MANUAL OF THE MICROSCOPIC DIAGNOSIS OF MALARIA

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.

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.

In addition to the laboratory techniques, the Manual sets forth a minimum of basic information on the life cycles of plasmodia.

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

MICROSCOPES

Three kinds of microscopes are used in malaria eradication programs:

(1) The compound microscope;

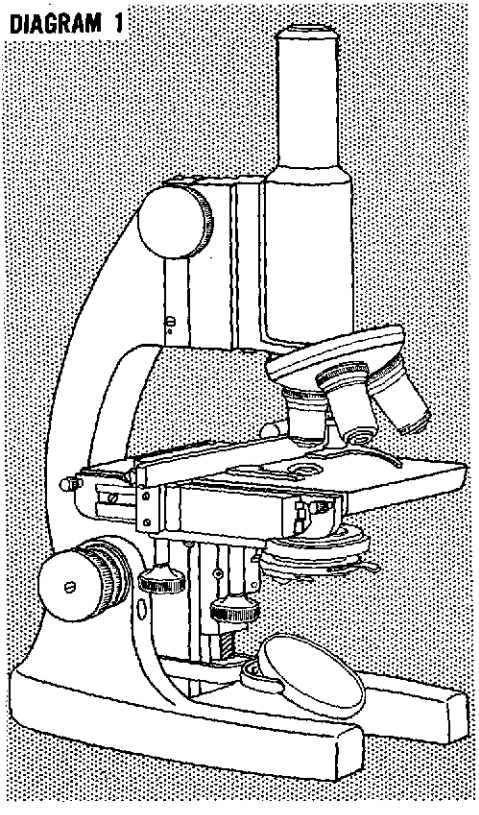
(2) The stereoscopic microscope;

(3) The hand lens or lupa with magnification of 2x to 10x.

The lupa, which is primarily considered a tool of entomology, is of great usefulness in the examination of 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 may complement the hand lens for this purpose and is indispensable for the examination of objectives whose resolution is impaired.

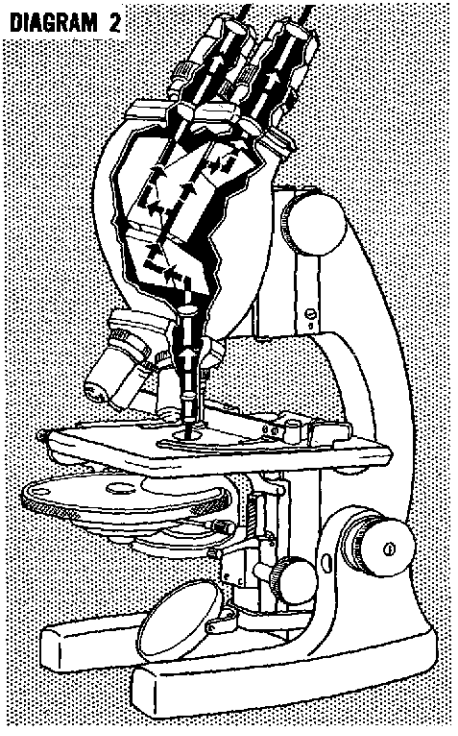
Compound microscopes are supplied with either monocular or binocular bodies. Some makers also supply a monocular tube with the latter.

Diagram 1 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. 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 2) permits the observer to use the microscope for prolonged periods with much less fatigue than with a monocular. It will be seen from the same diagram that a considerable amount of additional optical equipment is required in order to obtain the same image in both eyes. It may be noted that not only is the

DIAGRAM 2



total light reaching the slide via mirror and condenser divided in two, but each lens or prism in the binocular system reduces the amount reaching each eye. It should be clearly understood, then, that the amount of light required to give adequate results in the monocular microscope may be hopelessly inadequate for the binocular.

Again, where simple focusing of the fine adjustment is sufficient to obtain maximum resolution in the monocular eyepiece, 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. The use of the binocular microscope is complicated by the extra weight of the binocular body, which has a tendency to slip down and not remain in focus after some time. The manner of tightening the adjustment should be learned early and a constant tension should be maintained.

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. One European importation without increased magnification in the binocular body is marked 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 secured. The platform should have upon it, or incorporated in it, some type of slide holder (mechanical stage) which can be mechanically and accurately moved 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 is in definite relation to the inferior surface of the slide carrying the specimen. 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 for operations in which the condenser is removed.

The function of any microscope is to enlarge the image to a size that will permit the observer to see considerable detail with maximum clarity.

The total magnification obtained in the compound microscope using the oil immersion objective (usually 90x to 100x) may vary from 450x to 1,500x depending on the eyepieces or oculars available. Experience has shown that increased magnification of 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, staining, as well as the distribution of leukocytes. Little use has been found in malaria work for the 43x objective and it may be eliminated. Its place in the revolving nosepiece may be taken by some type of object marker for circling interesting microscopic objects for further examination. The total magnification may be estimated by combining the magnifications of optical elements involved—namely, ocular, body (if any), and objective. Technicians in training will appreciate more detail with an *optimum 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 conveniently used with body magnification over 1x. Other ocular magnifications are 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 a custom to treat all air-

exposed surfaces of lenses and prisms with a "coating" * which compensates for the unequal wave length of the colors of the spectrum. The result is a substantial reduction in dissemination of the light so that lenses and prisms so treated may function adequately with considerably less light than those uncoated. When viewed with incidental light, coated surfaces may be distinguished by the appearance of a bluish-violet luster. This treatment is becoming increasingly frequent with many modern microscopes (see 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 that the temperature will not exceed 35° C. This simple procedure will prevent the development of fungal mycelium on the surface of lenses and prisms.

It sometimes happens that 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 it may be good at first, suddenly becomes impaired. When the warmth of the examiner's face has warmed 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 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 in the sweat is transferred to the upper surface of the ocular. Frequent face washings with soap and water will reduce this minor inconvenience; the constant use of small pieces of Kleenex will control this considerably.

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. The oculars convert the rays of light into an image recognizable by the eye. Such light is called "trans-

* Anti-reflexion film.

mitted" light, whereas the light 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 that can be demonstrated with any 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 it is desired 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 the confused reflexion of different wave lengths to a minimum and permits a smaller light source to work as well as when lenses are not so coated. A bluish-violet luster seen by incident light on the surface of a lens distinguishes a coated surface from an ordinary one. Some manufacturers indicate that this treatment has been applied by placing a small pink or violet dot on the metal holder of the part.

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 thick blood film. Usually, tiny bacteria or other organisms are stained one color and easily distinguished in a background stained in a contrasting color. For malaria it is most desirable that the background be as clear and white

as possible so as to show up tiny objects .5 to 2 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 and 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. It is essential to arrange the microscope, lamp, and filters in such a way as to obtain the maximum amount of light possible, after which, through use of the iris diaphragm of the condenser, the light may be reduced to suit the individual worker.

At one time, only daylight was thought to be 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 can provide a constant source of uniform light. However, electricity does add an additional factor; electric light is frequently quite yellow.

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

Therefore, illuminators which depend on special types of light bulbs should not even be considered. Only those which can be used with bulbs obtainable in the local market should be adopted. Existing lamps with special sockets should be converted whenever possible. "Built-in" illuminators should not be purchased. Rather, the usual mirrors should be ordered.

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 beneath 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 imperative 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. However, with proper ventilation, the heat from bulbs of 100 watts or less can be tolerated. 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.

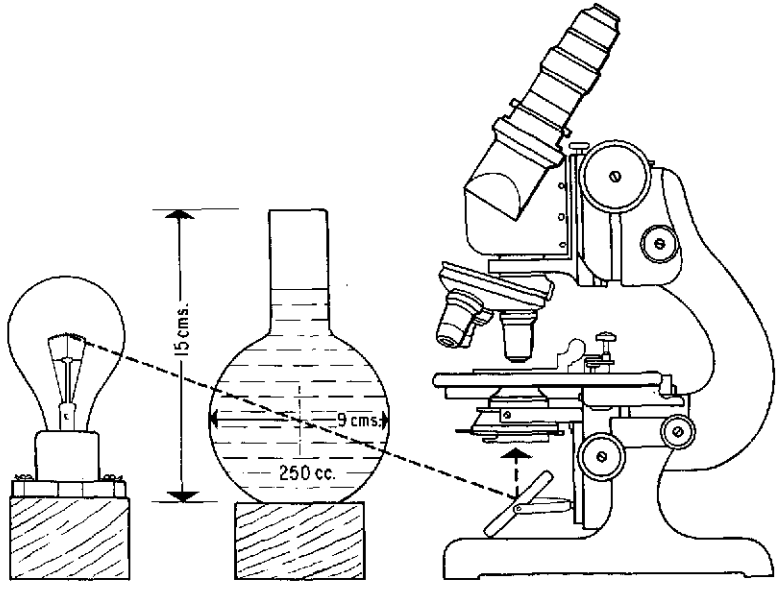
An example of good, adequate illumination *for a monocular microscope* is the ordinary blue-white 60-watt "daylight" bulb. This bulb, in a plain porcelain base and suitably screened on three sides, gives an abundant amount of blue-white light. No filters are required.

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 which uses a series of filters usually has a special transparent bulb as a light source and should not be considered. The model A.H.T. Cat. No. 6598 ($33.00) is an old German design for dark-field illumination. The 200-watt transparent bulb intended for dark

ground illumination is not necessary; nor are the ground-glass filter and its holder. If supplied, the latter should be removed. 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 .6 per cent—1 cc.) is added to the water in the flask to give the basic blue-white background. The water is changed as required.

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 glare and hold either the 60-watt or 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 3

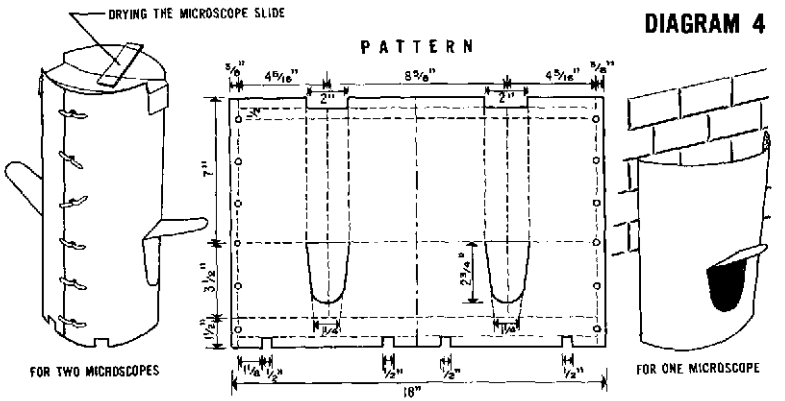


The above installations may readily be improvised by using a strong dark cardboard (insulating paper), a plain porcelain base or socket, and two meters of wire. As can be seen from Diagram 3 the relation of the brightest part of the bulb, the center of the spherical flask which acts as a lens, the mirror, and the relative heights of each in rela-

tion 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 convincing the student that his efforts to obtain maximum light can be improved upon. 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 Photovolt Corporation's light meter, model No. 200-M. A delicate photo-electric cell registers foot-candles on a scale of 100 divisions. When the switch is turned to "HI" the complete scale represents two foot-candles of 0.02 foot-candles per division; in "LO" the sensitivity is reduced 10:1. A special adapter to fit any ocular measures the light at the level of the pupil of the eye. With the switch set on "HI" the needle is "zeroed" at some point, which enables it to swing freely, such as 20 or 30. Results are stated in number of divisions covered or in decimals of a foot-candle. The average light reading generally used with a binocular microscope is from 1.5 to 4 divisions. This can frequently be improved from 8 to 12 divisions more by proper adjustment of all factors involved.

The same principle as seen in Diagram 3 can be applied to a homemade lamp, as represented on the left of Diagram 4. Diagram 4 is a scale drawing of the lamp shade which



is held together by brass paper clips. Two openings are used when two operators are seated opposite one another. When persons work against a wall, the entire apparatus is not always necessary; at the right (Diagram 4) a mask-like modification is shown.

One-hundred-watt pearl 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.

Degrees of color blindness impair the work of microscopists. Where possible, it is advisable to expose prospective candidates to one of the color vision tests.

PARASITOLOGY OF MALARIA DIAGNOSIS

Parasitology is that branch of science which deals with parasites of all kinds. Those which consist of a single cell are called protozoa, while those of more than one up to millions of cells are termed metazoa.

The position of the malaria parasites in the general scheme of protozoa can be seen in the wall chart, which is merely explanatory and not intended to be memorized.

The unicellular parasites or protozoa include not only the plasmodia of malaria of man, but also amebas, such as the one which causes dysentery *(Endamoeba histolytica)*; the trypanosomes, causing Chagas' disease and sleeping sickness; the leishmania, causing both skin and general body disease, such as chiclero's disease and kala-azar. In addition to the disease-producing ones, many parasites are quite harmless and special training is needed to distinguish them. Fortunately, the malaria parasites have no harmless counterparts to complicate their identification.

The ameba will serve as an example of one-celled, or unicellular, organisms of the large group or phylum called protozoa and a short description of it may help the student who wishes to read further on the subject.

The ameba consists of a mass of jelly-like substance called protoplasm, the peripheral or outer portion of which becomes more dense to form a limiting membrane. Lying in the protoplasm is a denser circular structure called a

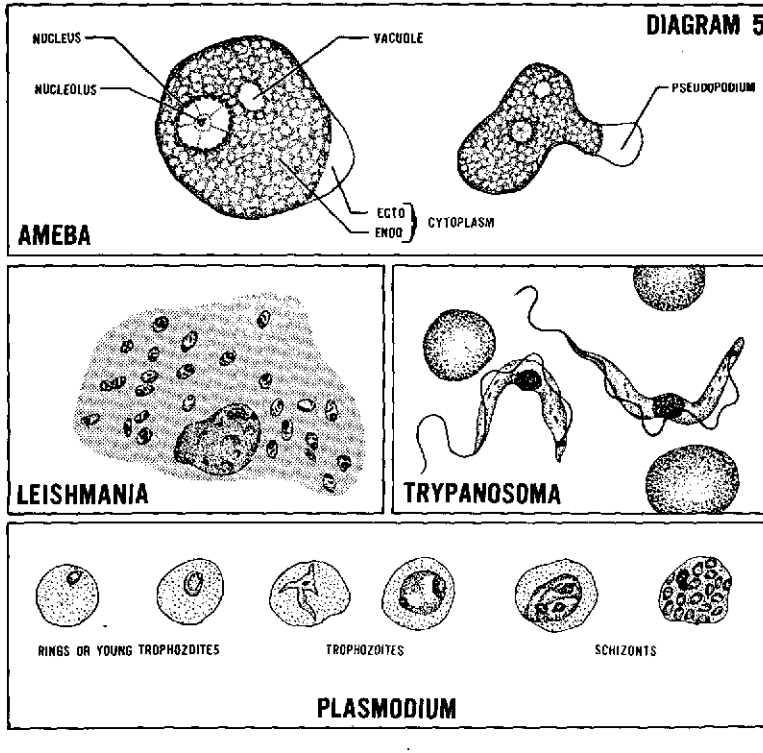
nucleus. The nucleus may show a central dense spot, or nucleolus.

The protoplasm around the nucleus is called cytoplasm; the granular portion is the endoplasm and the clear transparent part the exoplasm. There can be seen in the cytoplasm a clear space, called a vacuole, in which food particles engulfed by the cytoplasm are digested.

Amebas reproduce by binary fission; the nucleus divides in two and some of the cytoplasm accompanies each piece of nucleus.

The ameba moves and surrounds its food by putting out bulges in the membrane into which the cytoplasm flows. These protrusions are called pseudopodia. Malaria parasites are capable of moving in the same way. The nuclear material is called chromatin. When mature, their nuclei divide into many pieces (8-30), a process which is termed schizogony.

A diagram of amebas and other parasites follows.



BLOOD IN RELATION TO THE DIAGNOSIS OF MALARIA

Blood is the medium in which malaria parasites are found. If they 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. In one instance in Ghana, for example, women in a prenatal clinic were carefully followed by means of regular thick-blood-film examinations of 100 fields each. Falciparum gametocytes 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 more cases.

The only certain way to confirm that a person has no parasites in his blood is to subinoculate into a human volunteer 2-400 cc. of blood. If no parasitemia develops the blood was negative.

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, for a considerable period of time. Changes in the usual appearance of parasites may be produced by alterations in the red blood cells themselves, quite independent of the species of malaria infection.

Despite the appearance of the parasites in the *dehemoglobinized* thick blood film, malaria parasites are incapable of an independent existence. Except for the briefest periods when they are moving from one cell to another they are *intracellular,* for the most part in red blood cells. Since the blood is the vehicle which brings the parasite to our field of vision, it is highly desirable to know something about the blood itself.

Blood consists of a liquid called plasma, wherein are suspended the white blood cells (leukocytes), red blood cells (erythrocytes), and blood platelets. 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, con-

tracts to form a clot, clear yellow *serum* separates out after standing. A drop of blood on a slide, unless spread promptly, will clot in exactly the same fashion as in the tube but with considerable change in the plasma, and 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 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.

Cellular elements (erythrocytes, leukocytes, blood platelets) are developed in the bone marrow and released to the peripheral circulation as required.

Blood platelets are fragments of the cytoplasm of a bone marrow cell called a *megakaryocyte.* They may be irregular in size, shape, and density but all are variations of the same color in a given preparation. In defibrinated blood or blood that dries slowly, their appearance may vary enormously.

Erythrocytes are also derived from a type of bone marrow cell and the early stages contain a nucleus. Just before they are released into the circulation, the nucleus is lost and the young red blood cells of varying size may contain blue-staining elements (seen only with critical staining in the thin smear), variously described as reticulum (of reticulocytes), polychromasia, and punctuate basophilia, all of which disappear in 1-3 days.

A drop of *fresh blood* placed on a slide, covered with a coverslip, and examined with light greatly reduced by closure of the iris diaphragm, may be examined under both low-power objective (10x) and the high-power (43x). The oil immersion objective may also be used if the cells are not moved by the focusing action. The erythrocytes are biconcave discs which appear singly or in groups or rolls. When slight pressure is applied to the coverslip,

they are seen to be capable of great distortion 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.

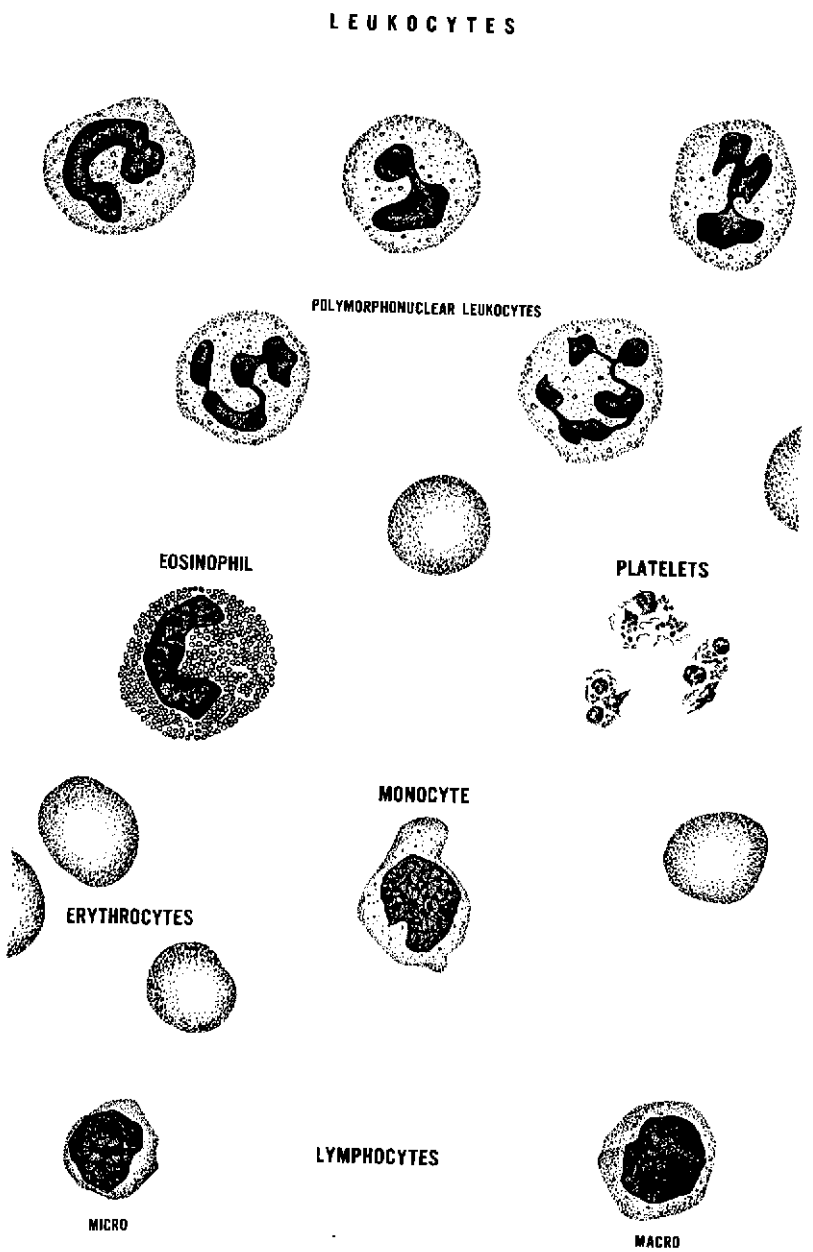
Under changing conditions of their surroundings, the red blood cells may alter their appearance greatly. In serum or 0.85 per cent solution of sodium chloride the smooth, round outline is preserved. If the amount of salt is decreased to 0.5 per cent, the cell shows little protuberances of its outer surface resembling a submarine contact mine. Such cells are termed "crenated" and may even retain this appearance after staining. Solutions which keep the red cells in their normal state are called normal solutions. Too much or too little salt content causes the cells to rupture and release their hemoglobin, and the water becomes and remains red.

Exposure to strong alcohols, 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. Ability to recognize the more common white blood cells when stained permits accurate description of the microscopic field of a blood preparation. In fresh blood preparations, the leukocytes are transparent, highly refractile, and may show movement of their cytoplasm. Their nuclei acquire a rich blue-violet color when stained. They vary in size and shape, and the cytoplasm may be clear or granular according to the type of cell. The life of a leukocyte is short, a few 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 6 gives a brief description of the common leukocytes.

DIAGRAM 6


LEUKOCYTES
POLYMORPHONUCLEAR LEUKOCYTES
EOSINOPHIL
PLATELETS
MONOCYTE
ERYTHROCYTES
LYMPHOCYTES
MICRO
MACRO

DEFINITIONS

Terms Relating to Forms of the Parasite in Man

Trophozoites —early undivided asexual parasites; the youngest trophozoites of each species are commonly called "rings."

Schizonts —all *adult* asexual forms with two or more divisions of the nucleus.

Mature schizonts —fully developed schizonts in which merozoites are completely formed.

Merozoites —product of segmentation of either a liver schizont or an erythrocytic schizont. They may be separated from, or contained in, the original schizont.

Erythrocytic —all stages of the parasite within red blood cells.

Exo-erythrocytic —liver stages in man. Pre-erythrocytic when derived from sporozoites; exo-erythrocytic when derived from other liver stages—the latter do not occur in falciparum infections.

Gametocytes —sexual forms developing and reaching maturity in the same host red blood cell. Gametocytes of *P. falciparum* are commonly called "crescents."

Sporozoites —infective forms 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 or Hemazoin—the name given to the *pigment* found in growing and fully developed parasites.

Chromatin —the red staining nuclear material of the parasite.

Cytoplasm —bluish staining protoplasm of the parasite.

Secondary terms:

Schüffner's dots Maurer's spots or clefts Ziemann's dots or stippling	Refer to appearances of the uninfected portion of the containing red blood cell of vivax, falciparum, and malariae infections, respectively.

They are visible only in thin blood smears in which the staining is of a superior quality.

THE MALARIA INFECTION IN THE MOSQUITO

The following description may be omitted if the sound movie edited by Shortt is shown. This movie is entitled "The Life Cycle of the Malaria Parasite" and is distributed by the Imperial Chemical Industries. There are both English and Spanish editions. A short black-and-white film produced by the Communicable Disease Center, U. S. Public Health Service, Atlanta, Georgia, depicts in cinemicrophotography the actual biting by the mosquito, and this should be shown, whenever available, in order to demonstrate that the injection of the sporozoites is essentially intravascular rather than as shown in the movie mentioned above.

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 malaria is well established, the blood will contain sexual forms of the parasite as well as the asexual forms which are responsible for the symptoms.

The latter forms, on reaching the mosquito's stomach, quickly die and are digested, but the former, the gametocytes, escape from their containing red blood cell. The smaller male gametocyte soon develops spermatozoa-like motile elements which detach themselves from its nucleus and actively move away in the surrounding liquid. The larger female gametocyte undergoes some maturation process which enables the motile microgamete to enter and move actively within the cytoplasm of the macrogamete.

Within a few hours an actively motile ookinete has de-

veloped and has moved between the cells lining the mosquito's stomach to come 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. A spherical oocyst becomes larger and larger, projecting farther into the cavity around the intestinal tract of the mosquito. After two to three weeks the oocyst, distended with 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. They come to rest in the cells of the salivary glands and await their destiny.

The brief motion-picture film of the mosquito in biting action shows that the proboscis probes repeatedly until it reaches the lumen of a capillary or tiny venule. Saliva is expressed during the biting and sucking operations, and the contained sporozoites are injected intravascularly as if by a needle in a vein. By subinoculation they can be shown to circulate up to 10 minutes, but rarely up to 30 minutes. Since the entire amount of blood passes through the liver every three minutes, it is relatively simple for numbers of them to enter liver cells immediately adjacent to blood-filled liver sinusoids. No doubt many are lost to phagocytes, but those which are successful begin their liver multiplication immediately and this lasts either 6, 8, or 9 full days. The growing form in the cytoplasm of a liver cell expands and its nucleus divides repeatedly until the large cyst-like, irregular-shaped mature schizont ruptures. Numbers of the liver merozoites find their way between neighboring liver cells to the nearest sinusoid that contains red blood cells. The pre-erythrocytic stage is over and the disease-producing erythrocytic stage begins. At the end of each 48-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.

TECHNIQUE OF MICROSCOPIC EXAMINATION

From the microscopic demonstrations it should be seen that considerably more light is required for the thick blood film than for the thin smear.

In order to avoid waste of valuable time due to inattention to seemingly insignificant details, the following routine procedure is suggested in the examination of each new blood specimen.

> Place a drop of immersion oil* near the edge of the thick blood film. Place the slide carefully in position between the jaws of the mechanical stage so that it is held firmly in place, as it is frequently necessary to re-examine doubtful or suspicious objects. Much time can be wasted relocating such objects lost from view because the slide was loosely placed on the stage.

When an area containing well-preserved leukocytes is encountered, one or more of the best of these should be moved to the center of the field. While these are being observed, the fine adjustment should be moved widely through a distance of 40 to 60 microns. If the leukocytes appear to move radially in any direction, the plane mirror must be adjusted until these cells, with such exaggerated movement of the fine adjustment, appear to hover over the same spot with no trace of radial movements. The same test maneuver may also demonstrate that the illumination under correct conditions is less yellow than before. If it is not, the condenser should be checked to see if it is at its highest point; if still yellow, the light source and filters should be checked to see if they are in the proper positions and of the proper color. Should the light be too blue, basic conditions should be altered in some way or other.

The colors of the nuclei of the leukocytes are now appraised. They should be a rich, blue-violet color; the cytoplasm of the lymphocytes should appear pale blue and the cytoplasm of the polymorphonuclear neutrophiles should show irregular-sized granules, varying in color from blue to red. The granules of the cytoplasm of the eosinophiles,

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* Cedarwood oil should never be used. There are several modern synthetics of the same refractive index, such as Cargille's (Shillaber), Crown, of thick and thin densities. A very satisfactory thin immersion oil may be made from heavy liquid petrolatum U.S.P. (Nujol), 82 parts, and Alpha-bromonaphthalene, 18 parts (obtainable from Distillation Products Industries, 775 Ridge Road West, Rochester 3, New York).

regular in size and shape, should be a rich copper-red hue rather than the usual bright pink of eosin-stained tissues. *Blood platelets* should be found in *groups* and in *twos and threes,* and their color in the particular preparation noted. This may vary from light red through violet to slightly blue. In certain bloods the clear area between the leukocytes may show small bluish areas that are either hazy or composed of dense blue dots. These are the remains of the younger red blood cells as they appear following simultaneous staining and dehemoglobinization.

Careful attention to this form of appraisal will save much time. Only when these colors are encountered with the normal blood elements can it be expected that any parasites present will reveal themselves in their appropriate colors. Thus, if the nucleus is excessively red in a particular area of a specimen, it is unlikely that any blue may be detected in the cytoplasm of parasites. On the other hand, if the leukocytes are too blue, with little violet in their nuclei, it is unlikely that the chromatin of the parasite will show much red color. Under these last two conditions, it is well to move widely over the thick film in search of an area where the leukocytes are more suitably stained. The latter may be found more rapidly by spreading oil over the whole area of stained blood and scanning same with the low-power (10x) objective.

During these operations it will be noted whether the various cells inspected for color are seen as good, clear images, that is, if the resolution of the outline of an intact polymorphonuclear is seen clearly and with good detail. Of course, if the images are obviously unclear, the cause will have to be determined and corrected before continuing.

We are now ready to examine the specimen. Since few microscopic fields with the oil immersion objective are entirely flat, it is very costly in terms of time to try to examine in detail every object in the illuminated area. Any object which requires careful examination should be moved to the center of the field, where the resolution obtained is the best. Therefore, the microscopic field *for searching purposes* may be limited to that central portion of the field that is in reasonably clear focus simultaneously with the central point. This may well include no more than two thirds of the actual illuminated area.

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 variation of individuals, the examination of a single specimen should be by a specific number of fields rather than by a certain number of minutes. Supervised workers have recorded from 39 to 215 fields examined during the same observation period. A thick blood film of approximately 3 to 4 cm^2 may contain 500 to 800 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 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.

If the observer has been examining slides with similar preparations during the same day, the recognition of what is chromatin will be automatic, but if the microscope has not been used for some days it is 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, but rather 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. 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.

If it is desired to investigate any *unusual* parasitic appearances in the thick blood film, a thin smear may be examined with 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 vivax and quartan. Attempts to diagnose species in the thin smear on less than 10 parasites can be unwise.

As will be discussed later, the diagnosis for any blood

specimen may be expressed by some combination of the following symbols:

(1) *P. falciparum*—rings only	(F)
(2) *P. falciparum*—rings and gametocytes	(F + g)
(3) *P. falciparum*—gametocytes alone	(Fg)
(4) *P. vivax*—all stages at once or at some time during the 48-hour cycle	(V)
(5) *P. malariae*—all stages at once or at some time during the 72-hour cycle	(M)
(6) *P. ovale*—all stages at once or at some time during the 48-hour cycle	(O)[1]

If two species are present, some type of numerical approximation should show which is dominant, e.g., 75V, 7Fg/100 fields; 75V means that vivax is the dominant infection, while falciparum is merely a secondary infection.

The use of the word negative is relative, but it usually indicates that no parasites were encountered in the examination of 100 fields or some other specified examination.

Steps for the Correct Microscopic Examination of a Thick Blood Film

1. Make certain that the seat is of an appropriate height.
2. Remove all traces of immersion oil, dust, and finger marks, etc., from the entire microscope, using for this purpose a soft cloth or fine Kleenex-type tissue. Ensure that the mechanical stage moves freely in both directions.
3. Check to see that the condenser has been located in its correct position, that is, as high as possible; that the iris diaphragm is completely open; and that the plane mirror is adjusted in such a way as to reflect the maximum amount of light.
4. Clean perfectly the upper glass surfaces of the condenser and the oculars.
5. Ensure that the height of the bulb in the lamp is optimal in relation to the blue flask and that the height

[1] Not applicable to the Western Hemisphere. In countries where **P. ovale** is present, the word "nil" should be used for negative.

and proximity of each provide the maximum amount of light on the mirror. Check daily to see if the solution in the flask shows precipitate or loss of color. If it is desired to observe the slide with a 10x objective it will be necessary to close the iris diaphragm somewhat.

6. Locate an area in the thick blood film which shows the best staining with the 10x objective. Areas which are too red will fail to demonstrate blue color and areas which are too blue will not show any red color. Place a drop of immersion oil over this region and, looking from the left side of the microscope, carefully lower the oil immersion objective into the oil until it almost touches the slide.
7. Following step 6, bring the preparation into view by moving upward with the coarse adjustment.
8. Use the left hand for constant focusing of the fine adjustment and the right hand for changing the fields with the knobs of the mechanical stage. Note the color of the microscope field; if it is white, blue-white, yellow, or blue, move the fine adjustment rapidly, with the eyes applied to both oculars, through a wide movement of at least twenty divisions. If the leukocytes appear to move radially in any direction, it is imperative that the adjustment of the mirror or slight changes in the position of the lamp be made until these cells come in and out of focus without any apparent displacement. It is customary to examine a given preparation for a minimum of 100 microscopic fields.
9. At the end of the microscopic examination, take off the slide and remove the immersion oil both from the slide and from the oil immersion objective with Kleenex.
10. Move mechanical stage to center position.
11. Be sure to leave the 10x objective down and lower the body until checked by the automatic stop. *This is a must,* as the lenses of the oil immersion objective can easily be damaged if left down when not in use. Cover the instrument with its dust cover and return it to its numbered position in the warm cabinet.

BEHAVIOR OF THE INFECTIONS WITH P. FALCIPARUM and P. VIVAX IN THE LIVER OF MAN [1]

Because exo-erythrocytic stages of plasmodia were first discovered in reticuloendothelium of birds, it was presumed for a long time that these stages would be found in the reticuloendothelium of man. This has not been the case.

Several years before the discovery of the liver stages, schematically pictured in the movie prepared by Shortt, experiments carried out in Australia during World War II to determine the exact effects of the well-known drug atabrine, dramatically demonstrated everything about exo-erythrocytic stages of falciparum and vivax in man. So complete and exhaustive were these experiments that only the location of these 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 asexual forms in the peripheral circulation. This was the most important feature of the tests, since only 10 to 50 cc. had been inoculated previously into a very small number of subjects.

As briefly as possible, these experiments may be condensed as follows:

Falciparum. Mosquitoes with demonstrable sporozoites of falciparum were allowed to feed on the right arm of one volunteer while 300 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 after the termination of the biting constantly failed to infect a second recipient. The blood withdrawn and injected daily for 5 days did not show any sign of disease in the recipient. In fact, not until after 140 hours had passed was any subinoculation reported as successful. This means there was a period of 6 full days before the blood of the person bitten could induce an infection in

[1] falciparum—6 days
vivax—8 days
ovale—9 days
malariae—11 days

a subinoculated individual. Daily thereafter the subinoculations resulted in infection without interruption or exception until that person either received a curative treatment with atabrine or developed immunity adequate for a spontaneous cure. All of these patients were followed with weekly subinoculations and in no instance, where subsequent activity was later demonstrated, did these weekly tests fail to infect. *When a negative was obtained the blood never became positive again,* although observation was continued for one year.

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. Once the asexual infection ceased to exist in the peripheral blood, it never returned (the sexual stages could persist for approximately 30 days before disappearing spontaneously).

It was repeatedly demonstrated that an individual who received 100 mg. of atabrine daily for 7 days before the infective falciparum bites and continuously for a minimum of three weeks thereafter, never developed visible parasitemia or symptoms, despite the fact that the subinoculations made on days 9 to 13 occasionally were positive. This was the first experimental proof of a drug completely eliminating the falciparum infection. It also demonstrated that 3x100 mg. atabrine tablets administered daily for 8 days could produce a radical cure of this infection.

Although there were some cures of individual cases with quinine, this drug could not be depended upon to produce a cure of falciparum malaria. Proof of this observation is the virtual disappearance of blackwater fever from those areas in which quinine is no longer used, either for suppression or treatment.

Vivax. These same 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 falciparum-infected mosquitoes. Falciparum appeared on day 7 and day 8; day 9 produced both and soon the falciparum quickly predominated.

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 therapeutic 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 visible 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 atabrine, 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 atabrine 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.

The situation in patients infected *not* by mosquito bites but by blood inoculation was quite different; these individuals received only the asexual erythrocytic forms. As long as these persisted in the blood, subinoculations were naturally positive even though the number of parasites in the inoculum was submicroscopic. Such patients, with either falciparum or vivax, were readily cured with smaller amounts of drugs and 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 period it was apparently completely exhausted, and that once the asexual infection was eliminated the patient was completely cured.

In vivax the situation was completely different. The pre-erythrocytic stage lasted a full 8 days but evidently did not exhaust itself entirely, since after a period when no circulating forms could be detected in the blood, one or more renewals of parasitemia and clinical activity were frequently encountered, though these were not constant. 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 in-

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

Apparently the suggestion to explore the liver for these hidden stages originated in an observation made by Garnham in East Africa. There he noted tiny vesicles on the surface of the liver of monkeys in whose blood the gametocytes of *P.* (Hepatocystis) *kochi* had been found. 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* 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 all three 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 and ovale contain hundreds of merozoites, whereas the falciparum cyst 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 cyst 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.

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

(1) Falciparum malaria is entirely curable by a single dose of appropriate remedies.

(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 persist more than 3 years.

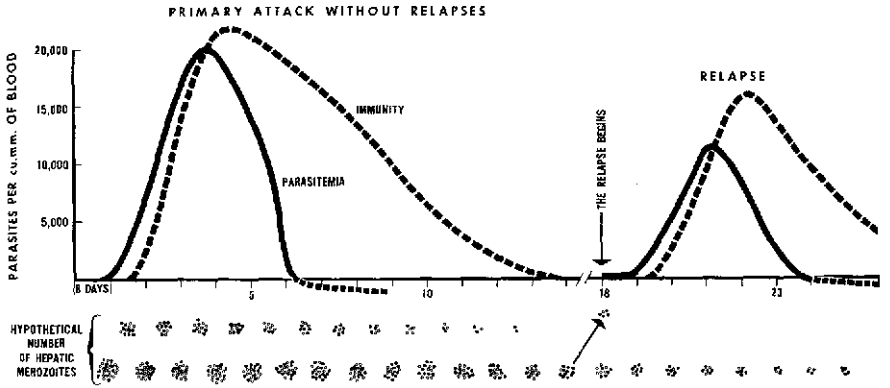
(4) Prolonged suppressive medication may also eradicate falciparum, but vivax is prompt to appear clinically when same is discontinued.

In 1959, after many unsuccessful attempts, the pre-erythrocytic stage of *P. malariae* was shown to be 11 days—identical with that of *P. inui* in monkeys.

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.

DIAGRAM 7

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



The above wholly imaginary schematic diagram is an attempt to show the role of the exo-erythrocytic stage of vivax malaria in:

(a) A solitary primary attack.

(b) One relapse following a primary attack.

The solid line shows the parasitemia as measured by the horizontal lines, which represent differences of 5,000 parasites per cubic millimeter. The parasite level is submicroscopic where the line becomes interrupted.

The dotted line suggests the degree of immunity; the perpendicular lines mark the duration of one complete exo-erythrocytic cycle, or 8 days.

The upper row of diminishing numbers of small dots suggests the number of liver merozoites reaching the circulation after successive liver schizogonies. This number decreases with each cycle until the formation of liver schizonts ceases.

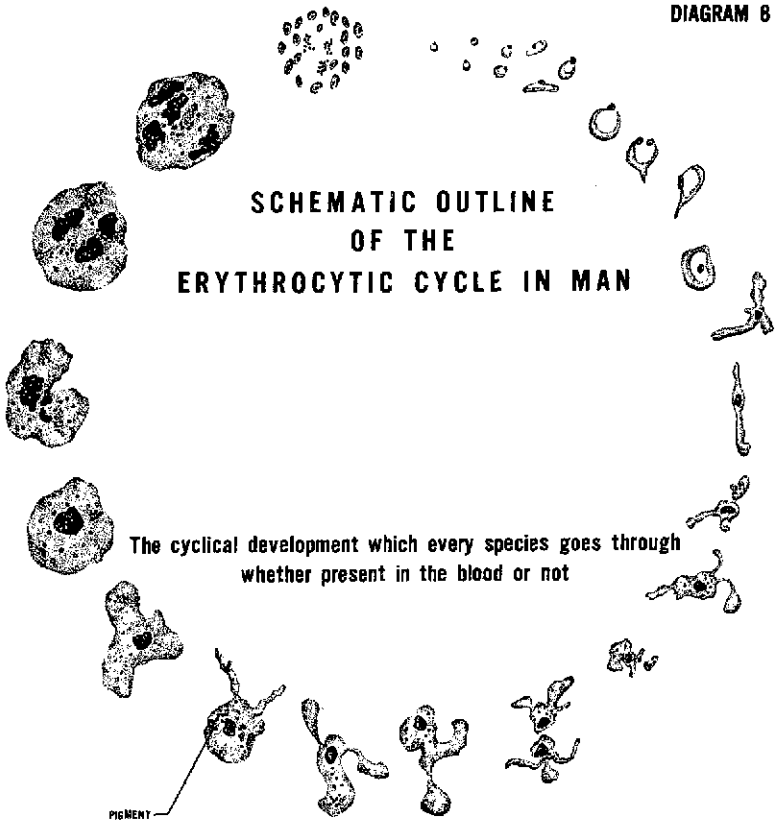
The lower row of clusters of dots represents a much larger number of liver merozoites released every 8 days. Only a fraction of these are required to initiate the parasitemia of a relapse (as at the end of the 18th liver cycle), provided the immunity is so low that the progeny of the erythrocytic schizogony escape destruction and successfully invade new red blood cells.

GENERAL BEHAVIOR OF THE DIFFERENT 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 vivax gametocyte-like stages, may appear identical with larger stages of malariae. In fact, the sole form of the parasite that may be considered *unique* is the *falciparum gameto-*

DIAGRAM 8



cyte. In order to achieve an accurate species diagnosis, it is necessary to have 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 what are the possible variations of forms that can occur, not only in each of the different species but with different conditions of the blood (Diagram 8).

The examiner is always asking 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 knowledge of the variation of parasitic forms that can be expected in the species tentatively suspected?

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 to be a genuine organism.

Once it has been decided to consider the object a true parasite, the next question is: Does its developmental stage fall within the range of development which, as learned by observation and experience, can be present at a certain point in the cycle of "X" species?

Often the unspoken question arises: Can this be a stage that is never present in the cycle of "X" species?

A minimum of 50 fields of a positive blood 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. As is shown rather concisely for vivax in the motion-picture film, all species may have more than one brood of parasites at the same time. Although falciparum, vivax, and ovale require 48 hours for maturation of the asexual forms, it is also possible and not uncommon for them 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. It seems as though the body could only have a single complete reaction in the same 24 hours, or that once the dominant brood is established all others are suppressed.

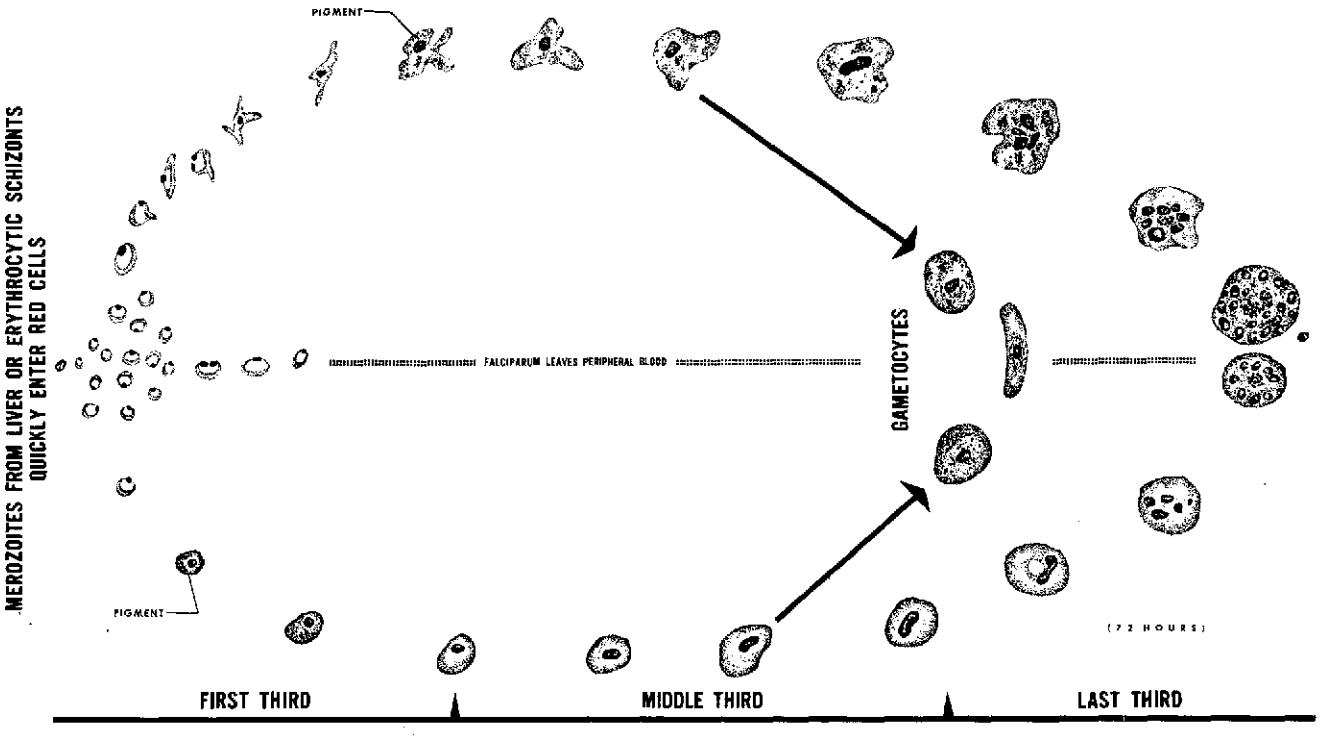
Different broods arise from the liberation of liver merozoites at different times in the primary attack of a mosquito-induced infection. They may also appear spontaneously in a continuing infection which has shown a regular single-brood periodicity for some time. The infection need not be of sporozoite origin for the development of a second brood. Apparently at the schizogony of a single-brood infection, at the end of each 48-hour period, the majority of the parasitized cells rupture within the same hour, and the balance complete their division at various intervals before or after the majority. If the entire schizogony requires five hours to complete and the majority rupture during the third hour, some do not divide until two hours later. Those which began the schizogonic process did so two hours before the majority.

One cycle later, the offspring group which began schizogony is now four hours ahead of the majority (the last groups complete the process four 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 9 presents, in a condensed schematic manner, the entire development cycle of the three common species of man's malaria. Although stretching from one side of the diagram to the other, the cycle is a continuous one, and the smallest form is encountered immediately after the largest schizont ruptures. The diagnosis of species will be greatly aided if a newly-found organism is mentally transferred to its appropriate position on this diagrammatic cycle.

THE ERYTHROCYTIC CYCLE IN MAN

DIAGRAM 9



INDIVIDUAL BEHAVIOR OF THE DIFFERENT SPECIES IN THE PERIPHERAL BLOOD

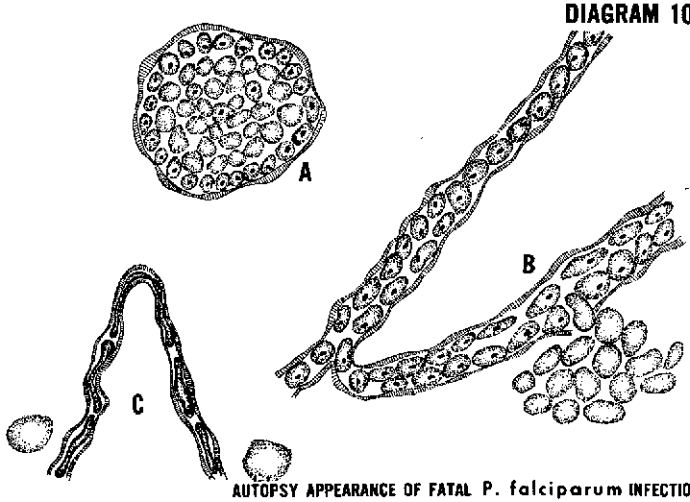
There are differences in the behavior of the individual species which are responsible not only for the symptoms produced in the patient but for the forms that are present in the blood as well.

Falciparum. Apparently when the falciparum merozoite enters a red blood cell, its very presence produces some inherent change in the red blood cell itself, which might be described as a stickiness of its external layer or membrane.

This condition is possibly responsible for the frequency with which young falciparum parasites are found at the periphery of the cell. 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 10 shows the autopsy appearance of a fatal falciparum infection. Perhaps it should first be explained that the well-known appearance of parasites and para-

DIAGRAM 10



AUTOPSY APPEARANCE OF FATAL P. falciparum INFECTION

sitized 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.

A survey of any tissue, particularly the brain, will demonstrate the increased frequency with which parasitized red blood cells are found along the walls of smaller 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 show its border completely lined with parasitized red blood cells, while few if any show the presence of parasites in the lumen of the vessel crowded with red blood cells (A). 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 reticuloendothelial 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 escape through the opening which followed too severe damage to some of the lining cells. As long as the reticuloendothelium 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 so deprived of essential oxygen, electrolytes, etc., that death follows closely thereafter, 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 such an amount of reticuloendothelium that the effect cannot be supported. Death therefore occurs probably 15 to 24 hours after that final schizogony.

It is most probable that the difference in pathology 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 occurs in a center equipped with the appropriate facilities, needle biopsies of the liver and punch biopsies of the large bowel may be attempted with the aim of demonstrating the pathological appearance during life.

If the foregoing is well understood, it is easy to understand why only the young forms of the parasite are encountered in the peripheral blood. If shock occurs, the obstructive phenomena sometimes mentioned in books can and do occur, since evidently some change takes place in the endothelium which releases all the sticky red blood cells from their sites.

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

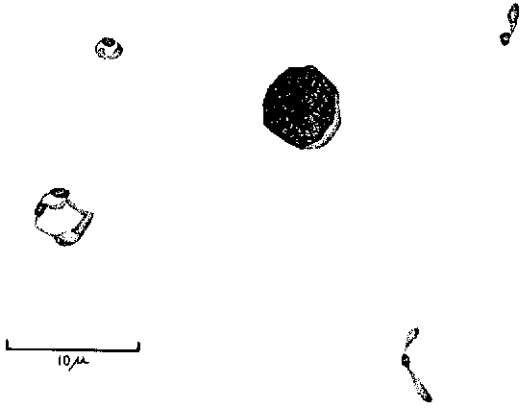
The characteristic high parasitemia is possible since these blood vessels, whose lining is occupied by parasitized cells, are also packed with uninfected cells. Schizonts rupturing in their fixed position liberate merozoites which are literally pressed against hundreds of cells. With their indifference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

In a primary attack of falciparum malaria, the parasitemia appears about the 10th to 12th day, followed within 24 hours by the appearance of symptoms. If the individual has a capacity to manufacture gametocytes, these will not begin to appear until 8 to 10 days later; left 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 these already present may continue to circulate from 2 to 4 weeks. An appropriate method of reporting blood findings

which accurately describes the phase of the infection in the individual examined is therefore: (a) ring forms only: F; (b) ring forms plus gametocytes: F+g; and (c) gametocytes alone: Fg.

The beginner should have access to and should spend the maximum amount of time in the inspection of as large a

P. falciparum **DIAGRAM 11**

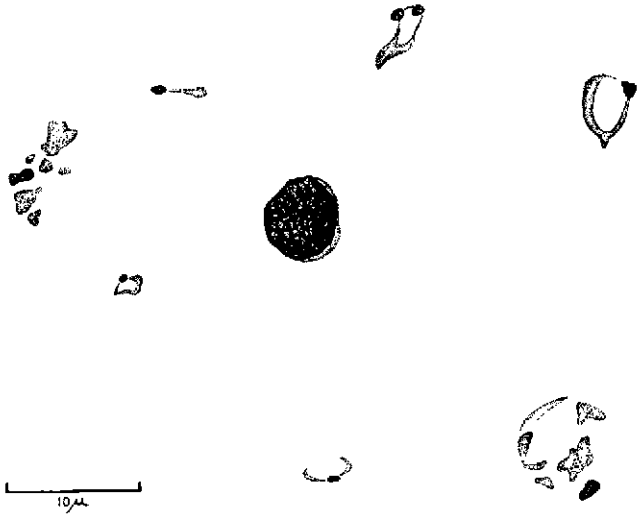


number of falciparum parasites as possible. Effort should be made to recognize the variation of those encountered. An appreciation should be gained of the smallest as well as the largest rings that can be seen. Falciparum rings are described as small, medium, and large. The nearer to the time of schizogony that the blood is taken, the more numerous will be the proportion of tiny rings. If the proportion of large rings is greatest, it may be assumed that the total number of parasites will diminish remarkably within six 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 indicated in Diagram 9. 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 competing, with the result that parasites are never absent from the peripheral blood, although their numbers may vary considerably.

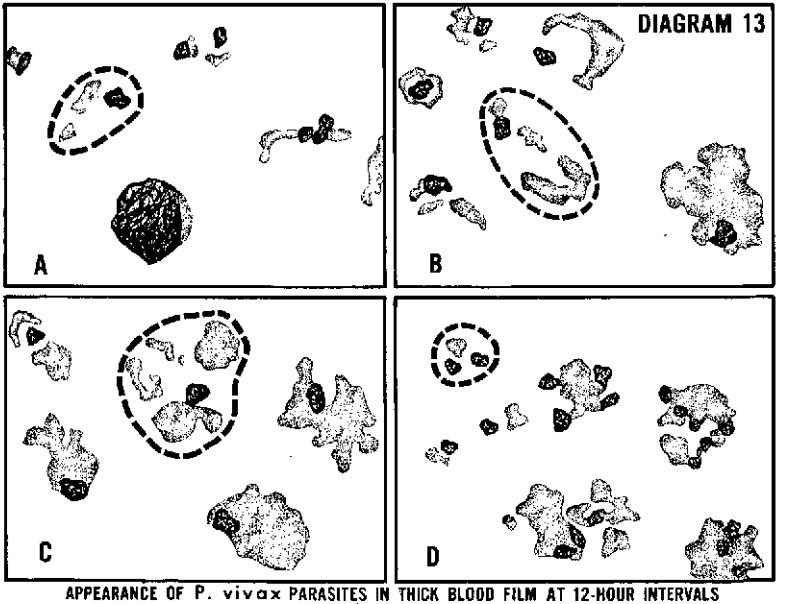
Vivax. The word vivax signifies lively and it well describes the almost frenzied activity which the species frequently exhibits. The vivax merozoite shows a distinctive preference for younger red blood cells, which are more elastic than the more mature ones and, as a result, are able to enlarge to better accommodate the growing parasite. However, there is no stickiness of the containing red cells as in falciparum, so 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 open circulation. The period during which the liberated merozoites are seeking a new host cell is much more prolonged

P. vivax **DIAGRAMA 12**



than with falciparum. Even then, it probably does not exceed five minutes, and so the number of parasites lost to phagocytosis is much greater. Total 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. In their division it is not uncommon to find more than one piece of chromatin in the new parasite; the second chromatin piece is often smaller than the first. Although these relatively early parasites appear already to be undergoing division, such is not the case. Moreover, within the newly-

entered red blood cells, the vivax parasite moves throughout the whole cell, putting out cytoplasmic pseudopodia which 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 picture more than a fraction of them. The result is that a large, irregular parasite may not be recognized as to its entirety in the thick blood film. Frequently a portion of the parasite is compact and may perhaps contain all the pigment for the entire amount of 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 currently seen in the containing blood. The confusion so frequently encountered in the diagnosis of vivax and malariae is due to these large dense portions being considered as the entire parasite. The tiny threads of cytoplasm connecting the individual fragments are frequently too fine to be seen (Diagram 13).



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

Since no stickiness occurs in the red blood cells infected with vivax, they continue to circulate without interruption. Instead of becoming sticky the red blood cell undergoes some change which, when the cell is stained in the thin

smear, produces a series of reddish granules in the cell envelope, regular in size, shape, and distribution. Fine at first, they become larger and more prominent. They are known as Schüffner's dots, 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 containing red blood cell. Schüffner's staining occurs only 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 about 10 per cent of routine stains show these changes.

As the vivax parasite matures it becomes rather 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, they do not persist for more than 12 to 48 hours after the disappearance of the asexual forms, as do the gametocytes of malariae. Unlike the case of falciparum, any medicine which removes the asexual forms from the circulating blood drives out the gametocytes as well.

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 of a V 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. Most likely, if looked for carefully, advanced dividing forms will be encountered as well.

As already mentioned, falciparum dominates vivax and vivax dominates malariae, but it is unusual to find any other combination 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. Falciparum consistently dominates vivax and vivax regularly dominates malariae, i.e., F>V>M.

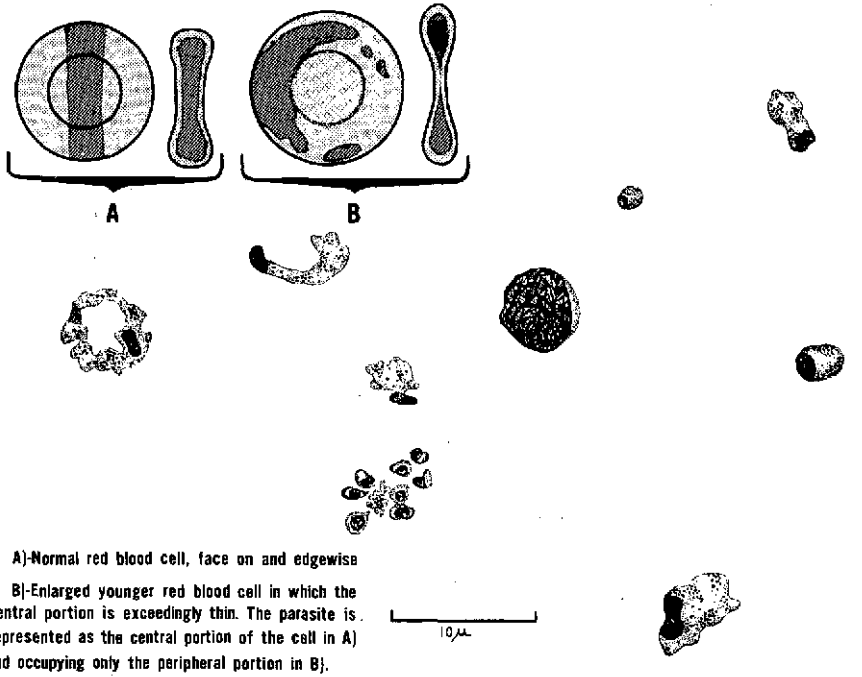
Some indication of the proportionate numbers would give a truer picture. The subordinate species may be indicated by enclosing its symbol in (), e.g., F+++ (M17/100) fields. In the clinical sense, at least, a mixed infection almost never happens.

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

P. malariae infections differ from those of the two more important species in having side effects not experienced 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 motionless and picks up its food by osmosis. There are no extruding pseudopodia. Advanced pre-segmenting and segmenting forms occasionally show considerable irregularity to doughnut formation as a result of the entire parasite being contained in the 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 portion of the cycle is exhibited in a single drop of quartan blood than is the case in vivax (Diagram 14). Three broods exist when all stages of the cycle of *P. malariae* are present in the same specimen of blood.

P. malariae **DIAGRAM 14**



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).

From shortly after the termination of ring stages to actual mature schizonts, the general characteristic of the malariae parasites 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 time is given for the parasite to round up. Particularly if these forms are somewhat numerous will they be miscalled quartan parasites.

However, true mixed infections do not occur with suf-

ficient frequency 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.

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 to another person whose blood is in good condition, either by needle or mosquito, and the resulting new infection follows the ovale pattern, may an ovale diagnosis be considered.

P. ovale is found most frequently in the West Coast of Africa but also occurs in East Africa, the Middle East, Malaya, Indonesia, and the Philippines. It is essentially a quartan infection with a 48-hour cycle and the presence of Schüffner's dots in the uninfected portion of the red blood cell containing the parasite. In fact, if the staining is not 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 inocuous, some strains have shown a degree of severity equal to vivax. Unlike vivax, Negroes are generally susceptible to ovale.

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 when in humid places and promptly dried. Accessory chromatin dots are as frequent as in vivax. An oblong appearance to the large

chromatin masses of the schizonts is said to have diagnostic significance.

THE MAKING OF THICK BLOOD FILMS

As indicated elsewhere in this Manual, thick blood films are not made as thick as formerly nor are they stirred or defibrinated.

Clean slides are packaged in lots of 5, 10, or 15. The package of 5 is probably more appropriate for field work (see Microscopic Slides). The end of the package is torn across, exposing the ends of the slides. Two slides are placed on the package and a clean, clear flat 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 bench is not flat, the cardboard may be held flat by a volunteer assistant from the ever-present onlookers. A flat piece of white paper is useful when spreading the blood. Beginners may be provided with a template or guide to the position on the slide of single or multiple thick films.

Blood is taken from the lobe of the ear if there is sufficient flesh, from the great toe of an infant, or from the finger. The side rather than the ball of the finger is punctured by a sharp sticker, which may be either of the type which is used once and disposed of or of the type designed for repeated use. The most common form of the latter type is one half of several shapes of common steel pen points. Special spring-type needles are seldom sharp enough and cause discomfort out of proportion to the magnitude of the operation. For continuous daily work, the pointed type of Bard-Parker blade merits consideration. It is stuck through the cork of a moderately wide-mouthed bottle of 30-50 cc. capacity. It may be kept completely clean and sharp if rubbed on fine (No. 0 or 00) emery paper at the beginning of each day's work or when required. A piece of this emery paper 4 x 6 cm. is placed around the bottle, emery surface to the glass, and held by string or rubber band, where it is constantly available. Ninety per cent alcohol is carried 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 paper until all traces of dried blood, rust, etc., are removed and the edge and point are so sharp that they are not readily seen without a hand lens.

The skin to be punctured is firmly wiped with cotton or gauze moistened with the alcohol to remove gross dirt and dried sweat, and is then wiped *with dry cotton or gauze.* 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 probably most satisfactory. The blade or pen is moistened with alcohol, wiped, and lightly and quickly stuck 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 punctured, the tip of the finger is gently squeezed until the blood wells up in a spherical drop on the dry skin (Diagram 15).

The edge of a clean slide is immediately pressed firmly against the forefinger of the operator and the surface of the slide is lowered 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 by the corner and first few millimeters of a second slide until a square or rectangular patch of approved thickness is obtained. A thinner streak of blood or partial thin smear is made from the blood remaining at the site of puncture to be used for a label written later, with a soft No. 1 pencil, when the blood has dried. Adequate thickness of a thick film is of course the maximum thickness which can be seen through under the oil immersion after staining. This can be estimated by first making one definitely too thick. It is spread insufficiently and the slide is turned perpendicular or on its edge; a drop is immediately formed at the dependent border. With the next slide, a similar amount of blood is spread wider and the slide turned up again. If the drop forms quickly, it is spread still wider until at last 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.

HOW TO TAKE A THICK BLOOD FILM

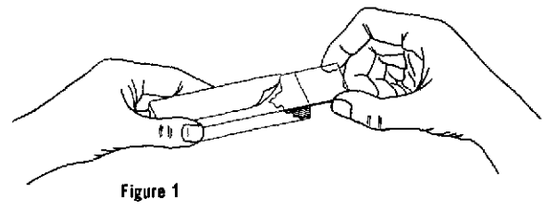
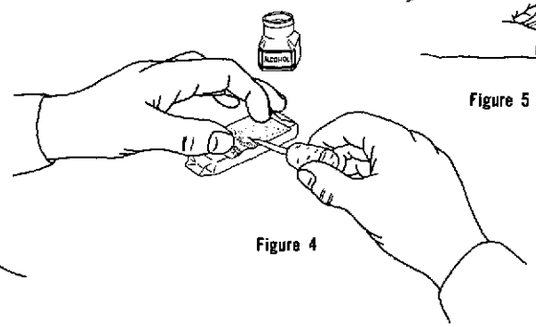


Figure 1


Figure 3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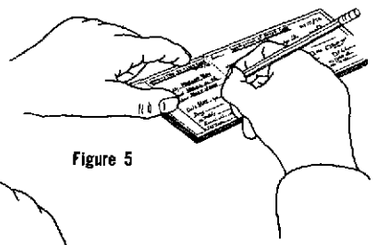
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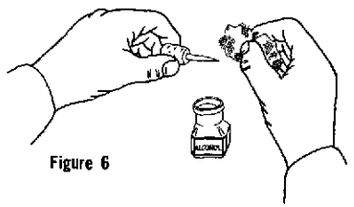
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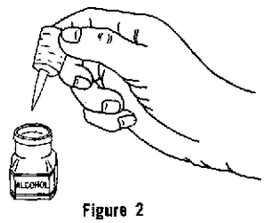
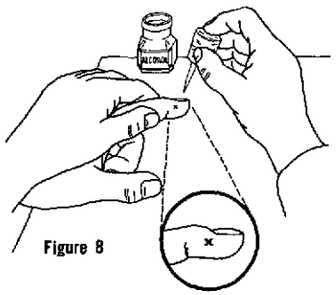


Figure 2


Figure 7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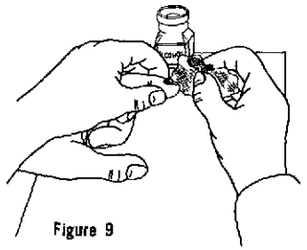
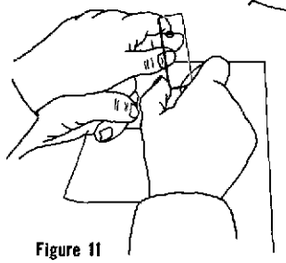


Figure 9


Figure 10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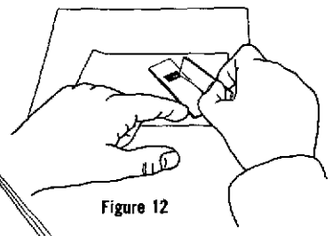
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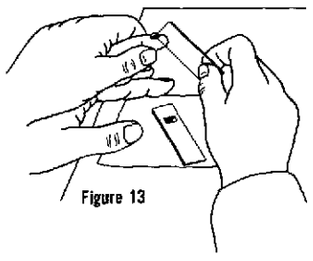
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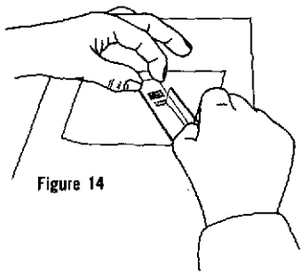
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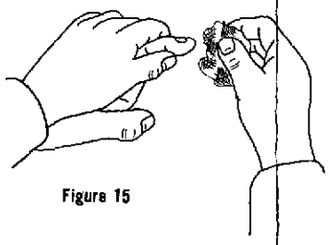
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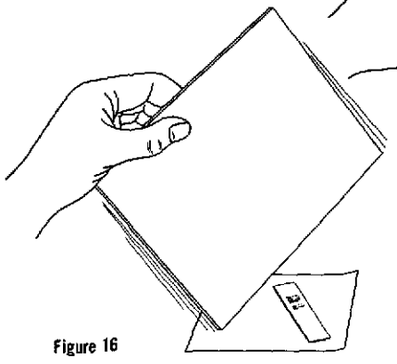
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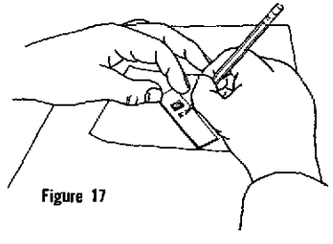
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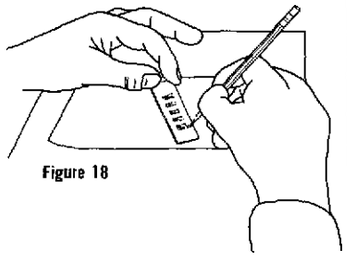
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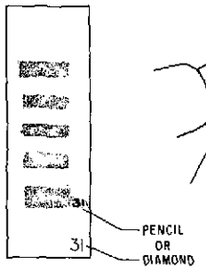


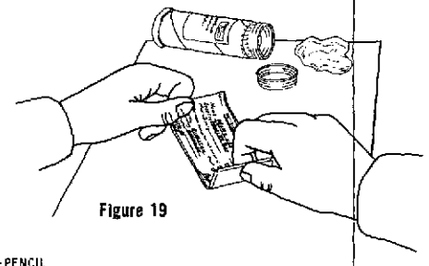


Figure 19

INSTRUCTIONS

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. When not in use, the sticker should be inserted in the under side of the cork of a low, wide-neck bottle and kept constantly immersed in 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.

Figure 4 When sharpening the sticker, hold the emery paper over a package of slides or other solid 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; otherwise, replace the sticker in the bottle, without exerting pressure.

Figure 7 Before the 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 carefully be wiped off the finger with a piece of dry gauze or cotton.

Figure 10 With a squeezing motion similar to that used in milking, another drop of blood is forced onto 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 left hand which is holding the punctured finger; the slide is then gently lowered 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 ¾" 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.

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 form a thin smear.

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 pencil, write in over 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 are to be taken at the same time, five transverse, narrow and 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 may be placed together and packed tightly in a slide wrapper. The identification slips are then wrapped around the compact package.

The place for writing the identification may be spread with blood that has remained some time on the surface of the skin but not the thick film. It should be spread immediately. 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 No. 1 lead pencil, using Roman numerals to indicate the month, viz: K L M 17 V 8 for 17 May 1958.

Where the humidity is high, drying may be accelerated by brisk fanning with a piece of cardboard. The inexpensive Humigraph* is useful in showing humidity changes which affect the drying of slides.

Five transverse, narrow thick films may be placed on the same slide if desired 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, the blood may be streaked to the edge of the slide.

THEORY OF BLOOD STAINS

Blood staining has always been complicated by the inherent great 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 if the same lots of methylene blue are used in identical procedures it is possible to obtain widely varying results, then it can be realized that no Giemsa, Wright's, or Leishman's stain can possibly turn out always the same. In some instances the difference may be so great as to render useless individual lots of stain.

The Biological Stain Commission was formed in the U.S.A. in 1925 to test each lot of every stain with the hope of eliminating those that were unsatisfactory. It is therefore most desirable in acquiring the blood stains 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 for the type of work to be done.

* A. Daigger and Co., 159 West Kinzie St., Chicago 10, Illinois.

With regard to the Giemsa stain, which is of maximum interest in malaria work, a number of laboratory workers still prepare their stains from the original dye components used, often with surprisingly good results. The beginner, however, except under exceptional circumstances, 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 on individual brands or types of stains by some of the elder workers. It may also explain how some Giemsa stains are satisfactory if dissolved in one brand of pure methyl alcohol and distinctly less so with others. The dissolved elements are evidently in such delicate chemical equilibrium that very slight changes of reaction may have 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, after which they begin to precipitate out of the 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 daily 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. If the access of air is permitted to the alcoholic solution, especially in the tropics where the humidity is high, this may occur quite rapidly. This 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 these are put on the bottles since the accumulation of dried stain may prevent the bottles from being properly stoppered.

To prevent the occurrence of this undetectable and continuous damage to the Giemsa stain, it is advisable to use small working bottles appropriate for usage during one or two days only, attached to which is a small straight test tube to hold a dry dropper *used only* to withdraw the liquid Giemsa stain directly. Small plastic bottles with a dropper opening and tightly closed by a screw cap, used with perfumes and some medicines, make ideal Giemsa work bottles. Stock bottles are therefore only opened 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 commonly 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 with this method exposure to the atmosphere is unusually prolonged. Moreover, lumps of moistened stained powder invariably adhere to the sides of the mortar and the face of the pestle, where they are lost to the final product. In place of this method the procedure has been recommended, and successfully used for several

years, whereby the dry powder is added directly to a bottle of convenient size, containing the proper amount of this alcohol-glycerin mixture, and a minimum of 50 scrupulously clean, small-sized solid glass beads, not to exceed 5 mm. in diameter, are also added to the bottle. This bottle is thoroughly shaken 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 each and every one of them, 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:

tap water
stream water
well water
spring water
rain water
distilled water
double and triple distilled water in ampules
buffer water
neutralized 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 in 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 lime-

stone, it may be quite useless. Water which has acquired aerogenic bacteria, yeasts or algae may no longer be suitable. 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.

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 rapidly prepared 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, a distilled water appropriately neutralized for the occasion may be superior to buffer 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 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.

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 the individual cells 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 by one of various methods from the red cells, separately or during the staining process. In the past, weak solutions of acetic 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 developed of having the dehemoglobinization take place in the stain solution.

The methods of staining thick films and thin smears are therefore diametrically opposed. 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 will be the dehemoglobinization; and the longer they are allowed to remain without staining, the less clear will be the preparations. 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. *The Giemsa stain was virtually the only one used and the dilution of one drop to 1 cc.* of distilled water became the custom. 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 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. In those packages, if a single, very heavy falciparum infection was present on one of the slides, transfer of parasites might take place occasionally to adjacent slides because of the debilitated character of the blood. Careful rinsing or use of a detergent may avoid 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

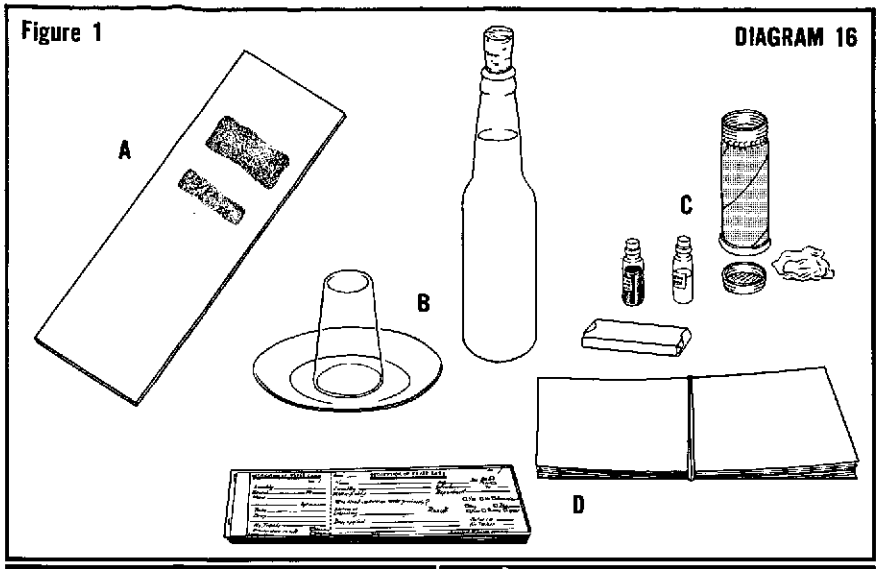
distilled water, and a similar dip in Solution B (a mixture of eosin and phosphates). The rapidity of the method did not allow time for thorough dehemoglobinization, but showed both leukocytes and parasites brightly and clearly colored. A modification of this same stain was later developed in India and is known as the J.S.B. stain.

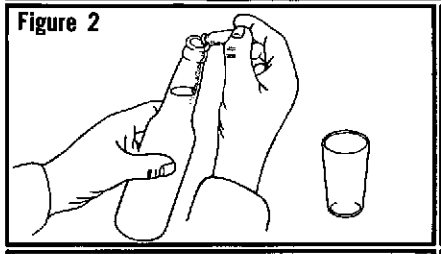
During the 1920's a London manufacturer made a staining plate slightly curved in such a manner that when a slide was inverted across the curvature of this plate, a space 3 to 5 mm. deep remained below the slide into which the stain was directed. Because of the particularly heavy weight of the hemoglobin 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. The adoption of a one-second dip in a methylene blue phosphate mixture (Methylene blue, medicinal, 1.0 gm., Na_2HPO_4 anhydrous, 3.0 gm., KH_2PO_4, 1.0 gm., thoroughly mixed in a mortar, ONE GRAM of the mixture is dissolved in 300-350 cc. distilled water), 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 17-E. It was found that this pre-treatment permitted excellent staining with Giemsa to occur after exposure of only 6 to 10 minutes to the Giemsa solution.

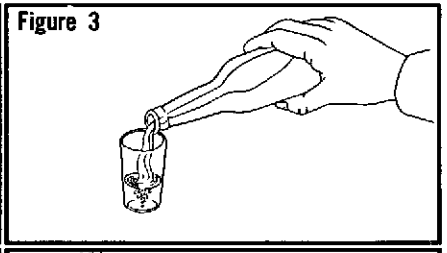
The staining of the combined thick film-thin smear requires that the thick smear be separately "fixed" with methyl alcohol before the whole slide is stained with some variation of the afore-mentioned techniques.

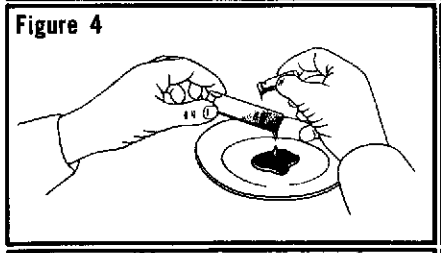
Thin blood smears alone may be stained with Giemsa stain following previous fixation, or by any of the May-Grünwald, Wright's or Leishman's stains whereby fixation is obtained by the application of the undiluted stain followed by the addition of the diluent.

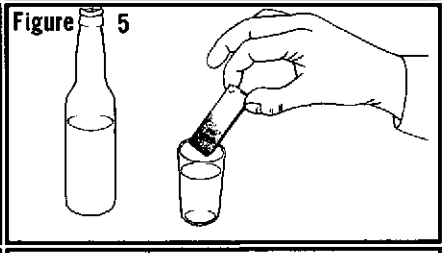
When thick blood films are long delayed in arriving at a laboratory capable of using Giemsa techniques, it is possible to pre-treat these slides with methylene blue phosphate solution and brief immersion in buffer water so that when they arrive at the laboratory some weeks later, it is possible to stain them with Giemsa and still obtain preparations of reasonable quality for examination. Diagram

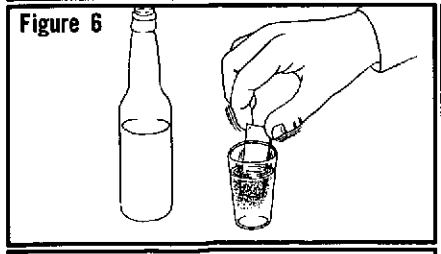


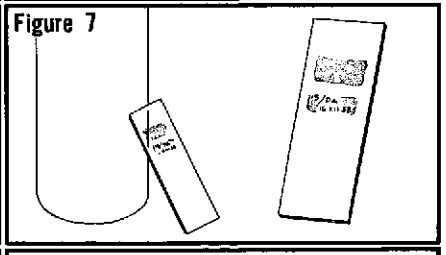


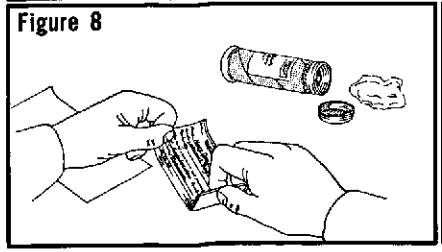


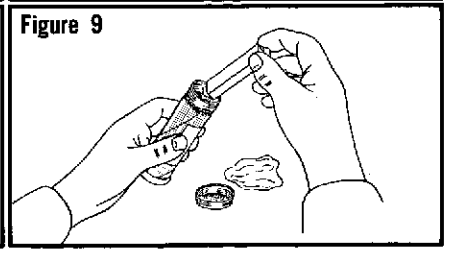












PRE-TREATMENT OF THICK SMEARS BY VOLUNTARY COLLABORATORS

Fig. 1

(a) Appearance of the thick-smear slide before pre-treatment.

(b) Simple equipment used by collaborators to pre-treat thick-smear slides 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 dish or tray.

(c) Cylindrical cardboard mailing tube, which when returned 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 of Fever Case" forms and a package of the wrapping paper supplied regularly to the collaborator.

Fig. 2

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

Fig. 3

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

Fig. 4

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

Fig. 5

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

Fig. 6

Move the slide gently to-and-fro in the buffer solution until the thick-blood smear loses all trace of red color. Whenever the solution becomes markedly blue, substitute by fresh solution from the bottle.

Fig. 7

Lean the slide on end against a bottle or any other suitable surface, to drain dry. The figure shows the slide as it appears after treatment (quite transparent).

Fig. 8

Wrap the slide in its "Notification of Fever Case" form.

Fig. 9

Place the wrapped slide in the cylindrical mailing tube for shipment to the laboratory.

16 shows how a very few slides may be treated in the home of a collaborator. For numbers of slides (Diagram 17) only steps A and B are required; the slides are gently moved to and fro in the second buffer water until only a trace of red color remains. Thick blood films many days old are probably so fixed by time that the methylene blue phosphate solution has no effect. Under these circumstances this step may be omitted.

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

Recently, plastic dropping bottles of from 30 cc. to 150 cc. capacity have become available. Giemsa stain, when placed in them, is more completely protected from contamination with moisture than in any previous container which required a separate pipette for delivering the appropriate number of drops. Therefore, evaluation personnel can be equipped to stain slides in the field, and pretreatment only of slides may be done by the collaborators. All that is needed to equip evaluation personnel beyond the already recommended equipment would be a small additional supply of buffered water, and a clear surface with a depression, as shown in Diagram 17-E. All slides taken by evaluation personnel may thus be stained in the field when a convenient number have been accumulated, say on a daily basis, or every other day. This will 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 obtained. For example, if one Giemsa gives good results when it is dissolved in a certain proportion with glycerin and alcohol, these quantities can be used. When the alcoholic solution is very strong and the leukocytes are overstained, 5 to 6 drops in 10 cc. of the diluent should be sufficient. It is better to dilute the stain with more alcohol and glycerin in order to maintain the universal proportion of one drop of alcoholic solution to 1 cc. of buffer solution.

Similarly with the buffer solutions, that proportion of salts which gives optimum results should be found. 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. 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 giving the best results are used. The proportion 6:5 is probably the most frequently used.

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, 1 drop to 1 cc. of distilled or buffered water, was poured on the slide 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.

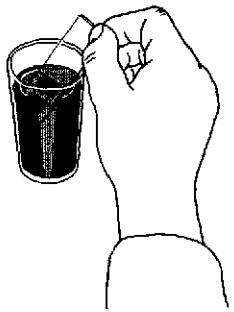
Staining times may vary with each lot of stain and should be tried out carefully. Dehemoglobinization is not as complete with the Field method as with the methylene blue-Giemsa technique, but the colors at the margin of the thick film are approximately the same with the two methods.

Staining of Thick Blood Films (Walker)
(Diagram 17)

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.
2. Dip for one second—no more—(count aloud "one-one-thousand") in the methylene blue phosphate solution; the slide may touch paper towel or other absorbent surface to *quickly* remove excess blue. 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 cotton cloth or paper towel that has been thoroughly wet and then squeezed to remove excess water.

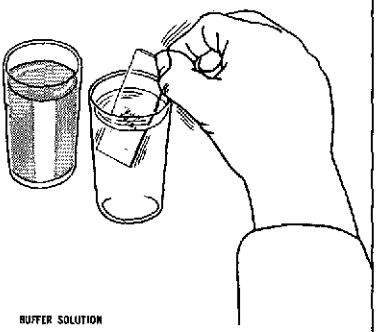
DIAGRAM 17

A



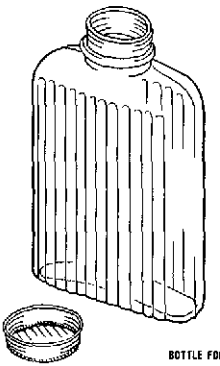
METHYLENE BLUE-PHOSPHATE SOLUTION

B



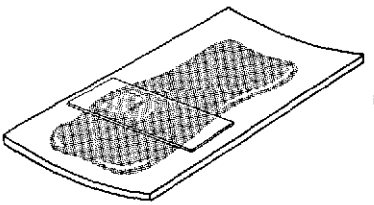
BUFFER SOLUTION

C



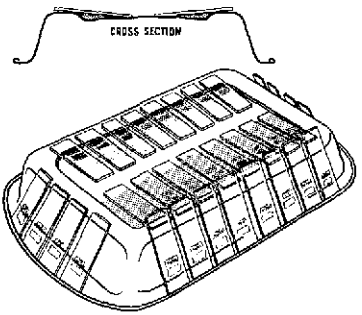
BOTTLE FOR SOLUTIONS

D



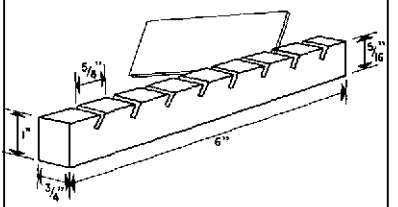
CURVED PLATE FOR STAINING

E



ENAMEL TRAY

F



RACK FOR DRYING SLIDES

3. Dip five to ten 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. Distilled water may be used if buffer is scarce.
4. Place the slides upside down over the 4-6 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.

Staining of Thin Blood Smears

The staining of thin blood smears is usually carried out in one of two ways:

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 more elegant 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 per cent) 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.

Steps to be Followed in Method A

1. Fix thin smears with pure methyl alcohol 2 inches apart.
2. Place slides across horizontal glass rods.
3. Flood with fresh Giemsa stain prepared by using 1 drop Giemsa to each cc. 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.

Steps to be Followed in 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 with copious amounts of same diluent.
6. Drain or blot.
7. Dry with gentle heat.

Stained by either method, red blood cells are buff.

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 1 gram quantities are placed in small well-stoppered vials. The contents of 1 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 3 times daily until it is thoroughly mixed. Keep tightly stoppered at all times. Filter if necessary. If Giemsa powder is not available, Wright's powder can be used in the same proportion.

3. *Buffer water*

Disodium monohydrogen phosphate anhydrous (Na_2HPO_4) 6.0 gms.

Monopotassium dihydrogen phosphate (KH_2PO_4) 5.0 gms.

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 1=Azure B	0.5 gm.
Na_2HPO_4 anhydrous	5.0 gms.
KH_2PO_4	6.3 gms.
Distilled water	500 cc.

Solution B:

Eosin, yellow w.s.	1.0 gm.
Na_2HPO_4 anhydrous	5.0 gms.
KH_2PO_4	6.3 gms.
Distilled water	500 cc.

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 if necessary.

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 different countries vary slightly in thickness, length, and width. Before placing an order for microscope slides we should review 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 and 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 and those showing even beginning of corrosion 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, non-corrosive, 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 slightly bevelled (appreciably reducing the number of finger cuts during wiping). The list price is $1.80 per gross. The No. 7030-B, "clinical," are identical with the above without bevelled edge. The list price is $1.45 per gross.

Several brands of "pre-cleaned" slides are on the market. If they come near to the above specifications and prove to be clean enough 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.

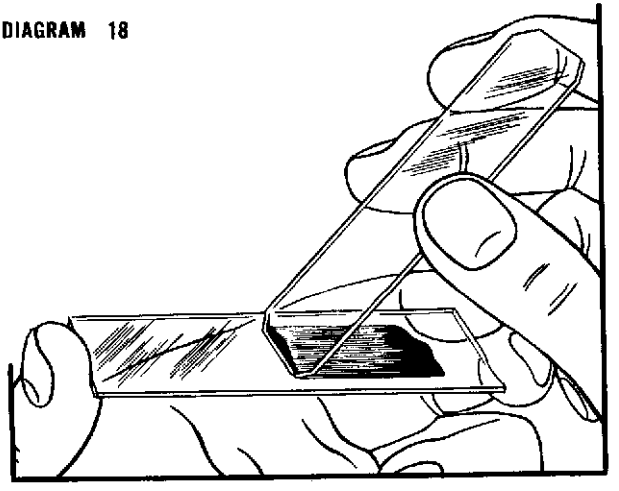
New slides which have never been used but which have been stored for months under favorable conditions may show varying degrees of corrosion. If at all possible, these should not be used for blood work, but rather diverted to some other activity.

New slides are commonly 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 ade-

quate number of clean slides—usually 3 to 8—the whole 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.

Where thin blood smears are indicated (unusual hematology, bone marrow) to demonstrate some particular feature of a malaria infection, Clay-Adams "margin-free" blood smear slides No. A-1463 are highly desirable for the preparation of consistently good thin smears.

DIAGRAM 18



"MARGIN FREE" SLIDE

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.

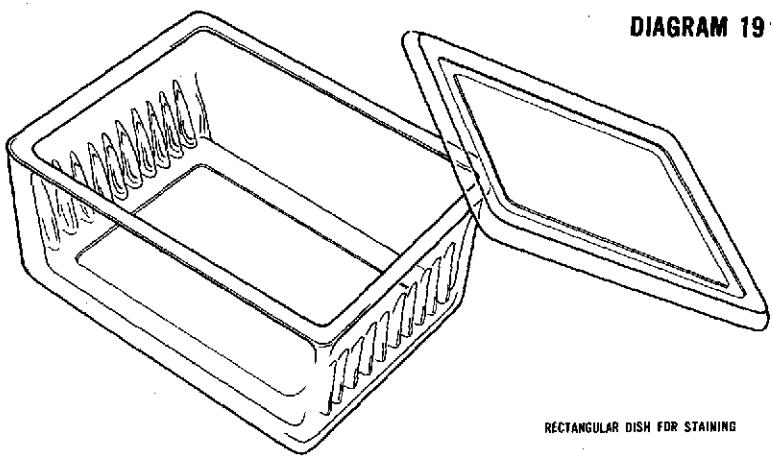
For this reason there has not been as yet any "short cut" to the slide-cleaning routine. It is not enough that their surfaces be highly polished; they should also always be firmly wiped with a clean cloth until nothing is adhering to the glass. In fact, so much pressure is exerted to achieve this end that novices frequently break numbers of slides

in learning to wipe them properly. Well-cleaned slides also reduce the number of confusing artifacts that may appear in any blood preparation.

The assumption that new slides from a freshly opened box are the best to use 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 from each other during the washing, soaking, and rinsing process are indispensable (Diagram 19). 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.

DIAGRAM 19



RECTANGULAR DISH FOR STAINING

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 by dissolving 100 gm. potassium dichromate in 10 per cent sulfuric acid and diluting to 1 liter. This solution is poured via a wide funnel back into the bottle, and the slides, still lying on their edges, are thoroughly rinsed under a faucet with running water, or if not available, in repeated changes of water until all traces of the acid have gone. If distilled water is plentiful (rain water collected in a clean enamel pan raised two feet from

the ground *is* distilled water) there is a final rinse in distilled water before the slides are wiped dry by firm pressure of the fingers in a longitudinal direction. These slides are laid out individually on a clean piece of cardboard, table, or bench to complete drying of the edges.

If this operation has been done properly there is no need for further immersion in good-quality alcohol to render them completely grease-free. If alcohol is used, the slides must be dried before being placed in it. The dry slides are packaged tightly wrapped in blocks of 10, 15, or 20 (if thin) to each block and are then ready for use. It is advisable to mark the date the package was made, since slides kept in packages for several months, depending on the humidity and contamination of the air by motor exhaust gases, might require washing again before use.

Used Slides. After being used for examination, all slides have some amount of immersion oil on them. If it is desired to preserve the slide for further examination, a few drops of a good-quality toluene are allowed to drop on the slide, which is placed in a wood drain block until dry. Slides are then wrapped in onion skin paper 4½ x 8½ inches, and their identification is written on the outside with lead pencil. Stained slides covered with oil are soon spoiled if left scattered about on the bench or exposed to sunlight.

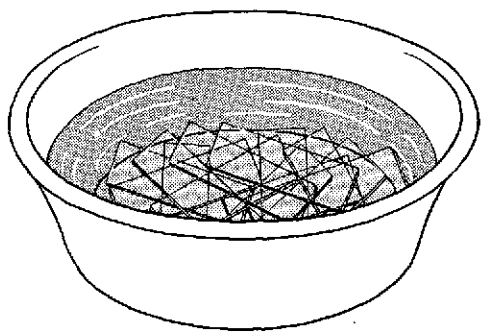


DIAGRAM 20

Provide a bowl of glass, enamel, or plastic (Diagram 20) at least 4 inches 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 soak-

ing in shallow vessels such as plates or flat pans. These are almost always left aside 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 must be separated individually or handled in the above-mentioned glass staining 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 may seem like 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 render 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 the same quality alcohol. They are wiped with special clean cloths used for glassware and packaged before use.

Importance of Cleanliness of Towels Used for Glassware.

To obtain the condition of scrupulous cleanliness required with glassware, it is well to obtain a generous sup-

ply of towels of suitable cotton material with as little lint as possible. 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 out in pieces of 40 x 60 cm. and the cut ends are sewn. The resulting 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.

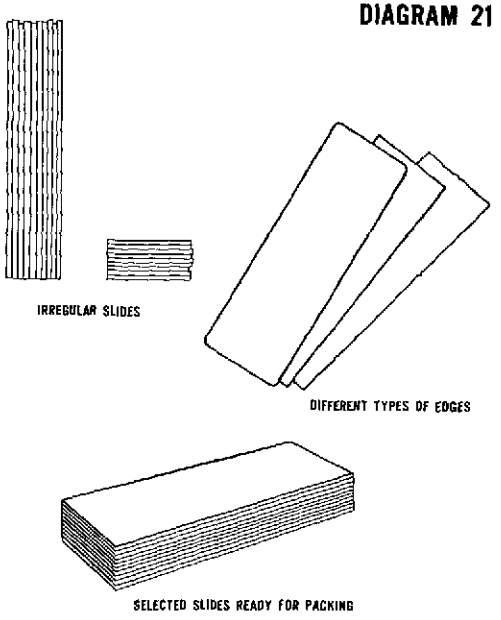
It should be understood that these cloths are rarely soiled when used only for glassware, but since the hands are constantly sweating at a degree depending on the climate, insensible perspiration is constantly absorbed by these cloths. They should therefore be washed frequently.

Soiled towels are soaked in water and a good soap is rubbed over the whole surface and well into the material, or they 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 where dirt is visible, they 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 distilled water is advisable. Starch should never be used and towels washed by commercial laundries should always be rinsed well before being used. Ironing is superfluous except when done to accelerate drying.

When used slides are washed and wiped dry, they should be separated into groups of equal sizes (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 21 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 particularly require extra reinforcement 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 for ready reference.

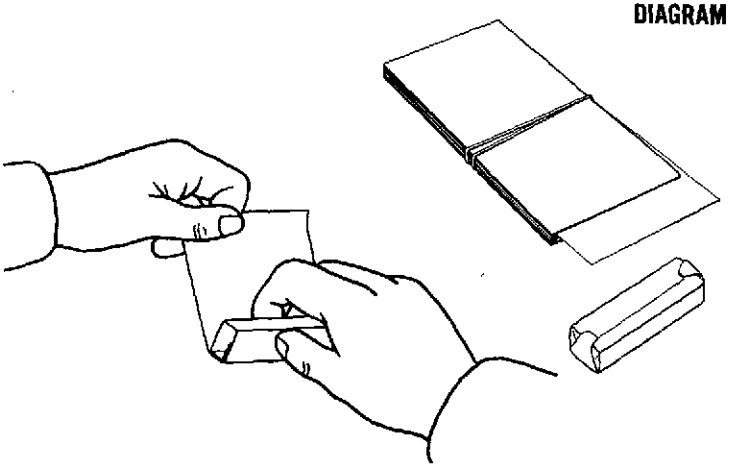
DIAGRAM 21



Handling, Storing, and Transportation of Microscopic Slides.

Modern packaging of merchandise arranges the contents in such a way that it reinforces the container. 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.

DIAGRAM 22



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 22.

Any tough, thin copy paper such as onionskin is cut, preferably in a printer's cutter, 4½ x 8½ inches, together with pieces of firm cardboard of the same dimensions. Fifty of 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 width may be 3½ inches if individual packs of five slides are made).

The necessary or 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 held so firmly 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, as the firm tight package can stand alone.

Any attempt to introduce paper between the individual slide 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 carry better if packaged in this fashion, which reduces movement between slides to a minimum.

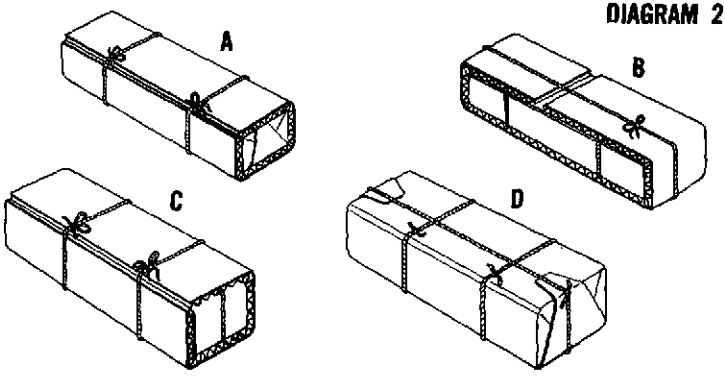
Any necessary identification may be written in soft pencil on the "edge" side of these packages, so they need not be opened to be identified. Packages of newly cleaned slides may very well bear the date they were cleaned. Such blocks or packages of slides are stored in the empty cardboard boxes in which they were received, either in the whole box or in the upper and lower half of same. Small metal cooking pans 18 x 28 x 3.5 cm. will hold as many packages of slides as can be conveniently handled, taking the 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 required to cover all sides, which are tightly tied with string before the carton is wrapped in tough wrapping paper.

Single packages or less than carton amounts of slides may

be packaged separately, using appropriate widths of carton board. Strips of corrugated cardboard are cut with a suitable Gem razor blade, razor-blade holder, or preferably with a heavy paper cutter, in varying widths. There are two types of sizes, approximately 2.5 cm. to 7.5 cm. wide with successive pieces each 0.5 cm. wider than the previous ones. The 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 (see Diagram 23). 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 23



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.

Adams one-slide mailers of cardboard A-1630, $26.00 per M.

Adams two-slide mailers of cardboard A-1625, $37.00 per M.

Mailing envelopes, $12.50 per M.

It is probably safer to apply a single layer of cardboard to each side of these mailers, held in place by tough wrapper paper, than to rely solely on the envelopes.

It should always be remembered that the specimen of blood on a slide represents 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 by loss or breakage of slides.

Adams four-slide mailers of cardboard A-1615, at $0.55 each can be very useful to field workers in protecting slides from flies, etc., until dry, at which time the same slides are incorporated into temporary packages.

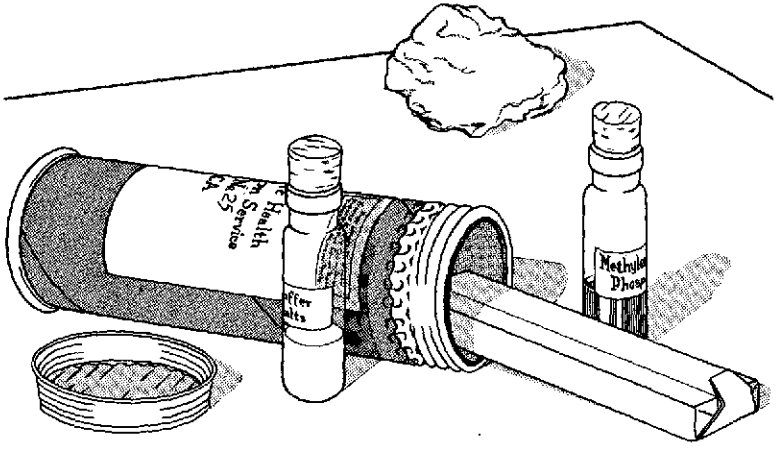
With Adams map-form slide trays for 20 slides A-1605, $12.60 per dozen, student or training slides may be quickly distributed and collected.

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

Positive slides and other exceptional material of special interest should be stored in a uniform manner and plainly labelled as individuals and groups in such a way that individual slides 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.

DIAGRAM 24



This same container may be used for slides and tubes destined for collaborators whose location does not permit the slides with blood to 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″ (see Diagram 24). One vial contains 3-5 cc. of methylene blue phosphate solution and the other sufficient dry buffer salts (0.2 gm.) to dissolve in a Coca-Cola bottle of distilled or rain water, which can be poured into a clean cup or glass. The former is poured over the blood specimen for one second only and the slide is then rinsed in the buffer water until only a trace of the red color remains.

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, in order that no undue amount of time is lost in the useless scanning of negative bloods.

In order for a laboratory service to function satisfactorily, it is important that all supervisory personnel know just 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 general quality of the work is affected. One example of this is when a worker calls for or uses something from an unlabelled bottle 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 and should be selected for aptitude and energy, rather than for degrees and formal training.

He should devote the 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, and he should appreciate the limitations of this equipment. He should insist on a high degree of cleanliness; he should understand, and attempt to straighten out the difficulties involved in maintaining the same. He should personally investigate any failure of methods or materials and correct them himself. He should be the key person in recruiting 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.

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

Cleaners. This personnel should be well instructed in special precautions required for 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—General.

The training of personnel should not be limited to a formal introductory course; it should be a continuous process. The student requires at least six months at a bench looking at routine slides in order to become acquainted with all the variable appearances of normal blood. The central laboratory can function as a training ground or as a sort of place of 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 minimize 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 more precise diagnosis. The latter includes three distinct stages of falciparum. Instead of reporting "mixed infections," the dominant and subordinate species may be clearly indicated with abbreviations, e.g., F/8M/100=two plus falciparum with 8 malariae forms per 100 fields. Since the non-falciparum species all show the various stages of development in the peripheral blood no matter when the blood is taken, and moreover 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, M, and O.

The beginner should be exposed to only the best possible study material until species diagnosis is thoroughly understood. The quality of specimen slides may 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 of understanding that the individual parasites are dynamic, growing, individual animals which react to their environment like any other animals and can vary greatly in appearance, but that the behavior of the infection as a whole has very definite patterns.

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 entomological laboratory work is also done. Whenever possible, the diagnosis laboratory should be apart from all other activities because of the na-

ture of the work done and because a quiet environment is most desirable.

The objective of such a laboratory is the competent examination of as many blood specimens as possible. Because of the increasing absence of positive bloods to stimulate the interest of the workers, the work becomes progressively tedious. Within limits, everything possible should be done for the comfort of those engaged in this rather tiring work.

A large, well-ventilated and well-illuminated room is essential. 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 pursued. In humid climates, a "warm closet" should be provided from the beginning. This should be of a size adequate to store at night the number of microscopes in use. It should be equipped with electric bulbs or some other heating device so as to maintain a consistent temperature not to exceed 35° C. This will effectively prevent the deposition of fungus on lenses or prisms.

Solidly constructed tables and/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 desk or bench 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 incident to constant movement.

Provision for a constant and adequate supply of distilled water should be made. 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.

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.

1.5x and 1.6x
1.25x
1x
5x eyepieces
6x
7x, 7.5x, or 8x

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); 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, less 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 4).

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.

1 small bottle for Shillaber's B. immersion oil or Crown oil (ordinary 20 cc. applicator bottles, with glass rod applicator, 75¢ to $1 per doz., 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 in. (see Diagram 19).

2 soft No. 1 lead pencils.

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

1 pointed Bard-Parker blade in cork of square 2 oz. bottle with ¾ in. mouth.

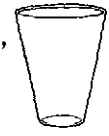
1 sheet 00 or 000 emery paper to maintain sticker bright, clean, and *sharp*.

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 straight table (or plastic) glasses, with perpendicular  rather than inclined sides,  for buffer wash water.

1 30-cc. bottle *good* Giemsa stain, or plastic dropping bottle.

1 small plastic dropper, held upright and dry in small heavy bottle.

1 10-25 cc. graduate (preferably plastic) test tube; test tubes marked at 5, 10, and 15 cc. work just as well.

1 curved staining plate or equivalent with 4-6 mm. depression, or 1 white enamel rectangular basin (see Diagram 17-D and E).

1 ordinary glass or plastic bottle to hold 500, 750, or 1,000 cc.

1 bottle mixed phosphate salts and 6 small tubes with corks to contain 0.5, 0.75, or 1.0 gm. of mixture.

1 block hard wood ¾ x 1 x 6 in. with transverse saw cuts about 1.3 mm. in width and 5 mm. deep.

1 package paper towels.

1 *plastic box* for 25 slides *(if possible)* to hold demonstration thick blood films.

1 Gem type razor blade.

2 pieces ordinary package cardboard (corrugated). Sundry pieces 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 miscellaneous microscopes which lack intermediate oculars are to used.

1 object marker to circle microscopic objects.

12-24 250-300 cc. Florence flasks.

1 Ishihara-type color vision test chart.

A collection of wood blocks 1 cm. and 2 cm. thick—6-8 cm. x 6 cm. long.

Spare corks for all bottles, especially small Giemsa work bottles.

1 30-60 cc. dropping bottle for pure toluene for each three students.

1 oblong enamel pan for staining, 14 x 10 x 3 in. for each three students.

1 pint Cargille or Crown oil (thinner grade).

Giemsa—either stock solution of a proven high grade Giemsa or:

- 4 x 5 gms. of a tested lot of Giemsa powder such as National Aniline NGe16.
- 3 x 1 lb. best quality acetone-free pure methyl alcohol, $2.00 each.
- 2 lbs. best quality pure glycerin.
- ½ lb. solid glass beads.

Buffer Salts:

8 x ¼ lb. anhydrous Na_2HPO_4.

1 lb. fine powder or crystals KH_2PO_4.

10 grams methylene blue, medicinal.

1 2-liter bottle containing chromic acid (potassium dichromate and sulfuric acid).

1 7.5 cm. funnel for pouring back same 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.

THE "DEADLY SINS" IN THICK FILM MICROSCOPY

1. POOR RESOLUTION
2. INADEQUATE ILLUMINATION
3. FAILURE TO CONSTANTLY MOVE THE FINE ADJUSTMENT
4. ALLOWING LEUKOCYTES APPARENTLY TO MOVE RADIALLY
5. FAILURE TO FAMILIARIZE YOURSELF WITH THE APPEARANCE OF LEUKOCYTES, PLATELETS, AND REMAINS OF YOUNG RED BLOOD CELLS SINCE THESE WILL BE 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