erythrocytic	Liver stages in man. Pre-erythrocytic when derived from sporozoites (the only stage formed in *P. falciparum* infections); exo-erythrocytic if derived from other liver stages.
Gametocytes	Sexual forms developing and reaching maturity while still in the same red blood cell. *P. falciparum* gametocytes are usually called "crescents."
Sporozoites	Forms infective for man resulting from the ultimate division of the oocyst in the mosquito.
Oocyst	Develops from the fertilized gamete between the lining cells of the mosquito's stomach.
Hematin	The *pigment* found in all but the earliest forms of the parasite; it may also be called hemazoin.
Chromatin	The red staining nuclear material of the parasite.
Cytoplasm	The bluish staining protoplasm of the parasite.

DEFINITIONS (*cont.*)

Pre-patent period — The time which elapses between the infective mosquito bite and the appearance of detectable parasites in the thick blood film.

Incubation period — The time which elapses between the infective mosquito bite and the appearance of symptoms. It includes the duration of the pre-erythrocytic stage plus the number of days required for the parasites to cause symptoms.

Paroxysm — The bodily manifestations which follow the schizogony of a sufficient number of parasites of any species. The commonest of these are chills, fever, and sweating. A paroxysm may last from ½ hour to 18 hours.

Attack — Can include one or several paroxysms and can terminate spontaneously or after the administration of medicine; it may last from one to many days.

Anemia — The loss of red blood cells from any cause. In malaria, it results from the rupture of mature schizonts at the time of each schizogony. An increase in the number of bluish staining erythrocytic elements in the thick blood film was first noted by Barber as an indication of anemia.