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Laboratory Diagnosis of PROTOZOAN DISEASES

BY

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PREFACE TO THE SECOND EDITION

DURING World War II it is safe to say that hundreds of thousands of our soldiers became infected with parasitic protozoa causing disease in man. This is especially true of the malaria plasmodia and *Endamœba histolytica*, causing amebiasis. The return of these men undoubtedly will cause an increase in such infections in this country and will render a prompt diagnosis imperative if we are to prevent their spread. Fortunately, during the war years there was a great increase in research upon diagnostic methods and a great many new and improved methods were discovered which have proven invaluable in the diagnosis of protozoan infections. This, together with the exhaustion of the first edition of this work has rendered it necessary to prepare a second edition, and it is believed that in this edition there are included all methods of diagnosis evolved during the war that have proven of value in protozoan infections. If the new edition proves as useful to physicians and laboratory technicians as the old the writer will be much gratified.

Grateful appreciation is given all who have aided the writer in the preparation of the new edition, to the authors from whose works he is indebted for the material contained in this book, and to his publishers for their whole-hearted coöperation. Special thanks are due the National Institute of Health and its Director, Dr. R. E. Dyer, U. S. Public Health Service, for permission to use the color plates illustrating thick blood film preparations of the various malaria plasmodia.

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PREFACE TO THE FIRST EDITION

THIS book is intended as a manual of laboratory methods for the diagnosis of diseases caused by protozoan organisms. These diseases are among the most important infections affecting man and include amebiasis and amebic dysentery, kala-azar, Oriental sore, espundia, African sleeping sickness, Chagas' disease, the malarial fevers and balantidiasis, and all of them are dependent upon laboratory methods for an accurate diagnosis. In an experience covering over fifty years, the author has often felt the need of a work devoted entirely to laboratory methods available for the diagnosis of these diseases and, for this reason, has undertaken the preparation of this volume. Many valuable diagnostic methods, in various languages, are hidden in the pages of medical journals and have never reached our text-books upon clinical diagnosis, or texts upon bacteriology and parasitology. In addition, the descriptions of even well-known methods of diagnosis in many of our texts upon clinical diagnosis are so brief, owing to lack of space, as to be difficult of interpretation and application, and this is true especially of protozoölogical methods, which differ greatly in technique from those of bacteriology.

In this work the author has endeavored to include all methods that have a real claim to value in the diagnosis of protozoan diseases, and has included critical remarks regarding their usefulness, as judged by his own experience and that of others. At the end of each section he has also added a "Critique of Diagnostic Methods" and a suggested scheme for a diagnostic procedure based upon his own experience. The opinions therein expressed are his own and he is responsible for them.

It is unnecessary to state that, in a work of this character, it has been necessary to compile from that of many authorities and the author has endeavored to give credit where due, in every instance. If this has not been done it is because of an oversight and not intentional. To all the many scientists who have developed the various diagnostic methods included in this volume the author desires to express his obligations. He is especially indebted to Dr. Ernest Carroll Faust for

permission to use illustrations from our text-book entitled "Clinical Parasitology" and to his publishers, Lea & Febiger, for many courtesies and for valuable assistance in the preparation of the book.

It is the earnest wish of the author that the book may be of real value to physicians who conduct their own clinical laboratories, to public health and other laboratories and, especially to laboratory technicians upon whom devolves the responsibility of preparing the material necessary for an accurate diagnosis of protozoan diseases.

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LABORATORY DIAGNOSIS OF PROTOZOAN DISEASES

INTRODUCTION

PARASITES belonging to the Protozoa are responsible for several important diseases in both man and animals, the most important infections in man caused by them being amebiasis; African sleeping sickness, or African trypanosomiasis; Chagas' disease, or South American trypanosomiasis; kala-azar; Oriental sore; espundia; and the various types of malaria infection. As the diagnosis of these diseases must usually be based upon the demonstration of the respective causative organism, a knowledge of the morphology of these protozoa and of the laboratory methods available for their diagnosis is of great importance to the physician, public health officer and laboratory technician.

The Protozoa are animal organisms composed of a single cell in which are performed all of the functions of life. For the classification, epidemiology, symptomatology, life-cycle, and relation to disease of the protozoa living in man the reader is referred to the work by the author and Faust, entitled "Clinical Parasitology," for in this volume only the morphology of these parasites will be considered and the methods available for the laboratory diagnosis of the infections of which they are the causative agents.

Morphology of the Protozoa.—The cell forming the body of a protozoön is composed of a mass of protoplasm, known as the *cytoplasm*, which is divided into an outer portion, the *ectoplasm*, and an inner portion, the *endoplasm*. The ectoplasm performs the functions of motion, respiration, ingestion of food, excretion and protection, and from this portion originate the organelles concerned in motility, as *pseudopodia, flagella* and *cilia*. The endoplasm fulfills the functions of nutrition and reproduction and contains the *nucleus, food vacuoles, chromatoidal bodies*, and ingested material, as starch granules, vegetable cells, red blood corpuscles, bacteria, or other ingested material.

From a diagnostic standpoint, in certain infections the most important body contained within the endoplasm is the *nucleus*, and as some species of the Protozoa, especially the amebæ, are classified almost entirely upon the structure of the nucleus, it is essential that the morphology of this body be clearly understood by the diagnostician. While the nucleus varies considerably in structure in different genera and species of the Protozoa, the following description of a typical protozoan nucleus will serve as a basis for the understanding of the

structure of this important part of the protozoan cell. It should be understood that the various structural elements mentioned are visible only in properly stained preparations and not in the living, unstained organisms.

The nucleus lies within the endoplasm, is circular in shape, and surrounded by a definite membrane, varying in thickness in different species, which is called the *nuclear membrane*. Upon the inner side of this membrane *chromatin granules* may be collected, either in the form of a uniform layer or as irregular, larger masses. At or near the center of the nucleus is a very deeply stained granule or rounded mass, known as the *karyosome*, composed of plastin and chromatin, which differs markedly in morphology in different species of the Protozoa. Between

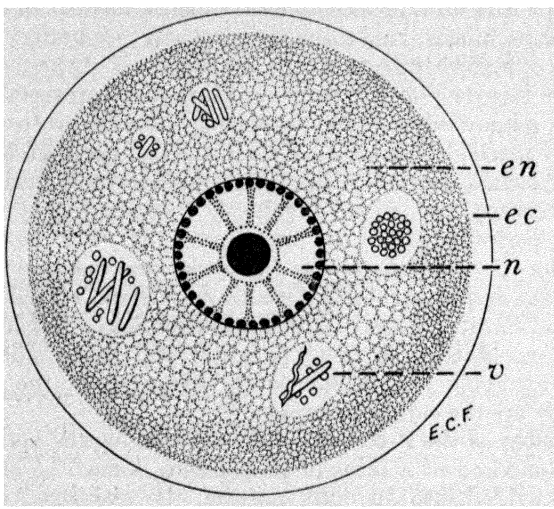


FIG. 1.—Schematic diagram of a protozoan cell, showing *n*, *nucleus*, with central *karyosome*, chromatin beads within nuclear membrane, and *achromatic fibrils* extending from karyosome to periphery; *en*, *endoplasm*, made up of a denser, granular meshwork and intermediate lighter cytoplasm, with numerous food vacuoles, *v*; and *ec*, *ectoplasm*, consisting of agranular or very finely granular cytoplasm. (Original, Faust.) (From Clinical Parasitology, Craig and Faust.)

the karyosome and the nuclear membrane, and connecting these structures, there is a delicate network of fibrils, known as the *linin network*, and chromatin granules may be observed upon the nodal points of this network in some species. A centrosome is sometimes present either within the nucleus or close to it within the endoplasm.

In many protozoa, in addition to the nucleus, the cytoplasm contains an accessory body, called the *kinetoplast*, often referred to as the *micronucleus*, while the *nucleus* is called the *macronucleus*. The kinetoplast consists of two portions, a minute oval or round granule, known as the *blepharoplast*, and a larger round, oval or rodlike body, known as

the *parabasal body*, the two being united by a delicate fibril. If a flagellum is present it arises from the blepharoplast and the portion of the flagellum so arising is called the *axoneme*, or root of the flagellum.

Chromatoidal bodies sometimes occur in the endoplasm of certain species of protozoa, especially within the cysts of the parasitic amebæ. These bodies present the staining reactions of chromatin and may be distributed throughout the endoplasm or collected in certain portions in the form of rodlike bodies with rounded ends or as filamentous or crystalline-like bodies. *Vacuoles* occur within the endoplasm of many species of protozoa which are known as *food-vacuoles* but contractile vacuoles do not occur in any of the parasitic species. As already stated, the endoplasm may contain *ingested material*, as red blood corpuscles, tissue cells, leukocytes, bacteria, starch granules, vegetable cells and crystals.

Certain organelles have been developed in the Protozoa which have to do with motility and the procurement of food. These vary in structure in the various genera and species but all originate in the ectoplasm. In the class Rhizopoda these organelles consist of prolongations of the ectoplasm known as *pseudopodia;* in the class Mastigophora long whip-like filaments are present, which are called *flagella*, while in the class Ciliata, the entire body of the organism is covered with short, thread-like filaments, known as *cilia*.

Reproduction in the Protozoa.—As the morphology of the Protozoa during reproductive activities is important in the diagnosis of genera and species it is necessary to briefly mention the various methods of multiplication that occur in the parasitic species.

All methods of reproduction are initiated by the division, or fission, of the parent cell, which is preceded by the division of the nucleus. In the Protozoa reproduction may be *asexual* or *sexual* in character. In *asexual reproduction* simple fission of the cell occurs, the nucleus first dividing, followed by the division of the organism into two or more organisms, known as *trophozoites*. If more than two organisms result from this method of division the process is known as *schizogony*. In *sexual reproduction* there usually occurs an alternation of generations and a life-cycle completed in two different hosts, reproduction in one host being asexual in type while in the other it is sexual. So far as man is concerned, the protozoan parasites having such a method of reproduction pass the asexual cycle in a vertebrate host and the sexual cycle in an invertebrate host. The union of the sexually differentiated cells is called *syngamy*. The male cells are known as *microgametocytes* and the female cells as *macrogametocytes*, while collectively they are called *gametocytes*, and the cell resulting from the union is known as a *zygote*. Prior to the formation of the latter, the macrogametocyte undergoes certain changes fitting it for fertilization and is then called a *macrogamete*, while the microgametocytes extrude slender, active motile filaments, known as *microgametes*, which penetrate and fertilize the

EXPLANATION OF PLATE I.*

Partly schematic. Rearranged and drawn by Williams. All stained by Giemsa.

I. FLAGELLATES.

FIG. 1.—Illustrating one flagellum Leishmania: *A*, Intracellular forms; *B*, cultural forms.

FIG. 2.—Illustrating undulating membranes: *A*, Trypanosoma lewisi; *B*, Trypanosoma brucei; *C*, Trypanosoma gambiense.

FIG. 3.—Illustrating two flagella. Bodŏ lacertæ (after Prowazek).

FIG. 4.—Illustrating four flagella. Trichomonas.

II. AMŒBÆ.

Illustrating points considered differential in the two common types of amœbæ (endamœbæ) described as parasitic in human beings.

FIG. 1.—Endamœba coli, vegetative stage.

FIG. 2.—Dividing nucleus.

FIG. 3.—Endamœba coli cyst containing eight nuclei.

FIG. 4.—Endamœba histolytica, vegetative stage.

FIG. 5.—Four-nucleate cyst.

III. SPOROZOA.

A, description of Figs. 1 to 16. (After Schaudinn.) The life cycle of Eimeria schubergi. In 1, the sporozoites, becoming free by bursting the sporocysts, pass out through an aperture in the wall of the oöcyst, and are ready to enter the epithelial cells of the host. 2 to 6 represent the asexual reproduction or schizogony, commencing with infection of an epithelial cell by a merozoite or a sporozoite; the merozoite after stage 6 may start again (5) at stage 2, as indicated by the arrows, or it may go on to the formation of gametocytes (9 to 11). 9 to 11 represent the sexual generation, the line of development becoming split into two lines—male (♂) and female (♀) culminating in the highly differentiated gametes, which conjugate and become again a single line, shown in 12–14. The zygote thus formed goes on to the production of spores, 15 and 16. 2 and 3 represent epithelial cells showing penetration of a merozoite or a sporozoite and its change into a schizont; 4, the nucleus of the schizont divided into numerous daughter-nuclei; 6, segmentation of the schizont into numerous merozoites, about a central mass of residual protoplasm, which in this figure is hidden by the merozoites; 5, merozoites passing to reinfect host cell and repeat the process of schizogony; 7, 8, merozoites to be differentiated into male and female gametocytes; 9, the two gametocytes within a host cell (the microgametocyte (♂) has fine granulations; the macrogametocyte (♀) has coarse granulations); 11, a female gametocyte undergoing maturation; 13, mature macrogamete, free from the host cell, and sending a cone of reception toward an approaching microgamete. In 12 the nuclei of the last stage have become microgametes, each with two flagella. The free microgametes are swimming to find a macrogamete. In 14, the zygote (fertilized macrogamete) is surrounded, by a tough membrane or oöcyst, which allows no more microgametes to enter and contains the female chromatin, which is taking the form of a spindle, and the male chromatin in a compact lump; in 15, the nucleus of the zygote divided—the nuclei of the sporoblasts; in 16 the four sporoblasts become distinct, leaving a small quantity of residual protoplasm; each sporoblast has formed a membrane, the sporocyst. Within each sporocyst two sporozoites form about a sporal residuum.

B, Babesia infecting red blood cells: 1, pear-shaped bodies; 2, dividing forms; 3, eight pear-shaped bodies in a cell; 4, irregular ring-like bodies; 5, large, irregular body; 6, body with a flagellum-like projection.

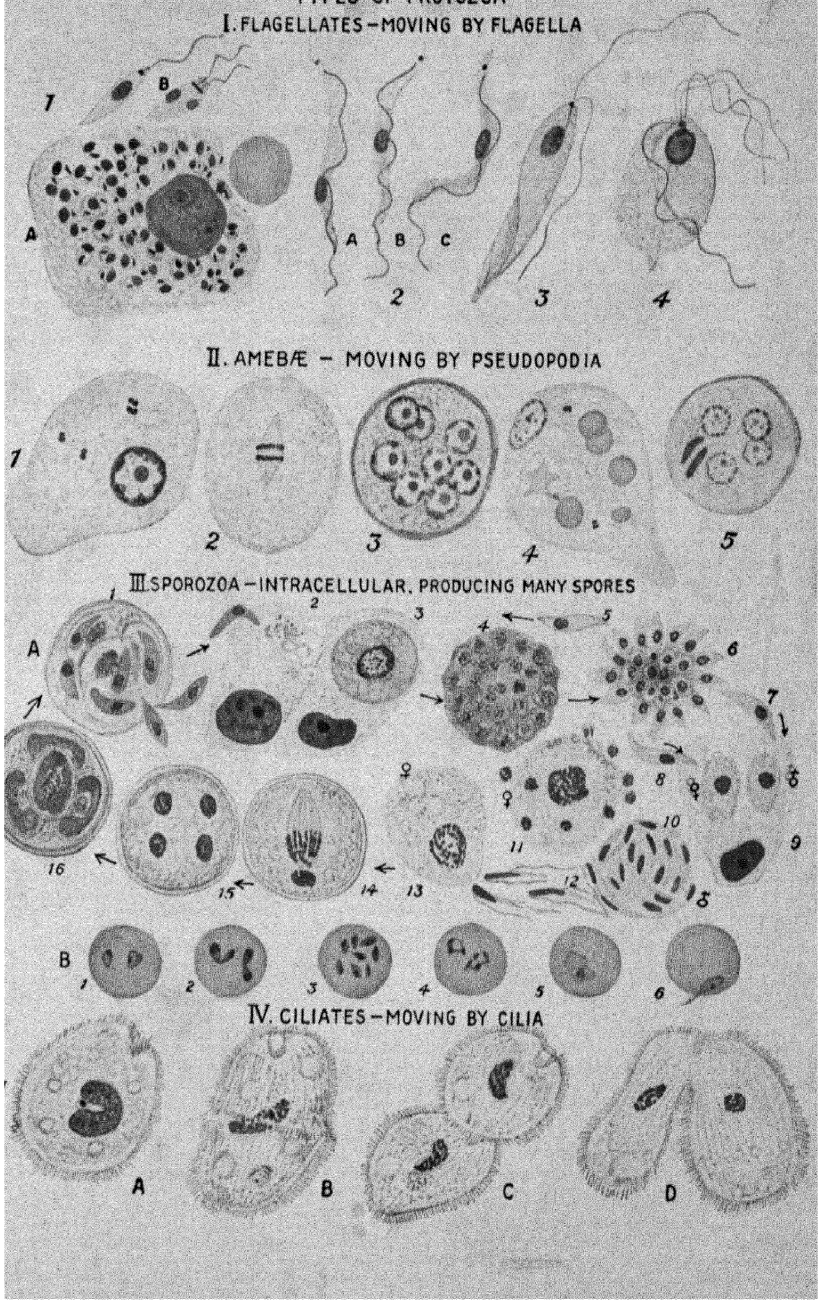
IV. CILIATES.

FIG. 1.—Balantidium coli (after Hartmann): *A*, adult form; *B*, *C*, dividing forms; *D*, conjugating forms.

* From Park and Williams' Pathogenic Microörganisms.

PLATE I

TYPES OF PROTOZOA



macrogametes. These bodies are collectively called *gametes*. After fertilization the zygote encysts and in it are produced many motile spindle-shaped bodies known as *sporozoites*. The latter bodies, reaching a suitable host, develop eventually into trophozoites and initiate the asexual cycle of reproduction.

In many of the Protozoa reproduction occurs within a *cyst*, formed by the trophozoite secreting a resistant wall about itself, the division of the nucleus occurring after the cyst-wall is fully developed. Cyst formation occurs when conditions are unfavorable for simple fission of the trophozoites and may be purely protective in nature, but in the parasitic protozoa division usually occurs within the cyst or following the exit of the organism from the cyst when conditions become favorable for asexual development.

During the various methods of reproduction mentioned the morphology of the various organisms varies greatly and will be described in the discussion of the morphology of each parasite in the following pages.

PART I

Laboratory Diagnosis of Amebiasis and Intestinal Flagellate Infections

CHAPTER I

THE LABORATORY DIAGNOSIS OF AMEBIASIS. MORPHOLOGY OF THE INTESTINAL AMEBÆ OF MAN

Introduction.—Amebiasis, or infection of man with *Endamœba histolytica*, includes all conditions produced in the human host by this ameba, from the non-symptomatic, so-called "carrier state" to the clinical entities known as amebic diarrhea, dysentery, hepatitis, abscess of the liver or other organs, and amebic invasion of the skin. In order to accurately diagnose this infection it is essential that one be thoroughly familiar with the morphology not only of *Endamœba histolytica* but of the other amebæ living in the intestine of man, *i. e.*, *Endamœba coli*, *Endolimax nana*, *Iodamœba bütschlii* and *Dientamœba fragilis*. While there is little or no evidence proving that any of the latter organisms are pathogenic, their differentiation from *Endamœba histolytica* is always involved in the diagnosis of amebiasis.

1. MORPHOLOGY OF ENDAMŒBA HISTOLYTICA

The importance of an accurate knowledge of the morphology of *Endamœba histolytica* cannot be overestimated, for while other diagnostic methods for infection with this parasite are available it is still true that the most reliable diagnostic method consists in the demonstration of the parasite by means of a study of its morphology.

Endamœba histolytica has four distinct stages in its life-cycle in man and other animals, *i. e.*, a vegetative, or trophozoite, a precystic, a cystic and a metacystic stage. The trophozoite is the motile stage and is present in the intestine when conditions are favorable for multiplication, while the precystic and cystic stages are present when such conditions are absent, at which time the trophozoite becomes motionless, rounds up, and secretes a cyst wall, eventually becoming a cyst. The morphology varies in all of these stages with the exception of the metacystic stage and it is necessary that one be acquainted with these variations in order to be able to differentiate this species from the other species of amebæ occurring in man.

The morphology of *Endamœba histolytica* and of the other intestinal amebæ may be studied in freshly prepared, unstained preparations or in preparations stained by various methods.

Morphology of Endamœba Histolytica in Unstained Preparations

The Trophozoite or Motile Ameba.—The motile form, or trophozoite, of *Endamœba histolytica* usually occurs only in semi-fluid or fluid stools although it may sometimes be observed in the mucus occurring upon fecal masses which have passed over acute amebic ulcerations, removing infected material from such lesions.



FIG. 2.—*Endamœba histolytica*. Unstained preparation of stool. Motile trophozoites, four in number, the three situated in the lower portion of the photomicrograph containing red blood corpuscles. Note clear pseudopodia. × 750. (Army Medical Museum Collection. Preparation by Craig. Craig and Faust, Clinical Parasitology.)

The trophozoite of this species is a hyaline, colorless body varying in size from about 15 to 60 microns in diameter, the average being from 20 to 25 microns in diameter. Smaller amebæ of this species may rarely be observed in the stools of symptomless patients while the stools of patients suffering from very acute amebic dysentery may contain very large amebæ, but the vast majority of amebæ of this species average about 23 microns in diameter.

In freshly passed fecal material the trophozoite of *Endamœba histolytica* is actively motile, at which time the ameba is divided into two distinct portions, a very refractile outer portion forming a pseudopodium which is projected in front of the organism, known as the *ectoplasm*, and a less refractile portion, comprising about two-thirds of the organism, known as the *endoplasm*, which during motility flows into

the ectoplasmic pseudopodium, producing the form of motion known as *ameboid motion*. The ectoplasm is homogeneous in appearance while the endoplasm is granular, resembling ground-glass. In this portion of the ameba there may sometimes be seen a ring of minute refractile granules representing the *nucleus* which may change its position in the endoplasm with the movements of the organism. In rapidly moving organisms the nucleus is not visible but may become visible as motility decreases and the parasite begins to degenerate, while in amebæ that have undergone marked degeneration the remains of the nucleus may be seen as a refractile mass lying within the granular and vacuolated endoplasm.

Besides the nucleus the endoplasm may contain various objects that have been ingested by the ameba, but in this species, unless degeneration is occurring, the endoplasm never contains bacteria, which helps in differentiating it from the other intestinal amebæ which invariably contain bacteria. The endoplasm may also contain starch granules, as well as red blood corpuscles, if free blood be present in the intestine. The occurrence of red blood corpuscles within this ameba is a very valuable diagnostic feature, for while these cells have been very rarely observed within other species of intestinal amebæ, it so rarely occurs that their presence within an ameba is practically diagnostic of *Endamœba histolytica*. Organisms containing red blood corpuscles are most frequently seen in the stools of patients suffering from acute amebic dysentery, in which much mucus and blood are present, but sometimes amebæ containing these cells are observed in the stools of patients in whom only occult blood can be demonstrated in the stool.

Motility in *Endamœba histolytica* is accomplished through the protrusion of pseudopodia, formed by the ectoplasm. Such pseudopodia are long and finger-like, or shorter and rounded, and are very refractile. Movement is caused by the endoplasm flowing into the protruded pseudopodium and if motility is marked the normal distinction between the ectoplasm and endoplasm is lost and the organism resembles a slug moving across the microscopic field. When motility is active the ameba moves in a definite direction, the anterior extremity being rounded while the posterior is attenuated and often drags behind it a mass of bacteria or detritus. In such amebæ the nucleus is usually invisible in unstained preparations.

The typical motility of *Endamœba histolytica* can be observed only in the organisms in freshly passed stools, as exposure to room temperatures quickly slows motility and eventually results in loss of motion and in the degeneration of the organism. When motility is sluggish the pseudopodia may be slowly extruded from any portion of the periphery of the ameba and immediately retracted, without resultant motion, or the resultant motion is not in a definite direction and the organism shows no polarity.

The differential diagnostic features of the trophozoites of this species in unstained preparations are the clear distinction between the ectoplasm and endoplasm and the glasslike appearance of the former; the absence of bacteria within the endoplasm; the presence of red blood corpuscles within the endoplasm; the comparatively rapid motility and the marked polarity of the ameba when in motion.

The Precystic Ameba.—Prior to encystment the trophozoites of *Endamœba histolytica* lose their motility, round up and extrude ingested material from the endoplasm. At this time they are known as precystic amebæ and their morphology is not diagnostic, such precystic forms being frequently mistaken for those of other species of intestinal amebæ. The distinction between the ectoplasm and endoplasm is lost, pseudopodia are not extruded, and the nucleus may become distinctly visible, either as a ring of very refractile granules or a rounded refractile mass within the endoplasm. In some of the precystic amebæ oval or rodlike refractile bodies may be seen within the cytoplasm, probably identical with the chromatoidal bodies frequently observed within the cysts of this species.

Normally, the precystic forms eventually become cysts but if they are passed in the stools before the formation of the cyst-wall, they do not undergo any further development so far as is known.

The Cystic Ameba.—When conditions are unfavorable in the intestine for the reproduction of the trophozoites they finally encyst, after passing through the precystic stage described above. In unstained preparations the distinctive morphological features of the cysts are obscured but in stained preparations the morphology is very distinctive and the cysts of this species may be easily differentiated from those of other intestinal amebæ.

In unstained preparations the cysts measure from 6 to as much as 20 microns in diameter, are spherical in shape unless distorted by pressure, and may have a clearly defined cyst-wall presenting a double outline. Some strains of this species produce cysts of small size and others large, but both are equally virulent so far as is known. The cytoplasm is colorless and finely granular in appearance but the nuclei are very poorly differentiated in unstained preparations, although rarely they may be seen as rings of refractile granules or as circular refractile bodies without definite structure. In the younger cysts chromatoidal bodies may sometimes be seen, appearing as refractile, hyaline, oval or thick, rodlike bodies with rounded ends. Ingested material is not present and the cysts never contain red blood corpuscles.

A diagnosis of *Endamœba histolytica* should not be based upon the morphology of the unstained cysts as those of other species of intestinal amebæ closely resemble those of this species in unstained preparations.

The Metacystic Ameba.—The metacystic stage of development consists of the recently liberated organisms from the cyst. These are smaller than the fully developed trophozoites of the same strain, but

otherwise their morphology is the same as that of the trophozoites. They initiate the infection in the human intestine.

Morphology of Endamœba Histolytica in Stained Preparations

The Trophozoite.—In properly stained preparations the trophozoite of this species is easily differentiated from trophozoites of other amebæ. The distinctive morphology is best observed in wet-fixed preparations stained with one of the hematoxylin stains. In such preparations the cytoplasm of the trophozoite stains a grayish color

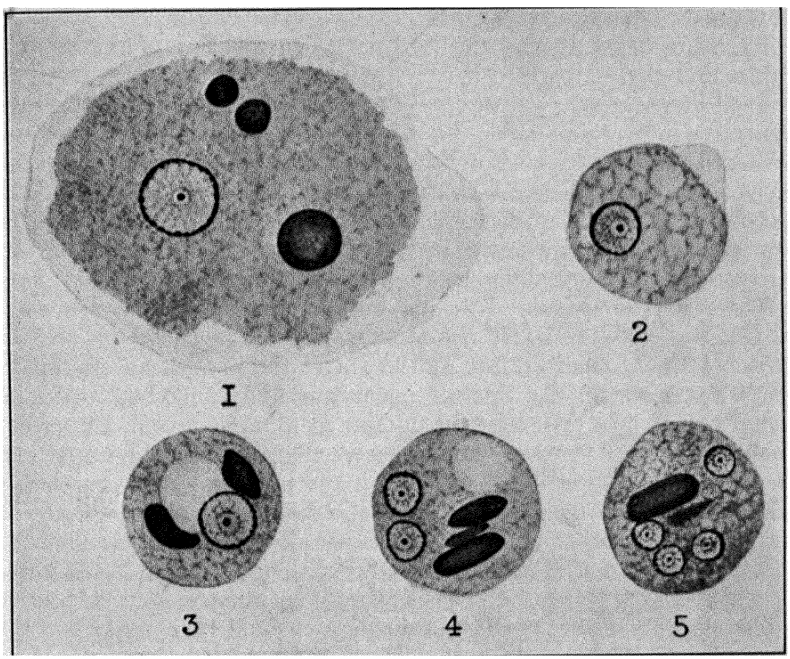


FIG. 3.—Stained specimens of *Endamœba histolytica*. *1*, Trophozoite, containing 3 red blood corpuscles; *2*, precystic form; *3*, *4* and *5*, uninucleate, binucleate and quadrinucleate cysts of *Endamœba histolytica*. (After Dobell and O'Connor, in "The Intestinal Protozoa of Man," courtesy of John Bale and Staples, Ltd., London.)

and the nucleus is well differentiated. The nuclear membrane appears as a very thin black ring, not over a line in thickness, upon the inner surface of which minute black stained chromatin granules are arranged in a single layer in a uniform manner. The karyosome of the nucleus consists of a minute black dot usually situated at the center of the nucleus but sometimes slightly to one side of the center. In very well stained specimens a delicate network of fibrils, stained brownish or black, may be observed between the karyosome and the nuclear

membrane, a narrow unstained area surrounding the karyosome. Chromatin granules are not present in this network as in some other amebæ.

Unless the trophozoites are undergoing degeneration, bacteria are not present in the endoplasm, but red blood corpuscles, appearing as yellowish-green bodies, may be present in the endoplasm and are practically diagnostic of *Endamœba histolytica*. The occurrence of these cells, together with the characteristic morphology of the nucleus, *i. e.*, the delicate nuclear membrane and the minute central karyosome, are the diagnostic points of importance in the differentiation of the trophozoite of this species from those of the other intestinal amebæ, in stained preparations.

Exposure to room temperature causes degeneration of the trophozoites and in such organisms the normal structure, as observed in stained preparations, is often greatly distorted, the nuclear membrane appearing much thicker, the chromatin granules lining it are larger and arranged in irregular masses instead of in a uniform layer, while the karyosome stains diffusely and appears much larger. Between the karyosome and the nuclear membrane chromatin granules may be present and the entire nucleus resembles quite closely that of *Endamœba coli*, rendering a differentiation impossible.

The Precystic Ameba.—The staining reactions of the precystic stage of *Endamœba histolytica*, in hematoxylin stained preparations, are like those of the trophozoite, or motile stage, the cytoplasm staining a grayish color while the nuclear membrane and karyosome stain intensely black. In this stage the nuclear membrane appears somewhat thicker and the karyosome a little larger than in the trophozoite, and in organisms undergoing degeneration the nucleus may very closely resemble that of *Endamœba coli*, so that a differential diagnosis may be impossible. Most of the precystic forms do not show any ingested material and do not contain red blood corpuscles. The precystic forms should be disregarded in diagnosis as many of them resemble those of other amebæ, and dependence in diagnosis should be placed upon the trophozoites or cysts which are always present along with precystic forms.

The Cystic Ameba.—The stained cysts of *Endamœba histolytica* are very characteristic and easily differentiated from those of other amebæ living in the intestine of man. In preparations stained with the hematoxylin stains, after wet-fixation, the cytoplasm stains a grayish color while the nuclear membrane, karyosome and chromatoidal bodies stain black, the cyst-wall remaining unstained and appearing as a hyaline capsule surrounding the cyst.

The size of the cysts varies from 6 to 20 microns in diameter in stained preparations and large and small cysts may occur in the same preparation, although usually the cysts approximate one another in size in each individual case. The shape of the cyst is usually spherical,

but oval cysts may sometimes be observed and cysts also occur of irregular shape probably produced by pressure during the fixing or staining of the specimen.

When the hematoxylin stains are employed the nuclei within the cysts stain distinctly and usually vary in number from 1 to 4, the fully

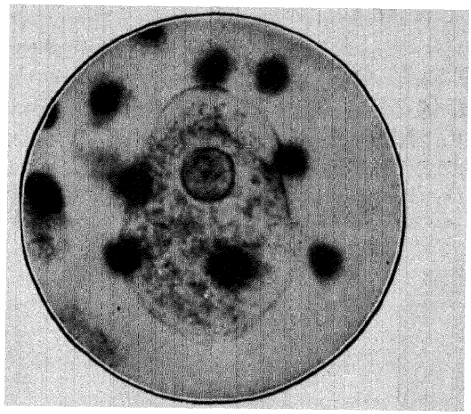


FIG. 4.—*Endamœba histolytica.* Trophozoites stained with iron hematoxylin, showing the typical nuclear structure of this species. Note the delicate nuclear membrane, lined with minute chromatin granules, and the minute central karyosome. × 680. (Army Medical School Collection Photomicrograph. Preparation by Craig. Craig and Faust, Clinical Parasitology.)

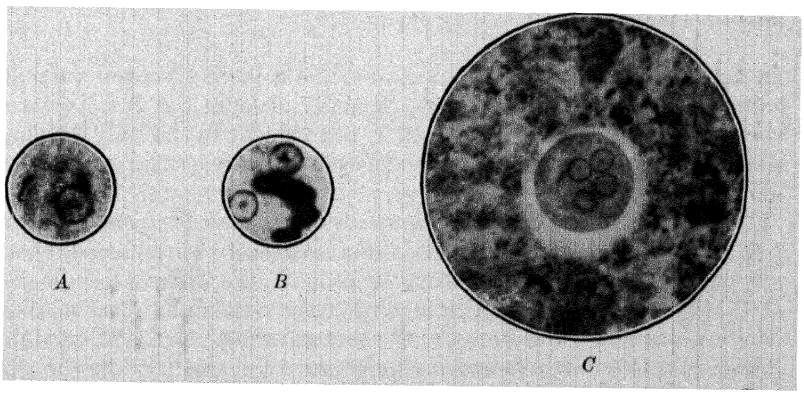


FIG. 5.—*Endamœba histolytica.* Stained with iron hematoxylin. × 750. *A*, Cyst containing one nucleus and chromatoidal body; *B*, cyst containing two nuclei and large chromatoidal mass; *C*, cyst containing four nuclei. (Army Medical School Collection Photomicrograph. Preparation by Craig. Craig and Faust, Clinical Parasitology.)

developed and typical cyst containing 4 nuclei. Cysts containing 1, 2 and 3 nuclei are also observed and, in very rare instances, cysts containing as many as 8 nuclei have been observed.

In the uninucleate and binucleate cysts the nuclear structure is like

that of the trophozoite, the nuclear membrane being about a line in thickness and staining intensely black while the karyosome consists of a minute black dot usually located at the center of the nucleus but sometimes slightly to one side of the center. Very minute black stained granules of chromatin may be seen upon the inner side of the nuclear membrane in well stained preparations. In the cysts containing 3 to 4 nuclei the nuclear structure is not as well defined owing to the smaller size of the nuclei. In such cysts the nuclear membrane is very delicate and the karyosome very minute, while definite chromatin granules cannot be distinguished lining the nuclear membrane. As the cyst is a spherical body it is generally impossible to demonstrate all of the four nuclei in the same microscopic plane so that it is necessary to focus up and down upon the cyst in order to bring all of the nuclei into view.

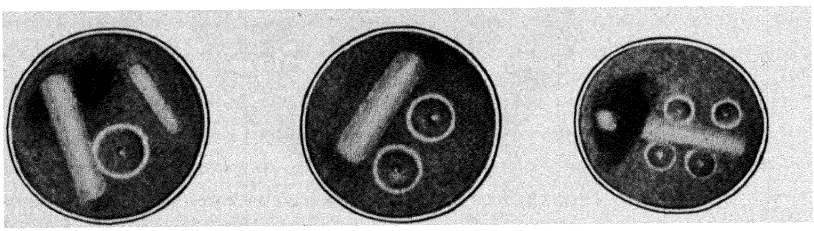


FIG. 6.—*Endamœba histolytica.* A uninucleate, binucleate and quadrinucleate cyst as they appear in iodine-stained preparations. The cytoplasm of the cysts stains a greenish-yellow while the nuclear membrane, the karyosome and the chromatoidal bodies are unstained, appearing as hyaline bodies. (Boeck and Stiles, Bull. No. 133, Hygienic Laboratory, U. S. Public Health Service, Gov't Printing Office, Washington, D. C.)

In the uninucleate and binucleate amebæ a large glycogen vacuole is sometimes observed, which may partially obscure the nuclei or crowd them to one side, but this vacuole is not present in the fully developed four nucleate cyst. Ingested bodies are not present in cysts of this species.

In a certain proportion of the cysts of *Endamœba histolytica*, most frequently in the uninucleate and binucleate cysts, chromatoidal bodies are present which are of great importance in the differential diagnosis of this species. These bodies lie within the cyst and appear as plump rods with rounded ends, or oval or short spindle-shaped bodies, stained intensely black with hematoxylin stains. In small cysts the chromatoidal bodies may appear as thinner rods with rounded ends. In the writer's experience, these bodies vary from one to three in number and are sometimes merged together into an irregular black mass within the cyst. Red blood corpuscles never occur in the cysts of *Endamœba histolytica.*

In *iodine stained* preparations, which are usually employed in routine diagnosis, the cysts of *Endamœba histolytica* can be differentiated from those of other intestinal amebæ by their characteristic appearance. In

such preparations the cytoplasm of the cysts stains a lemon- or greenish-yellow while the nuclear membrane appears as a refractile, delicate hyaline ring surrounding the nucleus and the karyosome as a minute refractile hyaline dot at the center of the nucleus. If a glycogen vacuole is present it stains a yellowish-brown and chromatoidal bodies appear as unstained refractile bodies within the cytoplasm. The cyst-wall remains unstained.

The characteristic and diagnostic features of stained cysts of *Endamœba histolytica* are the delicate nuclear membrane, the minute central karyosome, the number of nuclei, from 1 to 4, and the morphology of the chromatoidal bodies that may be present in the cysts. Size is of little importance as the cysts of other species of intestinal amebæ may be similar as regards their size.

The Metacystic Ameba.—The morphology of the metacystic amebæ in stained preparations is identical with that of the trophozoites but these organisms are much smaller than the fully developed trophozoite of the same strains.

2. MORPHOLOGY OF ENDAMŒBA COLI

Endamœba coli is a non-pathogenic species of ameba occurring in from 15 to as high as 70 per cent of normal individuals, being most common in tropical and sub-tropical countries where insanitary conditions are frequently observed. This species often occurs in conjunction with *Endamœba histolytica*, hence the importance of differentiating it from the latter species. It has four stages in its life-cycle in man, a trophozoite or motile stage, a precystic stage, a cystic stage and a metacystic stage, in each of which the morphology differs.

Morphology of Endamœba Coli in Unstained Preparations

The Trophozoite or Motile Ameba.—In unstained preparations the trophozoite of this species of ameba appears as a colorless, somewhat hyaline cell, usually measuring from 20 to 30 microns in diameter, although smaller and larger amebæ of this species are frequently observed. In freshly voided stools this ameba is sluggishly motile, as compared with *Endamœba histolytica*, moving very slowly about the microscopic field in an indeterminate direction, seldom exhibiting long-continued polarity. Frequently the trophozoite remains stationary, the pseudopodia being protruded and withdrawn without resultant motility. The pseudopodia are less glasslike in appearance than those of *Endamœba histolytica* and are rather short and blunt instead of long and finger-like as in the latter species. There is very little distinction between the ectoplasm and endoplasm during motion and the endoplasm is much more granular in appearance than that of *Endamœba histolytica*. In this species the nucleus is usually visible in the unstained organism, appearing as a ring of large refractile hyaline granules, repre-

senting the nuclear membrane, and containing a large, refractile hyaline mass to one side of the center of the nucleus, representing the karyosome.

Ingested material is present in the endoplasm of this species in the form of bacteria, vegetable cells, starch granules and crystalline material but red blood corpuscles are not normally ingested by *Endamœba coli.* Very rarely these cells have been observed within this ameba but, from a practical diagnostic standpoint it is a perfectly safe rule *to regard any ameba containing red blood corpuscles as a trophozoite of Endamœba histolytica.* In *Endamœba coli* bacteria are invariably present in the endoplasm of the trophozoite, thus differentiating it from the trophozoite of *Endamœba histolytica,* in which bacteria are never present unless the organism is undergoing degeneration. *Endamœba coli* ingests and apparently feeds upon bacteria while in the case of *Endamœba histolytica* bacteria invade the ameba after degeneration of the cytoplasm occurs.

Numerous food vacuoles are usually present in the cytoplasm of *Endamœba coli,* many of the vacuoles containing bacteria or other ingested material. In degenerating organisms of this species the nucleus often appears as a large collection of hyaline refractile granules lying in the endoplasm, which is filled with vacuoles. In such amebæ progressive motility is entirely absent although pseudopodia may be protruded and retracted if degeneration has not progressed sufficiently to kill them.

The diagnostic features in the morphology of unstained trophozoites of *Endamœba coli* are the lack of distinction between the ectoplasm and endoplasm when the organism is moving, the presence of a visible nucleus, the absence of red blood corpuscles from the endoplasm when blood is present in the stool, the presence of numerous bacteria within the ameba, and the sluggish ameboid activity as compared with the activity of *Endamœba histolytica.*

The Precystic Ameba.—In unstained preparations the precystic forms of *Endamœba coli* are smaller than the trophozoites, usually measuring from 12 to 35 microns in diameter. Such forms are circular in shape, motionless, and no distinction can be seen between the ectoplasm and the endoplasm. All ingested material has been extruded and the nucleus may be obscured or may be seen as a refractile ring of large hyaline granules enclosing a glistening colorless dot situated to one side of the nucleus. At this stage of development it is often impossible to differentiate this species from *Endamœba histolytica* and a diagnosis should never be based upon the morphology of precystic forms alone.

The Cystic Ameba.—In unstained preparations the cysts of *Endamœba coli* have a well-defined cyst-wall with a double outline, appearing as a colorless, hyaline capsule surrounding the organism. The cysts are usually larger than those of *Endamœba histolytica,* measuring from

10 to 33 microns in diameter, some strains producing large cysts while others produce small ones. The cysts have a granular appearing cytoplasm and ingested material is not present. Nuclei may be visible, especially in the uninucleate and binucleate cysts, and chromatoidal bodies may be seen, appearing as refractile filamentous or needle-like bodies or refractile thin rods, within the cytoplasm. The nuclei present a refractile nuclear membrane and a large refractile dot situated to one side of the center of the nucleus. The number of nuclei usually varies from 1 to 8, but cysts have been observed containing as many as 16 to 32 nuclei. A large glycogen vacuole is often present in the cysts of this species, especially in the uninucleate and binucleate cysts.

The diagnostic features of the unstained cysts of *Endamœba coli* are their comparatively large size, the very definite cyst-wall having a double outline, and the presence within the cyst of from 1 to 8 nuclei, having an eccentrically placed karyosome.

The Metacystic Ameba.—The morphology of the metacystic ameba in unstained preparations is the same as that of the trophozoite but it is smaller and less motile.

Morphology of Endamœba Coli in Stained Preparations

The Trophozoite.—In preparations wet-fixed and stained with the hematoxylin stains the cytoplasm of this ameba stains a bluish-gray, while the nuclear structures stain an intense black. The nucleus presents a rather thick black nuclear membrane upon the inner surface of which black stained granules of chromatin, arranged in an irregular manner or in irregular masses, may be observed. The karyosome stains black and appears as a comparatively large, circular mass lying to one side of the center of the nucleus, and surrounded by a distinct unstained halo. In the area between the nuclear membrane and the karyosome, black stained granules of chromatin may be present, while in very well stained preparations traces of a linin network may be observed.

The cytoplasm contains food vacuoles and bacteria, staining black, are invariably present. The constant presence of bacteria within the cytoplasm of *Endamœba coli* is a very valuable diagnostic feature as between this species and *Endamœba histolytica*, as, in the latter species, bacteria are never present unless the organism is undergoing degeneration.

The Precystic Ameba.—The morphology of the precystic forms of *Endamœba coli* in stained preparations is similar to that of the trophozoite but in such forms ingested material is not noticeable, although bacteria are present. Many of these forms so closely resemble those of *Endamœba histolytica* that a differential diagnosis should not be based upon such forms alone.

The Cystic Ameba.—In the cysts of *Endamœba coli* stained with the hematoxylin stains the cytoplasm is colored a bluish-gray while the nuclear structures and chromatoidal bodies stain black. The morphology of the nuclei in the cysts is similar to that of the nucleus in the trophozoite but the nuclei become smaller as division occurs and in the smaller nuclei the morphology is somewhat different from that of the trophozoite nucleus. In the uninucleate and binucleate cysts the

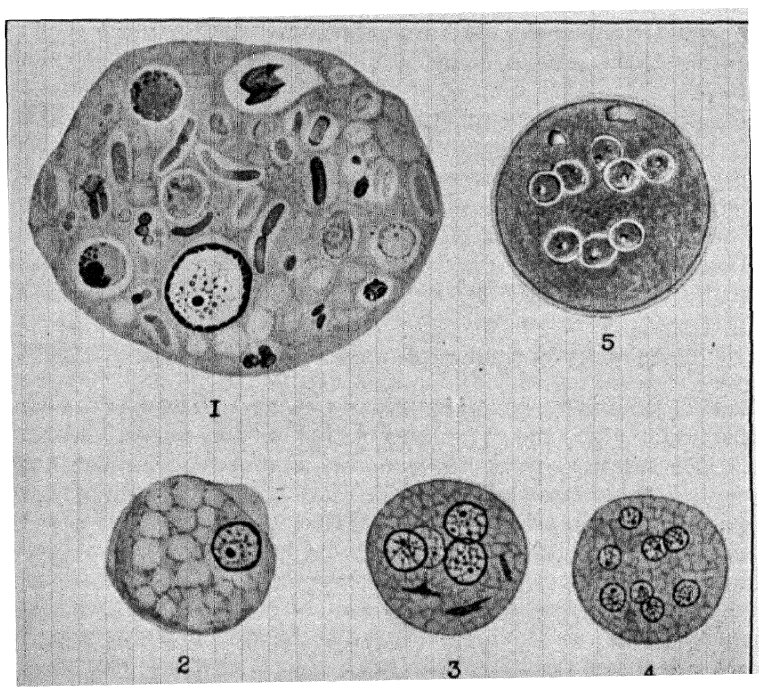


FIG. 7.—*Endamœba coli.* *1*, Trophozoite of *Endamœba coli: 2*, precystic form of *Endamœba coli; 3*, four nucleated cyst with chromatoidal bodies; *4*, eight nucleated cyst; *5*, iodine stained cyst, the nuclear membrane and karyosome of each nucleus remaining unstained and appearing clear and hyaline. *1*, *2*, *3* and *4* stained with iron-hematoxylin. (After Dobell and O'Connor, courtesy of John Bale and Staples, Ltd.) (*5*, after Boeck and Stiles.)

nuclear membrane is thick and stains intensely black and contains upon its inner surface granules of black chromatin arranged in an irregular manner. The karyosome consists of a large black dot situated to one side of the center of the nucleus. In the cysts containing a larger number of nuclei the membrane appears thinner and the karyosome smaller, but even in such cysts the membrane and karyosome are more massive than in *Endamœba histolytica*. The eccentric situation

of the karyosome in the nuclei of the cysts of this species is a most valuable differential feature as the karyosome in *Endamœba histolytica* is central in location in the nucleus. The number of nuclei in the cysts of *Endamœba coli* usually varies from 1 to 8, but rarely cysts containing larger numbers of nuclei are observed. When division is unequal cysts may be seen containing 3, 5 or 7 nuclei, one of the nuclei often being twice the size of the others due to the fact that this nucleus has not undergone division.

The chromatoidal bodies within the cysts of *Endamœba coli* differ markedly from those occurring in the cysts of *Endamœba histolytica* in stained preparations. Such bodies are most frequently observed in cysts containing from 1 to 4 nuclei and are rare in the fully developed cyst containing 8 nuclei. They consist of filamentous, acicular or delicate rodlike bodies, stained black, distributed throughout the cytoplasm. In some instances the chromatoidal bodies occur in collections resembling a sheaf of acicular crystals. Ingested material is not present in the cysts. A large glycogen vacuole may be present which remains unstained in hematoxylin stained preparations. This vacuole often crowds the nuclei to one side of the cyst and occurs most frequently in cysts containing from 1 to 4 nuclei.

With the *iodine stains*, used routinely in diagnosis, the nucleus or nuclei in the cysts are clearly visible, the nuclear membrane appearing as a rather thick colorless hyaline border surrounding the nucleus while the karyosome appears as a glistening hyaline dot situated to one side of the center of the nucleus. With this stain the glycogen vacuole stains a distinct brown or brownish-red while the cytoplasm of the cyst stains a greenish-yellow.

With all stains the cyst-wall remains unstained, appearing as a colorless, hyaline doubly outlined capsule surrounding the cyst.

In the stained cysts of *Endamœba coli* the differential diagnostic features are the very definite cyst-wall having a double outline; the presence of from 1 to 8 nuclei; the comparatively thick nuclear membrane; the large, eccentrically situated karyosome and the characteristic chromatoidal bodies. In addition the cysts of this species are usually considerably larger than those of *Endamœba histolytica*.

The Metacystic Ameba.—The morphology in stained preparations is the same as that of the trophozoite but the organism is smaller than the trophozoites of the same strain.

3. MORPHOLOGY OF ENDOLIMAX NANA

Endolimax nana is a common intestinal ameba of man, occurring in from 10 to over 30 per cent of normal individuals throughout the world. It has four stages in its life-cycle, the trophozoite or motile stage, the precystic stage, the cystic stage and the metacystic stage. It is a nonpathogenic parasite in man, so far as is known.

Morphology of Endolimax Nana in Unstained Preparations

The Trophozoite or Motile Ameba.—The size of the unstained trophozoite of *Endolimax nana* is of importance in differential diagnosis, as this ameba is usually smaller than either *Endamœba histolytica* or *Endamœba coli*. The trophozoites measure from 6 to 15 microns in diameter, about half the size of the two other amebæ just mentioned, although amebæ of this species measuring as much as 16 microns in diameter may be observed. Motility is present, being intermediate in rapidity between that of *Endamœba histolytica* and *Endamœba coli*, but pseudopodia are often extruded and withdrawn without resultant motion. The cytoplasm of the trophozoites is refractile and colorless and the pseudopodia are rather short and blunt and fairly well differentiated from the endoplasm. Ingested material, especially bacteria, is present in the endoplasm and food vacuoles containing bacteria are often present. Red blood corpuscles are never present in this ameba even though the stools may contain blood. The nucleus may sometimes be seen, the nuclear membrane being refractile and hyaline, while the karyosome may be visible as a refractile dot or two dots, sometimes in contact with the nuclear membrane.

In unstained preparations the characteristic morphological features of the trophozoite of *Endolimax nana* are the comparatively small size, the character of the nucleus, the presence of bacteria and food vacuoles, the absence of red blood corpuscles, and the morphology of the pseudopodia.

The Precystic Ameba.—The morphology of the precystic forms of *Endolimax nana* is similar to that of the trophozoite but these forms are usually smaller than the trophozoites, measuring from 5 to 10 microns in diameter, although large precystic forms may sometimes be noted. The precystic forms are of no value in diagnosis, which should be based upon either the trophozoites or the cysts.

The Cystic Ameba.—The cysts of *Endolimax nana* are colorless, oval, ellipsoidal or spherical bodies enclosed in a cyst-wall and usually measuring from 5 to 14 microns in diameter, although larger cysts of this species not infrequently occur. The average cyst measures about 7 to 8 microns in diameter, or about the size of a red blood corpuscle. The cysts contain from 1 to 4 nuclei but these are usually indistinct in the unstained trophozoite. Rarely in the uninucleate or binucleate cysts the nuclei may be seen as round masses of refractile material. The cysts of this species are much smaller on the average than those of either *Endamœba histolytica* or *Endamœba coli*, although some of the small cyst strains of *Endamœba histolytica* may produce cysts as small as the average *Endolimax nana* cysts.

The characteristic features of the cysts of this species of ameba in unstained specimens are the oval, ellipsoidal or spherical shape, the

small size, and the absence of definite nuclei. A specific diagnosis of *Endolimax nana* from the unstained cyst alone is usually impossible.

The Metacystic Ameba.—The morphology is the same as that of the trophozoites.

Morphology of Endolimax Nana in Stained Preparations

The Trophozoite.—In wet-fixed and hematoxylin stained preparations the staining reactions of *Endolimax nana* are like those of the other intestinal amebæ, the cytoplasm staining a bluish-gray and the nuclear structures black. The characteristic feature of the morphology is the nucleus, the structure of which clearly differentiates this species of ameba from the other species occurring in the human intestine. In

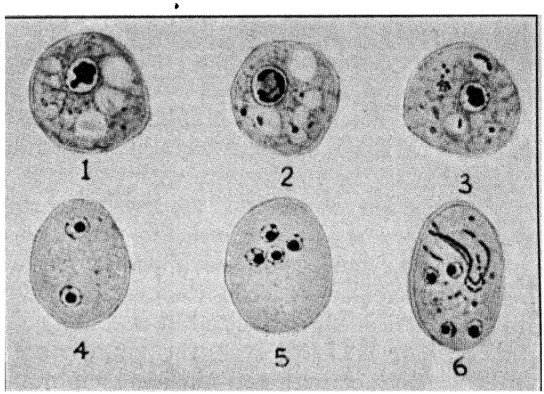


FIG. 8. *Endolimax nana*. *1*, *2*, *3*, Trophozoites, showing different types of nuclear structure; *4*, two nucleated cyst; *5*, *6*, four nucleated cysts. Stained with iron hematoxylin. (After Dobell and O'Connor, courtesy of John Bale and Staples, Ltd.)

well-stained hematoxylin preparations the nuclear membrane appears as a moderately thick membrane having no chromatin granules upon its inner surface. If the stain has been poor or extracted too much during the staining process, the nuclear membrane may be invisible or may appear very thin and poorly stained. The karyosome stains black and varies greatly in appearance in different organisms. Sometimes, and most often, it appears as a comparatively large round black mass at the center or to one side of the nucleus, frequently in contact with the nuclear membrane. Sometimes, it appears as two black masses of unequal size, connected by a delicate black filament, and lying in contact with the nuclear membrane, or as an irregular black mass lying within the nucleus. In routine preparations the karyosome usually appears as a single round mass either at the center of the nucleus or at one side near, or in contact with, the nuclear membrane.

In stained preparations food vacuoles containing bacteria may be present in the endoplasm, the bacteria staining black, while rarely a species of *Sphærita* may be seen within the ameba, appearing as a round collection of black granules, representing the spores of this organism.

The characteristic morphological features of stained trophozoites of *Endolimax nana* are the comparatively small size, the peculiar variation in the morphology of the karyosome in different organisms, and the absence of red blood corpuscles.

The Precystic Ameba.—The morphology of the precystic amebæ of this species in hematoxylin stained preparations resembles that of the stained trophozoite but ingested material and vacuoles are less frequently present.

The Cystic Ameba.—The stained cysts of *Endolimax nana* are slightly smaller than the trophozoites and contain from 1 to 4 nuclei, thus resembling the cysts of *Endamœba histolytica*. Ingested material is not present and the cytoplasm appears finely granular and stains a grayish color. The nuclei usually show an indistinct, thin nuclear membrane and there are no chromatin granules upon the inner surface of this membrane. In the uninucleate cyst the morphology of the nucleus resembles that of the trophozoite with the same variations in the morphology of the karyosome, but as division occurs the nuclei become smaller and then it is sometimes impossible to demonstrate any nuclear membrane, the karyosome appearing as a black mass lying in an unstained, circular area. In the cysts the karyosome may be divided into two parts of unequal size but is usually single. In the fully developed, or four nucleate cyst, the karyosomes of the four nuclei may all lie in contact with the nuclear membrane or may be central in position in one or more of the nuclei and in contact with the membrane in the others.

Chromatoidal bodies comparable with those occurring in the cysts of *Endamœba histolytica* or *Endamœba coli* do not occur in the cysts of this species but granular or bacilliform bodies, stained black, are sometimes seen which are apparently of chromatoidal nature.

In preparations stained with the routine iodine stain the cytoplasm of the cysts stains a yellowish-green, the cyst-wall remaining unstained. The nuclear membrane and the karyosome of the nucleus appear as refractile structures, especially distinct in the uninucleate cysts.

The characteristic diagnostic features of the stained cysts of *Endolimax nana* are the oval shape of many of the cysts, the presence of from 1 to 4 nuclei, the single or divided karyosome of the nuclei, centrally situated or in contact with the nuclear membrane, and the absence of chromatoidal bodies comparable with those of *Endamœba histolytica* or *Endamœba coli*.

The Metacystic Ameba.—The morphology of this stage is the same as that of the trophozoites, in stained preparations, but the ameba is smaller than the trophozoites of the same strain.

4. MORPHOLOGY OF IODAMŒBA BÜTSCHLII

Iodamœba bütschlii is a non-pathogenic ameba occurring in the intestine of a considerable number of normal individuals in all parts of the world. It has a trophozoite, or motile, stage, a precystic, cystic and metacystic stage in its life-cycle, and because of a superficial resemblance to *Endamœba histolytica* in some of its morphological features it is important that it be differentiated from the latter species. Only the trophozoite and cystic forms will be described as these are of importance in differential diagnosis.

Morphology of Iodamœba Bütschlii in Unstained Preparations

The Trophozoite or Motile Ameba.—As observed in freshly voided stools, the trophozoite of this species of ameba varies in size from 8 to 20 microns in diameter, the average being from 10 to 15 microns in diameter. The cytoplasm is colorless and in freshly passed stools the trophozoites are sluggishly motile, the motility being progressive in type, but exposure to room temperatures quickly stops motility, at which time the pseudopodia are slowly protruded and withdrawn without resultant change of position. The pseudopodia are broad and hyaline in appearance, with rounded ends, and are clearly differentiated from the endoplasm. In most of the trophozoites a nucleus is not visible in the unstained preparation but may sometimes be seen as a large refractile mass surrounded by a hyaline halo. The cytoplasm may contain ingested substances and food vacuoles containing bacteria or other ingested material, as crystals, starch granules, and vegetable cells may be present. Red blood corpuscles never occur within this species of ameba, even though the stools may contain blood. The characteristic features of the morphology of the trophozoites of *Iodamœba bütschlii* are the sluggish progressive motility, the large hyaline mass representing the nucleus, if visible, and the absence of red blood corpuscles from the cytoplasm if the stools contain blood.

The Cysts.—The unstained cysts of *Iodamœba bütschlii* measure from 5 to 20 microns in diameter and appear as refractile colorless bodies which vary greatly in shape. Instead of being oval and round, as in the other species of amebæ described, they are irregular, ellipsoidal, rhomboidal, triangular, almost square or even fusiform in shape, this great variation easily distinguishing them from the cysts of the other intestinal amebæ of man. The cysts are surrounded by a very well-defined cyst-wall, of considerable thickness, and having a double outline.

The cysts vary much in size but the average unstained cyst measures about 9 microns in diameter. Characteristic of many of the cysts is the presence of a large, round or oval colorless body lying in the cytoplasm, known as the "idiophilic body" or glycogen vacuole. This vacuole is much larger, as a rule, in the cysts of this species than in those of the other amebæ living in the human intestine. More than one glycogen vacuole may rarely be present.

The nucleus is not usually well seen in the unstained cysts and only one nucleus is present while all ingested material has been extruded.

The characteristic morphological features of the unstained cysts of *Iodamœba bütschlii* are the great variation in the shape of the cysts, the thick double outlined cyst-wall, the very large glycogen vacuole and the presence of but one nucleus in the cyst.

Morphology of Iodamœba Bütschlii in Stained Preparations

The Trophozoite.—The staining reactions of this ameba are similar to those of the amebæ already described. The cytoplasm stains a dull gray and the nucleus, in well-stained preparations, shows a black,

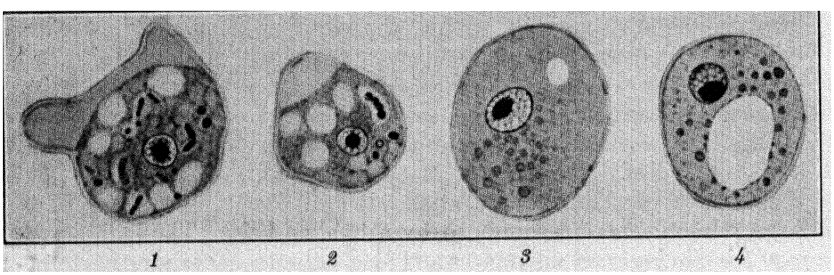


FIG. 9.—*Iodamœba bütschlii*. *1*, *2*, Trophozoites showing nuclear structure, vacuoles and inclusions; *3*, precystic form; *4*, cyst, showing the large glycogen vacuole. Stained with iron hematoxylin. (After Dobell and O'Connor, courtesy of John Bale and Staples, Ltd.)

rather thin nuclear membrane with no chromatin granules upon its inner surface, as a rule, although black granules of chromatin may be seen in this situation in some organisms. The karyosome is composed of rows of almost black granules surrounding a more deeply stained central body, the whole lying in a network, the background of which stains diffusely gray. The karyosome is comparatively large in this species and usually is situated centrally in the nucleus of the trophozoite. If the preparations have been overstained, or imperfectly differentiated, the karyosome will appear as a homogeneous black mass of considerable size lying within the nucleus, while the nuclear membrane is not visible or is poorly differentiated. In such specimens the karyosome appears to lie within a vacuole in the cytoplasm. There

The characteristic features of the stained cysts of *Iodamœba bütschlii* are the great variation in the shape of the cysts, the presence of but one nucleus, the peculiar structure of the nucleus, the comparatively large karyosome, and the presence of the very large glycogen vacuole staining an intense mahogany color with the iodine stain.

With the routine *iodine stain* the cyst-wall appears hyaline and colorless and shows a very distinct double outline. The large glycogen body stains a deep mahogany brown or brownish-red and is the most striking object within the cyst. In this species the glycogen body stains much more intensely with this stain than do the glycogen bodies of other species of intestinal amebæ and is much larger, often crowding the nucleus to one side of the cyst. The nuclear membrane appears hyaline and colorless while the karyosome consists of a refractile hyaline circular mass situated to one side of the nucleus and often in contact with the nuclear membrane. The cytoplasm stains a greenish-yellow and is free from ingested material.

5. MORPHOLOGY OF DIENTAMŒBA FRAGILIS

Dientamœba fragilis is a rather common species of ameba occurring in the intestines of from less than 1 per cent to as high as 20 per cent of normal individuals in most parts of the world. This species of ameba is generally believed to be non-pathogenic but a very few observers claim to have found it in patients having diarrhea or dysentery and have thought that it may have been responsible for these symptoms. At the present time it is true that the evidence available is insufficient to prove that *Dientamœba fragilis* is pathogenic for either man or any of the lower animals. This species is peculiar in that only the trophozoite, or motile ameba, is known, as cysts have never been proven to occur and the cystic stage of development is apparently absent.

Morphology of Dientamœba Fragilis in Unstained Preparations

The Trophozoite or Motile Ameba.—In unstained preparations, *Dientamœba fragilis* appears as a colorless organism varying somewhat in size but is comparatively small, measuring upon the average about 10 microns in diameter. It is an actively motile ameba, the motion being progressive in character in freshly voided stools. The pseudopodia are very characteristic, being well differentiated from the endoplasm and broad and leaflike in contour with indented or serrated margins. The nuclei are usually two in number but are not visible as a rule in unstained preparations. The cytoplasm contains ingested material, especially bacteria, but red blood corpuscles are never present.

When first described this ameba was believed to very quickly degenerate after exposure to room temperature but later observations have shown that it is more resistant to external influences than at first

believed and motile trophozoites have been found in the stools for some time after passage.

The characteristic morphological features of the unstained trophozoite of *Dientamœba fragilis* are the comparatively small size, the presence of two nuclei, the broad, leaflike pseudopodia with indented or serrated edges, the absence of red blood corpuscles; and the active progressive motility in freshly voided stools.

Mortality of Dientamœba Fragilis in Stained Preparations

In hematoxylin stained preparations the staining reactions of this ameba are like those of the other amebæ described. In the stained preparations it will be noted that the vast majority of the organisms contain *two nuclei*, a most important diagnostic feature. It has been the writer's experience that over 80 per cent of the trophozoites of *Dientamœba fragilis* contain two nuclei, while in the other species of amebæ dwelling in the human intestine it is very rarely that a trophozoite is seen containing more than one nucleus.

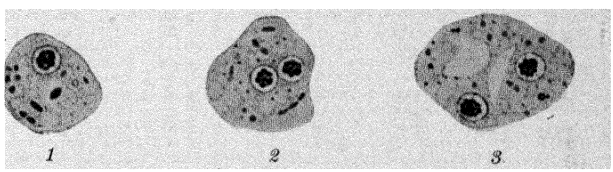


FIG. 10.—*Dientamœba fragilis*. *1*, Uninucleate ameba; *2*, *3*, binucleate amebæ. Stained with iron hematoxylin. (After Dobell and O'Connor, The Intestinal Protozoa of Man, courtesy of John Bale and Staples, Ltd., London.)

The structure of the nuclei is characteristic and is identical in each of the two nuclei. The nuclear membrane is very delicate, not over a line in thickness and is often invisible so that the karyosome appears to be lying in a vacuole in the cytoplasm of the organism. If the nuclear membrane is visible it will be noted that there are no chromatin granules upon its inner surface nor in the space between the membrane and the karyosome. The karyosome is centrally located in the nucleus and has a very characteristic morphology. It is composed of definite, comparatively large-sized chromatin granules, stained an intense black color, and lying in a dimly stained matrix. The granules are variously arranged, usually in a circular manner and very frequently as a group of 4 separate granules resembling a tetracoccus, but they vary much in number and arrangement. The number of granules may vary from 4 to 8 and they may be arranged as mentioned above or in an irregular mass, in a crosslike formation, or in a heaped-up circular mass in the center of the nucleus. Except in very rare instances the karyosome is located at the center of the nucleus and the arrangement of the karyosomal granules is identical in the two nuclei of the ameba. The

TABLE 1.—DIFFERENTIAL DIAGNOSTIC FEATURES OF THE INTESTINAL AMEBÆ OF MAN
(Slightly modified from Craig and Faust's Clinical Parasitology)

	Endamœba histolytica	*Endamœba coli*	*Endolimax nana*	*Iodamœba bütschlii*	*Dientamœba fragilis*
		"Vegetative" or Trophozoite Stage. Unstained			
Size in microns	15 to 60μ	15 to 50μ	6 to 15μ	8 to 20μ	5 to 12μ
Motility	Active; progressive and directional	Sluggish; rarely progressive and directional	Sluggishly progressive	Sluggishly progressive	Active and progressive
Pseudopodia	Finger shaped; hyaline and glass-like; rapidly extruded	Shorter and more blunt; more granular; slowly extruded	Blunt and hyaline; very rapidly extruded	Blunt and hyaline; slowly extruded	Blunt and leaf-like; hyaline
Inclusions	Red blood corpuscles; no bacteria in fresh specimens	Bacteria and other material; no blood corpuscles	Bacteria; no blood corpuscles	Bacteria; no blood corpuscles	Bacteria; no blood corpuscles
Nucleus	Invisible usually	Visible	Visible	Invisible	Invisible
		"Vegetative" or Trophozoite Stage. Iron Hematoxylin Stain			
Nuclear membrane	Delicate; inner surface has single layer of minute chromatin dots	Thicker; inner surface lined with coarse chromatin dots	Intermediate in thickness; chromatin seldom present on inner surface	Thick; chromatin dots may be present on inner surface	Very delicate; no chromatin dots on inner surface; two nuclei present
Karyosome	Minute and in center of nucleus	Much larger and eccentrically situated	Large and may be in center or to one side of center of nucleus	Large and granular, in center of nucleus or somewhat eccentrically placed	Large and composed of definite chromatin granules lying in a dimly stained matrix
Inrtanuclear chromatin	No chromatin between karyosome and nuclear membrane	Chromatin grains between karyosome and nuclear membrane	No chromatin between karyosome and nuclear membrane	No chromatin between karyosome and nuclear membrane	No chromatin between karyosome and nuclear membrane
Inclusions	Red blood corpuscles; no bacteria unless degenerated	No red blood corpuscles; many bacteria and other material	No red blood corpuscles; many bacteria	No red blood corpuscles; bacteria	No red blood corpuscles; bacteria

	Cystic Stage.	*Iodine Smear Preparations*			
Size in microns	6 to 20μ	10 to 33μ	5 to 14μ	5 to 20μ	No cysts have been demonstrated
Shape	Usually spherical	Usually spherical	Spherical, ovoidal or ellipsoidal	Irregular	
Cytoplasm	Bright greenish-yellow	Yellowish-brown	Pale green with numerous refractile vacuoles	Yellowish-green	
Glycogen mass	Diffuse and reddish-brown	Dark brown and indefinite central mass with indistinct border	Usually absent, brownish and either diffuse or defined	Usually present, dark brown and sharply outlined	
Nuclei	1 to 4; minute central karyosome very refractive; nuclear membrane beaded and refractive	1 to 8 or more; nuclear membrane refractive and granular; karyosome eccentric	1 to 4; indistinct	Indistinct; one usually present	
		Cystic Stage.	*Iron Hematoxylin Stain*		
Size in microns	6 to 20μ	10 to 33μ	5 to 14μ	5 to 20μ	No cysts have been found
Shape	Usually spherical	Spherical	Ovoidal or ellipsoidal	Irregular	
Cytoplasm	Alveolar, often vacuolated	Granular and vacuolated	Vacuolated with chromatin granules	Vacuolated, large glycogen vacuole usually present	
Chromatoidal bodies	Bar, oval or thick rod-like masses with rounded ends	Filamentous, thread-like or splinter-like with square or pointed ends	Small, spherical or bacilliform, often in a vacuole	Usually absent; when present small round or granular	
Nuclei	1 to 4; delicate membrane lined with minute chromatin granules; karyosome minute central dot	1 to 8 or more; thick nuclear membrane lined with large dots of chromatin or irregular masses; karyosome eccentrically placed and large	1 to 4; nuclear membrane indistinct; karyosome in a single or divided mass on or near nuclear membrane	1, rarely 2; nuclear membrane very thin, often indistinct; karyosome placed centrally or laterally and surrounded by large granules	

peculiar structure of the nuclear karyosome in this ameba renders a differential diagnosis between it and the other intestinal amebæ of man a matter of little difficulty.

The cytoplasm stains a dim gray color and may contain food vacuoles filled with black stained bacteria, but red blood corpuscles are never present even though the stools may contain blood.

The characteristic morphological features of the trophozoite of *Dientamœba fragilis* in stained preparations are the comparatively small size, the presence of two nuclei, the peculiar granular structure of the centrally situated karyosomes, the delicacy of the nuclear membrane, the absence of chromatin granules between this membrane and the karyosome, the presence of bacteria in the cytoplasm, and the absence of red blood corpuscles within the ameba.

DIFFERENTIAL DIAGNOSIS OF THE INTESTINAL AMEBÆ OF MAN

The differential diagnosis of the intestinal amebæ of man depends upon the microscopic study of unstained and stained preparations of the stools containing them. As no less than five species of amebæ live in the intestine of man, and as only one of these species, *i. e.*, *Endamœba histolytica*, is pathogenic, the differentiation of this species from the non-pathogenic species is most important, especially as these non-pathogenic species may occur in a large proportion of healthy individuals. Such a differentiation is usually possible by the study of unstained material containing the trophozoites, and of material containing the cysts after the use of the routine iodine stain, but in some instances it may be found necessary to employ the hematoxylin or other stains after wet-fixation, in order to make a specific diagnosis, and such stains should always be employed if one desires to study the minute structure of the nucleus or to prepare specimens for class use or for reference. If possible wet-fixed and hematoxylin preparations should be employed in diagnosis especially if the material to be examined has come from a long distance or is over a few hours old. A diagnosis of amebiasis should always rest, if possible, upon the demonstration of *Endamœba histolytica* in the stools of the suspected individual, as other methods of diagnosis are more difficult and of less specific value.

Table 1 gives the principal diagnostic features of the trophozoites and cysts of *Endamœba histolytica*, *Endamœba coli*, *Endolimax nana*, *Iodamœba bütschlii* and of the trophozoites of *Dientamœba fragilis*, and will be found to be useful in the differential diagnosis of these amebæ.

CHAPTER II

COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION—STAINING METHODS—DIAGNOSTIC VALUE OF A STUDY OF THE EXUDATE

1. COLLECTION OF MATERIAL FOR EXAMINATION

THE amebæ living in the human intestine are demonstrable in the stools but as these organisms are easily affected by temperature and other physical and chemical conditions, it is most important that properly collected and freshly voided material be examined, so that great care should be taken in the collection of such material. A most important fact should always be remembered in the examination of the feces for amebæ, *i. e.*, that the *motile forms*, or *trophozoites*, usually occur only in semi-fluid or fluid stools, while the *cysts* occur only in semi-formed or formed stools. An exception is sometimes noted in instances in which formed stools have passed over open amebic ulcers, the mucus from which may adhere to the stool, which, when examined, may show motile trophozoites. It is usually time wasted unless a cathartic has been administered to examine a fluid stool for cysts or a solid stool for motile amebæ, and in the case of "cyst-passers" or "carriers" of *Endamœba histolytica*, who pass formed stools, the motile trophozoites can only be demonstrated after the administration of a cathartic capable of producing a liquid stool. It should also be remembered that the *motile trophozoites* quickly lose their motility and degenerate after exposure to room temperature so that it is essential that the stool be examined as soon after passage as is possible, or a negative result may be obtained in positive cases. On the other hand, the *cysts* of the various intestinal amebæ are much more resistant to external influences and may be differentiated in formed stools for several days after passage but even in such cases it is preferable to examine the stool immediately after passage. It is an excellent rule to insist upon the patient passing the stool in the laboratory where the examination is to be made and this is usually possible in the case of cyst-passers or patients having such mild symptoms as not to interfere with their coming to the laboratory for examination.

The stools should always be collected in absolutely clean, dry receptacles. Antiseptics should never be employed in cleaning such utensils and no water should be left in, or added to, the bed-pan or other vessel in which the stool is to be passed. Many mistakes in diagnosis have been caused by the presence of antiseptics in the collecting utensil, which may cause the immediate disintegration of the amebæ or loss of motility, while free living amebæ or other protozoan

organisms may be present in water left in such utensils. Care should also be taken that no urine be passed at the time of the collection of the stool as urine frequently causes degeneration and loss of motility of the amebæ.

The first examination should always be made of a natural stool, whether it is formed or liquid in character. The entire stool should first be examined by the naked eye for the presence of pus, mucus, or blood, and portions of such material should be later submitted to a microscopic examination. If the stools are formed and are negative for cysts, another stool should be examined for motile trophozoites after the administration of a saline cathartic, preferably magnesium sulphate, as in some infections the amebæ are so few in number that cysts may not be found in the small portions of the formed stools examined. Repeated stool examinations should always be made if the first stool examined is found negative, for not infrequently several stools may have to be examined before the amebæ are demonstrated. The author has examined as many as 12 stools before finding *Endamœba histolytica* and it has been estimated by several authorities that not more than one-third of positive infections are detected by one stool examination while after six such examinations practically all infections are discovered. If a saline cathartic be administered a larger percentage of the positive infections will be found and Magath (1934) recommends that magnesium sulphate be administered as a routine in all cases and considers that it is possible to detect *Endamœba histolytica* in 80 per cent of infected individuals with but one stool examination after this cathartic, although only about one-third of the infections can be detected by a single stool examination if the feces are formed. Andrews (1934) also found that a much higher percentage of positive results is obtained after the administration of a purgative. He found that 88.9 per cent of infections were discovered by a single stool examination after a purgative while six daily examinations of the same individuals were necessary in order to detect 75 per cent of the infections without using a purgative.

Sawitz and Faust (1942), as the result of a very careful and extended research upon this subject have determined that the examination of a single preparation stained with the hematoxylin or iodine stains proved successful in less than one of five known positive infections and that ten preparations had to be examined before a negative result could be accepted as "reasonably reliable." On the other hand, when a combination of the zinc sulphate centrifugal flotation and staining with hematoxylin or iodine was employed "about one out of three or four infections were detected in a single examination, while five such examinations provided a probability of 70 to 90 per cent." These observations demonstrate forcibly the value of the use of repeated examinations, employing the most efficient technique, in the diagnosis of amebiasis.

The importance of repeated examinations of stools in suspicious

cases has been stressed by Dennis and Lund (1937) who instance a patient in whom there was a question of a differential diagnosis between an amebic abscess of the liver and a non-malignant tumor, in which 18 stool examinations were made before *Endamœba histolytica* was found and a diagnosis of amebic liver abscess could be made. This diagnosis was later confirmed by operation and the demonstration of the ameba in the contents of the liver abscess.

In collecting stools for examination for amebæ it should be remembered that any oil or oily emulsion, or bismuth or barium, if present in the stools, renders them unsuitable for examination by obscuring the organisms and patients taking any of these materials should cease doing so and the stools should not be examined until they are free from such substances.

If the stools cannot be passed in the examining laboratory, and semi-fluid or fluid stools are being voided, they should be kept at body temperature and sent at once in a suitable container to the laboratory for examination. This can best be done by placing the material in a thermos bottle, or, if such a bottle is not available, by placing the material in a tightly sealed container and placing this, in turn, in a container filled with water at body temperature. If the stools are formed it is not necessary to keep them at body temperature as the cysts of the amebæ are not easily affected by external temperatures for many hours after passage. In fact, such stools may be placed in the ice-box for preservation in hot weather without injuring the cysts which will remain perfectly normal in appearance after many hours at ice-box temperature.

If it be necessary to send portions of the stool to a distant laboratory for examination they can be mailed in special containers that are available for the purpose and two methods of sending such material are recommended. If the stool be fluid or semi-fluid in consistence the trophozoites will quickly lose their motility after passage and degenerate and such material should never be sent by mail as it is useless for examination when reaching the examining laboratory. Instead, smears should be made upon microscopic slides, fixed with Schaudinn's fluid (see page 52) before they become dry, and immediately placed, without drying, in mailing tubes filled with alcohol and sent to the laboratory. Such smears can then be stained at leisure and examined. If the stools are formed a small portion is enclosed in a mailing tube and sent at once to the laboratory. The cysts of *Endamœba histolytica* will be distinguishable in such material for a period of at least three or four days, and sometimes longer, so that they can usually reach the laboratory in ample time for a differential diagnosis to be made. Water should never be added to such specimens and the mailing tubes used should be dry and clean and free from all traces of antiseptics or other chemicals that might injure protozoan organisms. Care should be taken that the specimens are properly labelled with

the name of the patient, the date of collection, and the information desired by the sender.

In collecting material from an amebic abscess of the liver for examination it is important to remember that *Endamœba histolytica* occurs much more frequently in the tissues of the wall of the abscess than in the abscess contents, especially if a secondary bacterial infection complicates the picture. In an amebic abscess of the liver uncomplicated by a bacterial infection the appearance of the abscess contents is very characteristic, the color being a reddish-brown or chocolate and the consistence being thick, the whole somewhat resembling anchovy sauce. In such material motile forms of *Endamœba histolytica* are frequently observed if it be examined immediately after it has been obtained. On the other hand, if a bacterial infection is also present, the abscess contents resemble ordinary pus, are of a yellow or greenish color and rarely contain motile amebæ. In such cases the examination of material scraped from the abscess-wall will usually result in the demonstration of the parasite, if present. In all negative cases scrapings from the abscess-wall should be examined before it is concluded that the abscess is not amebic in character.

In the case of an amebic abscess of the lung the sputum contains motile trophozoites of *Endamœba histolytica* and if it is examined as soon as expectorated one will usually find the organisms in this material.

In mailing material for examination for the intestinal amebæ or flagellates it is necessary that it be placed in containers which are accepted by the Post Office Department and such containers may be obtained from any laboratory supply house. It is useless to mail the contents of an amebic abscess of the liver or lung for examination for *Endamœba histolytica* as the cysts of this parasite never occur in invaded tissues and the motile trophozoites will have disintegrated long before the specimen reaches the examining laboratory.

2. PREPARATION OF MATERIAL FOR EXAMINATION

For the examination of material for the intestinal amebæ and flagellates the following apparatus is necessary: A good compound microscope, fitted with a mechanical stage, and having periplanatic oculars × 5 and × 10, and acromatic or flourite 16 mm., 4 mm., and 1.9 mm. objectives; microscopic cover-slips and slides; a good daylight blue electric lamp for the source of illumination; a bottle of iodine solution; wooden toothpicks and applicators and one or more of the special stains which will be described in the following pages.

The material to be examined, whether stools, liver abscess contents or sputum, should first be studied in the unstained condition, after which iodine preparations should be studied if cysts are present. Finally, special staining methods may be employed if the diagnosis is in doubt, if it is desired to preserve the preparations for class work or

demonstration purposes, or if it is desired to study the finer structure of the organisms. For routine diagnosis it is seldom necessary to employ anything but the unstained and iodine stained preparations. In mounting material for the examination coverslips measuring 22 mm. square and not over 18 microns in thickness and clean white glass microscopic slides preferably 40 mm. by 75 mm. should be used.

A small portion of the material to be examined should be picked up on the end of a wooden toothpick, mixed with a drop of normal salt solution, and rapidly streaked across a microscopic slide for a space covering the width of two cover-slips and one-half of this smear covered immediately with a cover-slip. A drop of iodine solution, preferably D'Antoni's (see page 50) is then mixed with the material on the other half of the smear and this, in turn, covered with a cover-slip. Thus we have upon one slide material that can be examined unstained and stained with the iodine stain.

The following method of preparing stool specimens for microscopic examination is a little more time-consuming than that described above but it is believed gives better results:

A small portion of the stool is thoroughly mixed with a small drop of normal saline solution which has been placed upon a microscopic slide, and carefully emulsified, after which a cover-slip should be placed upon the mixture. Care should be taken that the resulting mixture is not too thick and as a guide, newspaper print should be visible through it. A too-thick mixture will obscure the protozoa that may be present and is useless for examination. Upon another microscopic slide a drop of iodine stain should be placed and a small portion of the stool to be examined added, thoroughly mixed, and covered with a cover-slip. After allowing the preparation to stand for at least five minutes, in order to let the iodine penetrate the cysts, it is ready for microscopic examination. The iodine preparation should only be employed in the case of formed stools, as already noted.

In preparing material from the stools for microscopic examination for *Endamœba histolytica* the presence of bloody mucus or of mucus should be noted and, if present, small portions of this material should always be examined for the motile trophozoites by adding it to normal saline solution, mixing, and covering with a cover-slip.

Hakansson's Aqueous Smear Method.—Hakansson (1942) recommends the following method of examination in addition to saline and iodine preparations, in order to eliminate *Blastocystis hominis*, which frequently confuses the differential diagnosis of the amebæ and to aid in the diagnosis of *Dientamœba fragilis:*

A drop of water is placed upon a microscopic slide and a minute particle of the feces to be examined is stirred in the water by means of a wooden tooth-pick. A cover-glass is then carefully applied, avoiding the formation of air-bubbles, and the preparation is then allowed to stand for a few minutes before being examined. During this period

osmosis causes the distention, rupture and disappearance of the blastocysts, thus greatly facilitating the finding of the cysts of *Endamœba histolytica* and other amebæ. The trophozoites of *Dientamœba fragilis* also distend and the organism becomes a refractive spherical shell-like object. This process may be followed under the microscope and Hakansson states that no other trophozoites of the intestinal amebæ are thus affected, rendering the diagnosis of this species much less difficult.

Quensel's method is also valuable for differentiating trophozoites. (See page 63.)

Concentration Methods.—If, as often happens, the examination of the unstained and iodine stained preparations prepared as recommended above, results negatively for *Endamœba histolytica* or other amebæ, some form of concentrating the stool should be employed, as the chances of finding the cysts of these organisms are greatly increased when concentrated material is examined. The most simple form of concentration which is of value is the following:

A portion of the formed stool about the size of a pea is emulsified in 10 cc. of normal salt solution or distilled water in a test tube, strained through two layers of cheese cloth which has been placed in a small funnel, and the filtrate placed in a centrifuge tube which, in turn, is filled with normal saline or water. The tube is then shaken and centrifugalized at a moderate speed for not to exceed five minutes. The supernatant fluid is then poured off and portions of the sediment prepared for microscopic examination in the same manner as already recommended for material prepared directly from the stools. Both unstained and iodine stained preparations should be examined.

While the concentration method just described will give good results, much better ones will be obtained by the use of the zinc sulphate flotation method devised by Faust and his co-workers (1938), and this method is the one recommended by the writer.

The Zinc Sulphate Flotation Method of Faust, et al.—This method depends for its success upon the concentration and flotation of the cysts of the intestinal amebæ and flagellates and is only applicable to the examination of formed stools, for the motile trophozoites of these organisms would be destroyed during the process and these, as already noted, occur in fluid or semi-fluid stools. The various steps involved in this valuable method are as follows:

1. Thoroughly mix one part of the formed stool, about the size of a pea, with about 10 parts of lukewarm water in a clean glass container.
2. Strain 10 cc. of this mixture through one layer of wet cheese cloth, previously placed in a small funnel, into a Wassermann tube.
3. The filtrate in the tube is then centrifugalized for forty-five to sixty seconds at the top speed of an International clinical centrifuge (about 2500 revolutions per minute). The supernatant fluid is poured off, 2 or 3 cc. of distilled water added, the tube thoroughly shaken to

distribute the sediment, and additional water added to fill the tube. The tube is then centrifugalized as before and the process is repeated until the supernatant fluid is clear.

4. After the last centrifugalization, the clear supernatant fluid is poured off, and 3 to 4 cc. of a 33 per cent zinc sulphate solution, which should have a specific gravity of 1.180, are added. The sediment is thoroughly mixed with this solution and enough of the zinc sulphate solution added to fill the tube to within one-half inch of the rim.

5. The tube is then centrifugalized for at least ninety seconds at top speed.

6. Several platinum loopfuls of the material which will be noted floating upon the surface of the solution in the tube are removed and placed upon a microscopic slide, one drop of D'Antoni's iodine stain is added and mixed.

7. The preparation is covered with a cover-slip and examined.

Remarks.—This method has been found to be successful in demonstrating the cysts of *Endamœba histolytica* when other methods have failed and it is equally valuable in examining stools for the eggs of the various helminthes. When one has mastered the technique it consumes but little time and is to be recommended in all cases in which a simple stool examination has resulted negatively.

Otto, Hewitt and Strahan's Zinc Sulphate Levitation Method.—This is a modification of the Faust flotation method in which straining and centrifugalization of the fecal specimen is omitted.

A zinc sulphate solution is made up by dissolving 331 grams of the salt in sufficient distilled water to make one liter of the solution, or 371 grams may be dissolved in 1 liter of water. After the solution has stood for twenty-four hours the specific gravity is adjusted to 1.18 by the addition of minute amounts of water, or the salt, if necessary.

In using the method a sample of feces about the size of a kidney-bean is carefully emulsified in the zinc sulphate solution in a glass shell vial 5 cc. deep and 1.8 cc. in diameter, the mixture reaching to the top of the vial. A $\frac{7}{8}$ inch cover-glass is placed in contact with the mixture, just covering the mouth of the vial. After one hour the cover-glass is carefully removed to a glass microscopic slide upon which one drop of D'Antoni's iodine (see page 50) has been placed and examined for cysts, which, if present, should have collected upon the surface of the cover-glass in contact with the mixture of zinc sulphate and fecal material.

Remarks.—The authors of this method claim that it is almost as efficient as the method of Faust, *et al.* and obviates straining and centrifugalization, and is adequate for diagnostic work in the ordinary laboratory. The method deserves a thorough trial but it is probable that when cysts of *Endamœba histolytica* or other protozoa are present in small numbers this method will not be nearly as efficient in demonstrating them as that of Faust, *et al.*

Sinha's Copper Sulphate Flotation Method.—This method has been recommended by Major H. S. Sinha, of the Indian Medical Service (1945). It is as follows:

1. Place 1 cc. of normal saline in a centrifuge tube.
2. Emulsify a portion of stool about the size of a pea in the saline solution.
3. Fill the centrifuge slowly to the brim with copper sulphate solution of specific gravity of 1.035.
4. Bring the under surface of a microscopic slide into direct contact with the surface of the fluid in the tube, being careful that no air-bubbles occur between the slide and the emulsion.
5. Allow to stand for ten minutes.
6. Hold the nearer end of the slide with two fingers and push the tube toward the left and quickly turn the slide upward.
7. Examine the slide with or without a cover-glass. Lugol's iodine solution may be at once added to the material upon the slide, if desired.

Remarks.—This method is stated by Sinha to be very effective in the detection of low grade infections with *Endamœba histolytica*. He found that of 100 stools that were negative for this parasite upon direct microscopic examination, no less than 11, or over 10 per cent, were positive for the cysts with this method. The nuclear structure was better defined and the differentiation of *Endamœba histolytica* cysts from those of other amebæ was rendered much easier.

This method merits careful attention and appears to be a comparatively simple one as compared with other flotation methods.

The Examination of Preparations.—The proper employment of the microscope is of the utmost importance in the examination of preparations containing the intestinal amebæ or flagellates. One should not attempt the differential diagnosis of the intestinal protozoa unless one had been well trained in the use of the compound microscope and in the recognition of these organisms by a thoroughly qualified teacher.

It should be an invariable rule in the examination of unstained preparations, and of preparations stained with the routine iodine stain, to use as little light as is possible for a clear view of the organisms. It should be remembered that all of the protozoa are colorless and hyaline in appearance and if the microscopic field is flooded with light they will be invisible. It is thus essential that the iris diaphragm of the microscope be closed as much as is possible and still admit enough light to enable one to clearly distinguish them. The sub-stage condenser of the microscope should also be carefully raised and lowered until the maximum definition of the object being examined is obtained and neglect of the proper use of the condenser often results in poor definition and consequent failure to secure the best results of which the microscope may be capable.

The preparations should first be examined with the low power objective and suspicious objects afterwards studied with the higher

power objectives. Use as high power an ocular as will give enough light with the objective that is being used as to clearly define the object that is being examined.

A systematic method of examining the preparations should be followed, beginning at the right upper corner of the cover-slip and working back and forth, using the mechanical stage of the microscope, until every portion of the preparation has been examined. While a systematic examination may be made by pushing the preparation back and forth by hand, instead of using a mechanical stage, it is less accurate, more tiring, and more time consuming, and is comparable to one using a horse and buggy when an automobile is available. The added expense of a good mechanical stage is more than compensated for by its convenience and technical accuracy.

After the preparations have been examined they should be placed in a vessel containing a 5 per cent solution of lysol and the feces should be likewise disinfected. Tubes containing fecal or other material should be placed in the lysol solution and afterwards emptied and thoroughly cleaned. Both the preparations and fecal tubes may contain infective material, as the cysts of *Endamœba histolytica* or pathogenic bacteria, and should be handled accordingly.

3. STAINING METHODS FOR ENDAMŒBA HISTOLYTICA

Various more or less complicated staining methods have been recommended for the differentiation of *Endamœba histolytica* and all of them require technical knowledge and experience in their use to be of real value. Fortunately, it is seldom necessary to resort to these methods in the differential diagnosis of this ameba, as unstained preparations and preparations stained with the simple iodine stain are usually sufficient for diagnostic purposes. However, it is sometimes necessary to use one or more of these methods and if one desires to preserve material for study or future reference, some method of staining which will give a permanent preparation is essential. Some authorities use stained preparations for routine diagnostic work but the writer has never found it necessary to do so provided fresh fecal material is examined and the examination is made within a short time after the stool has been passed.

In view of the excellent results that have been obtained with staining and flotation methods in which examination of unstained material has resulted negatively, it is recommended that in the examination of suspected individuals in whom negative results have been obtained from the study of unstained material, staining and flotation methods be employed if possible. In surveys for *Endamœba histolytica*, where many carriers without symptoms are examined, and in whom the amebæ may be few in number, the use of such methods will undoubtedly greatly increase the number of positive results and give a much

more accurate picture of the actual incidence of this infection in any locality.

The Routine Iodine Stain for Cysts.—The method of using this stain in the diagnosis of amebic and flagellate cysts has already been described (see page 45). The iodine solution used varies with different authorities but that devised by D'Antoni is the best as it is stable in stock solution and is superior to Lugol's solution or other iodine solutions. The method of preparing the stain is as follows:

D'Antoni's Standardized Iodine Stain.—The stain is made with a standardized 10 per cent potassium iodide solution which is prepared as follows:

One hundred grams of potassium iodide (Merck's or Baker's) are weighed and placed in a chemically clean 1000 cc. volumetric flask and distilled water added to the mark.

A 25 cc. volumetric flask, clean and absolutely dry, is weighed to the fourth decimal point and the weight recorded. This flask is filled to the mark with the above potassium iodide solution and is again weighed to the fourth decimal place. By subtracting the weight of the empty flask from that of the flask plus the solution, the weight of 25 cc. of the 10 per cent potassium iodide solution is determined. Theoretically, this weight should be 26.925 grams. However, due to the deliquescence of potassium iodide, the actual weight of the solution will be found to be less than the theoretical weight. This difference in weight, when divided by the theoretical weight and the quotient, in terms of percentage, is subtracted from 10 (the percentage desired), represents the actual percentage of the above solution. Thus:

$$100 : \text{actual percentage of solution tested} = x : 10 \text{ per cent}$$
$$(x = \text{grams of potassium iodide})$$

One hundred subtracted from the number of grams obtained from x gives the number of grams of potassium iodide to be added to the above solution to give a standardized 10 per cent potassium iodide solution.

Having secured the standardized solution the stain is prepared by adding to each 100 cc. of a 1 per cent solution obtained from the standardized 10 per cent solution, 1.5 grams of powdered iodine crystals. The staining solution should then be allowed to stand for four days before it is used. It must be filtered before using and should not be allowed to remain unstoppered, as volatilization of the iodine will occur. The stock solution, if tightly stoppered, can be kept for long periods of time without deterioration.

Lugol's Iodine Solution.—While the standardized iodine stain of D'Antoni gives the most satisfactory results, excellent results may also be obtained by the use of Lugol's iodine solution, and this is the stain that is usually employed by protozoölogists and laboratory workers.

It has the disadvantage of deteriorating rather quickly. The formula for Lugol's solution is as follows:

Iodine crystals powdered	5 gm.
Potassium iodide	10 gm.
Distilled water	100 cc.

The potassium iodide is dissolved in the distilled water in a chemically clean flask and the iodine crystals slowly added, shaken until dissolved, and the resultant solution filtered and placed in a tightly stoppered bottle. This solution loses its staining qualities with time and should be stored in the dark and freshly prepared at least every two weeks.

Dobell and O'Connor recommended (1921) a weaker iodine stain than that of the Lugol solution, *i. e.*, 1 per cent iodine in 2 per cent potassium iodide solution, made with distilled water. Lugol's solution may be diluted if it overstains to secure the weaker iodine stain.

Donaldson's Iodine-Eosin Stain.—Many authorities prefer the stain recommended by Donaldson (1917) which combines iodine with eosin in a single staining solution. It is prepared by saturating a 5 per cent aqueous solution of potassium iodide with iodine crystals, the resulting solution being then mixed with an equal volume of a saturated aqueous solution of eosin (yellow aqueous eosin). A small drop of the mixture is mixed with a small portion of fecal material on a microscopic slide, covered with a cover-slip, and examined. The cysts of the intestinal amebæ and flagellates appear as yellowish or greenish-yellow bodies, upon a red background. Glycogen bodies within the cysts are stained brown.

The various iodine stains that have been described are useful only in the examination of material containing the cysts of the intestinal amebæ and flagellates and preparations stained with these solutions cannot be preserved.

STAINS FOR PERMANENT PREPARATIONS OF INTESTINAL PROTOZOA

Many different stains have been proposed for permanent preparations of the intestinal protozoa and all of them require considerable practice before good preparations will be obtained. The first, and most important factor in securing good permanent stained preparations, is proper fixation of the specimen, as it is absolutely necessary that the specimen be fixed before it is allowed to dry upon the microscopic slide or cover-glass. Another most important factor is that the material examined be freshly voided, especially in the case of fecal material containing trophozoites, or motile forms, of the organisms. If the specimen is allowed to dry before the fixing solution is added all definite structure will be destroyed, and the preparation rendered worthless for diagnostic purposes.

Methods of Preparing Smears for Staining.—Smears of fecal material for staining may be prepared in various ways, either upon microscopic slides or cover-glasses. The writer would recommend that microscopic slides be used for this purpose as they are easier to manipulate during the staining process and a much greater amount of material is available for study.

The most commonly used method in making the fecal smears is to spread the material in as uniform a layer as possible with a camel's-hair brush upon the microscopic slide. Another method commonly used is to place a drop of the material to be stained upon one end of the microscopic slide and then smear it along the length of the slide by means of the end of another slide. Still another method is to place the material upon one end of the slide and then smear it along the slide by means of a wooden toothpick. Whatever method is used, the resulting smear should not be too thick or too thin and the amount of material used has to be learned by experience. The smears must be placed in the fixing solution immediately, before any drying can occur or they will be worthless.

Fixing Solutions.—Among the many fixing solutions that have been recommended that of Schaudinn is probably the most generally employed and useful.

Schaudinn's Fixing Solution.—This solution is prepared as follows: To 200 cc. of a saturated solution of mercuric chloride in distilled water add 100 cc. of 95 per cent or absolute alcohol and 15 cc. of glacial acetic acid. This solution will keep indefinitely.

Method of Using.—Just before use the solution should be heated to about 60° C. (140° F.). Fecal smears are rapidly made upon microscopic slides and immediately immersed in the Schaudinn solution and allowed to remain for from two to ten minutes, at the termination of which time the fecal smears are fixed as well as the organisms that may be present. The smears should then be removed from the solution and immersed in 50 per cent alcohol, rinsed in this to remove most of the fixative, and then placed for from ten minutes to half an hour in 70 per cent alcohol to which enough iodine solution has been added to give the mixture a rich port wine color. The smears should then be placed in 70 per cent alcohol where they can be kept until one is ready to stain them. The washing in alcohol and in iodized alcohol is absolutely essential to remove the sublimate of mercury from the specimens, which, if allowed to remain, would ruin the staining reactions of the amebæ and flagellates. *At no time during the process should the smears be allowed to dry upon the microscopic slides or the preparations will be worthless.*

The following fixing solutions have also been found to be useful in the preparation of permanent specimens of the intestinal amebæ and flagellates:

Bouin's Non-alcoholic Fixative. This fixative should be prepared just before using. The formula follows:

Picric acid, saturated aqueous solution	30 parts
Formol (40 per cent)	10 parts
Acetic acid, glacial	2 parts

The solution should be lukewarm when used and the smears of fecal or other material placed in it while still wet and allowed to remain for fifteen to thirty minutes, and can then be stained.

Bouin's Alcoholic Fixative.—The formula of this fixative is as follows:

Picric acid	1 gm.
Acetic acid	15 cc.
Formol (40 per cent)	60 cc.
Alcohol (80 per cent)	150 cc.

A stock solution of the picric acid and alcohol should be kept on hand and the other chemicals added in the proper amounts at the time the fixative is used. The smears are allowed to remain in the solution for from fifteen to thirty minutes and then placed in 70 per cent alcohol until stained, at which time they must be carried through the various graded alcohols into distilled water before staining.

Carnoy's Fixative.—This fixative is recommended by Stitt (Stitt), *et al.* (1938) as excellent for the fixation of amebæ. The formula follows:

Alcohol, absolute	6 parts
Chloroform	3 parts
Acetic acid, glacial	1 part

The moist smears are placed in this solution and allowed to remain for ten to twelve minutes, then placed in absolute alcohol for ten to fifteen minutes, in 95 per cent alcohol for ten to twenty minutes, and finally in distilled water for ten to twenty minutes, after which they should be stained.

Flemming's Fixative.—James (1914) states that this fixative gives excellent results. The formula is as follows:

Osmic acid, 2 per cent in distilled water	4 cc.
Chromic acid, 1 per cent in distilled water	15 cc.
Glacial acetic acid	1 cc.

The smears are fixed with this solution for from five to fifteen minutes and then washed in running water for half an hour. After washing, the smears are hardened in alcohols of increasing strength as after the use of the Schaudinn's fixing solution. It is best to prepare this fixative immediately before use.

Gilson's Fixative—This excellent fixing solution is prepared and used as follows:

Mercuric chloride	5 gm.
Nitric acid (80 per cent)	4 cc.
Acetic acid (glacial)	1 cc.
Alcohol (70 per cent)	25 cc.
Distilled water	220 cc

Smears of the material to be stained should be fixed with this solution for five to ten minutes and then passed through 50, 60 and 70 per cent alcohol. To the 70 per cent alcohol enough iodine should be added to give it a port wine color and the smears should be allowed to remain in this mixture for ten to fifteen minutes after which they should be rinsed in 70 per cent alcohol and stained or preserved in this solution for future staining. Tissues may be left in the fixing solution for as long as twenty-four hours.

Mann's Fixative.—This fixative, according to James (1914) has the following formula:

Mercuric chloride, saturated solution in ¾ per cent sodium chloride solution	50 cc.
Osmic acid, 1 per cent solution in distilled water	50 cc.

The smears are fixed in the dark with this solution for fifteen minutes, then transferred to 50 per cent alcohol in the dark, and changed twice during the first day; after which the smears are taken through 60, 70, 80, 90 per cent and absolute alcohol, and back to 80 per cent alcohol, after which they should be stained.

Zenker's Fixative.—This is a favorite fixative with some authorities. The formula is as follows:

Mercuric chloride	5 gm.
Potassium bichromate	2 5 gm.
Sodium sulphate	1 gm.
Distilled water	100 cc.

The above forms a stock solution to which 5 per cent of glacial acetic acid is added to the amount to be used for fixation. The moist smears are placed in the fixative and allowed to remain for ten to fifteen minutes, then placed in distilled water for ten minutes, in distilled water to which enough iodine solution has been added to give it a port wine color for ten minutes, and finally thoroughly rinsed in distilled water, after which they should be stained.

Staining Solutions and Methods.—For the staining of permanent preparations of the intestinal amebæ various staining solutions and methods have been devised and all of them require considerable experience in their use before good preparations are obtained. Perhaps the most frequent source of failure is allowing the material to become dry at some stage of the fixing or staining process and it cannot be too much emphasized that *it is absolutely necessary to be sure that at no stage of fixation, extraction, staining, differentiation, dehydration or clearing do the preparations become dry.* It is also essential that the dehydration process carried out after fixation of the preparations be very carefully conducted and that the preparations be allowed to remain in the various solutions for a sufficient period of time, and this is also true of the dehydration process after staining. It should also be remembered that the trophozoites of both amebæ and flagellates

stain more easily than do the cysts and this should be allowed for in estimating the time any particular stain is allowed to act, or overstaining will result.

With all of the methods of staining after wet-fixation it is essential that the smears be overstained at first after which the nuclear structures are brought out by certain methods of decolorization or differentiation, as it is called. The amount of differentiation required varies with the thickness of the smear and other factors and can only be ascertained by experience. The decolorization, or differentiation, should be done with the preparation under the microscope and as soon as nuclear details are distinctly visible should be discontinued.

The following staining methods have all been found to give excellent results:

Alli's Staining Method.—This method employs a special fixative and stain.

Fixative.—The fixative has the following formula:

Tannic acid, C.P.	4 gm.
Ethyl alcohol, 70 per cent	90 cc.
Glacial acetic acid	5 cc.
Phenol crystals	1 gm.

Stain.—The stain is prepared by adding to boiling distilled water hematoxylin crystals to a 0.5 per cent concentration and then allowing the mixture to boil until a cherry red color develops. The mixture should then be placed in a suitable container and allowed to ripen for several days after which it is securely corked and stored. The older it is the better.

Method of Staining.—The following is the method of staining:

1. Smears of the fecal material are made upon cover-glasses or slides and are dropped face downward into a Petri dish containing the fixative and allowed to fix for five to seven minutes or longer. If slides are used a suitable staining receptacle should be employed.

2. Wash the preparations in tap water three or four times and then place, face up, in a mordant consisting of a 4 per cent aqueous ferric ammonium sulphate solution and allow to remain for a few seconds, and then wash well in tap water.

3. Stain in the hematoxylin solution for three to five minutes and wash in tap water.

4. Decolorize in 70 per cent alcohol for one half to one minute or just long enough for a distinct blue color to appear and then wash well in tap water.

5. Pass through 50, 70, 85, 95 and absolute alcohol, leaving in each at least one minute.

6. Clear in xylol and mount in Canada balsam. At no stage in the process should the preparations be allowed to dry.

Remarks.—This method is recommended for the staining of the intestinal amebæ and flagellates and is claimed by the originator to be

comparatively simple, economical, rapid and to give excellent results so far as morphology is concerned. It is a rapid method as compared with some others and deserves a more extended use than it has been given by parasitologists.

Dobell's Staining Methods.—The following staining methods have been recommended by Dobell (1942):

Method 1.—Tungstic-hematoxylin Method.

The smears are fixed in Schaudinn's fluid and are then placed in a 2 per cent solution of phosphotungstic acid and left for ten minutes. They are then washed in several changes of distilled water to remove the excess acid, then placed in a ripened 0.2 per cent aqueous solution of hematoxylin for fifteen to thirty minutes or longer and then placed in tap water until of a purple color and mounted as usual.

Method 2.—Molybdic-hematoxylin Method.

The smears, after fixation, are placed in a 2 per cent solution of ammonium molybdic acid and the rest of the procedure is the same as in Method 1.

Remarks.—The above methods are very simple, as a mordant is not necessary, time-saving, and economical, and, as shown by Dobell's results, give excellent morphological detail, thus rendering them of great value in differential diagnosis.

Faust's Iron Hematoxylin Staining Method.—This method is recommended for routine preparation of permanent specimens as it gives good results and is not as time consuming as some of the other methods of staining. The technique is as follows:

1. Fix smears of fecal or other material with Schaudinn's fixing solution which has been heated to a temperature of 60° C. (140° F.). Allow smears to remain in the solution for two minutes.

2. Take smears from fixing solution and immerse in 70 per cent alcohol; 70 per cent alcohol to which enough iodine solution has been added to give the mixture a port wine color; 70 and 50 per cent alcohol, leaving in each for two minutes.

3. Wash smears in gently running water for two minutes.

4. Immerse smears in a 2 per cent aqueous iron-alum solution at 40° C. (104° F.) and leave for two minutes.

5. Wash smears in gently running water for three minutes.

6. Stain in 0.5 aqueous hematoxylin (Grubler's or National Anilin) for two minutes.

7. Wash in gently running water for two minutes.

8. Differentiate in *cold aqueous* iron-alum solution.

9. Wash in gently running water for from ten to fifteen minutes.

10. Immerse smears for two minutes each in 70, 80, 90 per cent and absolute alcohol.

11. Clear the smears with xylol.

12. Mount smears in xylol-balsam.

At no time during these various steps in the staining process should the preparations be allowed to dry.

Remarks.—This is an excellent diagnostic staining method and can be recommended for routine use for the differentiation of *Endamœba histolytica* from other species of amebæ. It is rapid and gives clear nuclear pictures in which the morphology is typical. It is to be preferred to more complicated and time consuming staining methods for diagnostic purposes.

Heidenhain's Iron Hematoxylin Staining Method.—This method is time consuming but gives beautiful results in the hands of a trained technician. Two solutions are employed, the *staining solution* and *the mordant.* The formulæ follow:

Staining Solution:	Hematoxylin crystals (Grubler)	1 gm.
	Alcohol, 90 per cent	10 cc.
	Distilled water	90 cc.

The hematoxylin crystals are dissolved in the alcohol aided by gentle heating and the distilled water is added. The mixture is placed in a tightly corked flask and allowed to ripen, in the sun, if possible, for ten days, after which 100 cc. of distilled water is added and the stain is ready for use. When ripe the mixture should be brown in color.

Mordant Solution: This consists of 2 grams of violet crystals of iron alum (sulphate of iron and ammonium) in 50 cc. of distilled water.

The steps involved in using this method are as follows:

1. Fix smears in Schaudinn's fixing solution.
2. Pass smears through 70 per cent alcohol, 70 per cent alcohol to which enough iodine solution has been added to give a port wine color, 70, 50 and 30 per cent alcohol, leaving in each five minutes.
3. Place in distilled water for ten minutes.
4. Place in the mordant solution and leave for six hours.
5. Rinse smears quickly with distilled water.
6. Place in the staining solution and leave for at least six hours. May be left overnight. At end of this time the smears are a jet black color.
7. Thoroughly wash smears in distilled water.
8. Transfer the smears to a 1 per cent iron-alum solution, made by diluting some of the mordant solution with three parts of distilled water. This will gradually dissolve some of the black color and this decolorization, or differentiation, as it is technically called, is continued until the nuclear structure of the amebæ or flagellates is clearly visible. This is ascertained by washing the smear in distilled water and examining it under the microscope while still in distilled water. When differentiation is adequate proceed as follows:
9. Wash smears in gently running water or distilled water.
10. Dehydrate smears by placing them in 70, 80, 95 per cent and absolute alcohol, leaving in each for five minutes.
11. Clear the smears with xylol.
12. Mount in xylol balsam.

Remarks.—This is an old and reliable staining method for amebæ and flagellates and, when carefully used, gives excellent morphological pictures, but it is very time consuming and requires considerable experience before good results are obtained. For this reason it is not recommended for use in surveys or where the element of time is important, or unless one has had experience in its use.

Johnson's Iron Hematoxylin Staining Method.—This is a comparatively rapid method of staining with hematoxylin and is said to have the advantage that the differentiation, or decolorization, does not need to be controlled by microscopic examination of the preparations. The technique is as follows:

1. Make *thin* films of the material to be stained.
2. Place in Schaudinn's fixing solution, heated to 60° C. and allow to stay for ten minutes.
3. Place in 95 per cent alcohol to which enough iodine solution has been added to give it a port wine color, and allow to stay for five minutes.
4. Place in 70 per cent alcohol for five minutes.
5. Rinse smears in tap water for from one to two minutes.
6. Place in a 4 per cent iron-alum solution and allow to stay for fifteen minutes.
7. Rinse in tap water for one or two minutes.
8. Stain with 0.5 per cent aqueous hematoxylin (Grubler's or National Anilin) for ten minutes.
9. Differentiate in 0.25 per cent iron-alum solution for twelve minutes.
10. Wash in running water for twenty to thirty minutes.
11. Dehydrate by passing through 70, 80, 95 per cent and absolute alcohol, two minutes each. Clear in xylol and mount in xylol balsam.

Remarks.—This method of staining gives good results and is not time consuming. It is useful in differential diagnosis but the morphological details are not as well brought out as in some other staining methods, although enough so that one can use it in the differential diagnosis of the various species of amebæ.

Kessel's Staining Method.—The following staining method is recommended by Kessel as an excellent one for staining the intestinal amebæ and flagellates.

Stain.—The formula of the stain employed is as follows:

Hematoxylin crystals	1 part
Ethyl alcohol	10 parts

Place this mixture in the incubator and allow to ripen for two weeks.

Method of Staining.—1. Fix in Schaudinn's fixative for three minutes at 40° C. temperature.

2. Place in each of the following for two minutes: 70 per cent alcohol, 70 per cent iodine alcohol, 70 per cent alcohol and 50 per cent alcohol.

3. Wash in running tap water for two minutes.
4. Transfer to 2 per cent iron-alum at 40° C. for two minutes.
5. Wash in running water for three minutes.
6. Transfer to 0.5 per cent aqueous hematoxylin at 40° C. for two minutes or longer according to density of the stain.
7. Wash in water for two minutes.
8. Decolorize in cold 2 per cent aqueous iron-alum from one to five minutes. Watch this under the microscope and stop when necessary.
9. Wash in running water for ten minutes.
10. Transfer through 50, 70, 80, 90 and 100 per cent alcohol for two minutes each.
11. Place in xylol for two minutes.
12. Mount in Canada balsam.

Remarks.—This is an excellent staining method and comparatively time-saving. It is essential that the hematoxylin solution should be well ripened and the preparations should not be allowed to dry at any stage in the process of staining.

Mann's Method of Staining, as Modified by Dobell.—This method is recommended by Dobell (1919) and gives very beautiful preparations if properly applied. Unlike the various hematoxylin methods, by which the nuclear membrane, chromatin granules, karyosome and chromatoidal bodies are stained black, and the cytoplasm of the amebæ a grayish-blue, Mann's stain colors the nuclear membrane, chromatin granules, karyosome and chromatoidal bodies a brilliant red while the cytoplasm of the amebæ stains blue. Ingested red blood corpuscles stain red with the Mann stain.

The formula of the staining solution is as follows:

Aqueous solution of methyl (not methylene) blue, 1 per cent	35 cc
Aqueous solution of eosin, 1 per cent	45 cc.
Distilled water	100 cc.

The steps in the staining process are as follows:

1. Fix smears in warm Schaudinn's fixing solution for ten minutes.
2. Place in 50 per cent alcohol and rinse thoroughly.
3. Transfer smears to 70 per cent alcohol to which enough iodine solution has been added to give a port wine color and allow them to remain for ten minutes.
4. Place smears in 70 per cent alcohol and allow to remain for ten minutes.
5. Place in distilled water and allow to remain for ten minutes.
6. Stain smears with the staining solution (methyl blue-eosin) allowing them to stain for from four to twelve hours. The exact time varies with different smears and must be determined by experience.
7. Wash smears thoroughly in distilled water.
8. Differentiate in 70 per cent alcohol containing a few drops of a saturated solution of Orange G (10 drops of the saturated Orange

G solution to 100 cc. of 70 per cent alcohol). The differentiation must be controlled under the microscope.

9. Wash in distilled water.

10. Dehydrate by passing the smears through 70, 80, 95 per cent and absolute alcohol, leaving in each at least five minutes.

11. Clear smears in xylol.

12. Mount in xylol balsam. (At no time during the staining process should the smears be allowed to dry or they will be useless.)

Mayer's "Hæmalum" Staining Method.—This is an excellent staining method for amebæ and flagellates and is comparatively rapid. The formula for the staining solution is as follows:

Hematoxylin crystals (Grubler's or National Anilin)	1 gm.
Sodium iodate	0.2 gm.
Potash alum	50 gm.
Distilled water	1000 cc.

The hematoxylin crystals are dissolved in the distilled water and the sodium iodate and potash alum then added, and the mixture filtered.

This stain is used as follows:

1. Fix smears in Schaudinn's fixing fluid as in methods described above.

2. Pass smears through 95, 70, 50 and 30 per cent alcohol, leaving in each for ten minutes.

3. Place smears in distilled water and leave for ten minutes.

4. Stain with the hematoxylin staining mixture for from five to twenty minutes. Trophozoites for five to ten minutes and cysts for twenty minutes.

5. Immerse smears in gently running tap water and leave until they appear blue in color.

6. Pass smears through 30, 50, 70 and 90 per cent alcohol, leaving in each at least five minutes.

7. Immerse smears in absolute alcohol and leave for ten minutes.

8. Immerse smears in equal parts of absolute alcohol and xylol and leave for five minutes.

9. Clear smears in xylol and mount in neutral balsam.

Mallory's Chloride of Iron-Hematoxylin Stain.—James (1914) recommends this stain for the identification of *Endamœba histolytica*. The reagents employed in the staining process are the following:

1. Hematoxylin, 1 per cent solution in distilled water, freshly prepared.

2. Iron-alum, a $2\frac{1}{2}$ per cent solution in distilled water.

3. Ferric chloride, 10 per cent solution in distilled water.

4. Ferric chloride, $\frac{1}{2}$ to 1 per cent solution in distilled water.

The hematoxylin solution is prepared by heating a test tube of distilled water and dissolving in it enough hematoxylin crystals to give the mixture a cherry-red color, which will result approximately in a 1 per cent solution. The various solutions should be freshly prepared.

The method of employing this stain, according to James, is as follows:

1. After fixation and dehydration the smears are placed in distilled water.

2. Place the preparations on glass rods or over a staining jar and cover at once with the 10 per cent aqueous solution of ferric chloride and mordant in this for ten to fifteen minutes.

3. Drain the slides and cover preparations with the 1 per cent solution of hematoxylin, when a dense black precipitate appears. The solution should then be poured off and fresh staining solution should be added until the preparations are flooded and are visible through the solution. They are then stained for from five to ten minutes.

4. Wash the preparations in two changes of distilled water.

5. Decolorize in a $\frac{1}{4}$ to 1 per cent solution of ferric chloride until nuclear structure is clearly visible. It is best to use the weaker solution first and increase to the stronger, as decolorization may be too rapid if the 1 per cent solution be employed at first.

6. Rinse thoroughly in distilled water.

7. Wash in running water for at least fifteen minutes, pass through 60, 70, 80 and 95 per cent alcohol clear with xylol and mount in neutral balsam.

Mallory's Phosphotungstic Acid-Hematin Stain, James Modification.—As James (1914) says: "This excellent stain has two advantages; it does not require a mordant, and it is ready for use in twenty-four hours." The writer has seen very beautiful preparations made by James, in which this staining method was used and can confirm his further statement that "it is particularly valuable for demonstrating the chromatin of the nucleus, especially in separating the chromatin of the karyosome from the achromatinic substances." This method of staining, as modified by W. M. James, is as follows:

The *staining solution* has the following formula:

Hematin, preferably hematin ammonium	1 gm.
Phosphotungstic acid (Merck) 10 per cent aqueous solution	20 cc.
Distilled water	100 cc.

After mixing the above the stain is ripened by adding 5 cc. of a $\frac{1}{4}$ per cent aqueous solution of potassium permanganate and allowing the mixture to stand for twenty-four hours.

The following are the steps in the staining process:

1. Place smears in distilled water after fixing and dehydrating.

2. Stain in the phosphotungstic acid hematoxylin for from twelve to twenty-four hours.

3. Rinse smears in distilled water.

4. Decolorize in a strong solution of Orange G, in distilled water. (James uses a nearly saturated solution.) Usually about half an hour is required for decolorization. The preparations should be examined under the microscope at intervals by washing them off in distilled water, mounting in this, using a cover-glass.

5. Wash thoroughly in two changes of distilled water in order to remove all of the Orange G.

6. Dehydrate by placing the smears in 80 per cent alcohol for five minutes; in two changes of 96 per cent alcohol, for five minutes each; and in two changes of absolute alcohol, in the first for five minutes and in the second for ten minutes.

7. Clear the smears in xylol and mount in neutral balsam.

If the smears are very thin, James recommends that instead of decolorizing in the Orange G, the preparations be taken directly from the staining solution into 96 per cent alcohol, allowed to remain in this for five minutes and then taken through absolute alcohol, two changes, and xylol, and then mounted in neutral balsam.

Remarks.—While this staining method gives good results, it is too time consuming for routine diagnostic work or for use in making surveys.

Phosphotungstic Acid-Hematoxylin Method.—The staining solution employed in this method is prepared as follows:

One gram of hematoxylin crystals is added to 80 cc. of distilled water, and dissolved by heat over a Bunsen burner. To the solution so obtained add 20 cc. of a 10 per cent solution of phosphotungstic acid, dissolved in distilled water by heating. The mixture is allowed to ripen for two weeks before it is used.

Method of Staining.—Thin smears of the feces to be examined are made upon microscopic slides and fixed in Schaudinn's fixative, as usual. Then proceed as follows:

1. Wash the films in distilled water.

2. Rinse the films in distilled water and add enough iodine solution to color the water a distinct port wine tint and allow them to remain in this mixture for from ten to fifteen minutes, after which they should be washed again in distilled water.

3. Place the smears in water in which one crystal of hyposulphite of soda has been dissolved and leave long enough for the brown color of the smears to disappear, and then wash in water.

4. Immerse the smears in the phosphotungstic-hematoxylin staining solution and leave for twelve hours, after which they are washed in distilled water.

5. Wash the smears twice in absolute alcohol and absolute alcohol and xylol, equal parts, and mount in neutral Canada balsam.

Remarks.—This staining method gives excellent results in staining the nuclei of trophozoites and cysts but while it is a good diagnostic method, it is very time consuming, and not as useful as many others in survey work or routine diagnostic work. It is also true that the morphology of the stained nuclei becomes indistinct after a few days so that the method should not be employed in the preparation of permanent smears for class use or future reference.

Quensel's Method for Staining Living Trophozoites.—This method was introduced by Quensel in 1918 but has been neglected or lost sight of in recent years. It has been in use in the laboratories of the School of Hygiene and Public Health, Johns Hopkins University, since 1936, after being introduced there in 1936, by Dr. Justin Andrews. It is the only method available which gives good results in the staining of trophozoites in fresh fecal specimens but it does not stain the cysts of the amebæ or the trophozoites or cysts of the flagellates.

Staining Solution.—The staining solution is prepared as follows:

1. Mix 20 cc. of a saturated solution of Sudan III in 80 per cent ethyl alcohol.
2. Mix the above with 30 cc. of a saturated and filtered aqueous solution of methylene blue (Medicinal).
3. Filter the above mixture into 50 cc. of a 10 per cent solution of cadmium chloride C. P. in distilled water.
4. Shake gently now and then for twenty minutes, during which time a flocculent precipitate forms and the mixture becomes practically colorless.
5. After shaking, filter and remove the fluid from the precipitate by placing the filter paper containing it upon another filter paper and leave over night to dry.
6. Transfer the precipitate to a fresh filter paper and wash rapidly with 25 to 30 cc. of distilled water.
7. Dissolve the washed precipitate in 250 cc. of distilled water, and filter again if crystals of cadmium chloride form within a few days.

This staining solution may be kept for several months, if desired.

Method of Staining.—A small amount of the fecal material to be stained is mixed upon a microscopic slide in a large drop of the staining solution. From ten to thirty minutes are required for staining. Overstaining occurs after this period of time. Cover with a cover-glass and examine at intervals during staining.

Remarks.—This is a most valuable method of staining the trophozoites of the intestinal amebæ in fresh fecal material and should be of great value in differentiating the trophozoites of the various species. The nuclei stain blue and the nuclear membrane and karyosomes are clearly differentiated with the exception of the nucleus of *Dientamœba fragilis*. As the cysts do not stain this method is of no value in survey work but is a most useful clinical method in studying the trophozoites, especially those of *Endamœba histolytica* and *Endamœba coli*. *Blastocystis hominis* and leukocytes also stain with this method.

Ratcliffe and Parkin's Staining Method.—The following staining method is recommended by Ratcliffe and Parkins as a substitute for the Heidenhain method.

1. Fix the fecal smears in Schaudinn's fixative or a modification of the same which contains 5 per cent of acetic acid, for one to twenty-four hours.

2. Remove the $HgCl_2$ with iodine solution and the iodine with 70 per cent alcohol and then wash the smears in water.

3. Stain with phosphotungstic-acid-hematin (Mallory's) for twenty to thirty minutes. (See page 61.)

Wash with water until the smears become blue, and then dehydrate in 95 per cent alcohol and absolute alcohol. The dehydration should be done slowly. Be careful to prevent drying of the smears during any stage of the operation.

Remarks.—With this stain the cytoplasm of the amebæ stains blue and the nuclei a darker blue, the morphological details being well brought out. The length of staining required with Mallory's phosphotungstic acid solution will vary with each batch of stain and has to be ascertained by actual trial. While this method gives excellent diagnostic results, it is one that requires much experience before such results are obtained with it and, therefore, it is not as generally useful in diagnosis as some others which are described in this section.

Rosenbusch Method of Staining.—This is a method of hematoxylin staining that gives excellent results but requires considerable practice before such results can be obtained. Three solutions are employed in this staining method, as follows:

Solution 1:	Hematoxylin crystals	1 gm.
	Alcohol, 95 per cent	100 cc.

The hematoxylin crystals are dissolved in the alcohol, the mixture placed in a tightly stoppered flask and allowed to stand for ten days before using.

Solution 2:	Saturated solution of lithium carbonate in distilled water.	
Solution 3:	Sulphate of iron and ammonium (iron-alum)	3.5 gm.
	Distilled water	100 cc.

The staining mixture is prepared by adding 5 to 6 drops of Solution 2 to each 10 cc. of Solution 1, just before staining, while Solution 3 is the mordant, and when diluted, the differentiating solution.

The steps involved in the staining process are the following:

1. Fix the smears in Schaudinn's fixing solution for five to ten minutes.

2. Immerse the smears for five minutes in 70 per cent alcohol; 70 per cent alcohol to which enough iodine solution has been added to give a port wine color; 90 per cent alcohol; and distilled water.

3. Place the smears in the 3.5 per cent solution of iron-alum (Solution 3) and allow to remain for one-half hour or longer.

4. Wash well in distilled water.

5. Stain smears in the hematoxylin-lithium-carbonate mixture (Solutions 1 and 2) for from five to twenty minutes. Cysts require longer staining than trophozoites.

6. Wash thoroughly in distilled water.

7. Differentiate with the iron-alum solution used in Step 3 which has been diluted 3 parts with distilled water.

8. Wash thoroughly in distilled water.

9. Immerse in 95 per cent alcohol and absolute alcohol for ten minutes each.

10. Clear smears with xylol.

11. Mount smears in xylol balsam.

At no time during the staining process should the smears be allowed to dry or they will be worthless.

Walker's Method of Staining.—This is a rapid method of staining with hematoxylin and gives good diagnostic results but not as beautiful preparations as the Mann or Heidenhain iron-hematoxylin methods. For routine diagnostic work this is an excellent staining method. The staining solution has the following formula:

Hematoxylin crystals (Grubler's or National Anilin)	1 gm.
Saturated aqueous solution of ammonia alum	100 cc.
Distilled water	300 cc.
Thymol	a crystal

The hematoxylin crystals are dissolved in the distilled water by aid of gentle heat and the ammonium alum and thymol crystal then added. The mixture should be allowed to ripen for ten days, after being placed in a loosely stoppered flask, using cotton for this purpose. After it is ready for use it should be kept in the dark.

The stain is used as follows:

1. The smears are fixed in the Schaudinn fixing solution for five to ten minutes.

2. Wash thoroughly in distilled water. Several changes of distilled water should be used.

3. Immerse smears in the aqueous alum-hematoxylin staining mixture and allow to stain for from three to five minutes.

4. Immerse smears in 50, 60, 70, 90 and 95 per cent alcohol, leaving in each for at least five minutes.

5. Immerse smears in absolute alcohol and leave for ten minutes.

6. Clear smears in xylol.

7. Mount smears in xylol balsam.

Preparation and Staining of Sections of Tissue.—For the study of the distribution of *Endamœba histolytica* in the lesions produced by it, and the effect of this parasite upon the invaded tissues, it is necessary to prepare and stain sections of the intestine, liver or other organ infected and in doing so the technique usually employed in pathological studies is followed. For the best results the pieces of tissue should be fixed in Zenker's fluid for twenty-four hours and washed in running tap water for the same period of time, and then embedded in paraffine. *Serial* sections should be cut and stained preferably with hematoxylin and eosin, dehydrated and cleared with xylol in the usual manner, and mounted in neutral balsam.

Objects Which May Be Mistaken for Amebæ in Unstained and Stained Preparations.—The feces of man contains numerous cells that may be mistaken in both unstained and stained fecal smears for *Endamœba histolytica* or other intestinal amebæ by one who has not

had a wide experience in the diagnosis of these organisms, and if the feces are allowed to stand exposed to the air other confusing elements may contaminate them from dust, as various pollens and the cysts of free-living amebæ or flagellates, as well as the trophozoites derived from the cysts.

Among the objects that may cause confusion in freshly voided stools are the various *helminth ova*, some of which might be mistaken by the

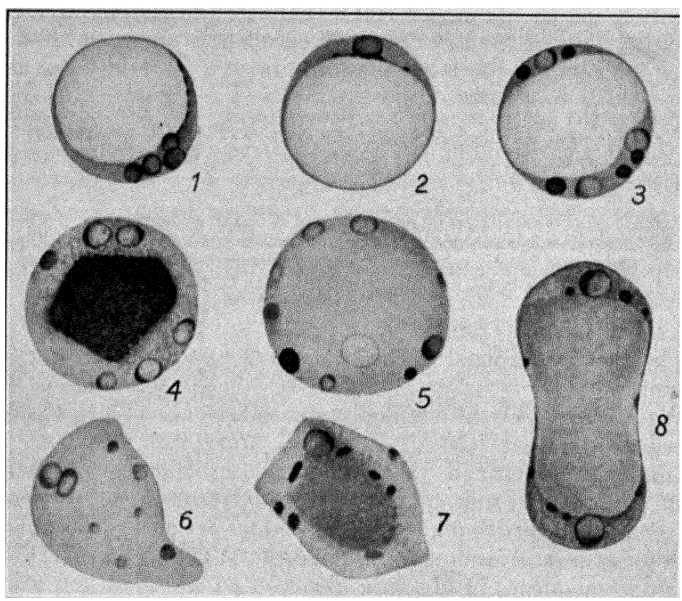


FIG. 11.—*Blastocystis hominis*. Stained with iron hematoxylin. × 2000. *1–7*, ordinary forms; *8*, dividing form. (After Wenyon, in "Protozoölogy," courtesy of Baillière, Tindall and Cox.)

beginner in fecal examination for the cysts of amebæ. Such a mistake can be avoided by measuring the suspected body, for the eggs of helminths are usually larger than the largest amebic cyst, while most of these eggs are covered with a thick shell, either smooth or mammillated, and often colored by the bile. In addition, the embryo within the helminth eggs differs entirely in structure from that of the contents of any amebic cyst.

Blastocystis hominis, a vegetable organism commonly occurring in human feces, is probably more often mistaken, especially in unstained preparations, for the cysts of the intestinal amebæ, than any other one object, and many erroneous diagnoses of infection with *Endamœba histolytica* have been based upon finding this organism in the patient's stools. *Blastocystis hominis* in unstained fecal preparations occurs as a

colorless, hyaline, refractile body of oval or spherical shape, and possessing no ameboid motility. It measures from 5 to as many as 40 microns in diameter, the average organism measuring from 10 to 15 microns. The shape is usually spherical but dividing forms are common resembling an hour-glass in shape. The most common organisms consist of a large central mass of finely granular hyaline material surrounded by a narrow band of more refractile hyaline material containing highly refractile granules and one or more larger, round or oval bodies, the nuclei. The entire cell is surrounded by a delicate refractile membrane which may be mistaken for a cyst wall. Some of the blastocysts appear to be filled with oval or round nuclei, resembling spores. The central material, which usually comprises at least two-thirds of the organism is referred to by some writers as "reserve material" and the outer cytoplasmic border as the "protoplasmic layer." The relative proportions of these two parts of the blastocyst varies considerably and rarely the central reserve material is smaller in size than the protoplasmic border. In some instances a capsule is present surrounding the entire cell.

Some authorities believe that several species of blastocysts exist and Lynch (1922) described no less than three distinct species based upon differences in morphology. While the great variation in the morphology of *Blastocystis hominis* supports the possible existence of several species it cannot be said that the observations of Lynch and others, in this respect, have been confirmed.

In preparations stained with the hematoxylin stains, after wet-fixation, the central reserve material stains a grayish color, while the peripheral band of cytoplasm remains unstained but contains well-stained nuclei, the central karyosome of which is of considerable size and stains intensely black.

Blastocystis hominis, while often confused with the cysts of the intestinal amebæ, should be easily distinguished if attention is paid to the entirely different structure of this organism.

A method of eliminating *Blastocystis hominis* from fecal preparations has been described by Hakansson (see page 45).

Other objects occurring in the feces which may possibly be mistaken for amebæ are goblet cells and epithelial cells from the wall of the intestine, and *endothelial cells* from the blood vessels, which sometimes contain red blood corpuscles (see page 69). Neutrophilic leukocytes have also been mistaken for small cysts of *Endamœba histolytica* in unstained fecal preparations but such a mistake could be made only by a tyro in clinical pathology.

An excellent general rule to follow in the diagnosis of the trophozoite, or motile, stage of the intestinal amebæ is to never regard any cell as a trophozoite in unstained preparations, unless it is moving or extruding well-defined pseudopodia and to never diagnose such an organism as *Endamœba histolytica*, if the stool contains blood, unless

red blood corpuscles are present in the cytoplasm, if one is not expert in the differential diagnosis of the intestinal protozoa.

Charcot-Leyden Crystals and Their Diagnostic Value.—Many of the older writers upon amebic dysentery stress the diagnostic value of Charcot-Leyden crystals in the stools of individuals infected with *Endamœba histolytica*, if diarrhea or dysentery be present. While it is true that these crystals are often seen in amebic dysentery stools, they occur in other conditions, especially in helminthic infections, so that their presence can be regarded as only suggestive at most, although it should lead to a very careful examination for *Endamœba histolytica*. (These crystals are illustrated in Figure 12.)

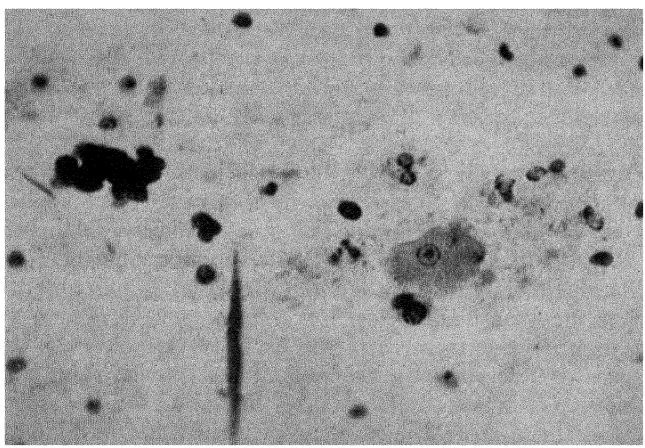


FIG. 12.—Exudate in amebic dysentery, showing non-cellular character. Note the agglutination of the red blood corpuscles, the pyknotic nuclei of the few leukocytes present, the Charcot-Leyden crystal and the small trophozoite of *Endamœba histolytica*. (Army Medical Museum Collection. Photomicrograph by Callender.) (From Craig's "Amebiasis and Amebic Dysentery," courtesy of Charles C Thomas, Springfield, Ill.)

The Morphology of the Cellular Exudate in the Diagnosis of Amebiasis.—The characteristic morphology of the cellular exudate in amebic dysentery and its importance as a distinguishing diagnostic feature between amebic and bacillary dysentery, was first called attention to by Willmore and Shearman, in 1916, and their observations have been confirmed and extended by several investigators, notably by Bahr and Willmore (1917), Manson-Bahr (1919), Haughwout (1921–1924), Haughwout and Callender (1925), and Callender (1925–1934). The latter authority, especially, has urged the value of a study of the cytology of the dysenteric exudates in the differentiation of amebic and bacillary dysentery. It is in the latter type of dysentery that such a study is most valuable in diagnosis, owing to the difficulty of isolating

and identifying the various dysentery bacilli but even in amebic dysentery the cytology of the stool is so characteristic as to be of considerable diagnostic value, although in this infection the diagnosis should rest upon the demonstration of *Endamœba histolytica* in the exudate.

In uncomplicated cases of amebic dysentery the stools contain blood, the corpuscles being usually collected in clumps of irregular shape, while in the stools of bacillary dysentery the red blood corpuscles are distributed singly throughout the exudate. Charcot-Læyden crystals

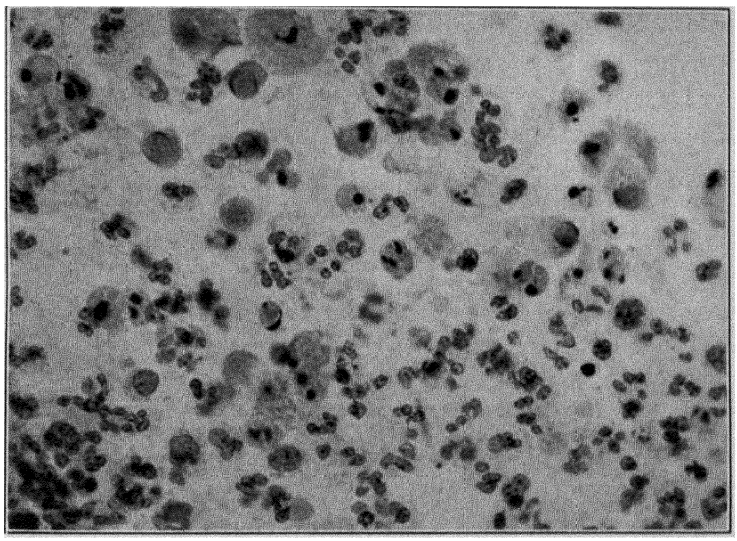


FIG. 13.—Exudate in acute bacillary dysentery, showing cellular character, in contradistinction to that of amebic dysentery, as shown in Figure 12. Exudate consists of very numerous pus cell and macrophages and no agglutinated red blood corpuscles. (Army Medical School Collection. Photomicrograph by Callender.)

may be present in the amebic dysentery exudate but do not occur in the stools of bacillary dysentery. In the amebic dysentery stool, pus cells are very few in number and the leukocytes and tissue cells that may be present show marked evidence of cytolysis, a very large percentage of them consisting of the nucleus surrounded by very little or no cytoplasm. These cells are known as "pyknotic bodies" and are very characteristic of the amebic dysentery exudate. In the stools of bacillary dysentery very large numbers of pus cells occur and form at least 90 per cent of the cells present. Such cells consist of degenerated leukocytes, the nuclei of which present evidences of toxic degeneration. In addition to the pus cells there occur in the bacillary dysentery exudate very large, mononuclear cells, endothelial in origin,

which are known as macrophages, and which often contain red blood corpuscles which have been ingested by these cells. The resemblance of these cells to trophozoites of *Endamœba histolytica* that have ingested red blood corpuscles has led to countless mistakes in diagnosis and the writer has repeatedly been shown these cells as trophozoites of this species of ameba by well-trained technicians. The mistake is excusable

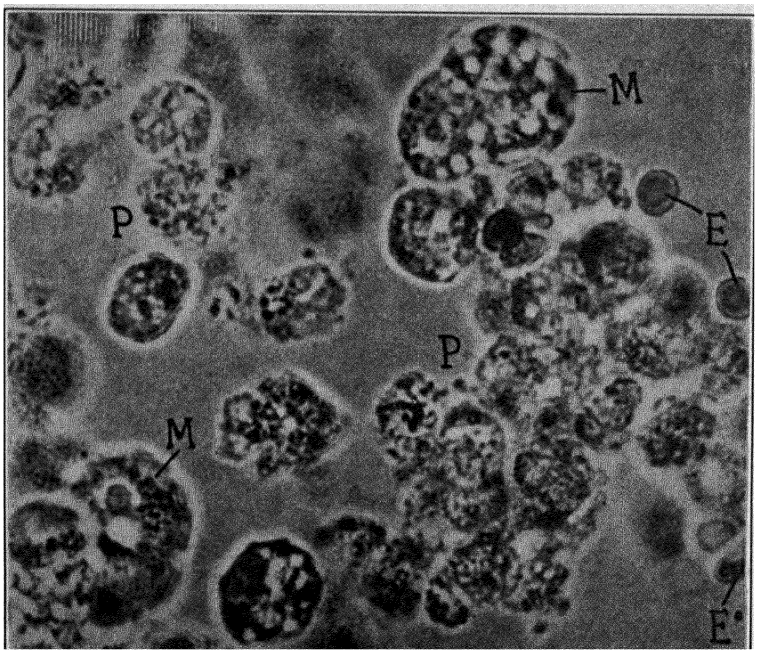


FIG 14.—Exudate in acute bacillary dysentery. High magnification. Note granular appearance of the cells which is very characteristic. *M*, macrophages. *P*, polymorphonuclear leukocytes. *E*, erythrocytes. Photomicrograph and preparation by Callender. (After Haughwout, in Bulletin No. 3, 1924, Institute for Medical Research, Federated Malay States. Manila, P. I. Bureau of Printing.)

and emphasizes the importance of the rule already mentioned: *i. e.*, to never make a diagnosis of *Endamœba histolytica* in the case of a cell containing red blood corpuscles unless pseudopodia are extruded and the cell possesses progressive motility. The macrophage cells in bacillary dysentery stools have no progressive motility.

Another cell occurring in the bacillary dysentery stool of diagnostic importance is the so-called "ghost-cell." These cells consist of macrophage cells or leukocytes in which degeneration has occurred to such an extent that nothing but the periplast and a few chromatin granules are left, the nuclei having been entirely destroyed.

It is, as Callender (1925) states, perfectly possible to make a pre-

sumptive differential diagnosis between amebic and bacillary dysentery by a study of the cellular exudate in the stools and such a study can be made by anyone who is trained in the use of the microscope and is familiar with the morphology of the cells occurring in fecal material. However, one should, if possible, demand the demonstration of *Endamœba histolytica* or of one of the dysentery bacilli before accepting as final a diagnosis of either infection. In addition, combined infections with this ameba and the dysentery bacilli not infrequently occur and in such cases the cellular exudate is of little value in arriving at the diagnosis. Under certain conditions, where the services of a protozoölogist or bacteriologist are not available, the study of the cytology of the stools in patients having diarrhea or dysentery will furnish very valuable information as to the nature of the infection and should never be neglected.

CHAPTER III

THE CULTIVATION OF ENDAMŒBA HISTOLYTICA

TECHNIQUE OF CULTIVATION—MAINTENANCE OF *Endamœba histolytica* IN CULTURES—MORPHOLOGY OF *Endamœba histolytica* IN CULTURES—VIRULENCE OF *Endamœba histolytica* IN CULTURES—CULTURE METHODS AND MEDIA

ALTHOUGH many investigators endeavored to cultivate *Endamœba histolytica* prior to 1918, it was not until that year that this ameba was apparently successfully cultivated upon artificial culture media by Cutler. He employed media containing blood clot and eggs and, in view of later observations it is believed that he was really the first to obtain cultures of this parasite, although this is disputed by some authorities, as his work was not confirmed. However, Boeck and Drbohlav (1925), using special culture media devised by them, were successful in obtaining cultures of *Endamœba histolytica* in conjunction with the bacteria occurring in the stools along with this ameba, and were able to maintain such cultures for weeks and months without special difficulty. Pure cultures of *Endamœba histolytica* have been obtained by several observers but unless bacteria are added to such cultures the amebæ perish and the cultures cannot be maintained. Rees (1941) has succeeded in obtaining pure cultures by inoculating a single washed cyst obtained with a micro-manipulator into suitable media but found that unless he added certain bacteria to the cultures the amebæ reproduced for only a short time and then perished.

It is needless to state that it is most desirable that pure cultures of this ameba be obtained if possible, as the employment of such cultures in diagnostic and research work would solve many of the problems of amebiasis but even mixed cultures of amebæ and bacteria are very useful in diagnosis and in the study of the morphology, life-cycles and pathogenicity of this organism.

DIAGNOSTIC VALUE OF CULTURES

The value of routine cultures of *Endamœba histolytica* in the diagnosis of amebiasis was first stressed by St. John (1926) who found that he obtained a higher percentage of positive results in a survey by employing routine culture methods, combined with a microscopic examination of the stools, than with a microscopic examination alone. Later, St. John and the writer (1927) surveyed 71 physicians for *Endamœba histolytica*, comparing the results obtained from the microscopic examination of a single stool, and of a concentrate, with those

obtained from a single routine culture examination, and found that 39 were positive for one or more species of amebæ by the culture method, while but 27 were positive with the concentration method and only 19 by the direct microscopic examination. *Endamœba histolytica* was found in 11, or 15.49 per cent of the persons examined by cultures; in 9, or 12.67 per cent by the concentration method, and in only 6, or 8.45 per cent by the direct microscopic examination of the stools. None of those examined presented definite symptoms of amebiasis at the time of examination.

Other observers have confirmed the value of cultures in the diagnosis of amebiasis. Thus, Svensson and Linders (1934) obtained positive results in 94 per cent of known infections with *Endamœba histolytica* by culturing a single stool obtained by catharsis, while Poindexter (1933) states that in his hands cultural methods were superior to the direct microscopic examination of the stool in the detection of the parasite. Tonney, Hoeft and Spector (1933) found that the cultural method of diagnosis greatly increased the percentage of positive findings for *Endamœba histolytica* in their survey of food-handlers in hotels in Chicago during the epidemic of amebiasis in that city, and Nauss and Salinger (1935), in their examination of 270 apparently healthy individuals in New York City, found 3, or 1.1 per cent positive by direct microscopic examination and a study of stained preparations, while with culture methods they found 9, or 8.3 per cent, of these individuals positive for *Endamœba histolytica*.

Tsuchiya (1942), employing his culture medium (see page 81) in comparing the relative value of the culture method of diagnosis, direct fecal examination, and a concentration method, found that in 100 cases of known amebiasis the microscopic examination of unconcentrated preparations revealed 86 per cent of the cases, the concentration method 91 per cent, and the culture method 97 per cent. He found that the culture method was especially valuable in the case of carriers of *Endamœba histolytica* showing very small numbers of cysts in the stools. He recommends that cultures be made as a routine procedure in the examination of stools for this parasite as it increases the percentage of positive findings and confirms negative findings by other methods.

While some authorities have reported less favorable diagnostic results from the use of cultures, it has almost invariably been found that in such instances serious errors in technique occurred or the media used were unfavorable for the cultivation of *Endamœba histolytica*. As a result of his experience the writer believes that if a direct microscopic examination of the stool results negatively, preparations secured by the flotation method of Faust should be next examined, and if these also be negative, cultures of the stool should be made and examined at the end of twenty-four and forty-eight hours, as a routine procedure.

TECHNIQUE OF CULTIVATION

For the cultivation of *Endamœba histolytica* standard test tubes should be employed, while the inoculation and transfers of the material to be cultured should be made with a 1 cc. pipette having a good sized bore. The technique used in making bacteriological cultures will absolutely fail when applied to the cultivation of protozoa, especially amebæ. Thus, the inoculation of the culture medium with a platinum loopful of material, as in the inoculation of bacterial cultures, will invariably result in failure to obtain a growth of amebæ, as the amount of material so inoculated is altogether insufficient, and the same is true as regards transfers of cultures. It is also useless to spend time in the microscopic examination of a loopful of material from the culture for the ameba, as the chances of finding the organism in so small an amount are practically negligible, as cultures of *Endamœba histolytica* do not contain countless organisms as in the case of bacteriological cultures and seldom show more than a few amebæ to a microscopic field even when a large drop of material from the culture is being examined.

In culturing stools or other material for *Endamœba histolytica* aseptic precautions are not as essential as in making bacteriological cultures but as it has been found that certain bacterial contaminations may harmfully influence the growth of this parasite in cultures, it is best to observe ordinary aseptic precautions in inoculating and transferring the cultures, although the inoculating and transferring pipettes should never be heated as is the platinum loop in bacteriological technique.

Both fluid and solid slant cultures covered with a nutrient liquid are employed in the cultivation of *Endamœba histolytica*, and the fecal and other material to be cultured should be as fresh as possible, especially if stools of fluid or semi-fluid consistence are to be cultured. It is useless to inoculate culture media with material from fluid or semi-fluid stools that have been voided for more than fifteen minutes or exposed to cold temperatures for any length of time after passage, as the trophozoites of *Endamœba histolytica* will have been killed or so injured as to be incapable of reproduction in the culture media. For routine diagnostic purposes a small portion of the fecal mass about the size of a pea, if the stool is formed or semi-formed, or about 0.5 cc. of the fluid or semi-fluid stool, is thoroughly mixed with the liquid medium or with the fluid covering the slant, if slant cultures are used, and the tubes placed in the incubator at 37° C. (98.6° F.). At the end of twenty-four hours the cultures should be examined microscopically and if found negative for the amebæ should be replaced in the incubator, and again examined at the end of forty-eight hours. If still negative the cultures should be discarded and reported as *negative.*

In making preparations from the cultures for microscopic examination a 1 cc. glass pipette, having a good sized bore, should be used for procuring the material from the culture for examination, and a large

drop of the sediment at the bottom of liquid cultures or the same amount of material from the junction of the liquid and slant *at the bottom* of slant cultures, should be removed, placed upon a microscopic slide, covered with a cover-glass, and examined unstained for the amebæ. In securing material from the slant cultures one should gently rub the surface of the slanted medium with the pipette and mix the material so loosened with the fluid at the base of the slant, as amebæ are usually present in considerable numbers upon the surface of the slant. In either fluid or slant cultures it is useless to examine material from the upper portion of the culture medium and in the case of fluid cultures most of the amebæ will be found in the sediment at the bottom of the culture tube.

It has already been stated that the amebæ seldom occur in very large numbers in routine diagnostic cultures and the failure to recognize this fact has been responsible for poor results in the hands of inexperienced observers, who have considered cultures as negative because they did not find numerous amebæ present upon a casual examination, as is the case with bacteria in bacteriological diagnostic work. In the average twenty-four hour culture upon the ordinary media employed for the purpose it is not usual to observe more than 2 to 4 amebæ in a microscopic field, and many fields do not contain a single organism. In fact, no culture should be considered as negative unless at least 3 preparations from it have been thoroughly examined at intervals of twenty-four and forty-eight hours. The cultures at the end of twenty-four hours are sometimes apparently negative but when reëxamined at the end of forty-eight hours amebæ may be found present in small numbers. Usually, primary cultures of *Endamœba histolytica* do not show many amebæ, and then only in scattered fields, but transfers from such cultures may be rich in amebæ and the writer has often observed scores of amebæ in a single field of the microscope in cultures that have been maintained for some time in the laboratory, transferring every other day.

MAINTENANCE OF ENDAMŒBA HISTOLYTICA IN CULTURES

For purposes of experimental research it is necessary to maintain cultures of *Endamœba histolytica* in the laboratory and this can be easily accomplished and various media, hereafter described, have been found excellent for maintenance. However, one must observe certain technical rules if success is to be expected and it will be found that certain strains of the parasite are more easily maintained in culture than are others. The first essential for the maintenance of the ameba in cultures is the use of a uniform and carefully prepared culture medium which should not vary in the quantity or quality of its constituents. Another important detail is the temperature of incubation, which should not exceed 37° C. (98.6° F.), and should not fall below

36° C. (96.8° F.), at any time. In making transfers, aseptic precautions should be observed for it has been found that the introduction of new bacteria into the culture medium frequently results in the death of the amebæ. The transferring pipette should have been sterilized by heat and allowed to cool before using and a separate pipette should be employed for each culture tube. As much of the sediment in the culture should be transferred as is possible and a pipette with a large bore should be used for the purpose. Transfers should never be made with the platinum loop used in bacteriological technique as failure will usually result, the amount of material so transferred being altogether inadequate. The time of transfer varies with different media but is usually at the end of every forty-eight hours with the ordinary media employed for the culture of *Endamœba histolytica*. In inoculating and transferring material for culturing *Endamœba histolytica* suitable pipettes for routine use are the 1 cc. graduated pipettes used in serological technique. Such pipettes have a bore sufficiently large and in using them enough material can be obtained through capillary attraction, thus obviating the employment of a rubber bulb attached to the end of the pipette for suction purposes, as advised by some authorities. A separate pipette should be used for each culture and as soon as used the pipette should be placed in a jar containing an antiseptic solution.

It has been the experience of the writer, and others, that the number of the amebæ in cultures varies greatly from day to day and there often appears to be a cyclical variation in the growth and reproduction of these parasites in cultures. At times the cultures will have so few amebæ in them that one believes that the organisms are dying out but perhaps the next day's examination will show large numbers of actively motile amebæ and this may continue for several days, when again they will almost disappear from the cultures. For this reason one should not discard cultures that are apparently negative but should make transfers as usual and it will frequently be found that the transfers show numerous amebæ upon examination.

MORPHOLOGY OF ENDAMŒBA HISTOLYTICA IN CULTURES

In cultures the morphology of *Endamœba histolytica* is practically the same as in the stools. Both trophozoites and cysts are present in cultures and do not vary from normal in appearance except that the trophozoites always contain numerous bacteria in contrast to the absence of bacteria in the trophozoites observed in stools unless the amebæ are undergoing degeneration. Indeed, it has been found that it is impossible to cultivate *Endamœba histolytica* for any length of time except in the presence of bacteria, as pure cultures of this organism quickly perish unless bacteria are added to the culture medium.

The trophozoites, or motile forms, of *Endamœba histolytica*, as observed in cultures, are usually larger than the average amebæ seen

in the stool, unless diarrhea or dysentery are present, and are actively motile if the culture be examined in a warm room. When first removed from the culture tube the trophozoites are rounded up and motionless but motility begins within five minutes, the amebæ sending out rounded pseudopodia succeeded by progressive motility. The trophozoites from cultures exhibit marked polarity, the entire organism being sluglike in shape, the anterior, or advancing end being rounded while the posterior end is more pointed and often has adhering to it more or less granular detritus, which is dragged along with it when the ameba moves from place to place upon the microscopic slide. Frequently the progressive motility is so marked that the advancing pseudopodium cannot be distinguished, the endoplasm flowing into it instantly, so that the entire organism appears to be moving along in a determinate direction without the aid of pseudopodia.

The cysts of *Endamœba histolytica* are not usually observed in cultures kept at incubator temperatures but if such cultures are removed from the incubator and kept at room temperature the trophozoites will usually encyst and will excyst if the cultures are replaced in the incubator or if transfers are made and kept in the incubator. Methods have been devised to favor encystment in cultures and will be discussed in describing various culture media in the following pages. In cultures inoculated with material from stools containing cysts excystment occurs in the cultures after being placed in the incubator and motile trophozoites are found a few hours after the inoculation of the material containing the cysts.

The cysts of *Endamœba histolytica* in cultures present the same morphology as in the stools but cysts containing more than four nuclei are much more frequently observed in the cultures. Brug, in 1928, was the first to call attention to the presence in cultures of this ameba of cysts containing as many as 30 to 40 nuclei and his observations have been confirmed by Thomson and Robertson (1929) and others. However, their occurrence is so rare as to be of no diagnostic importance.

In routine diagnostic cultures the differentiation of *Endamœba histolytica* from other intestinal amebæ of man should be based upon the morphology of the motile trophozoite, especially upon the character of the motility. Some authorities have experienced difficulty in distinguishing between *Endamœba coli* and *Endamœba histolytica* in cultures, and their morphology is sometimes very confusing, but the motility of *Endamœba histolytica* which is so much more rapid and progressive than that of *Endamœba coli*, and the very distinctive polarity of *Endamœba histolytica* should serve to easily differentiate it from *Endamœba coli*.

While it is possible to cultivate *Endamœba coli*, *Endolimax nana*, *Iodamœba bütschlii* and *Dientamœba fragilis* in the same media as *Endamœba histolytica*, none of these amebæ can be maintained in cul-

ture for any great length of time, disappearing after a few transfers, whereas *Endamœba histolytica* may be maintained without difficulty for many years in artificial culture media. The writer has cultivated the other species of amebæ but has never been able to carry them through many transfers and it may be stated as a working rule that any ameba from the human intestine that has been maintained in cultures for more than a few weeks, is, in all probability, *Endamœba histolytica.*

VIRULENCE OF ENDAMŒBA HISTOLYTICA IN CULTURES

It has been demonstrated by many observers that most strains of *Endamœba histolytica* that have been studied do not lose their virulence for susceptible animals despite prolonged cultivation. Certain strains do lessen in virulence and others become avirulent upon cultivation but this is the exception rather than the rule. The writer has found that most strains with which he has worked have retained their virulence under cultivation and one strain had lost practically nothing in its virulence for kittens after more than five years' cultivation upon the Boeck-Drbohlav-Locke egg serum medium. In a recent study of this subject Meleney, Frye and Leathers (1939) record the results of the study of 14 strains of *Endamœba histolytica* cultivated for a period of four years in a medium containing egg, Ringer solution, horse serum and rice and found that in no strain was pathogenicity lost for kittens, and that a marked decrease in virulence was present in but one strain after this period of time. At the beginning of the observations the strains varied in their pathogenicity for kittens and it was found that the same variations were retained throughout the four years during which the amebæ were under cultivation. In a still later contribution, Deschiens (1929) states that if the cultivated strain of *Endamœba histolytica* be allowed to encyst periodically by exposure to a temperature of 4° C. (39.3° F.) there will be no loss of virulence upon prolonged cultivation and has found this to be true even after six years' cultivation.

The results of the observations mentioned and of others are of importance from the standpoint of experimental research upon amebiasis as they demonstrate that cultures of *Endamœba histolytica* may be maintained for years without losing their capacity to infect susceptible animals which one may desire to use in research problems.

CULTURE METHODS AND MEDIA

Many media and methods of using them have been devised for the cultivation of *Endamœba histolytica* and the most important and useful will be described in the following pages, the media being arranged in alphabetical order under the names of the authorities who have described and employed them.

Balamuth and Sandza's Medium.—The following medium is recommended by the originators as a simple, easily prepared and efficient one for the cultivation of *Endamœba histolytica*. It is prepared as follows:

Two eggs are boiled for fifteen minutes and, after cooling, the yolks are separated and crumbled into 125 cc. of a 0.8 per cent sodium chloride solution and the mixture then boiled for ten minutes. After the evaporated water has been replaced the mixture is filtered by suction, again brought up to 125 cc. by the addition of water and then autoclaved for twenty minutes at 15 pounds pressure. After cooling, the slight precipitate is removed by simple filtration and to the filtrate is added 125 cc. of M/15 phosphate buffer (pH 7.5), making the total salt concentration M/30 phosphate solution in 0.4 per cent sodium chloride.

The mixture is distributed in 5 cc. amounts in suitable test tubes, autoclaved, and stored in the ice-box. Before inoculation a loopful of rice starch, which has been sterilized, is added to each tube.

The authors state that a richer growth of the ameba may be obtained by doubling the concentration of egg yolk or by adding 0.5 per cent of Wilson Liver Concentrate made by the Wilson Laboratories, of Chicago.

Remarks.—The writer has had no experience with this medium but its simplicity recommends it and the authors state that the growth of *Endamœba histolytica* upon it is uniformly good; that maximum growth is obtained in from the second to the fourth days after inoculation and that motile trophozoites persist for more than nine days after they first appear in the medium.

Boeck and Drbohlav's Media.—The media devised by these observers have been found to be very valuable in the cultivation of *Endamœba histolytica*, especially when one desires to maintain the cultures for a long period of time. These media were described by the authors in 1924–1925 and were the first media described upon which it was possible to culture this ameba indefinitely. They recommended two media for the purpose, the Locke-egg-serum medium and the Locke-egg-albumin medium.

Locke-egg-serum or L. E. S. Medium.—This medium is prepared as follows:

Four eggs are carefully washed, brushed with alcohol, and broken into a sterile flask containing glass beads. Fifty cc. of Locke's solution (see below) are added and the mixture thoroughly shaken until a homogeneous mixture is secured. This is then distributed into test tubes, using enough of the mixture in each tube to produce a slant about 1 to 1.5 inches when coagulated by heat. The tubes are then slanted and placed in an inspissator and kept at 70° C. (158° F.) until the slants are solidified. The tubes are then autoclaved at 15 pounds pressure for twenty minutes. After autoclaving the slants are covered

to a depth of about 1 cm. with a mixture of 8 parts of sterile Locke's solution and 1 part of sterile inactivated human blood serum. To insure sterility the mixture of Locke's solution and blood serum should be passed through a Berkefeld or other filter and incubated at 37° C. for at least twenty-four hours before it is used.

LOCKE-EGG-ALBUMIN OR L. E. A. MEDIUM.—Drbohlav modified the preceding medium, by using crystallized egg albumin instead of blood serum. A 1 per cent solution of crystallized egg albumin in Locke's solution is sterilized by filtration and then added to the culture tubes containing the egg slants as described for the Locke-egg-serum medium.

The initial reaction of both of these media varies from pH 7.2 to pH 7.8 and needs no adjustment.

LOCKE'S SOLUTION.—The Locke solution employed in preparing the Boeck-Drbohlav media has the following formula:

NaCl	9.0 gm.
$CaCl_2$	0 2 gm.
KCL	0 4 gm.
$NaHCO_3$	0 2 gm.
Glucose	2 5 gm.
Distilled water	1000 0 cc.

This solution should be sterilized in an Arnold sterilizer or in the autoclave.

Remarks.—The most frequently used of these two media is the Locke-egg-serum medium but *Endamœba histolytica* grows well upon either of them if the tubes are kept in the incubator at 37° C. (98.6° F.). If it is desired to maintain the cultures, transfers should be made every forty-eight hours, about 0.5 cc. of the sediment at the junction of the covering fluid and the slant being transferred with a sterile 1 cc. pipette.

Cleveland and Collier's Medium.—This medium was recommended by Cleveland and Collier, in 1930, as practically a specific medium for the cultivation of *Endamœba histolytica*. It is prepared as follows:

To a liter of distilled water there are added 30 grams of liver infusion agar (to be obtained from the Digestive Ferments Company of Detroit, Michigan) and 3 grams of $Na2HPO_4$. This mixture is autoclaved and slants are prepared as usual. The slants are then covered with a mixture consisting of fresh horse serum, 1 part, and 0.8 per cent NaCl solution, 6 parts, which has been sterilized by filtration. No adjustment of the reaction is necessary. Just before inoculating the tubes a little sterilized rice flour is added to each tube.

Remarks.—This medium has been used by many observers with satisfactory results but the claim that it is practically specific for *Endamœba histolytica* has not been substantiated. In the hands of the writer this medium has not given as consistently good results as the Boeck-Drbohlav Locke-egg-serum medium.

Craig's Media.—The writer, in 1926 and 1927, demonstrated that for routine diagnostic work the solid egg, agar or serum slants recommended by most authorities are not necessary for the cultivation of *Endamœba histolytica* and that very simple media may be used for this purpose. It was also found that these comparatively simple media could be employed in the maintenance of cultures for weeks and months, if transfers were made every forty-eight hours. Three such media have been used by the writer with success and are prepared as follows:

MEDIUM A: LOCKE-SERUM MEDIUM.—This medium is prepared by mixing a modified Locke solution with either inactivated human, horse or rabbit blood serum, which is not over forty-eight hours old at the time of making the medium.

The modified Locke solution used in preparing this medium has the following formula:

Sodium chloride	9 0 gm.
Calcium chloride	0 24 gm.
Potassium chloride	0 42 gm.
Sodium bicarbonate	0 20 gm.
Distilled water	1000 0 cc.

The Locke solution is filtered and autoclaved at 15 pounds pressure for fifteen minutes and allowed to cool, after which 1 part of blood serum is added to each 7 parts of the Locke solution. The mixture is then filtered through a Berkefeld or other bacteria proof filter until it comes through clear, which may necessitate passing it through the filter several times. After filtration, the medium is tubed, placing about 10 cc. in each tube and incubated at 37° C. (98.6° F.) for thirty-six hours to determine sterility. If the tubes are found to be sterile they should be kept in the incubator at 37° C. until used. The reaction of this medium does not require adjustment and before mixing it with the Locke solution the blood serum should be inactivated by heating it at 56° C. (132.8° F.) for one-half an hour. Just before inoculating the medium a minute amount of rice starch or flour should be added to each culture tube to be inoculated.

MEDIUM B: RINGER-SERUM MEDIUM.—This medium is prepared in the same manner as Medium A except that a modified Ringer solution is used instead of the Locke solution. The Ringer solution used has the following formula:

Sodium chloride	8.0 gm.
Calcium chloride	0.2 gm.
Potassium chloride	0 2 gm.
Distilled water	1000.0 cc.

MEDIUM C: NORMAL-SALINE-SERUM MEDIUM.—This very simple medium is prepared by mixing 1 part of inactivated human blood serum with 7 parts of normal salt solution (0.85 per cent) and from 500 to 1000 cc. of this mixture is filtered through a Berkefeld or other

bacteria proof filter and tubed, placing about 10 cc. of the medium in each tube. The tubes are then incubated at 37° C. (98.6° F.) for forty-eight hours to determine sterility, and if found sterile are stored in an incubator at the same temperature until used.

Remarks.—In the hands of the writer these simple media have been found to be as useful for *diagnostic* purposes as any of the more complex media and can be recommended for that purpose. For maintenance of cultures of *Endamœba histolytica* they are not as efficient as the Boeck-Drbohlav or some other media but if transfers are made every twenty-four to forty-eight hours the ameba will live in such media for as long as three months. There is very little difference between the results obtained with either of the three media described and for routine diagnostic purposes the simplest of them, *i. e.*, the Normal-saline-serum medium is recommended.

Dobell and Laidlaw's Media.—The two following media were devised by Dobell and Laidlaw, in 1926, for the cultivation of *Endamœba histolytica* and have been used with excellent results by many investigators.

MEDIUM 1.—This consists of an egg serum mixture made as recommended by Boeck and Drbohlav (see page 79), but with Ringer's instead of Locke's solution. The Ringer solution recommended has the following formula:

Sodium chloride	9 0 gm.
Calcium chloride	0 2 gm.
Potassium chloride	0 2 gm.
Distilled water	1000 0 cc.

The covering fluid for the slants is made by adding egg albumin or inactivated horse serum to the Ringer solution in the proportion of 1 part to 8 of the solution. If egg albumin is used instead of horse serum, the whites of 4 eggs are mixed with a liter of Ringer solution and sterilized by filtration and the mixture of horse serum and Ringer solution, if chosen as the covering fluid, should also be filtered through a bacteria proof filter before being used. After adding the egg albumin or serum covering fluid to the culture tubes containing the slants, and just before inoculation, a very small amount of rice starch is added to each tube and mixed gently with the fluid covering the slant. The rice starch should be sterilized by heating it in a dry oven at 80° C. (180° F.) for one-half an hour.

MEDIUM 2.—In this medium undiluted horse serum is used for making the slants instead of egg and Ringer solution. The horse serum is sterilized by passing it through a bacteria proof filter, placed in tubes, slanted, and inspissated at 80° C. (176° F.) for not to exceed one hour and ten minutes. One hour is usually sufficient to secure good slants. The tubes are then cooled, incubated at 37° C. (98.6° F.) for forty-eight hours to determine sterility, and the slants then covered with the egg albumin solution or the serum solution, described in

Medium 1. Rice starch is added at the time of inoculation as in Medium 1. No adjustment of the reaction of this medium is necessary.

Remarks.—These media, especially Medium 1, have been employed with success and are especially valuable if one desires to maintain cultures of *Endamœba histolytica* indefinitely. While Dobell claimed that these media were more favorable to the growth of this ameba than the Boeck-Drbohlav media, the writer has not found this to be true, although it is not necessary to make transfers as often as with the latter media. It is not necessary to make subcultures oftener than every six days instead of every two days as with the Boeck-Drbohlav media, but, in the writer's experience, the amebæ are not more numerous in the Dobell-Laidlaw media, and these culture media are not better for routine diagnostic work than the more simple media of the writer and other investigators.

Frye and Meleney's Flask Cultivation.—These investigators recommend the cultivation of *Endamœba histolytica* in Erlenmeyer flasks instead of in test tubes if one desires to obtain a large amount of material containing the amebæ, as in preparing antigen solutions for the complement fixation test (see page 95). They state that in tubes containing slants of coagulated egg-Ringer medium (see page 82) the amebæ multiply only in the mixture of rice starch and sediment at the bottom of the slant while in a flask the whole egg-Ringer surface at the bottom of the flask is available for multiplication. Among the other advantages of this method of cultivation are a saving in time in the preparation of the media and washing of glassware, a saving in glassware and a saving in media. Only one-fifth as much of the egg-Ringer medium and only three-fifths as much horse serum-Ringer are required for a 250 cc. flask as for 25 test tube cultures. In addition, the chances of contaminating the cultures are reduced as fewer utensils are handled and cultural variations in strains of the ameba are less likely to occur. Approximately 15 cc. of the medium are required for a 250 cc. flask and 25 cc. for a 500 cc. flask, these amounts being sufficient to cover the bottom of the flask with a thin layer of the medium.

Method of Preparing Flask Cultures.—The egg-Ringer mixture is placed in the flasks and coagulated by placing the flasks in a pan of boiling water, in an Arnold sterilizer or in an autoclave under live steam without pressure. As a smooth surface is desired as a base the heating should be carefully watched in order to prevent the formation of bubbles in the coagulating material. After coagulation is complete the flasks are autoclaved under pressure and may be stored for use as required.

Before inoculating the flasks the covering fluid, serum-Ringer and rice flour are added and the flasks incubated at 37° C. (98.6° F.) for forty-eight hours, after which the flasks may be inoculated. In adding the horse-serum-Ringer solution from 75 to 85 cc. are required

for a 250 cc. flask and 125 to 150 cc. for a 500 cc. flask. The egg serum base should be covered to the depth of about 1 inch with the horse serum-Ringer, and about 0.25 cc. of sterile rice flour is added to the covering fluid.

To inoculate the flask cultures 1 cc. of a test tube culture containing numerous *Endamœba histolytica* is transferred to each flask, being gently mixed with the fluid covering the solid egg-Ringer base.

Remarks.—The method of flask culture devised by Frye and Meleney is not suitable for diagnostic work or for maintaining *Endamœba histolytica* in culture, for which purposes test tube cultures are more practical and useful, but for obtaining a large amount of material for extraction in preparing antigen for the complement fixation test for amebiasis, the flask method is preferable for the reasons already mentioned.

Nelson's Alcohol Extract Medium.—The following very simple medium is stated by Nelson (1947) to be superior to other media employed in the cultivation of *Endamœba histolytica*. It consists of alcoholic extracts of various tissues and egg yolk combined with agar. Human liver, calf liver, beef liver, guinea-pig liver, cat intestine, and egg yolk all give about the same result when extracted although egg yolk is stated to be a little better than extracts of other tissues.

The extraction of the selected tissue is made with 95 per cent ethyl alcohol by adding 10 parts of the tissue or egg yolk to 90 parts of the alcohol. The tissue used is cut into small pieces and placed in the alcohol and if egg yolk is used the yolk is separated from the white and added to the alcohol. Egg white should not be extracted as it gives an inert extract.

The flask or bottle containing the alcohol and tissue selected is shaken several times a day and is ready for use in forty-eight hours. The stock extract at the time of use is placed in a water bath, the alcohol driven off by heat and the agar then added to the resulting material. Ten tubes of the medium are prepared as follows:

Place 10 cc. of the stock alcoholic extract in a small flask and drive off the alcohol by heating in a water bath; then add 20 cc. of a melted 2 per cent agar in buffered 0.5 per cent saline, and tube the mixture in 2 cc. quantities and slant. No serum supplement is required. Cover the agar slants with buffered 0.5 per cent saline and inoculate and incubate. The agar mixture can be autoclaved before slanting if desired. The pH of the medium if tissue extracts are used should be 7.4 and 7.6 if egg yolk extract be used. Rice starch or rice flour sterilized at 150° C. is added to the medium at the time of inoculation.

Remarks.—This medium would appear to be a most excellent one for the cultivation of *Endamœba histolytica* especially as *Blastocystis hominis* does not grow in it and because of its simplicity. It is also excellent for the maintenance of cultures and subinoculations do not need to be made oftener than every four or five days. In surveys made

by Nelson this medium gave better results than microscopic examination, or flotation and as good results as hematoxylin stained preparations. The reader is referred to Nelson's papers for an extended discussion of the technique and results obtained with the medium.

St. John's Medium.—This medium was recommended for the cultivation of *Endamœba histolytica* by St. John, in 1932, and is prepared and used as follows:

One gram of powdered heart muscle (Bacto Beef Heart Dehydrated, sold by the Digestive Ferments Company, Detroit, Michigan) is extracted in a modified Locke solution by boiling in 1000 cc. of the solution in a double boiler for one hour. The modified Locke solution has the following formula:

Sodium chloride	8 0 gm.
Calcium chloride	0 2 gm.
Potassium chloride	0 2 gm.
Magnesium chloride	0 1 gm.
Sodium phosphate (NaH_2PO_4)	0 1 gm.
Sodium bicarbonate	0 4 gm.
Distilled water	1000 0 cc.

The extract is filtered through filter paper, tubed in 5 to 10 cc. amounts and autoclaved at 15 pounds pressure for half an hour. Just before the tubes are inoculated 0.05 cc. of Ralston's whole wheat flour is added to each tube with a dry, sterile pipette and mixed with the medium. The flour should be sterilized before use and this is done by wrapping the amount needed in tinfoil, autoclaving it at 15 pounds pressure for thirty minutes, drying in a hot air oven at 55° C. (131° F.) overnight, and finally heating in the hot air oven at 80° C. (176° F.) for thirty minutes. The reaction of the medium does not need adjusting.

Remarks.—This is an excellent diagnostic medium and is also one of the best for the maintenance of cultures of *Endamœba histolytica*. St. John claimed for it the following advantages: (*a*) ease of preparation and low cost; (*b*) the comparatively small number of bacteria that develop in it; (*c*) the length of life of the amebæ; and (*d*) its availability for the study of the effect of drugs upon the amebæ. The writer has confirmed all of these claims and in addition, as stated by St. John, the other intestinal amebæ, *Blastocystis hominis*, and the intestinal flagellates do not grow in this medium, a great advantage in the study of cultures of *Endamœba histolytica*, which are frequently overgrown by *Blastocystis hominis*, while the presence of other amebæ complicates the diagnostic picture.

In sub-culturing *Endamœba histolytica* in this medium it is not necessary to make the transfers oftener than every fourth or fifth day instead of every twenty-four or forty-eight hours as with most of the other culture media that have been recommended.

The writer has used this medium extensively in the cultivation of *Endamœba histolytica* and recommends it for diagnostic use although it

does not give appreciably better results than does the simple Ringer-serum or the normal-saline-serum media already described (see page 81). For the maintenance of cultures the St. John medium has the advantage that transfers do not have to be made as frequently as with other media but it is not superior to the Boeck-Drbohlav media for this purpose.

It is well, if this medium is used for maintaining cultures, to add 1 per cent of horse serum to it, as a richer growth of the amebæ is thus obtained. The horse serum should be added after the medium has been autoclaved and allowed to cool and the resultant mixture should then be passed through a bacteria proof filter, tubed and stored for use as required. The addition of horse serum is not necessary in routine diagnostic work and is not recommended.

Tanabe and Chiba's Medium.—This medium was described by Tanabe and Chiba, in 1928, as an excellent one for the cultivation of *Endamœba histolytica* and has been used with success by numerous investigators. It is prepared as follows:

Agar slants are made with Ringer's solution and asparagin, the formula being as follows:

Agar	10 0 gm.
Asparagin	1 0 gm.
Ringer's solution	1000 0 cc.

This mixture is sterilized in an autoclave for thirty minutes under 15 pounds pressure and then tubed and slants made as usual. The slants are then covered with Ringer's solution containing 5 per cent of rabbit's blood serum (not inactivated), which is sterilized by passing the mixture through a bacteria proof filter. Just prior to the inoculation of the medium two or three loopfuls of rice starch which has been sterilized in the hot dry oven (see page 82) is added to each tube.

Remarks.—This medium gives good results in the cultivation of *Endamœba histolytica* and in the maintenance of such cultures but is no better for these purposes than the Boeck-Drbohlav media and is not as useful in routine diagnosis as the more simple liquid media already described.

Tsuchiya's S. C. Medium.—Tsuchiya introduced this medium in 1934 and it consists of Dorsett's egg slants covered with nutrient broth to which has been added a mixture of rice starch and charcoal. The medium is prepared and used as follows:

Dorsett's Egg Slants.—Wash and break the eggs into a flask and shake the flask until the whites and yolks are thoroughly mixed. Add 25 cc. of distilled water to every four eggs used, shake and strain through sterile gauze. Pour 10 cc. of the mixture into each culture tube, slant in an inspissator, and expose to a temperature of 73° C. (163.4° F.) for four to five hours on two days and on the third day raise the temperature to 76° C. (168 8° F.) or, if one desires to save

time, sterilization may be completed by a single exposure of 100° C. (212° F.) in the Arnold sterilizer for fifteen minutes.

NUTRIENT BROTH.—The nutrient broth used has the following formula:

Peptone	10.0 gm.
Beef extract	3.0 gm.
Sodium Chloride	5.0 gm.
Distilled water	1000.0 gm.

Adjust pH to 7 and autoclave at 15 pounds pressure for thirty minutes.

THE S. C. MIXTURE.—This consists of a thoroughly triturated mixture of rice starch (Heil Corporation) and charcoal (Merck's) in the proportion of 3 parts of rice starch to 1 part of charcoal. The mixture is sterilized, in small amounts, in a glass vial, by dry heat at 180° C. for forty-five minutes.

In using this medium, the egg slants are each covered with 5 cc. of the nutrient broth and to each tube is added a heaping 4 mm. loopful of the sterile mixture of rice starch and charcoal. The tubes are then inoculated with a small portion of the feces to be examined and incubated at 37° C. (98.7° F.) and examined in from twenty-four to thirty-six hours.

Remarks.—Tsuchiya has found this method of cultivation to be a most excellent one (see page 73) and it is recommended as one of the most valuable methods we possess for diagnostic purposes.

Fecal Extracts in the Cultivation of Endamœba Histolytica.—It has been found by Andrews, Johnson and Schwartz (1933) that the addition of extracts of fecal material when added to various culture media for *Endamœba histolytica* markedly increases the growth of this ameba and results in a much greater number of amebæ in the cultures. They have reported the results of their work in cultivating this ameba in cultures made with the media of Cleveland and Collier, St. John, and Tanabe and Chiba, but it is probable that the addition of the fecal extracts to other media would give approximately similar findings.

FECAL EXTRACT.—The fecal extract used by these investigators is prepared as follows:

One part of feces by volume is thoroughly mixed with 4 parts of the liquid portion of the particular culture medium which is being used for the cultivation of *Endamœba histolytica*, *i. e.*, Locke-serum, Ringer-serum, etc. This mixture is then allowed to stand for two hours, being repeatedly shaken during that time, after which it is filtered through cheese-cloth and centrifuged for one-half hour. It is then filtered through an infusorial earth filter and finally sterilized by passing the filtrate through a Berkefeld filter. If slant cultures are being used the resulting filtrate of the extract is used to cover the slant while if liquid media are desired the extract furnishes the medium. In both instances the extract is inoculated with the material being examined and the sediment examined at the end of twenty-four and forty-eight hours.

These observers found that the length of life of the amebæ was not affected by the addition of the fecal extract but that there was an increase in number amounting to over 100 per cent in some cases, and that in all the media tested there was a marked increase in the number of the amebæ when fecal extracts were added to the media.

Stone's Method of Eliminating Blastocystis Hominis from Cultures of Endamœba Histolytica.—All who have endeavored to cultivate *Endamœba histolytica* in artificial media have been greatly hampered by the development of large numbers of *Blastocystis hominis* in the cultures if this organism was present in the material inoculated. Frequently this organism multiplies to such an extent in the cultures that it so influences the medium as to render it unsuitable for the multiplication of *Endamœba histolytica* or obscures the latter parasite because of the immense number of *Blastocystis hominis* present. Failure to cultivate *Endamœba histolytica* is often due to the growth of *Blastocystic hominis* so that the elimination of this organism from cultures becomes most important. Stone has devised a method for this purpose which he states is invariably successful if carried out as he recommends.

The method, as recommended by Stone (personal communication) is as follows:

1. Cultures of *Endamœba histolytica* which contain *Blastocystis hominis* have added to the fluid over the egg slants enough sterile 1 to 10,000 neutral acriflavine in Ringer's solution to make a 1 to 50,000 solution. The cultures are then incubated for twenty-four hours at 37° C. (98.6° F.), transferred, incubated for another twenty-four hours and then transferred to the lactic acid medium described below. The Ringer solution employed has the following formula:

Sodium chloride	8 0 gm.
Potassium chloride	0 2 gm.
Calcium chloride	0.2 gm.
Distilled water	1000 0 cc.

2. *Lactic Acid Medium.*—The lactic acid medium is prepared as follows:

A Locke solution, having the following formula, is sterilized in the autoclave at 15 pounds pressure for fifteen minutes.

Locke Solution

Sodium chloride	9.00 gm.
Calcium chloride	0.24 gm.
Potassium chloride	0 42 gm.
Sodium bicarbonate	0.20 gm.
Lactic acid, I. O. N.	0.23 gm.
Distilled water	1000.00 cc.

With sterile precautions mix 1 part of sterile horse serum with 7 parts of the cooled Locke solution. Then add 5 cc. of the resulting mixture to previously prepared sterile egg slants, *i. e.*, the solid portion of the Boeck-Drbohlav medium, and inoculate with the material from the acriflavine cultures.

Transfers of the cultures on the lactic acid medium are made at twenty-four hour intervals for three successive days and the cultures are then transferred to the regular Boeck-Drbohlav Locke-egg-serum medium.

Before each inoculation and transfer throughout the entire procedure 0.05 cc. of a dry sterile mixture of 2 parts of powdered rice starch and 1 part of powdered animal charcoal is added to each culture tube.

Stone states that some *Blastocystis hominis* will still be present in the cultures after the transfers are completed but in a degenerated condition and will completely disappear upon successive transfers to the Boeck-Drbohlav medium mentioned above. If this organism should continue to multiply in the cultures the process is repeated and Stone states that the repetition has never failed to eliminate the organism.

Remarks.—For routine diagnostic work it would not usually be necessary to employ this method but if one desires to maintain strains of *Endamœba histolytica* for indefinite periods and *Blastocystis hominis* is present, the method is a most valuable one, as contaminations with this organism usually results in the loss of the strain under cultivation.

Methods for Producing Cysts in Cultures of Endamœba Histolytica.—For many research problems in the study of amebiasis, especially in studying the life-cycle of *Endamœba histolytica*, complement fixation, pathogenicity, variations in virulence and cultivation, it is desirable that cysts of this parasite be available for experimental purposes. Several methods have been devised which favor the occurrence of cysts in cultures of *Endamœba histolytica* but most of them do not give consistently regular results and cyst formation often fails to occur.

The most simple method of obtaining cysts in cultures is to remove cultures that have been maintained for several generations in the incubator at 37° C. (98.6° F.) from the incubator and keep them at room temperature in a dark place. Eventually cysts will appear in a considerable proportion of these cultures but frequently this procedure fails and the results are not uniformly successful.

Chang's Method.—This method was proposed by Chang (1942) and gave excellent results in his hands.

Cultures of *Endamœba histolytica* were made upon Boeck and Drbohlav medium (see page 79) and transferred every two days in this medium for four or five weeks. At the end of this time all except 3 to 5 cc. of the fluid in each tube of a forty-eight hour old culture is pipetted off without disturbing the sediment, and discarded. The remaining fluid is used to wash the surface of the egg slant and to suspend the sediment. When this is done, the sediment mixture from each tube is pipetted into a tube of the encystment medium described below and the tubes incubated at 37° C. (98.6° F.).

The *encystment medium* is composed of 15 to 20 cc. of M/30 phosphate buffered to pH 7.6; $\frac{1}{5}$ to $\frac{1}{6}$ cc. of liver extract fluid, and 2 to 3 loopfuls of rice starch powder. These are prepared as follows:

1. *M/30 Phosphate Solution.*—Dissolve 11.94 gm. of $Na_2HPO_4.12\ H_2O$ and 4.52 gm. KH_2PO_4 separately in 0.4 per cent NaCl solution to make 1 liter. Autoclave at 15 pounds pressure for twenty minutes.

2. *Liver Extract Fluid.*—Dissolve 40 gm. of liver extract powder (No. 343, Lilly) and 4 gm. of $Na_2HPO_4.12\ H_2O$ in distilled water to make 50 cc. of pastelike fluid. Autoclave at 15 pounds pressure for twenty minutes.

3. *Sterile Rice Starch Powder.*—Place a few grams of Difco Bacto-Rice Starch Powder in a culture tube and place the tube horizontally in a hot-air oven at a temperature of 160° to 180° C. for one hour and repeat twice at twenty-four hour intervals.

To prepare the encystment medium, pipette the desired amounts of both the buffered phosphate solution and the liver extract fluid into a culture tube and add 2 to 3 loopfuls of the rice starch. The pH value should be determined by the glass electrode.

Remarks.—In this medium, if the pH value is maintained, large numbers of cysts will occur but marked variations are observed with different strains of the ameba. It is an excellent method but fails at times, as have all other methods so far devised for obtaining cysts.

Dobell's Method.—This method was described by Dobell, in 1928, and is generally successful in producing cysts. It consists in cultivating *Endamœba histolytica* in media which do not contain rice starch for several transfers, after which rice starch is added to the culture media. Using this method Dobell was able to trace the complete life-cycle of *Endamœba histolytica* in cultures but in the hands of the writer it has often failed to result in cyst formation.

Stone's Method.—In 1935, Stone described a method of producing encystment in cultures of *Endamœba histolytica* which has given excellent results and by the use of which he has been able to obtain multitudes of cysts for use in the preparation of antigen for the complement fixation tests for amebiasis. Cysts have been produced daily with this method by its originator without difficulty and it would appear to be the best method so far devised for the purpose. He states that from 1,800,000 to 2,000,000 cysts per culture was an average count over a period of six months.

TECHNIQUE OF METHOD.—Forty-eight hour old cultures of *Endamœba histolytica* on the Boeck and Drbohlav Locke-egg-serum medium, using sterile horse serum, are used as a basis for obtaining the cysts. The supernatant fluid covering the slants is pipetted off very carefully, so as not to disturb the sediment, until only about 1 cc. of the fluid remains in each tube. Employing aseptic precautions the remaining 1 cc. of fluid in the cultures is used to wash the egg slant free from amebæ and then the entire suspension is drawn up into a sterile

pipette and is ready for transference to the encystment medium, which is prepared as follows.

A modified Locke solution is made having the following formula:

Sodium chloride	8.00 gm.
Calcium chloride	0.20 gm.
Potassium chloride	0.20 gm.
Magnesium chloride	0.01 gm.
Sodium phosphate (secondary) (Na_2HPO_4)	2.00 gm.
Sodium bicarbonate	0 40 gm.
Potassium phosphate monobasic (KH_2PO_4)	0 30 gm.
Distilled water	1000.00 cc.

The above ingredients should be mixed and dissolved by means of heat. The resulting solution should then be allowed to cool, filtered through a medium filter paper and tubed in 10 cc. amounts. The tubes should then be placed in an autoclave and sterilized under 15 pounds pressure for twenty minutes. Just prior to the inoculation of this medium 0.05 cc. of sterile whole wheat flour (Ralston's) should be added to each tube and mixed with the medium by shaking. The whole wheat flour is prepared by grinding it in a mortar until it passes through a No. 50 sieve (50 wires to the inch), then tubed in 4 gram amounts, placed in an autoclave and sterilized at 15 pounds pressure for twenty minutes and then dried in a hot air oven at 58° C. (136.4° F.) for twenty-four hours.

The suspension of the amebæ from three of the Boeck-Drbohlav cultures is transferred to one tube of this encystment medium, thus giving an inoculation of many trophozoites of *Endamœba histolytica*. Tubes thus prepared are placed in an incubator at 37° C. (98.6° F.) and examined at twenty-four hour intervals. During the first twenty-four to thirty-six hours there is a great multiplication of the trophozoites but as the nutritive factors in the medium are used up the amebæ encyst and after forty-eight to seventy-two hours the tubes will be found to contain only cysts, the trophozoites having all encysted or degenerated. The cysts so produced are normal in appearance and will excyst when inoculated into a suitable medium.

Remarks.—The writer has had no personal experience with this method of causing encystment of *Endamœba histolytica* but it would appear to be the most efficient method for the purpose that has been published. He has used antigens for the complement fixation test prepared by extracting washed cysts of *Endamœba histolytica* obtained in the manner described and which were sent him by Major Stone, and found such antigens to be specific.

Pure Cultures of Endamœba Histolytica.—It has already been stated that, as yet, no one has been able to maintain *Endamœba histolytica* in culture media free from bacteria for more than a generation or two, although pure cultures of this parasite have been obtained by several observers. Three methods have been employed for this purpose, *i. e.*, the cultivation of trophozoites from pus from liver abscesses

free from bacteria, the employment of various chemicals which kill the bacteria without injuring the amebæ, and the culture of bacteria-free cysts transferred by means of the micromanipulator.

As regards the first method mentioned, Cleveland and Sanders (1930) obtained pure cultures by inoculating into media bacteria-free material containing *Endamœba histolytica* obtained from experimentally produced amebic liver abscesses in kittens. They found that reproduction of the amebæ occurred in such cultures and that the organisms remained alive in the cultures not longer than fourteen days, and transfer of the amebæ to bacteria free media could not be maintained indefinitely.

The employment of certain chemicals to destroy the bacteria in cultures of *Endamœba histolytica* with the idea of thus obtaining pure cultures of the organism has been tried by several investigators. St. John (1929), while working with the writer, was able to secure pure cultures by adding acriflavine to cultures containing the ameba but was unable to maintain such cultures, without adding bacteria, for more than two transfers. Cleveland and Sanders (1930), using washed cysts of *Endamœba histolytica*, immersing them in a 1 to 1000 bichloride of mercury solution, and then transferring the cysts to bacteria-free media, in three experiments obtained motile trophozoites but none of them lived for more than six days and transfers to other bacteria-free media were unsuccessful.

Recently, Snyder and Meleney (1941) have been successful in producing the excystation of washed cysts of *Endamœba histolytica* in bacteria-free media and their method will be described.

Snyder and Meleney's Method.—These investigators obtained cysts of *Endamœba histolytica* by following Dobell's method (see page 90). When cultures showing cysts are obtained the fluid portion is centrifugalized several times after adding distilled water in order to kill the trophozoites present and to remove rice particles. The cysts are then separated from the rest of the sediment by centrifugalization in a solution of zinc sulphate having a specific gravity of 1.13. The cysts will rise to the surface and should be removed and washed in distilled water to remove all traces of the zinc sulphate. The cysts are then treated with a 1 to 50,000 dilution of mercuric chloride for forty-five minutes and then washed several times with distilled water, which results in a suspension of numerous cysts usually free from living bacteria. The possible presence of bacteria may be ascertained by placing 0.1 cc. of the suspension in culture media and keeping in the incubator for twenty-four hours at 37° C. (98.6° F.).

Cysts so obtained will excyst in media containing bacteria but will not excyst in sterile media unless reducing agents are present or a condition of anærobiosis is produced by partial exhaustion of the molecular oxygen from the medium. As reducing agents Snyder and Meleney employed neutralized cysteine hydrochloride (0.1 per cent) and neutralized thioglycollic acid (0.3 per cent). Other methods that

favored excystation were æration with nitrogen gas, and absorption of oxygen with alkaline pyrogallol. Total exhaustion of the oxygen is not essential for excystation.

By using one or another of the methods described above, Snyder and Meleney have obtained pure cultures of *Endamœba histolytica* in modified Locke solution, 0.3 per cent meat extract, 2 and 0.2 per cent proteose peptone, and infusion of coagulated whole egg. The addition of heat-killed bacteria appeared to favor excystation but there was no evidence of multiplication of the excysted motile trophozoites.

Continued cultivation of the bacteria-free excysted amebæ was not possible unless living bacteria were added to the culture medium.

Choice of Culture Method for Diagnosis of Endamœba Histolytica. —The choice of a particular cultivation method for the diagnosis of *Endamœba histolytica* should be based upon the proven efficiency of the method and secondarily upon the ease of preparation and cost of the materials used in the medium. For routine diagnostic work the writer believes that the simple fluid media devised by him give as good results as do more complicated and expensive media and should be preferred to any medium requiring egg, serum or agar slants. Neither is the addition of rice starch or wheat cereal necessary for routine diagnosis although one or the other should be added if it is desired to maintain the cultures indefinitely.

Of the media recommended by the writer, the simple Normal-saline-serum medium has given excellent results in routine diagnosis in the hands of several observers as well as in the writer's, and Vogel (1928) and Kolle and Hetsch (1929) recommended this medium for the cultivation of *Endamœba histolytica*. Magath and Ward (1928), at the Mayo Clinic obtained better results with the writer's Normal-saline-serum medium than with the Boeck-Drbohlav L. E. S. medium. They cultured 115 stools using both media and obtained 23 positive results, or 20 per cent with the Normal-saline-serum medium and 20 positive results, or 17 per cent with the Boeck-Drbohlav L. E. S. medium.

Nelson's alcohol extract medium is an excellent diagnostic one and especially valuable for the maintenance of *Endamœba histolytica* in cultures.

Similar results have been obtained by the writer in using the Locke-serum medium and St. John's medium and it is recommended that in routine diagnostic work one of these simple and inexpensive media be employed rather than the more complicated and expensive media, as Boeck and Drbohlav's, in which solid slants have to be employed.

No culture method so far devised should replace the microscopic examination of the stools in the diagnosis of *Endamœba histolytica* but it has been shown that in cases in which the amebæ are very scarce in the stool and cannot be demonstrated microscopically, cultivation of such stools sometimes results in the demonstration of the

parasite. For this reason the writer recommends that in routine diagnostic work all stools that are negative microscopically be cultured in one of the simple fluid media described.

Choice of Medium for Maintenance of Cultures.—In the choice of a medium for the maintenance of cultures of *Endamœba histolytica* one should choose one that has either an egg, agar or serum slant covered with a fluid medium or the St. John liquid medium. All of the slant media described in the preceding pages have given excellent results and it is true here, as in many other technical matters, that "practice makes perfect." A medium used by one thoroughly trained in its use, and having a long experience with it, will give better results than one used by an untrained observer with little practical experience, and it is best to select a certain medium and use it indefinitely rather than to try one medium after another without learning how to use any to the best advantage.

For the maintenance of cultures of *Endamœba histolytica* the writer has relied upon the Boeck-Drbohlav Locke-egg-serum (L. E. S.) medium as it has given as good results in his hands as any of the other media recommended for the purpose. A little rice starch or whole wheat flour adds to the efficiency of this medium and should be placed in each culture tube before transfers are made. The writer has maintained cultures of *Endamœba histolytica* upon this medium for over five years, transferring the cultures every forty-eight hours, without any particular difficulty, and believes that for routine employment in the maintenance of cultures of this ameba it is more generally useful than any of the other media that have been recommended.

It has been found by Lamy (1944) that the addition of para-aminophenyl sulphamide to cultures of amebæ checks bacterial growth, so that it is not necessary to sub-culture for at least one month. The chemical should be added to the medium used in a concentration of 0.5 per cent, and he obtained excellent results with a medium composed of horse serum, Ringer's solution, and rice starch.

CHAPTER IV

COMPLEMENT FIXATION IN THE DIAGNOSIS OF AMEBIASIS

NATURE OF THE COMPLEMENT FIXATION REACTION—TECHNIQUE OF THE TEST—PRACTICAL VALUE OF THE COMPLEMENT FIXATION TEST IN THE DIAGNOSIS AND AS A CONTROL OF THE TREATMENT OF AMEBIASIS

Introduction.—In 1927 and 1928, the writer published the results of his observations demonstrating that complement fixation occurred in the blood serum of individuals infected with *Endamœba histolytica* in the presence of a suitable antigen, and in 1929, the technique of a complement fixation test which has proven to be of value in the diagnosis of amebiasis and as a control of the treatment of this infection.

That complement fixation occurs in amebiasis has been since confirmed by numerous investigators. The observations of Spector (1932), Menendez (1932), Sherwood and Heathman (1932), Heathman (1932), Tsuchiya (1934), Weiss and Arnold (1934–1937), Meleney and Frye (1935–1937), Shiraogawa (1935), Stone (1935), Yamamoto (1936), Paulson and Andrews (1938), Boe (1940), Magath and Meleney (1940), Rees, Bozicevich, Reardon and Jones (1942), Amaral (1944), Bozicevich, Hoyem and Walston (1946) and Thomen and Read (1946) have all confirmed the writer's findings that complement fixing substances are present in the blood serum of individuals infected with *Endamœba histolytica*, in that of animals experimentally infected, and that such bodies may be demonstrated by means of a complement fixation test.

NATURE OF THE COMPLEMENT FIXATION REACTION

While the exact nature of the complement fixation reaction in amebiasis has not, as yet, been determined, the evidence is all in favor of its being a true antigen-antibody reaction. Most of the investigators of this reaction have employed alcoholic extracts of cultures of *Endamœba histolytica* and it is demonstrated that the antigenic substance is extractable with alcohol. As it has been impossible, as yet, to use pure cultures of *Endamœba histolytica*, it follows that all of the antigens used, with the exception of that of Stone (1935), which is prepared by extracting washed cysts of *Endamœba histolytica*, contain not only extractives from the amebæ but also from the bacteria that may be growing in the cultures with the amebæ. This fact has led many to question the specificity of this reaction but the writer, in his initial

experiments upon complement fixation, demonstrated that such bacteria, either ærobic or anærobic, when cultivated either alone or together and then extracted in alcohol, did not cause complement fixation in sera which gave either negative or positive reactions with the amebic antigen. These results have been confirmed by Heathman (1932), Sherwood and Heathman (1932), Menendez (1932), Tsuchiya (1932), Yamamoto (1936) and others, and it may be definitely stated that the positive reaction obtained with the complement fixation test in amebiasis depends upon the presence in the antigenic extracts of a substance or substances which are extracted from the amebæ, and that the presence of extractives from the accompanying bacteria do not give a reaction or apparently interfere with the specific reaction.

The results obtained by Stone (1935) and confirmed by the writer and others, using a bacteria-free antigen, prepared by extracting cysts of *Endamœba histolytica* which have been washed free from bacteria, substantiates the specificity of the complement fixation reaction in amebiasis, as with this antigen as good results were obtained as with antigens prepared from cultures containing both amebæ and bacteria. In the writer's hands, Stone's antigen gave practically identical results in cases of amebiasis as were obtained with the culture antigens.

The results of the test in animals experimentally infected with *Endamœba histolytica* also point to the specific nature of the complement fixation reaction. Many authorities have found that the blood sera of susceptible animals, as rabbits, kittens and dogs, after inoculation with *Endamœba histolytica*, contain complement fixing bodies and Stone (1935) inoculated rabbits intraperitoneally with washed, living cysts of *Endamœba histolytica* and found that their blood serum gave strongly positive reactions with his antigen that was prepared by extracting washed cysts of this parasite. The writer and Kagy (1933) found that the inoculation into the ileum and cecum of dogs of material containing *Endamœba histolytica* trophozoites was followed by the appearance in their blood serum of complement fixing bodies, demonstrable by the amebic antigen. Thus, of 29 dogs experimentally infected with *Endamœba histolytica* in this manner, all but 1, or 96.5 per cent, developed a positive complement fixation reaction. The reaction appeared in the blood of some of these animals within a few days after inoculation and in the majority within fifteen days after colonic inoculation. Autopsies upon these animals showed that the earlier the reaction occurred the more severe were the lesions in the intestine, but all showed typical amebic lesions and motile trophozoites of *Endamœba histolytica* with but one exception, that of a sacrificed animal, in which healed lesions were found in the intestine. In addition *Endamœba histolytica* was present in the stools of all of the inoculated dogs. As a control, 48 normal dogs were tested for complement fixation for amebiasis and all gave negative reactions.

Similar results have been obtained by other observers, notably by Meleney and Frye (1937) who found that "Dogs experimentally infected with *Endamœba histolytica* uniformly developed a positive complement fixation reaction for amebiasis associated with active amebic dysentery." In the instance of *Macacus rhesus* monkeys they were unable to obtain complement fixation in either naturally or experimentally infected animals nor did the animals they used show any lesions in their intestine after inoculation. On the contrary, the writer and Swartzwelder (1938) found that after experimentally infecting four *Macacus rhesus* monkeys with *Endamœba histolytica*, all developed a positive complement fixation reaction and all showed amebic lesions in the intestine. Such diverse results are hard to explain but do occur in experimental research on amebiasis.

Another evidence of the specific nature of the complement fixation reaction in amebiasis is the fact that in both man and infected animals the reactions disappear after the infection with *Endamœba histolytica* has been eliminated. All observers agree that the positive reaction disappears within a variable time after the elimination of the infection. In experimentally infected animals autopsy has shown that when the positive reaction became negative the lesions in the intestine were healed and *Endamœba histolytica* could no longer be demonstrated in the stools, while in man the disappearance of the positive reaction has always followed the disappearance of the ameba from the feces of the individual.

The time of the disappearance of the positive complement fixation reaction after the elimination of the infection varies. In one series of cases observed by the writer, in which repeated complement fixation tests were made in order to determine the date of the disappearance of the positive reaction after treatment had been completed and the amebæ had disappeared from the stools, it was found that the time of disappearance varied from three to twenty-eight days. In 25 of these patients, or 86.6 per cent, it had become negative by the end of the second week.

In summing up the evidence that we possess regarding the nature of the complement fixation reaction in amebiasis, it is true that it all points to its specific nature, the reaction depending upon the presence of specific complement fixing bodies in the patient's blood serum which react with specific bodies extracted from *Endamœba histolytica* in the presence of complement. The constant presence of specific complement fixing bodies in the blood serum of naturally infected man and experimentally infected animals; the fact that bacteria-free antigens are efficient in producing specific complement fixing bodies in experimentally infected animals and give a positive reaction with the blood serum of individuals infected with *Endamœba histolytica;* the occurrence of specific complement fixation in animals after the injection of all of

the types of antigen that have been employed in making the test; and the fact that the positive reaction disappears in both naturally infected man and experimentally infected animals after the elimination of infection with *Endamœba histolytica*, would appear to conclusively demonstrate that the complement fixation reaction in amebiasis is a true antigen-antibody reaction and specific in nature. The fact that presumably false positive reactions are sometimes obtained with this test does not, in any way, negative this conclusion, for such false reactions may, and do, occur with every specific complement fixation test that has been employed in the diagnosis of disease.

TECHNIQUE OF THE COMPLEMENT FIXATION TEST FOR AMEBIASIS

In describing the technique of the complement fixation test for amebiasis that followed by the writer will be described. While several other techniques have been described in the literature, they are all modifications of the writer's technique and the results obtained with them are no better than obtained with the writer's method when properly performed, while false reactions are more frequently obtained with most of the modified methods.

Recently (1946) a modification of the complement fixation test for amebiasis has been devised by Bozicevich, Hoyem and Walston using an antigen that may be obtained from Hynson, Wescott and Co. and which is reported to give excellent results. The technique of this modification will not be given here as it accompanies each package of antigen.

Apparatus Required.—The following list of apparatus and reagents required for this test follows and includes all that is really essential.

1 Luer syringe, capacity 1 cc.
1 Luer syringe, capacity 5 cc.
1 Luer syringe, capacity 20 cc.
6 needles for collecting blood. (Special needles for this purpose can be purchased from any surgical supply house.)
1 electric centrifuge, International Instrument Company, having one four 15 cc. tube head and metal holders and one four 50 cc. tube head and metal holders. Type of current and voltage should be specified.
20 centrifuge tubes, plain, each 15 cc.
10 centrifuge tubes, graduated, 15 cc.
10 centrifuge tubes, plain, 50 cc.
1 incubator, either electric, gas or oil, with proper thermo-regulators, to run at 56° C. (132.8° F.).
1 water-bath, with thermometer, burner, thermo-regulator, and large enough to accommodate the required number of copper tube racks, to run at 37° C. (98.6° F.). The regular Wassermann water-baths are suitable.
1 set of copper Wassermann test tube racks for the particular water-bath purchased.
1 amboceptor cutter. (Made by Topham, Washington, D. C.)

1 set of 3 amboceptor markers, 4 mm., 5 mm., and 6 mm. (Made by Topham, Washington, D. C.)
1 paraffin bath to fit the incubator mentioned above, to hold tubes during inactivation of blood serum.
100 or more test tubes without lip, 100 by 12.5 mm.
4 test tube baskets, 6 in. high by 7.5 in. in diameter.
10 Mohr pipettes, 10 cc., graduated in tenths.
10 Mohr pipettes, 5 cc., graduated in tenths.
20 or more serologic pipettes, 1 cc., graduated in hundredths to tip. Should be of small bore and the graduations far enough apart to render accurate reading and delivery possible for quantities as small as one-one hundredth of a cc.
50 or more serologic pipettes, 1 cc., graduated in tenths, to be used for measuring the sera to be tested.
12 Erlenmeyer flasks, capacity 250 cc. each.
6 animal cages for rabbits and guinea-pigs.
20 pounds soft paraffin, melting point 43° C. (109.4° F.).
Sodium chloride. C.P. 500 gm.
Absolute alcohol. 500 cc.
6 small triangular files (3 inches long).

Guinea-pigs and rabbits must be kept for the production of complement and amboceptor.

Preparation and Titration of Reagents.—The following reagents are employed in the writer's complement fixation test for amebiasis and the utmost care should be exercised in their preparation and titration. The reagents are:

Complement.—The fresh blood serum of guinea-pigs.

Hemolytic amboceptor.—The blood serum of rabbits immunized to human red blood corpuscles.

Hemolytic antigen.—A suspension of human red blood corpuscles.

Amebic antigen.—An alcoholic extract of cultures of *Endamœba histolytica* or of mucus rich in this parasite obtained from the intestine of infected dogs.

Amebic amboceptor.—The infected patient's blood serum.

Normal saline solution.

Preparation of Normal Saline Solution.—For preparing the red blood cell suspension used in the complement fixation test for amebiasis and for making all dilutions of the various reagents that are used in the test, a normal salt solution should be used. This is prepared by dissolving 8.5 gm. of C.P. sodium chloride in 1000 cc. of distilled water, thus making a 0.85 per cent solution. This should be sterilized, preferably in an Arnold sterilizer, and tested after sterilization by adding a drop or two of washed red blood corpuscles to 5 cc. of the solution. If no hemolysis results at the end of half an hour the solution is suitable for use. The normal saline solution should be most carefully prepared as serious errors may result with this test when improperly prepared saline solutions are used.

Preparation of Complement.—Full grown, healthy guinea-pigs should be used as the source of complement, as it has been shown by

serologists that the blood serum of these animals is superior as a source of complement to the sera of other animals. However, it has been shown by Williams (1920) that the blood sera of some guinea-pigs contain natural hemolysins for the blood corpuscles of individuals belonging to Groups IV and II, of Jansky's blood group classification (AB and A, Landsteiner grouping) and in sufficient amounts to give rise to erroneous results if such sera be used for complement in complement fixation tests. To obviate this source of error, the blood cell suspension employed in the test should be made from the blood of individuals belonging to Group I, of the Jansky classification, or Group O, of the Landsteiner classification.

As the blood serum of different guinea-pigs varies considerably in complementary strength it is best to employ the serum of at least three guinea-pigs for each series of tests, and it is absolutely essential that the guinea-pig serum be titrated before each series of tests to determine its complementary strength.

To secure the serum the animals may be bled from the heart or the throat may be cut and the blood allowed to drain into Petri dishes. Bleeding from the heart is the usual method now used in securing blood serum for complement as it does not demand the sacrifice of the animal and the pigs may be used many times for bleeding by alternating them and not bleeding oftener than once in two weeks. To secure blood from the heart, the guinea-pig is etherized and the heart is punctured with the needle of a 10 cc. syringe which has been washed out with a citrate solution. The needle of the syringe may be connected with the syringe by a flexible rubber tube which much facilitates the handling of the syringe and making the puncture. While the operation requires considerable practice the technique when once mastered is by far the most satisfactory way of obtaining complement for this test.

After bleeding, the blood should be ejected from the syringe into a sterile Petri culture dish, allowed to stand at room temperature for two hours and then placed in the ice-box overnight. In the morning the clear serum is pipetted off and, if it is to be used at once, it is diluted with one and one-half parts of normal saline solution (0.85 per cent). If not to be used for several hours it should be placed at once in the ice-box undiluted and should not be employed in the test if it is over forty-eight hours old. The serum should be free from red blood corpuscles and not more than slightly blood-stained.

The syringe, needles, Petri dishes and all apparatus employed in collecting the blood should be sterilized before use and be perfectly dry when used, with the exception of the syringe which should be washed out with normal saline or citrate solution before the puncture is made. If the serum, after separation from the clot, contains numerous red blood corpuscles, these may be removed by centrifugalization, the clear serum being carefully pipetted off from the sedimented corpuscles.

If all of the diluted complement is not used up it may be kept in the ice-box for from two to three days without losing much of its strength but must be titrated before it is used again. Immediately after being drawn from the guinea-pig the complement is comparatively weak and is at its best from twenty-four to thirty-six hours after withdrawal, after which time it gradually becomes weaker and after forty-eight hours is usually unsuitable for use.

It should be titrated each time before use in the test.

Methods for the preservation of complement have been devised but have proven unsatisfactory in the writer's hands.

Preparation of Hemolytic Amboceptor.—The hemolytic serum, or amboceptor, employed in the complement fixation test for amebiasis, as recommended by the writer, is prepared by immunizing rabbits to human red blood corpuscles. Noguchi (1909) was the first to call attention to the advantages of a human hemolytic system in the Wassermann test and the same advantages are present in the complement fixation test for amebiasis. The chief advantage is that the employment of the human system obviates the danger of false negative results being obtained if the sheep system be used, due to the fact that many human blood sera contain natural hemolytic amboceptor for sheep red blood corpuscles and in such sera negative results may be obtained in positive cases of amebiasis if the sheep system be used in the complement fixation test, even more often than in the Wassermann test. If the sheep system be employed it is necessary that the natural sheep hemolytic amboceptor in the patient's blood serum be removed and while this can be done it is time consuming and may weaken the reaction in positive cases of infection with *Endamœba histolytica. The writer believes that some of the discordant results of the complement fixation test for amebiasis in the hands of different investigators have been due to the use of a sheep hemolytic system and would urge the importance of employing the human hemolytic system in this test.*

Various methods of immunizing rabbits for the purpose of securing a good amboceptor serum have been recommended but the two following methods have proven most successful in the writer's hands.

Method 1.—This is the method that has been successfully used as a routine at the Army Medical School for obtaining hemolytic sera for the Wassermann test and is just as applicable to this test:

Pure white, full grown rabbits are selected for immunization and are given repeated intravenous injections of washed human red blood corpuscles. The blood corpuscles are prepared for these injections as follows: The requisite amount of blood, allowing at least twice the amount of blood as the amount of corpuscles to be injected, is withdrawn from one of the large arm veins of a volunteer with a sterile glass syringe which has been washed out with a sodium citrate solution to prevent the clotting of the blood. The blood is then ejected at once into a flask containing from 200 to 250 cc. of normal saline solution (0.85 per cent) and distributed into 50 cc. centrifuge tubes and

centrifugalized until the red blood corpuscles are deposited and the supernatant fluid is practically colorless. The supernatant fluid is then poured off and the tubes again filled with normal saline, well shaken, and again centrifugalized. This process is repeated four times after which the supernatant fluid is tested for albumin; if the least trace is demonstrated, the washing is again repeated and continued until the supernatant fluid is free from albumin. Usually, four washings will be found sufficient. Centrifugalization should not be continued for so long that the corpuscles become packed in the bottom of the tubes, for the force that is necessary to dislodge them may break up some of the corpuscles and render them unsuitable for injection.

After the corpuscles have been washed as described, the supernatant saline solution is carefully decanted and the sediment of red blood corpuscles mixed with as little normal saline as is necessary to secure a suspension that will pass through the needle of the syringe used for injection purposes, which should have been carefully sterilized before use.

The rabbits selected for injection are given 3 cc. of the blood cell suspension intravenously, using the ear vein, for three successive days; they are then allowed to rest for twenty-one days, and then given daily intravenous injections of 0.5 cc. of the blood cell suspension for five days. From seven to nine days after the last injection, their blood serum is tested for hemolytic strength and will generally be found suitable for use in the test. If not, five more intravenous injections of 0.5 cc. each of the blood cell suspension should be given, when almost invariably a good amboceptor serum will result. Deaths of the rabbits from anaphylaxis seldom occur with this method of immunization and it is recommended for routine use. If suitable for use the rabbits are bled and the serum preserved as noted later (see page 104).

Method 2.—This method of immunization is more rapid than the one just described but is more laborious in that daily injections have to be made for a longer time. In this method daily intravenous injections of 0.05 cc. of the blood cell suspension are given for a period of twelve days and the blood serum of the animal titrated six days after the last injection, at which time the serum will usually be found to contain enough hemolysins to be suitable for use as the amboceptor in the test. If not, it is best to discard the rabbit and start another, as it is seldom that a greater number of injections will raise the titre sufficiently for use.

Preliminary Titration of Amboceptor.—The following simple method of determining the hemolytic strength of the serum of the immunized animals is recommended:

A small amount of blood is obtained from the rabbit's ear vein in a Wright tube, the serum allowed to separate, after which it is pipetted off and inactivated by heating it in a water-bath at 56° C. (132.8° F.) for one-half hour. One drop of the inactivated blood serum is mixed

with 39 drops of normal saline (0.85 per cent) and the mixture titrated for its hemolytic strength as shown in Table 2.

TABLE 2.—PRELIMINARY TITRATION OF AMBOCEPTOR SERUM

Tube No.	Amount of salt solution, 0.85%, cc.	Number of complement units*	Amount of blood suspension, 5%, cc.	Amount of amboceptor serum diluted 1-40, drops
1	0.9	2	0 1	1
2	0 9	2	0 1	2
3	0 9	2	0 1	3
4	0 9	2	0 1	4
5	0 9	2	0 1	5
6	0 9	2	0.1	None

Incubate in water-bath at 37° C. for one hour or in incubator at same temperature for two hours and read results.

* If the unit of complement has not been determined by a previous titration, use 0.1 cc. of a 1:1.5 dilution in salt solution.

After the incubation in the water-bath at 37° C. (98.6° F.) for one hour has been completed, the titration is read and if the tube containing one drop of the diluted rabbit serum shows complete hemolysis, the serum is strong enough in hemolytic amboceptor for use in the test and the animal should be bled at once. If not, and Method 1 has been employed for immunization, more injections of the blood cell suspension should be given, as directed above. The control tube, No. 6, should show no hemolysis.

BLEEDING IMMUNIZED ANIMALS AND PRESERVATION OF THE AMBOCEPTOR SERUM.—Bleeding is best accomplished by quickly cutting the carotid vessels while holding the rabbit over a large Petri dish which has been previously sterilized. The dish, containing the blood, should then be kept at room temperature for two hours, after which it is placed in the ice-box overnight. In the morning the serum is carefully pipetted off into small test tubes, inactivated by heating in the water or paraffin bath at 56° C. (132.8° F.) for one-half hour, and then kept in the ice-box until used in the test or impregnated in filter paper. If the blood serum is slightly tinged with hemoglobin, as often happens, it is still usable but if it is distinctly red it should not be used in the test.

The preservation of amboceptor serum is best accomplished by impregnating special filter paper with the serum as originally recommended by Noguchi (1912), but it may be preserved in ampules in the ice-box and used as required. The writer would recommend the preservation upon filter paper as the serum retains its hemolytic strength for a longer period of time than when kept in liquid form, the danger of bacterial contamination is entirely eliminated, while drying upon filter paper destroys almost entirely the hemagglutins which are often present in the serum without injuring the hemolysins. Even though some hemagglutins be still present after placing the serum on filter paper they will have disappeared if the paper is kept for a period of three weeks before it is employed in the test.

IMPREGNATION OF FILTER PAPER.—The most suitable filter paper for impregnation with the amboceptor serum is Schleicher and Schull's No. 597, but any filter paper of the same texture should serve the purpose. The paper should be cut into squares measuring 10 by 10 cm., and about 1.5 cc. of the amboceptor serum is required to saturate one of these squares of paper. The requisite amount of the amboceptor serum is placed in a sterilized Petri dish which will just accommodate the 10 by 10 cm. square paper and the number of squares of filter paper which the serum will saturate are added, one by one, until all of the blood serum has been absorbed by the paper. The squares of saturated paper are then carefully lifted with a pair of forceps, drawn across the edge of the dish several times to remove any excess of serum, and then placed upon a piece of unbleached muslin large enough to hold all of the squares of filter paper. The muslin containing the paper should then be placed under an electric fan and the paper rapidly dried, after which the papers should be placed in an air-tight glass container and kept at room temperature in a dark and dry place.

Amboceptor paper, thus prepared, will retain its hemolytic strength for many months but should be titrated monthly in order to be sure of its exact strength. The writer has used amboceptor paper over a year old in almost the same hemolytic dose as when it was first prepared whereas amboceptor serum kept in the fluid condition had long since lost its usefulness because of loss of strength.

HEMOLYTIC ANTIGEN OR BLOOD CELL SUSPENSION.—The hemolytic antigen used in the writer's complement fixation tests for amebiasis consists of a 5 per cent suspension in normal saline (0.85 per cent) of human red blood corpuscles obtained from an individual belonging to Blood Group O, of the Landsteiner classification (Group I, of the Jansky classification, Group IV, of the Moss classification). The observations of Williams, already mentioned, have shown that erroneous results may be obtained in complement fixation tests using human hemolytic antigen if the red blood corpuscles of individuals belonging to other blood groups be used in making the blood cell suspension, owing to the fact that an excess of amboceptor may result from natural hemolysins present in the guinea-pig blood serum used as complement in the test. In addition, isohemolysins in the serum of the individual tested may give rise to an excess of hemolytic amboceptor, so that in order to secure the greatest accuracy with the test the blood cell suspension should be prepared with cells from Group O only.

The quantity of blood to be collected for the complement fixation test will vary, of course, with the number of tests. One cubic centimeter of blood will furnish enough suspension for 100 tests, using 0.1 cc. of the suspension for each tube employed in making the tests. Under ordinary conditions a much smaller number of tests will be made and enough blood can usually be secured by pricking the finger just above the root of the nail with a sterile needle, the blood being allowed

to drop into a sterile graduated centrifuge tube of 15 cc. capacity, the tube being filled to the 9 cc. mark with 2 per cent sodium citrate in normal saline solution previously. Enough blood is added to bring the total to the 10 cc. mark. If only a few tests are to be made, a smaller amount of blood will be sufficient.

The tube is then thoroughly shaken by hand to distribute the blood and the contents then divided into two or more centrifuge tubes, which are filled with normal saline and centrifugalized at high speed until the red blood corpuscles are at the bottom of the tubes. Care should be taken not to centrifugalize for too long a time as the cells may become packed so firmly that it will be impossible to dislodge them from the bottom of the tubes without breaking them up and thus rendering them useless for the test. After centrifuging, the supernatant fluid is poured off and the tubes again filled with normal saline and again centrifugalized. This process is repeated until the supernatant fluid, upon testing, is perfectly clear and free from albumin. In practice, four centrifugalizations should be made before using the blood cells in the test, this number being sufficient.

After the last centrifugalization the supernatant fluid is pipetted off and enough normal saline solution is added to the sediment of red blood corpuscles in each tube to make a 5 per cent suspension of the cells. In making the test, 0.1 cc. of this suspension is added to each tube containing 0.9 cc. normal saline, thus making a half of one per cent suspension.

In making a small number of tests, say from 10 to 20, enough blood for the suspension can be secured by allowing 30 drops of blood from the finger to drop into the graduated centrifuge tube filled with citrate solution, and the entire process of centrifugalization may be carried out with this single tube.

Technique of Blood Grouping.—All individuals are classified, as regards their blood grouping, into four classes, variously designated by different authorities. At the present time there are three group classifications adopted, those of Landsteiner, Jansky and Moss. For the convenience of the reader the relationships of these classifications as regards the four blood groups is shown in the following table:

TABLE 3.—BLOOD GROUPING CLASSIFICATIONS

Landsteiner classification, Group	Jansky classification, Group	Moss classification, Group
AB	IV	I
A	II	II
B	III	III
O	I	IV

The Landsteiner classification should be preferred and will be followed in this description of the technique of blood grouping.

In the complement fixation test for amebiasis the blood cell suspension should always be made with the red blood cells of individuals

belonging to Group O, for the reasons mentioned previously (page 104). To determine the blood group of any individual proceed as follows:

Known blood serum from individuals belonging to Groups A and B are employed. A drop of each is placed at opposite ends of a glass microscopic slide, being very careful that the two do not come into contact. Puncture the finger or lobe of the ear of the person whose blood is to be classified and transfer in turn to each of the drops of serum approximately one-third of a drop of blood by means of a glass rod, using a different rod for each transfer. Mix the serum and blood thoroughly so as to form a uniform suspension. Observe the mixture for a minute or slightly more and if agglutination of the blood cells occurs it will be noted that flocculi, visible to the naked eye, appear in the mixture, while if no agglutination occurs the suspension remains uniform in appearance. The reaction may rarely be delayed for more than a moment.

If the blood of the individual tests is agglutinated by both A and B sera it belongs to Group AB; if it is agglutinated by A serum alone, it belongs to Group A; while if it is agglutinated by B serum alone, it belongs to Group B. If it is not agglutinated by either A or B serum, it belongs to Group O, and is suitable for use in the making of the blood cell suspension for the complement fixation test.

Approximately 45 per cent of individuals belong to Group O, in which the blood cells are not agglutinated or hemolyzed by the blood serum of individuals belonging to any of the other groups, so that by testing the personnel of any laboratory, one or more individuals will be found belonging to this group, and blood from these individuals should be used in preparing the blood suspension for the test.

If, for any reason, the blood cells used in making the suspension are from individuals belonging to other blood groups than Group O, the suspension should be tested with the blood serum of each guinea-pig used as a source of complement to ascertain whether any of the guinea-pig sera contain natural hemolysins to the blood cells in the suspension. To do this, add to a tube containing 0.9 cc. normal saline, 0.1 cc. of the blood cell suspension and 0.2 cc. of the guinea-pig serum which has been diluted with one and one-half times the amount of normal saline. Incubate in the water-bath at 37° C. (98.6° F.) for one hour and if no hemolysis results the guinea-pig serum may be used for complement. If hemolysis is present the serum should be discarded.

Preparation of Amebic Antigens.—Several types of amebic antigen have been devised for use in the complement fixation test for amebiasis and the most valuable of them will be described under the name of the investigator who originated the particular antigen.

Craig's Antigen.—This antigen consists of an alcoholic extract of cultures of *Endamœba histolytica* grown upon the modified Locke-egg-serum medium of Boeck and Drbohlav, which is described upon page 79 of this work. A culture upon this medium which is rich in

Endamœba histolytica is selected and a sub-culture is made from this. At the end of forty-eight hours two sub-cultures are made from this sub-culture and at the end of each forty-eight hours thereafter two sub-cultures are made from each of the sub-cultures, until there are available for extraction at least 120 cultures. At the time of making the sub-cultures care should be taken to remove all of the material at the junction of the egg slant and the covering Locke solution, so that one may secure a rich growth of the amebæ from day to day. Each day the cultures should be examined microscopically to be sure that the amebæ are growing satisfactorily, and if cultures are found in which the amebæ do not number at least two or three to the microscopic field, the culture is discarded and sub-culture made from a stock culture.

Upon obtaining the requisite number of cultures all of the material at the junction of the egg slant and the covering Locke solution is pipetted off with a 1 cc. pipette into suitable centrifuge tubes from all of the cultures. In pipetting off this material one should gently rub the surface of the egg slant with the pipette in order to loosen any amebæ that may be on the slant, and mix such material with the sediment before removing the mixture from the culture tube. The mixture in the tubes is centrifugalized and the sediment so obtained is all placed in a glass bottle and seven and one-half volumes of absolute alcohol is added. The mixture is then well shaken and placed in an incubator at 37° C. (98.6° F.) and extracted for fifteen days, the bottle being shaken several times a day during that period. After extraction the mixture is filtered through a fine filter paper and is then ready for use, after titration.

Remarks.—This is the standard antigen that the writer devised for this test and which he has used almost exclusively in routine practice. In his hands it has given excellent results but must be employed by a trained serologist as the antigenic strength is so weak that it may have to be used undiluted and, in the writer's experience a dilution of more than 5 parts of normal saline to 1 part of antigenic extract has not been possible. This fact tends to the occurrence of anticomplementary reactions and false positive results unless the test is performed by one well trained in serological diagnosis.

If antigens afe made by extracting cultures of *Endamœba histolytica* grown in flasks, according to the technique of Frye and Meleney (page 83), the antigenic strength is greatly increased because of the greater number of amebæ available for extraction and such antigens, according to Magath and Meleney (1940), have been antigenic in dilutions of from 1 to 65 to as high as 1 to 150, and are preferable for use owing to the lessened danger of anticomplementary reactions.

Craig and Scott's Antigen.—The antigen is prepared by extracting the mucoid material rich in amebæ obtained from the intestine of dogs infected with *Endamœba histolytica*. The method of inoculating the dogs will be found described on page 121 of this work.

In preparing this antigen a dog is selected that is passing stools containing many *Endamœba histolytica* and the mucoid material is obtained by aspiration of the material from the cecum and upper portion of the colon, using a glass pipette with a large bore to which a 25 cc. rubber bulb is attached. The material obtained consists very largely of bloody mucus very rich in amebæ and enough material can be obtained from the intestine of one acutely infected animal to make enough antigenic extract for many thousand tests.

After obtaining the material for extraction it is placed in a suitable glass flask and absolute alcohol is added in the proportion of 1 part of the material to 7 parts of absolute alcohol. The flask is then placed in an incubator, which should be kept at 45° C. (113° F.), and extracted for fifteen days, the flask being thoroughly shaken several times a day during that time. After extraction is complete the mixture is filtered, diluted with from 3 to 5 parts of normal saline (0.85 per cent) and titrated. Usually such antigens may be diluted with from 3 to 5 parts of normal saline for use in the test.

Remarks.—The writer has found that antigens prepared according to this method are as satisfactory for use in the complement fixation test for amebiasis as other antigens and the method obviates the cultivation of *Endamœba histolytica* which is very time consuming and technically difficult. Dogs are easily infected with *Endamœba histolytica* and the material obtained from the infected intestine is much richer in amebæ than are cultures and bacteria are much less numerous, while the antigenic extracts so obtained are more antigenic than those made by extracting tube cultures of *Endamœba histolytica*, but are not as antigenic as the extracts prepared from flask cultures after the method of Frye and Meleney.

Stone's Antigen.—This antigen, evolved by Stone, in 1935, is prepared by extracting the washed cysts of *Endamœba histolytica* obtained from cultures in which encystment has been produced by the method described by Stone and already mentioned (see page 90).

The method of preparation of this antigen is thus described by Stone: (American Journal of Tropical Medicine, Vol. XV, p. 485, 1935.)

1. *Collection of Cysts.*—Take 12 to 20 cultures (prepared as recommended for producing cysts), containing on an average 1,800,000 cysts per culture, and allow them to stand in a vertical position in an ice-box for twenty-four hours. All of the cysts will now be in the bottom of the tubes. Carefully draw off and discard all but 1 cc. of the supernatant fluid by means of a suction pump, large dropper or pipette. Great care should be taken not to disturb the cysts in the bottom of the tubes while handling and drawing off the supernatant fluid.

2. *Separation of the Cysts from Starch Granules.*—Mix the remaining contents of each tube and pipette and place the resulting suspension into a 250 cc. settling flask. Wash out each tube with 1 or 2 cc. of physiological salt solution and add these washings to the material in the settling flask. Add about 50 cc. of physiological salt solution to the material in the settling flask and then thoroughly mix the contents. Allow the mixture to stand ten minutes by the

clock so that the major portion of the starch included in the mixture will have time to settle out. Then carefully decant the upper 25 cc. of the supernatant fluid into another settling flask. Replace the saline decanted from the first flask with fresh saline, thoroughly stir the mixture and then allow it to stand ten minutes. Again decant the upper 25 cc. into the second flask. Continue this procedure until examination of the residue in the first flask shows few or no cysts remaining. The second flask will now contain about 250 cc. of fluid, all the cysts, and very little starch. Thoroughly mix the contents of this flask and then allow it to stand for fifteen to twenty minutes. Decant the fluid to a third settling flask and if the steps have been carefully carried out there should be a negligible amount of starch left in the suspension (in the second flask).

3. *Washing the Cysts.*—Pour the saline suspension of the cysts into 50 cc. centrifuge tubes and centrifuge at 1850 revolutions per minute for five minutes. Decant the supernatant fluid from the tubes and discard it. Add sufficient saline to each tube to fill it. Thoroughly mix the residue and the fluid in each tube and then centrifuge at 1850 revolutions per minute for five minutes. Repeat this process until the supernatant fluid is perfectly clear and examination of the residue shows only cysts and an occasional bacterial organism. The cysts should now be practically bacteria-free and there should be very little débris or starch present. If more than a minimum amount of starch is still present, return the material to a settling flask and bring the volume up to 100 cc. with saline. Mix thoroughly, and allow the mixture to stand fifteen minutes, decant the supernatant fluid, and recentrifuge. After the final centrifuging the sedimented cysts in the bottom of each tube are mixed with 2 cc. of absolute alcohol (ethyl) and then this material from all of the tubes is pipetted into a 120 cc. glass stoppered bottle. Add glass beads to the bottle until it is one-half filled, then add absolute ethyl alcohol until the bottle is two-thirds filled.

4. *Breaking Up the Cysts.*—The bottle containing the cysts, beads and alcohol is now placed in a shaking machine for two hours at the end of which time all of the cysts will be thoroughly broken up.

5. *Extraction of Cysts.*—Allow the mixture so prepared to remain at room temperature, with occasional shakings by hand, for one week. After filtration through ordinary filter paper the antigen is ready for titration and use. If the antigen should be hemolytic or anticomplementary, evaporation at room temperature to one-third or one-half of the original volume will lessen the size of the working dose and thereby reduces its alcoholic content, thus making it less anticomplementary and less hemolytic. Antigens prepared in this manner are usually well within the working range in regard to anticomplementary and hemolytic values. The amount of bacterial substance present is negligible; the antigen is made up almost entirely of amebic substance.

Remarks.—The writer has compared the results of the complement fixation test for amebiasis obtained with this antigen and the antigens prepared as recommended by him, and has found that they are practically identical. The Stone antigen has the advantage that it is bacteria-free and, therefore, a more ideal antigen for the test than those in which not only *Endamœba histolytica* but the bacteria growing in the cultures, is extracted. However, it has been shown by numerous observers that the bacterial content of the antigenic extracts does not interfere with the specificity of the reaction, so that a bacteria-free antigen is not essential, although it is certainly desirable, and, in this respect, the Stone antigen is the best that has as yet been devised.

Titration of Reagents Employed in the Test.—The complement, hemolytic amboceptor and antigen used in the test must be carefully titrated in order to ascertain the suitable amount of each to be employed. The complement, or guinea-pig serum, *must be titrated before every series of tests*, daily if necessary, while the amboceptor paper and antigens are titrated at less frequent intervals. Usually, the amboceptor paper does not need to be titrated oftener than once a month and this is also true of the antigens that have been described.

(*a*) TITRATION OF COMPLEMENT.—After diluting the guinea-pig serum, or complement, one and one-half times with normal saline solution (0.85 per cent) the titration is made in accordance with Table 4.

After incubating in the water-bath at 37° C. (98.6° F.) for one hour, the tubes being shaken every fifteen minutes to liberate the amboceptor from the paper (see titration of amboceptor), the first tube showing complete hemolysis is noted and the amount of complement in it is called a complement unit. Usually tube 4 will be the first in the series to show complete hemolysis and in this instance 0.05 cc. of complement would be one unit and in making the test twice this amount, or 0.1 cc. would be used. The control tube, tube 10, containing amboceptor paper without complement, should show complete inhibition of hemolysis.

TABLE 4.—TITRATION OF THE COMPLEMENT

Tube No.	Complement (40 per cent guinea-pig serum), cc.	Saline solution, cc.	5 per cent blood cell suspension, cc.	Amboceptor, units
1	0 02	0 9	0 1	2
2	0 03	0 9	0 1	2
3	0 04	0 9	0 1	2
4	0 05	0 9	0 1	2
5	0 06	0 9	0 1	2
6	0 07	0 9	0 1	2
7	0 08	0 9	0 1	2
8	0 09	0 9	0 1	2
9	0 10	0 9	0 1	2
10	0 10	0 9	0 1	0

Incubate in water-bath at 37° C. for one hour and read. The tube showing complete hemolysis contains one unit of complement. Two units are used in making the test. (Craig, Colonel Chas. F.; Am. Jour. Trop. Med., p. 485, 1935.)

(*b*) TITRATION OF AMBOCEPTOR PAPER.—The amboceptor, after having been impregnated into filter paper, should be titrated to determine its hemolytic strength. At the time that this is done, strips of the paper, 3 mm. wide, are cut from the sheet of amboceptor paper, and varying lengths of one of the strips are used in the titration. Table 5 shows the method of titrating the amboceptor paper.

The tubes should be incubated in the water-bath for one hour at 37° C. (98.6° F.) and should be thoroughly shaken every fifteen minutes in order to liberate the amboceptor from the paper. The titration is read at the end of the hour and the first tube in the series showing

complete hemolysis is noted and the amount of amboceptor paper in that tube is called one unit of amboceptor. An efficient amboceptor paper should show complete hemolysis in tubes 3, 4, or 5, or with pieces of the amboceptor paper measuring 3 by 3, 3 by 4, or 3 by 5 mm., but frequently complete hemolysis is observed in the tube containing the piece of paper measuring 3 by 2 mm. The control tubes, 9 and 10, should show no hemolysis. In making the test two units of amboceptor are used. Thus, if hemolysis is complete in tube 3, containing the piece of amboceptor measuring 3 by 3 mm., a piece of the paper measuring 3 by 6 mm. would be used in the test.

TABLE 5.—TITRATION OF AMBOCEPTOR PAPER

Tube No.	Salt solution, cc.	Complement, units	Blood cell suspension, 5 per cent, cc.	Amboceptor paper, mm.
1	0 9	2	0 1	3 x 1
2	0 9	2	0 1	3 x 2
3	0 9	2	0 1	3 x 3
4	0 9	2	0 1	3 x 4
5	0 9	2	0 1	3 x 5
6	0 9	2	0 1	3 x 6
7	0 9	2	0 1	3 x 7
8	0 9	2	0 1	3 x 8
9	0 9	2	0 1	None
10	0 9	None	0 1	3 x 16

Incubate in water-bath for one hour at 37° C. (Craig, Colonel Chas. F.; Am. Jour. Trop. Med., p. 485, 1935.

If the amboceptor serum has not been placed in filter paper graduated amounts should be placed in tubes and titrated with the same amount of complement, etc., and the same rules followed in reading the results of the titration.

(*c*) TITRATION OF AMEBIC ANTIGEN.—The antigen used in the complement fixation test for amebiasis must be titrated to determine its hemolytic, anticomplementary and antigenic qualities and because it is sometimes necessary to employ the antigenic extract undiluted the titration must be very accurate. Whether the antigen is used diluted or undiluted the titration procedures are the same and if one is titrating a new antigen it is well to set up a series of titrations employing the undiluted antigen and the same antigen diluted with from 1 up to 10 parts of normal saline (0.85 per cent) solution. Higher dilutions are sometimes possible but with the antigen prepared after the writer's methods, dilutions above 1 part of antigen to 5 parts of normal saline are seldom possible. The antigen is titrated for its *hemolytic* qualities as shown in Table 6 (Page 112).

After incubating in the water-bath for one hour the titration is read. If undiluted antigenic extract is being titrated complete inhibition of hemolysis should be present in the series of tubes up to tubes 6 or 7, while tube 8 may show considerable hemolysis. In practice it has been found that if no hemolysis is present in tube 6, the antigenic extract will give excellent results when used in the test. If the antigenic

extract has been diluted the same quantities are used in the titration and if no hemolysis occurs in tube 5 the antigen is suitable for use.

TABLE 6.—TITRATION OF ANTIGEN FOR HEMOLYTIC PROPERTIES

Tube No.	Amount of salt solution, cc.	Amount of complement, cc.	Antigenic extract, cc.	Blood cell suspension, 5 per cent, cc.
1	0 9	0 1	0 08	0.1
2	0 9	0 1	0 09	0 1
3	0.9	0 1	0 10	0 1
4	0.9	0 1	0.11	0 1
5	0.9	0.1	0.12	0 1
6	0 9	0 1	0 13	0 1
7	0.9	0 1	0.14	0 1
8	0 9	0 1	0 15	0 1
9	0 9	0 1	0 16	0 1
10	0 9	0 1	None	0 1

Incubate in water-bath for one hour at 37° C. (Craig, Colonel Chas. F.; Am. Jour. Trop. Med., p. 485, 1935.)

Titration for Anticomplementary Properties.—Antigens prepared according to the writer's methods are sometimes so anticomplementary as to be useless and it is most important that the anticomplementary qualities of each antigen should be ascertained by a careful titration. Table 7 illustrates the manner of titrating antigens for this purpose.

TABLE 7.—TITRATION OF ANTIGEN FOR ANTICOMPLEMENTARY PROPERTIES

Tube No.	Salt solution, cc.	Inactivated normal serum (human), cc.	Antigenic extract, cc.	Complement, units		Blood cell suspension 5 per cent, cc.	Amboceptor paper, units
1	0 9	0 1	0 02	2	Incubate in water-bath at 37° C. for one-half hr.	0 1	2
2	0 9	0 1	0 03	2		0 1	2
3	0 9	0 1	0 04	2		0 1	2
4	0 9	0 1	0 05	2		0 1	2
5	0 9	0 1	0 06	2		0 1	2
6	0 9	0 1	0 07	2		0 1	2
7	0 9	0 1	0 08	2		0 1	2
8	0 9	0 1	0 09	2		0 1	2
9	0 9	0 1	0 10	2		0 1	2
10	0 9	0 1	None	2		0 1	2

Incubate in water-bath at 37° C. for one hour, shaking every fifteen minutes.

After the last incubation in the water-bath at 37° C. has been completed the tubes are placed in the ice-box and the titration is read at the end of two hours. None of the tubes containing the antigen should show inhibition of hemolysis while the control tube 10 should show complete hemolysis. If all of the tubes containing antigen show complete hemolysis the antigen is suitable for use in the test but if any of the tubes show inhibition of hemolysis the antigenic extract is too anticomplementary and should not be used, especially if the antigen is undiluted.

Titration for Antigenic Properties.—This titration is made with a known positive serum from a patient whose feces contains *Endamœba histolytica.* If the antigenic extract is used undiluted it is necessary in practice to use an extract that does not require more than 0.1 cc.

to give a positive reaction with a known positive serum. If larger amounts are employed false positive results may occur. With the undiluted antigen prepared by the writer's methods amounts of from 0.07 to 0.1 cc. have given positive results which have been proven specific.

The amebic antigen is titrated for its antigenic properties as shown in Table 8.

TABLE 8.—TITRATION OF ANTIGEN FOR ANTIGENIC PROPERTIES

Tube No.	Salt solution cc.	Positive serum, cc.	Complement, units	Antigenic extract, cc.		Blood cell suspension, 5 per cent, cc.	Amboceptor paper, units
1	0 9	0 1	2	0 02	Incubate in water-bath at 37° C. for one-half hr.	0 1	2
2	0 9	0 1	2	0 03		0 1	2
3	0 9	0 1	2	0 04		0 1	2
4	0 9	0 1	2	0 05		0 1	2
5	0 9	0 1	2	0 06		0 1	2
6	0 9	0 1	2	0 07		0 1	2
7	0 9	0 1	2	0 08		0 1	2
8	0 9	0 1	2	0 09		0 1	2
9	0 9	0 1	2	0 10		0 1	2
10	0 9	0 1	2	None		0 1	2
11	0 9	0 1	None	None		0 1	2
12	0 9	None	2	None		0 1	2

Incubate in water-bath at 37° C. for one hour, shaking every fifteen minutes. (Craig, Colonel Chas. F.; Am. Jour. Trop. Med., p. 485, 1935.)

After the incubation for one hour in the water-bath has been completed the tubes are placed in the ice-box and the titration read at the end of two hours. Tubes 10 and 12 should show complete hemolysis and tube 11, no hemolysis. In the remaining tubes the smallest amount of antigen showing complete inhibition of hemolysis, *i. e.*, fixation of complement, is called the antigenic unit and is the amount that should be used in making complement fixation tests for amebiasis.

Technique of the Complement Fixation Test for Amebiasis.—After titrating the various reagents used in the test in the manner described, and having ascertained the proper amount of antigen to be employed, the technique of the test is as follows:

Two tubes are required for each blood serum to be tested and, in addition, there must be a control set of two tubes each for a known positive and a known negative serum. Racks made for two rows of tubes, anterior and posteriors, are used in making the tests. The complement should be titrated before each series of tests and all blood sera inactivated by heating in the water or paraffin bath at 56° C. (132.8° F.) for one-half hour to destroy the complement present in all blood sera.

Proceed as follows in making the tests: Place 0.9 cc. of normal saline (0.85 per cent) in all of the tubes used in the series of tests. In tube 1, anterior, place 0.1 cc. of the patient's serum that is being tested and the same amount in tube 1, posterior, and do the same in the case of every serum that is being tested. In tube 2, anterior and posterior,

place 0.1 cc. of a known positive serum, and in tube 3, anterior and posterior, 0.1 cc. of a known negative serum. These are the controls. To each tube add *2 units* of complement and to each anterior tube, *1 unit* of the amebic antigen, and incubate all of the tubes in the water-bath at 37° C. (98.6° F.) for one-half hour. At the expiration of this time add to each tube 0.1 cc. of the 5 per cent suspension of red blood corpuscles and *2 units* of the amboceptor paper. The tubes are now incubated in the water-bath at 37° C. for one hour, being thoroughly shaken every fifteen minutes during that period, in order to liberate the amboceptor from the paper. *This is very important and if not done, false positive results will be obtained.* At the end of the hour's incubation in the water-bath the tubes are placed in the ice-box for two hours and then the reactions are read, and, if the reagents have been properly titrated and used, the results should be as follows:

The negative serum tube, tube 3, should show complete hemolysis; the positive serum tube, tube 2, should show complete inhibition of hemolysis; while tube 1, containing the serum to be tested, will if negative, show complete hemolysis, and if infection with *Endamœba histolytica* is present will show varying degrees of inhibition of hemolysis, usually either complete (4 plus) or almost complete, if treatment has not been administered. All of the tubes in the posterior row which do not contain antigen, should show complete hemolysis. If there is any inhibition of hemolysis in the posterior tubes something is either wrong with the reagents used in the test or the serum that is being tested is anticomplementary. If several different sera are being tested it will be noted that at the end of the hour's incubation in the water-bath some will show complete hemolysis while others may show varying degrees of inhibition of hemolysis. If the tests were read at this time very erroneous results would be obtained but after two hours in the ice-box all of the tubes containing antigen should show complete hemolysis except those from individuals infected with *Endamœba histolytica*. The method of performing the complement fixation test for amebiasis is graphically illustrated in Table 9.

TABLE 9.—METHOD OF MAKING COMPLEMENT FIXATION TEST FOR AMEBIASIS

Serum for diagnosis	Positive control set	Negative control set
Front tubes	*Front tubes*	*Front tubes*
Patients serum, 0.1 cc	Positive serum, 0.1 cc.	Normal serum, 0.1 cc.
Complement, 2 units	Complement, 2 units	Complement, 2 units
Antigen, 1 unit	Antigen, 1 unit	Antigen, 1 unit
Salt solution, 0.9 cc.	Salt solution, 0.9 cc.	Salt solution, 0.9 cc.
Back tubes	*Back tubes*	*Back tubes*
Patients serum, 0.1 cc.	Positive serum, 0.1 cc.	Normal serum, 0.1 cc.
Complement, 2 units	Complement, 2 units	Complement, 2 units
Salt solution, 0.9 cc.	Salt solution, 0.9 cc.	Salt solution, 0.9 cc.

Incubate for one-half hour in water-bath at 37° C.

Add 2 units of amboceptor paper to each tube and 0.1 cc. of 5 per cent suspension of human red blood corpuscles.

Incubate for one hour in water-bath at 37° C. and let stand in ice-box for two hours, and then read reactions.

READING AND INTERPRETATION OF THE REACTIONS.—The reactions with this test are usually very definite and weak positive results are seldom seen. The reactions should be read as 4 plus (++++), 3 plus (+++), 2 plus (++), plus (+), plus-minus (+−), and negative (−), according to the degree of inhibition of hemolysis, a 4 plus reaction signifying complete inhibition of hemolysis. In the interpretation of the reactions, any reaction below a strong 3 plus reaction is not considered as of diagnostic importance unless treatment has been administered or *Endamœba histolytica* is present in the stools. In the latter case, partial reactions may be of confirmatory value but a diagnosis should never be based upon such reactions alone.

Results of the Complement Fixation Test in Amebiasis.—The writer has made this test in over 1500 individuals and the results obtained with it in 1000 individuals whose stools were checked for *Endamœba histolytica* at the time that the tests were made illustrate what may be expected from the test in the diagnosis of infection with this parasite.

Of the 1000 individuals tested, 175, or 17.5 per cent, gave a positive reaction (++++ or +++) and 825, or 82.5 per cent, gave a negative reaction. Of the 175 positive cases, *Endamœba histolytica* was demonstrated in the stools in 157, or 89.7 per cent, while of the 825 negative cases, only 12, or 1.4 per cent, showed this parasite in the stools. Positive reactions were not obtained in individuals showing other species of amebæ or protozoa in their stools, of which there were no less than 220 cases, or 32.5 per cent, while no less than 176, or 25.1 per cent, showed other species of amebæ in their stools. Thus, a high percentage of positive results may be expected with this test in individuals infected with *Endamœba histolytica;* a certain percentage of negative results in such individuals; negative results in cases of infection with other intestinal protozoa and a small percentage of false positive results in individuals apparently free from infection with *Endamœba histolytica.* In all of these respects this test is comparable with other specific antigen-antibody reactions, the results being as accurate as those obtained with most serological procedures employed in the diagnosis of disease.

In the writer's experience false positive results are not obtained in syphilitic patients or in individuals suffering from other disease conditions, with the exception of chronic ulcerative colitis, in which condition positive results have sometimes been obtained in cases in which *Endamœba histolytica* could not be demonstrated. The reason for such reactions is not known at present. While the writer has obtained false positive reactions in a very few cases of ulcerative colitis, in most instances in which such a reaction was obtained repeated examinations of the stools resulted in the demonstration of this parasite, as many as eight to twelve examinations being necessary in some cases. In every case in which an apparent false reaction is obtained with this test repeated stool examinations should be made for *Endamœba histolytica*

and, in the vast majority of such cases, will result in demonstrating its presence.

PRACTICAL VALUE OF THE COMPLEMENT FIXATION TEST

The complement fixation test is inferior, as a diagnostic test, to the microscopic examination of the stools for *Endamœba histolytica*, if such examinations can be made by well-trained personnel, and it should never replace such examinations. In cases in which properly conducted stool examinations cannot be made the complement fixation test should be employed in diagnosis, or in cases suspected of the infection in which stool examinations are negative, provided the test can be made by qualified technicians. The reagents used in the test, as the antigen, and the hemolytic amboceptor, demand most careful preparation, while their titration and the technique of the test is complicated and demands the knowledge of an expert serologist if confusing or false reactions are to be eliminated. For the various reasons mentioned, the writer has never urged the use of this test as a routine diagnostic measure as he is confident, that if used in this manner, so many false reactions would occur as to bring the test into disrepute. *If properly performed by trained serologists the complement fixation test for amebiasis is a valuable diagnostic procedure but in ignorant hands it is of little or no value.*

When expert microscopic examination of the stools is not possible or in suspected cases, especially of liver abscess, in which the stools are negative, the complement fixation test is of value; in detecting "carriers" or cyst-passers of *Endamœba histolytica;* in suspected cases of amebic abscess of the liver; in acute or chronic dysentery of unknown origin; in the determination of the results of specific treatment and in the control of the treatment of amebiasis.

(*a*) **The Diagnosis of "Carriers" or Cyst-Passers.** The complement fixation test for amebiasis is frequently positive in individuals infected with *Endamœba histolytica* who present no symptoms of the infection, the so-called "carriers" or cyst-passers. Where it has been possible to employ this test in hospitals it has been invariably found that its use has often resulted in picking up infections in patients whose stools would never have been examined because of the absence of any symptoms suggesting infection with *Endamœba histolytica.* Subsequent stool examinations have almost invariably demonstrated the presence of this parasite and such patients were proven to be symptomless "carriers." In hospitals where routine examinations are made of the stools of all patients, by a trained pathologist, this test would be of little service, but, unfortunately, routine stool examinations are not made of all patients, even in some of our best hospitals, and in such hospitals the complement fixation test would be of great value in detecting latent amebic infections or atypical symptomatic cases.

(*b*) **The Diagnosis of Amebic Abscess of the Liver.**—In almost all cases of amebic abscess of the liver the complement fixation test for amebiasis gives a positive reaction, and in suspected cases in which the stools are negative for *Endamœba histolytica*, and these are numerous, this test has proven of great value as a diagnostic measure. The writer has observed several cases of amebic abscess of the liver in which repeated examinations of the stools were negative but in which the symptoms were suggestive, in which the complement fixation test gave a positive reaction and proper treatment led to recovery. In all of these cases operation revealed an abscess of the liver and scrapings from the abscess-wall, or preparations of the contents of the abscess, were positive for *Endamœba histolytica*. It is scarcely recognized by the medical profession that a large proportion of individuals suffering from amebic abscess of the liver or lung fail to show *Endamœba histolytica* in the stools. Thus, Ochsner and DeBakey (1935) in 131 cases of amebic abscess of the liver observed by them found the stools negative for the ameba in no less than 63.9 per cent, and their experience is that of many others who have studied this phase of amebiasis. An amebic abscess of the liver may occur years after the initial amebic infection in the intestine has disappeared and a negative stool is of no value if suspicious symptoms of an abscess be present. It is in such cases that the complement fixation test is so often of value and a positive reaction with this test should be regarded as practically diagnostic of the presence of such an abscess of the liver.

(*c*) **The Diagnosis of Acute and Chronic Amebic Dysentery.**—The complement fixation reaction is of practical value in differentiating amebic from other forms of diarrhea or diarrhea when it is impossible to secure accurate stool examinations. Most cases of diarrhea and acute and chronic dysentery caused by *Endamœba histolytica* give positive results with the complement fixation test, and while the symptomatology of classical amebic dysentery is quite characteristic, many atypical cases occur, and a diagnosis based upon clinical symptoms alone is not reliable.

It is fortunate that in cases of acute amebic dysentery, or in the acute exacerbations of a chronic amebic dysentery, a microscopic examination of the stools will demonstrate the presence of actively motile trophozoites of *Endamœba histolytica*, many of them containing red blood corpuscles, and these are easily recognized by any one who has had experience in the examination of stools for this parasite, while, in addition, the difference in the character of the cellular exudate in the stools of amebic and bacillary dysentery already described (see page 69) will greatly assist in differential diagnosis. Stool examinations should never be omitted when facilities are available but in the absence of such facilities the complement fixation test will be found to be of practical value in the diagnosis of these conditions.

(*d*) **The Control of Treatment and the Evaluation of Amebicidal Drugs.**—Not the least valuable of the practical uses of the complement fixation test for amebiasis is its employment in the control of the treatment of this infection and in the evaluation of the specific effect of drugs that are proposed for the treatment of amebiasis.

1. CONTROL OF TREATMENT.—It has already been noted that a positive reaction with the complement fixation test becomes negative, usually within two weeks after the disappearance of the amebæ from the stools, following successful treatment (see page 97). If the reaction should remain positive, even though the stools become negative for the ameba, our experience has shown that the infection has not been eliminated and that, sooner or later, the amebæ will reappear in the stools. In such cases, further treatment is indicated and should be continued until the reaction becomes negative. By employing the test in this manner it can be used as a control of treatment, as a persistent negative reaction furnishes a definite proof of cure.

A single negative complement fixation reaction is not sufficient proof that the infection is eliminated but the test should be repeated at monthly intervals for at least three months. In some cases observed by the writer in which, following specific treatment, the stools become negative for *Endamœba histolytica* and the complement fixation reaction also became negative, a return of a positive reaction was noted before the amebæ reappeared in the stools. Such results are explained by the failure of the treatment to destroy all of the amebæ in the tissues of the intestine, a few still remaining which, multiplying, produced the positive reaction although they were in such small number in the stools as not to be demonstrable.

2. TESTING OF AMEBICIDAL DRUGS.—The use of the complement fixation test for amebiasis as an indicator of the efficiency of new drugs proposed for the treatment of this infection is rendered possible by the fact that a positive reaction with the test disappears permanently after successful treatment in experimental animals, as the dog, and by the fact that if treatment is not successful in eliminating the infection the reaction will again become positive even though it may have remained negative for some time, if the infection is still present in the tissues. Susceptible animals, especially the dog, could be used for this purpose and the value of the drug being used for the treatment of the infection could be ascertained by frequent complement fixation tests. That this is feasible has been proven by the work of the writer and Kagy (1933), and in a recent test by the writer of the value of a new drug proposed for the treatment of amebiasis it was found, by the use of the complement fixation test, that in experimentally infected dogs the dose recommended by the manufacturers should be increased at least six times, as judged by the results of the test and autopsy examinations.

The practical value of this test in the diagnosis of amebiasis depends upon efficient antigens and these are difficult to prepare and vary much in strength, so that very careful titration is necessary. In the writer's hands the test has proven of great value and he has used it as a routine test for several years but never to the exclusion of the microscopic examination of the stools, which, when done by one trained in the recognition of the various amebæ occurring in the human intestine, remains the most simple and accurate method of diagnosis. The difficulty of preparing good antigens and the necessity of the services of trained serologists in performing the test renders it impracticable for the average clinical laboratory but these difficulties will be eliminated in the future and, when it is possible to prepare antigens from pure cultures of *Endamœba histolytica* this test should take its place as a routine diagnostic procedure in all well-equipped clinical laboratories.

Bozicevich, Hoyem and Walston's Complement Fixation Test.—Through the researches of Rees, Bozicevich, Reardon, Jones, Hoyem and Walston's a complement fixation test for amebiasis has been developed that is giving good results in the diagnosis of amebiasis. This test is now known as the Bozicevich, Hoyem and Walston test and the technique is described in the circular which accompanies the antigen which may be procured from Hynson, Westcott and Dunning, of Baltimore, Md. The method of preparing the antigen is complicated and as it can be purchased in the open market it will not be described here. In a personal letter to the writer Dr. Bozicevich states "It has been our experience that the antigen will not usually give falsely positive reactions in the presence of other protozoan or helminth parasites, also such reactions are not encountered in the sera obtained from syphilitic individuals or those having malaria. Falsely negative reactions are encountered, however, in the case of most carriers of amœbic infection."

Remarks.—The fact that falsely negative reactions are frequently encountered in latent, or "carrier" infections with *Endamœba histolytica* sharply limits the usefulness of this test as a diagnostic agent in amebiasis, for in symptomatic infections the parasite can almost invariably be demonstrated in one way or another and a complement fixation test is seldom required for diagnosis. It is in the asymptomatic infections that such a test is most valuable when *Endamœba histolytica* cannot be demonstrated. The test could not be used in surveys to determine the incidence of amebic infection owing to the frequency of negative reactions in "carrier" cases.

CHAPTER V

INOCULATION OF ANIMALS WITH ENDAMŒBA HISTOLYTICA—THE SIGMOIDOSCOPE IN DIAGNOSIS—CRITIQUE OF DIAGNOSTIC METHODS FOR AMEBIASIS

THE INOCULATION OF ANIMALS

THE inoculation of animals is not necessary in the diagnosis of amebiasis and is not employed for this purpose, except that in the application of the complement fixation test some authorities use a positive control serum obtained by immunizing rabbits to *Endamœba histolytica*. However, in research problems connected with amebiasis the use of experimental animals is essential and most of our information regarding the biology of this parasite and its pathogenicity has been obtained by means of animal experimentation.

Natural infections with *Endamœba histolytica* have been observed in dogs by Kartulis (1891), Darling (1915), Ware (1916), Fisher (1918), Bauche and Motais (1920), Faust (1930), Boyd (1931), Andrews (1932), and Kubo (1936); in monkeys, by Mello (1923), Dobell (1931), Hegner (1932), and others; in rats by Lynch (1915), Chiang (1925), Nagahana (1934), Andrews and White (1936), Atchley (1936) and Awakjan (1936), and in the domestic pig by Kessel (1928).

Experimentally, infection with *Endamœba histolytica* has been produced in kittens by many investigators, the most extensive observations being those of Craig (1905), Wenyon (1912), Dale and Dobell (1917), Rees (1929), Martin (1930), and Meleney and Frye (1933–1936). Infections have been produced in the monkey by Musgrave (1906), Mello (1923), Dobell (1931), Hegner, Johnson and Stabler (1932), Craig and Swartzwelder (1938) and others; in guinea-pigs by Baetjer and Sellards (1914) and Chatton (1918); in rabbits by Huber (1909), Thomson (1926), Heathman (1932), Menendez (1932) and Stone (1935); in the domestic pig by Kessel (1928); in the rat by Lynch (1915), Brug (1919), Chiang (1925), Kessel (1928), Regendanz (1929), Kitabatake (1934), Nagahana (1934), Takemura (1934), Tanabe (1934), and Atchley (1936), and in the dog by Lösch (1875), Hlava (1887), Harris (1901), Dale and Dobell (1917), Faust (1931), and Craig and Kagy (1933).

Among the animals that are susceptible to infection with *Endamœba histolytica* the only ones that are of practical value in research are the dog, kitten and rabbit. The dog is the most suitable animal for the study of the pathogenesis of amebiasis, in the opinion of Faust and the writer, for in this animal the infection may present all of the clinical

types seen in man, from the symptomless carrier to the most acute type of amebic dysentery. The infection produced experimentally in this animal lasts for a much longer time than it does in kittens so that the lesions more nearly resemble those found in man, while the longer duration of the infection enables one to study problems relating to immunity, complement fixation, and the effect of amebicidal drugs to much greater advantage than in infections in kittens in which a fatal result is usually a matter of a few days rather than weeks, as in the dog. The rabbit is only useful as a source of positive blood serum for the complement fixation test. For the study of variations in the virulence of different strains of *Endamœba histolytica* the kitten is more valuable than the dog, as in these animals the infection is more severe and usually fatal within a short time and Meleney and Frye have shown conclusively that even slight variations in the virulence of different strains of *Endamœba histolytica* can be detected by their effect upon kittens.

Methods of Inoculation.—Animals may be inoculated with *Endamœba histolytica* either by the mouth or through the rectum. Direct inoculation of the ileo-cecal region by operative procedures is usually successful but this is a method that is not necessary under ordinary conditions. Intraperitoneal injections are used in immunizing rabbits for the production of an immune serum for use in the complement fixation test.

Inoculation of Dogs.—Faust (1931) was the first investigator to call attention to the fact that the dog is an excellent animal in which to study the pathology of amebiasis in not only the acute, but in the subacute and chronic stages of the infection, as this animal lives for a much longer time after infection than do kittens, while monkeys, aside from their cost, are more resistant to actual invasion of the tissues of the intestine and frequently are infected with much difficulty. It is also possible to produce the "carrier" condition in the dog and thus every stage of amebiasis, as observed in man, may be experimentally produced in this animal.

Prior to the observations of Faust, the dog had always been inoculated orally with cysts of *Endamœba histolytica*, either by feeding material containing the cysts or by injecting such material directly into the stomach by means of a suitable rubber catheter passed into the organ or by rectal injection of infective material. Faust devised a new method of inoculation which places the infective material directly in the cecum through the rectum. His method of inoculation is as follows:

A glass pipette measuring 30 cm. in length and having an outer diameter of 7 mm. is used for injection. It should be tapered at one end, which should have a smooth margin, and equipped at the other end with a 25 cc. rubber bulb. The material to be injected is aspirated into the glass pipette and the pipette is then inserted into the rectum and gently pushed up the large bowel until the ileocecal valve is

reached, when it is ejected by means of the rubber bulb. The dog should previously have had its bowel thoroughly emptied by means of an enema. In this way the amebæ are placed in the same position in which they begin to multiply after a natural infection, *i. e.*, in the lowest portion of the ileum and the region of the ileo-cecal valve.

If it is desired to transfer an infection with *Endamœba histolytica* from an infected to an uninfected dog, the pipette is gently passed into the rectum and through the colon and cecum of the infected animal until it comes into contact with the ileo-cecal valve. The rubber bulb is then compressed and the pressure of the air from the bulb is usually sufficient to cause the ileo-cecal valve to relax. The pipette is then slowly withdrawn, the pressure on the rubber bulb being gradually released which allows the mucoid exudate, rich in amebæ, to enter the pipette. By carefully regulating the relaxation of the pressure on the rubber bulb a large amount of material containing numerous amebæ may be withdrawn from the cecum and upper portion of the large intestine. This material is immediately injected into the uninfected dog in the manner already described.

The writer has employed Faust's method of inoculation and has seen it employed in scores of experimental dogs and recommends it as the best method of inoculating these animals with *Endamœba histolytica*. While the technique requires some practice it is easily mastered and the results are most satisfactory.

Inoculated dogs should be placed in proper cages and no food should be given for a period of twenty-four hours. Adult dogs may be used for inoculation and animals should be selected that are as free from other parasitic infections as is possible and of a size suitable for handling. Young dogs and puppies may be more susceptible to infection but are more apt to die either from the infection or from other disease conditions. Dogs suffering from a heavy hookworm infection are not suitable for inoculation.

Inoculation of Kittens.—Kittens have been the favorite experimental animals used in research upon amebiasis and the inoculation of these animals may be either oral or rectal, or by operative procedure. Half grown, healthy kittens should be selected for inoculation.

ORAL INOCULATION.—Kittens may be fed material containing the cysts or trophozoites of *Endamœba histolytica*. Prior to the cultivation of this ameba a small amount of fecal material containing either the cysts or trophozoites was mixed with milk or other food and fed the kittens after they had been kept without any food for a period of at least twenty-four hours. Usually the kitten would eat the infected food without hesitation but sometimes would refuse even though very hungry. Such feedings were usually followed by infection, even though trophozoites were fed, although most authorities believed that this stage in the development of *Endamœba histolytica* was not infective

because it was destroyed by the gastric juice. As long ago as 1905, the writer infected kittens by feeding the trophozoites and Walker and Sellards (1913) produced dysentery in 3 of 4 human volunteers by feeding them material containing motile trophozoites. More recently, Swartzwelder (1937) has produced infection in 5 of 13 dogs by feeding motile trophozoites in a medium proven free from cysts, thus confirming the writer's observations and proving that the gastric secretion does not always kill the trophozoites. The material fed the dogs by Swartzwelder consisted of blood and mucus containing numerous motile trophozoites obtained from the intestine of dogs infected with *Endamœba histolytica* by aspiration in the manner already described (see page 121).

Since the cultivation of *Endamœba histolytica* and the possibility of securing large numbers of cysts in culture (see page 90), the preferable method of inoculating kittens by mouth is to feed them material containing large numbers of cysts washed free from bacteria, as suggested by Stone (see page 90). If the kittens refuse to eat food mixed with a suspension of the cysts, which rarely happens, the material can be placed directly into the stomach by means of a rubber catheter to which is attached a syringe or large rubber bulb. The material is ejected into the stomach and is almost invariably followed by an infection. Material containing motile trophozoites may also be thus inoculated but the results as regards the infection of the animals are not as satisfactory.

Rectal Inoculation.—The most satisfactory method of producing infection with *Endamœba histolytica* in kittens is by means of rectal injections of material rich in motile trophozoites of this parasite. The injections are made with a rubber catheter to which is attached a glass syringe or rubber bulb. After aspirating into the catheter and syringe the material to be injected, usually cultures of *Endamœba histolytica*, the catheter is introduced into the rectum and gently pushed ahead until an obstruction is felt when the material is injected slowly into the intestine. The catheter is then slowly withdrawn and the rectum is sealed by a plug of cotton-wool soaked in a solution of collodion in ether. This prevents the ejection of the material, which frequently happens unless the anus is so sealed. After two or three days the cotton plug is softened with ether and removed. The transfer of the infection from kitten to kitten is best accomplished by killing the infected animal and injecting the material obtained by washing off and gently scraping the lesions in the intestine, such material usually containing many motile trophozoites of *Endamœba histolytica.*

In kittens infections produced in this manner are usually acute and dysenteric symptoms are pronounced. Death frequently occurs in a few days and at autopsy the lesions are frequently quite superficial although numerous amebæ are present. The severe, deep-seated

ulcerations often observed in dogs after experimental inoculation, are rarely observed in kittens unless the strain of *Endamœba histolytica* employed is of unusual virulence.

Inoculation of Rabbits.—In the application of the complement fixation test for the diagnosis of amebiasis it is necessary that a positive blood serum be available for control purposes. Usually such a serum is obtainable from an individual suffering from the infection if such tests are made in a laboratory connected with a hospital but if not, a positive control serum can be obtained by immunizing rabbits to *Endamœba histolytica*. This is best done by the method suggested by Stone (1935) which follows:

Half-grown rabbits are selected and their blood tested by the Wassermann test. If negative, the animals are inoculated intraperitoneally with approximately 4,000,000 washed bacteria free cysts of *Endamœba histolytica* suspended in normal saline solution (0.85 per cent). The serum of the inoculated rabbits is tested for its reaction with the amebic antigen a few days after injection and if positive, is also tested with the Wassermann test. If, as sometimes happens, a positive reaction is also obtained with this test, the animal is rejected. Stone states that he had no trouble in immunizing rabbits in this manner and that their blood sera gave strongly positive reactions with the amebic antigens.

Remarks.—In inoculating all animals with material from cultures of *Endamœba histolytica* a microscopic examination should be made of such material in order to be sure that numerous amebæ are present. If fluid cultures are employed, as the Locke-serum-medium, the sediment at the bottom of the tubes should be carefully collected with a 1 cc. pipette from several cultures and pooled, while if material from the Locke-egg-serum medium, or other slant media be used, it should be collected from the junction of the fluid and slant after gently rubbing the slant to loosen any amebæ that are present upon it. The material from several slant cultures should be pooled for the inoculation.

THE SIGMOIDOSCOPE IN THE DIAGNOSIS OF AMEBIASIS

Sigmoidoscopic examinations, while often useful in the diagnosis of amebiasis, should not be resorted to as a *routine* procedure. It is only when the microscopic examination and cultures of the stools are negative for *Endamœba histolytica* after repeated examination that it is necessary to use the sigmoidoscope and such cases are exceedingly rare in the writer's experience. Many gastro-enterologists place an unwarranted faith in the results of sigmoidoscopy in the diagnosis of amebiasis, forgetting that in the case of carriers, or cyst-passers, lesions are very frequently absent from the sigmoid and rectum and that, even in symptomatic amebiasis, these areas of the intestine may not be invaded. The statement, made by Manson-Bahr (1939) that in over

80 per cent of cases of infection with *Endamœba histolytica* lesions are present in the sigmoid and rectum is not confirmed by the autopsy experience of Clark (1925), Rogers (1922) and others, including the writer. Clark found that in 186 patients examined at autopsy, lesions occurred in the sigmoid and rectum in between 71 and 75 per cent, while Rogers, in 36 cases observed at autopsy, found that in 23, or 63.8 per cent, the lesions were limited to the cecum and ascending colon. In considering these figures it should be remembered that they are based upon autopsy experience with patients who died of amebic dysentery or its complications and that the percentage of involvement of the rectum and sigmoid in individuals having latent amebiasis and mild symptomatic amebiasis must be very much lower. The writer believes that if such infections are included the percentage of individuals showing lesions in the rectum and sigmoid will not exceed 30 per cent at the highest. It is thus evident that if one relied alone upon the result of sigmoidoscopy in diagnosis, many infections would be missed, and that it should not be a routine method of diagnosis to the exclusion of more reliable, simple and less painful methods of examination.

In some infections the lesions in the rectum, if present, are few in number, and in such cases, it is sometimes possible to demonstrate the amebæ by sigmoidoscopy when the stools are negative on repeated examinations. Thus, D'Antoni, in 1942, in the examination of 14 infections with *Endamœba histolytica*, employing direct examination of the stool after an enema, and sigmoidoscopy, found that in 4 cases the parasite could be demonstrated only in the aspirate obtained by sigmoidoscopy, but he also found that in 8 of the patients the ameba was recovered only in specimens obtained after a purgative and an enema. These results prove the value of a combination of the two methods of examination and in suspected cases in which stool examinations are negative, sigmoidoscopy should be done, but only in such cases.

In those instances in which a sigmoidoscopic examination is indicated, the diagnosis of amebiasis should rest upon the demonstration of *Endamœba histolytica* in scrapings removed from suspicious lesions by means of the Volkman spoon or material obtained through the sigmoidoscope, and not upon the character of the lesions that may be present. It is probably true that a diagnosis may be made upon the morphology of the lesions by one who has had a long experience in the study of lesions of the intestine as observed through the sigmoidoscope but gastro-enterologists who have had an extended experience state that such a diagnosis of amebiasis is not reliable and that the lesions of amebiasis cannot be distinguished from lesions due to other causes in many cases. Thus, Simon (1934), who had a very great experience with sigmoidoscopic examinations in amebiasis, stated: "The character of the ulcerative lesions in amebiasis, as viewed through the

proctoscope, can in no manner be considered pathognomonic. Moreover, in the vast majority of individuals who harbor *Endamœba histolytica*, no visible evidence of the infection can be found by rectal examination."

The material obtained by the sigmoidoscopic examination should be at once placed upon a microscopic slide and prepared and examined as already described (see page 44). A diagnosis of *Endamœba histolytica* should not be made unless *motile* trophozoites or cysts of this parasite be present.

CRITIQUE OF DIAGNOSTIC METHODS

It is evident that there are several diagnostic methods available for the demonstration of infection with *Endamœba histolytica* and their relative value should be understood by the diagnostician. It is fortunate that in this infection the more simple diagnostic methods are the most valuable and the more complicated methods, as cultivation or the complement fixation test do not need to be employed unless the simpler methods have failed. The first step in the diagnosis of amebiasis should be a microscopic examination of the stool of the patient, obtained after a saline cathartic and passed in the laboratory and examined at once. Both unstained preparations and preparations stained with the iodine or Quensel stain should be examined and several preparations should be made before a negative report is returned. In such stools motile trophozoites of *Endamœba histolytica* should be present if the individual is infected as well as cysts which have been washed out with the evacuation. It is best to examine material obtained from the second, rather than the first stool, following purgation. It has already been stated that stools obtained after purgation are more apt to show the amebæ than the normal stool and this method of diagnosis is recommended.

The employment of a concentration or flotation method greatly increases the chances of demonstrating the amebæ.

Repeated examinations of either iodine or hematoxylin stained preparations are necessary in order to obtain satisfactory results. Thus, Sawitz and Faust (1942) in a series of comparative tests found that a single iodine or hematoxylin stained fecal smear demonstrated less than one in five infected individuals and that ten such smears had to be examined before a negative result could be accepted as reasonably correct. On the other hand, using one of these methods combined with zinc-sulphate centrifugal flotation about one of every three or four infections were detected in a single examination, while five such examinations were successful in from 70 to 90 per cent of infections.

The examination of stools obtained without purgation should never be neglected if, for any reason, the patient cannot take a saline laxative, and it has been the experience of the writer that the simple

microscopic examination of the stool, repeated if necessary several times, will almost invariably result in the demonstration of *Endamœba histolytica* if that parasite be present.

The employment of *staining methods* with the exception of the iodine or Quensel's stain (see page 63) is not *essential* for diagnosis in the opinion of the writer, although some authorities prefer to rely upon stained preparations in the differentiation of *Endamœba histolytica* from the other species of amebæ occurring in the human intestine. If a staining method is employed the more rapid and simple ones of Dobell, Faust, Johnson, Kessel or Walker (see pages 56 58 65) are recommended.

In the hands of trained observers a higher percentage of positive results in examinations for *Endamœba histolytica* will be obtained from wet-fixed, hematoxylin preparations than from unstained preparations, in the opinion of some authorities. Thus, Wenrich (1941) in the examination of 700 college students obtained 34.5 per cent more positives for *Endamœba histolytica* by the study of stained slides alone than by the study of unstained preparations alone. While such extreme differences have not been the experience of the writer, it is true, that in the hands of experienced workers, stained preparations do give somewhat better results than unstained, but in the hands of the ordinary technician much poorer results would be obtained and, for this reason, unstained preparations are recommended for ordinary diagnostic work.

Culture methods are very useful in the diagnosis of amebiasis and should be employed if the microscopic examination of the stool is negative. It is good routine practice to inoculate cultures after each stool examination provided negative results have been obtained and to examine the cultures microscopically at the end of twenty-four and forty-eight hours. Not so very infrequently cultures will prove positive for the ameba when stool examinations have proven negative.

In employing *cultures for diagnosis* the more simple fluid culture media as the writer's Locke-serum and Normal-saline-serum media, the St. John's medium or the Nelson alcohol extract slant medium give as good results as the more elaborate slant media that have been recommended, and should be preferred. For the *maintenance of cultures* the slant media, as the Drbohlav Locke-egg-serum medium or the Nelson alcohol extract medium are essential, as the amebæ die out in the fluid media after several transfers.

If both microscopic examinations of the stools and of cultures are negative a *sigmoidoscopic examination* should be made and preparations from any suspicious lesions that may be observed examined for *Endamœba histolytica.* Only rarely will such examinations result in the demonstration of the parasite if stools and cultures have proven negative, in the writer's experience. The sigmoidoscope should not be employed as a *routine* aid in diagnosis as it is unnecessary.

The *complement fixation test* is a very valuable diagnostic method when properly performed by a trained serologist but should never replace stool examinations. When such examinations are not possible, for any reason, the complement fixation test should be employed and in the writer's experience, is specific for amebiasis if a positive reaction is obtained, except in certain cases of ulcerative colitis, in which a false positive reaction may rarely be obtained. In the diagnosis of amebic abscess of the liver and in cases in which stool examinations have proven negative, the complement fixation test is a most valuable diagnostic procedure and should never be neglected if available.

It goes without saying that training in the recognition of *Endamœba histolytica* is absolutely essential in the diagnosis of amebiasis and this training should have been received from an expert in the differentiation of this species of ameba from the other amebæ living in the intestine of man, *i. e.*, *Endamœba coli*, *Endolimax nana*, *Iodamœba bütschlii* and *Dientamœba fragilis*, as well as from the intestinal flagellates, *Giardia lamblia*, *Chilomastix mesnili*, *Embadomonas intestinalis*, *Trichomonas hominis* and *Enteromonas hominis*. Lack of such training will invariably lead to many mistakes in diagnosis and the diagnosis of amebiasis should not be undertaken unless such training has been received.

CHAPTER VI

THE DIAGNOSIS OF FLAGELLATE INFECTIONS—MORPHOLOGY OF THE INTESTINAL FLAGELLATES OF MAN

THE diagnosis of flagellate infections of the human intestine is important, for while these organisms are not proven to be pathogenic and no definite lesions have been proven to be caused by them, it is important that they be differentiated from *Endamœba histolytica* as they often occur with this ameba and may be confused with it, especially those that produce cysts which may be diagnosed as the cysts of *histolytica*. Thus, it is essential that one be conversant with the morphology of these flagellates from the standpoint of the differential diagnosis of amebiasis. In addition, if we are ever to understand the possible relationship of the different flagellates to symptoms of disease their diagnosis is essential and of definite importance.

The flagellates commonly found in the human intestine are *Giardia lamblia, Chilomastix mesnili* and *Trichomonas hominis*. Much less frequently encountered are *Embadomonas intestinalis* and *Enteromonas hominis*.

1. MORPHOLOGY OF GIARDIA LAMBIA

This flagellate, one of the most common in the human intestine, has a "vegetative," or trophozoite, and a cystic stage in its life-cycle.

Morphology in Unstained Preparations.—In unstained preparations of stool containing this flagellate, both the trophozoites and cysts appear as colorless, somewhat hyaline bodies. The *trophozoites* are pear-like in contour, the anterior end being broad and rounded while the posterior tapers to a point. They are actively motile, swimming about with a jerky progressive motility, and measure from 9.5 to 21 microns in length by 5 to 15 microns in breadth. They are convex dorsally and concave ventrally, an ovoidal concavity being present upon this surface, called the sucking disk, which may occupy almost three-quarters of the ventral surface. The trophozoites have 8 flagella which are not visible when the organisms are moving rapidly but which may be distinguished when motility becomes sluggish. The flagella are best studied in stained preparations but their motion may be well studied under dark-field illumination. When degeneration of the trophozoites occurs the flagella may be lost and the trophozoites may resemble small amebæ, being rounded up and sometimes apparently extruding minute pseudopodia.

The *cysts* of *Giardia lamblia*, in unstained preparations, appear as colorless, hyaline oval bodies, measuring 8 to 12 microns in length by

7 to 10 microns in breadth. There is a well-defined cyst-wall having a double outline and refractile bodies may be seen within the cysts which represent the nuclei of the dividing parasite.

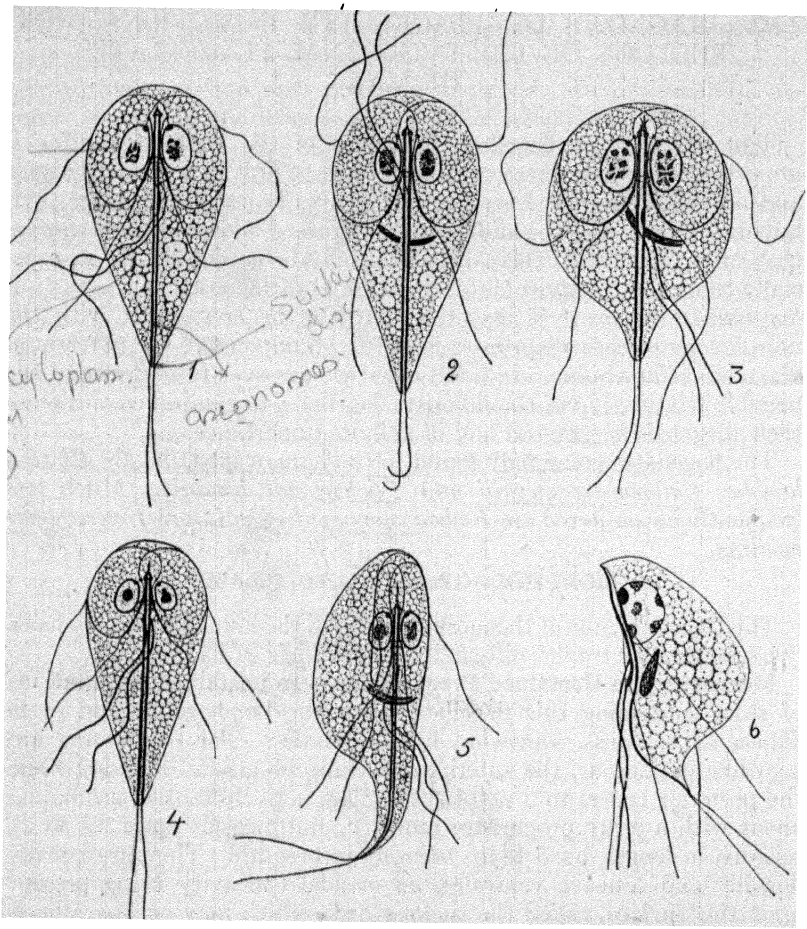


FIG. 15.—*Giardia lamblia*. *1–4*, Variations in morphology of trophozoites of *Giardia lamblia; 5*, partial side view of trophozoite; *6*, side view of trophozoite. Stained with iron hematoxylin. (After Wenyon in "Protozoölogy," courtesy of Baillière, Tindall and Cox.)

Morphology in Stained Preparations.—In order to demonstrate the morphology of *Giardia lamblia* preparations should be fixed with Schaudinn's sublimate alcohol and stained with one of the hematoxylin stains.

In stained preparations the trophozoites present a granular, alveolar cytoplasm which stains a bluish-gray color and the nuclei and other

structures are stained a deep black. There are two nuclei, one on each side of the body, oval in shape, and having a large round karyosome situated centrally or to one side of the center of the nucleus. Extending from the anterior to the posterior end are two parallel rodlike bodies, the axonomes, which end in two blepharoplasts, from

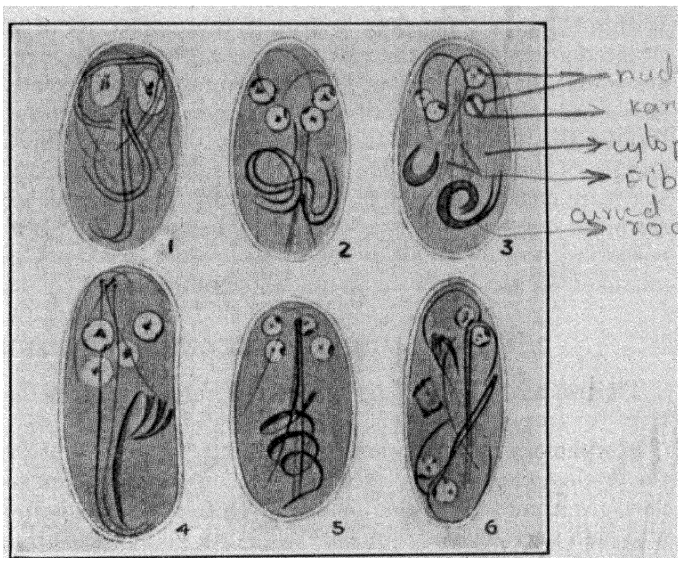


FIG. 16.—*Giardia lamblia*. Cysts showing various stages of development. *1*, Forms with two nuclei; *2–5*, forms with four nuclei; *6*, form in which there are two nuclei at opposite poles of the cyst and the organism is dividing within the cyst. Stained with iron hematoxylin. (After Wenyon, in "Protozoölogy," courtesy of Baillière, Tindall and Cox.)

each of which arises a flagellum which projects from the posterior end of the organism. Besides this pair of flagella there are three other pairs, one originating from two blepharoplasts at the anterior end of the axonomes; the second, from the same pair of blepharoplasts; and the third, from blepharoplasts situated on the axonomes near the posterior edge of the sucking disk. The anterior pair of flagella cross one another and after passing along the anterior and lateral margins of the sucking disk, become free flagella near the junction of the anterior and middle third of the body; the middle pair of flagella, after reaching the posterior margin of the sucking disk, diverge and become free flagella at the juncture of the middle and posterior thirds of the body of the organism; the third pair, originating from the axonomes near the posterior border of the sucking disk become free flagella near the center of the body. It will be noted that in *Giardia lamblia* the important structures are all paired, there being 2 nuclei, 2 axonomes and 4 pairs of flagella, which distinguishes this organism from any other occurring in the

human intestine. In addition to the structures mentioned one or two curved bodies, which stain black, may be observed posterior to the sucking disk, the nature of which is unknown. Stained trophozoites are slightly smaller than the unstained ones.

The *cysts*, in stained preparations, are oval in shape and show an unstained cyst-wall and a cytoplasm staining faintly blue or gray. Within the cyst there may be from 2 to 4 nuclei, all of which may be situated at one end of the cyst or 2 at each end of the cyst. The nuclei are round, have a delicate black membrane and a small round black karyosome situated centrally or to one side of the center of the nucleus. Besides the nuclei the cyst may contain short black fibrils arranged in groups of four, and 2 black curved rods, which form a V-shaped structure near the end of the cyst furthest from the nuclei, if the latter are all at one pole of the cyst, as they usually are. Some of the cysts resemble those of small cysts of *Endamœba histolytica* and could be mistaken for the latter by an untrained observer.

2. MORPHOLOGY OF CHILOMASTIX MESNILI

This rather common flagellate of the human intestine has a "vegetative," or trophozoite, and a cystic stage in its life-cycle.

Morphology in Unstained Preparations.—In unstained preparations, the *trophozoites* of *Chilomastix mesnili* are elongate pear-shaped and measure from 6 to 20 microns in length by 3 to 10 microns in breadth, although very slender forms are sometimes seen measuring from 3 to 4 microns in breadth. The organisms are colorless and the body may be apparently twisted upon itself due to a spiral groove which extends around the body diagonally from the dorsal side of the anterior extremity to the ventral side posteriorly. In fresh preparations motility is pronounced, the motion being of a jerky progressive character. When motility is slow flagella may be distinguished but these are best seen in stained preparations.

The *cysts*, in unstained preparations, are colorless, lemon-shaped, and have a well-marked, double-outlined cyst-wall. They measure from 7 to 10 microns in length by 4.5 to 6 microns in breadth. At the smaller end of the cyst there is a blunt prolongation which gives it a lemon-like shape. Little structure is visible in the unstained cysts and cysts having a spherical or irregular shape may rarely be observed.

Morphology in Stained Preparations.—In stained preparations employing wet-fixation and hematoxylin, the *trophozoites* are pear-shaped with a pointed posterior extremity. The groove already mentioned, which gives the twisted appearance to the body, is well differentiated in stained preparations, while at the anterior end there is a mouth, or cytostome, which extends posteriorly for about one-half the length of the body, and is characterized by having two lips and contains within it a very short flagellum. The nucleus is situated at

the anterior, or broad, end of the body and has a black, well-defined nuclear membrane and a small round karyosome situated to one side of the center of the nucleus. Between the karyosome and the nuclear membrane minute black chromatin granules are sometimes seen in very well-stained preparations.

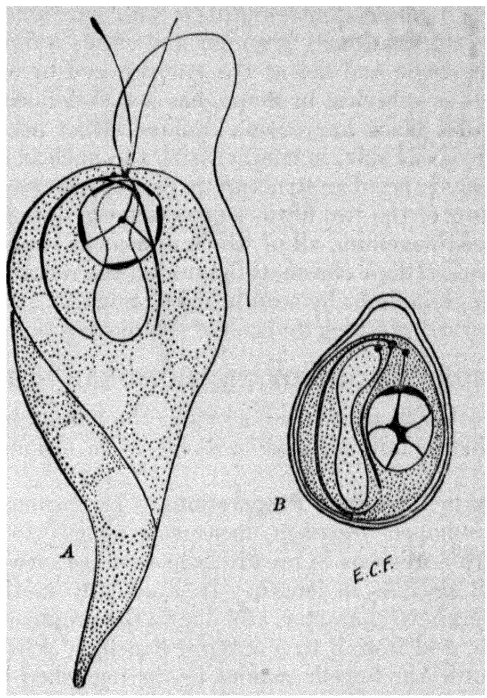


FIG. 17.—*Chilomastix mesnili*. *A*, Trophozoite; *B*, cyst. × 3200. The delicate flagellum within the cytostome is not shown in the drawing. (After Faust, in Jour. Lab. and Clin. Med.; courtesy of C. V. Mosby Company.)

At the anterior end of the body there is a black stained mass of granules, which, in well-differentiated specimens, is seen to consist of six separate chromatin granules, the blepharoplasts. The three anterior blepharoplasts each gives rise to a flagellum about the length of the body of the organism, which is projected anteriorly, while the three posterior blepharoplasts form the lips of the cytostome and the flagellum within this structure, in the following manner: One of the blepharoplasts gives rise to a fibril which supports the right lip of the cytostome; the second blepharoplast gives rise to a fibril supporting the left lip of the cytostome; while the third blepharoplast gives rise to the free flagellum within the cytostome which is directed posteriorly. The three anterior flagella are very delicate and it requires very careful staining and differentiation to clearly demonstrate them but the

flagellum within the cytostome is thicker and more easily demonstrated. Some authorities regard the latter as the edge of an undulating membrane.

The *cysts*, in hematoxylin stained preparations, are very characteristic. They are lemon-shaped and the cyst membrane does not stain, appearing as a hyaline double-outlined wall surrounding the cysts. The cytoplasm appears finely granular and stains a dim blue or gray. The nucleus is single and lies at the anterior end or near the middle of the cyst. It is spherical in shape, has a well-defined black nuclear membrane, and a black karyosome situated either near the center of the nucleus or to one side, in contact with the nuclear membrane. In some of the cysts a peculiar structure is present representing the cytostome, consisting of the two fibrils supporting the lips of the cytostome and the enclosed flagellum, all of which appear as black fibrils located near the nucleus. Black chromatin granules, the remains of the blepharoplasts, may sometimes be seen near the nucleus. Binucleate cysts are rarely observed in which division of the nucleus has occurred.

3. MORPHOLOGY OF TRICHOMONAS HOMINIS

Unlike the other flagellates occurring in the human intestine, *Trichomonas hominis* has but one stage in its life-cycle, the motile or trophozoite stage.

Morphology in Unstained Preparations.—*Trichomonas hominis* is a colorless, pear-shaped organism, measuring from 7 to 15 microns in length and 3 to 5 microns in breadth, the average measurement being from 10 to 12 microns in length. It is actively motile, the motion being of a progressive character, and due to the presence of an undulating membrane, and from 3 to 5 anterior flagella. When the organism is actively motile the flagella cannot be distinguished but as motility lessens they can be made out as very delicate, thread-like bodies projecting from the anterior end, while the undulating membrane appears as a series of coglike projections at the periphery of the body, this appearance being due to the fact that only the tips of the undulations of the membrane can be seen when motility is present. Extending through the middle of the body from the anterior to the posterior end is a hyaline appearing rodlike body, known as the axostyle, which forms a pointed, tail-like posterior extremity. At the anterior end there is a narrow cleft, the mouth, or cytostome. The nucleus is usually invisible in the unstained preparations but may rarely be seen as a round refractile mass at the anterior end of the trichomonas. The edge of the undulating membrane is formed by a flagellum which may be free posteriorly, forming a short posterior flagellum. If the stools contain blood, red blood corpuscles may sometimes be observed within the cytoplasm of these parasites.

Morphology in Stained Preparations.—The morphology of *Trichomonas hominis* in preparations fixed with Schaudinn's solution and

stained with hematoxylin, is very characteristic. The nucleus is situated at the anterior end of the body and has a large black karyosome, usually central in position, and a delicate black nuclear membrane. Just anterior to the nucleus is an oval or round black mass representing the blepharoplasts from which arise the anterior flagella. In very well stained and differentiated specimens this mass is seen to be really composed of from 3 to 5 dots, or blepharoplasts, from each of

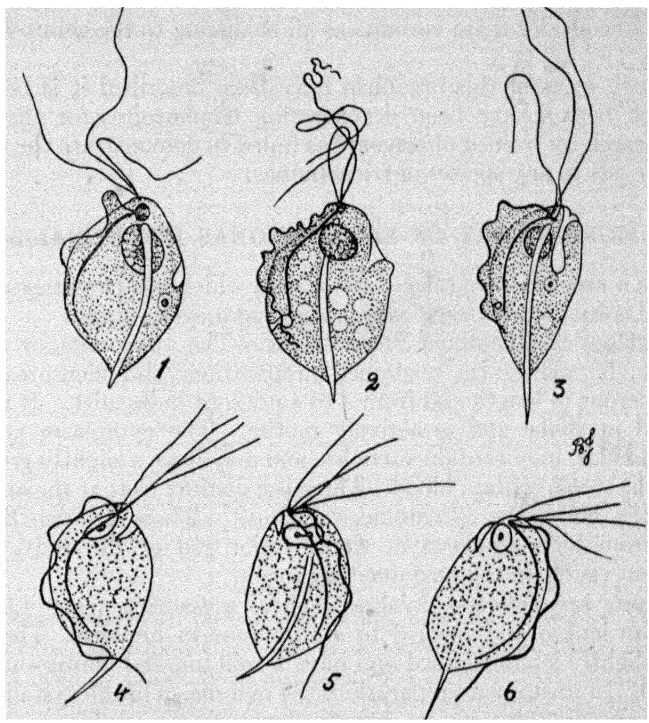


FIG. 18.—*Trichomonas hominis*. *1–3*, Forms with three anterior flagella, after Faust; *4–6*, forms with four anterior flagella, after Wenyon and O'Connor. (From Wenyon, in "Protozoölogy," courtesy of Baillière, Tindall and Cox.)

which arises a flagellum. The number of flagella varies from 3 to 5, the most frequently observed number being 4 in the writer's experience.

Arising from a blepharoplast at the anterior end of the body is an undulating membrane. The only portion of this membrane which stains is the flagellum forming its margin and prolonged posteriorly as a short free flagellum. This stains black and is regularly curved. In very well stained specimens the base of the undulating membrane is observed to be formed by a basal fibril.

The axostyle arises from a blepharoplast at the anterior end of the organism and remains unstained. It is a spikelike structure extending

backward and terminating in a sharp point forming the so-called "tail" of the trichomonas. In stained preparations it is somewhat refractile and uncurved, forming a straight "back-bone" for the parasite, but in living specimens examined under the dark-field microscope, the axostyle is observed to bend slightly with the movements of the organism.

Some authorities have considered that the varying number of flagella in this species indicates that three genera exist and have named them *Trichomonas, Tetratrichomonas* and *Pentatrichomonas* but it is best, at present, to consider these variants as all belonging to the genus *Trichomonas.*

Although cysts of this organism have been described it is believed that such bodies have been degenerating trophozoites for the most careful search by trained observers has failed to demonstrate the occurrence of cysts in any species of trichomonas.

4. MORPHOLOGY OF EMBADOMONAS INTESTINALIS

This is a rare intestinal flagellate of man which has two stages in its life-cycle, a motile, or trophozoite, stage and a cystic stage.

Morphology in Unstained Preparations.—The *trophozoite,* or motile organism, is colorless in unstained preparations and measures from 4 to 9 microns in length and from 3 to 4 microns in breadth. It is oval or round in shape and is actively motile. The cytoplasm appears finely granular, may contain vacuoles, and may have a slightly greenish color if the stools contain blood. There is a distinct cleft at the anterior end of the body, the cytostome, or mouth. There are two flagella arising from blepharoplasts at the anterior end of the body which are seldom visible in the unstained organism.

The *cysts* are colorless hyaline bodies, measuring from 4.5 to 7 microns in length and from 3 to 4.5 microns in breadth. They are oval or slightly lemon-shaped and have a well-marked double-outlined cyst-wall. In unstained preparations the cytoplasm of the cyst appears colorless and granular and no definite structures are visible.

Morphology in Stained Preparations.—In preparations fixed with Schaudinn's solution and stained with hematoxylin, the *trophozoite* of *Embadomonas intestinalis* presents a nucleus having a delicate black nuclear membrane and containing a central black stained karyosome. The nucleus is situated in front of the cytostome. There are two dotlike blepharoplasts lying close to, or in contact with the nucleus, from which arise the two flagella, one of which, long and very delicate, immediately becomes a free flagellum projecting from the anterior end of the body, and arising from the anterior blepharoplast, while the other, arising from the posterior blepharoplast, is short and much broader, and is contained within the cytostome for part of its length, afterwards becoming a free flagellum directed posteriorly. Bacteria may be seen within the cytoplasm in some of the trophozoites.

The *cysts*, in stained preparations, are oval or slightly lemon-shaped bodies, having an unstained cyst-wall and containing a single nucleus having a delicate black nuclear membrane and a small central black karyosome. No other structures are visible in the majority of the cysts but in some there may be seen two black fibrils situated near the nucleus, or a black threadlike loop may surround the nucleus. It is believed that these are the remains of the structure forming the lips of the cytostome. No multiplication occurs within the cysts of this species of flagellate.

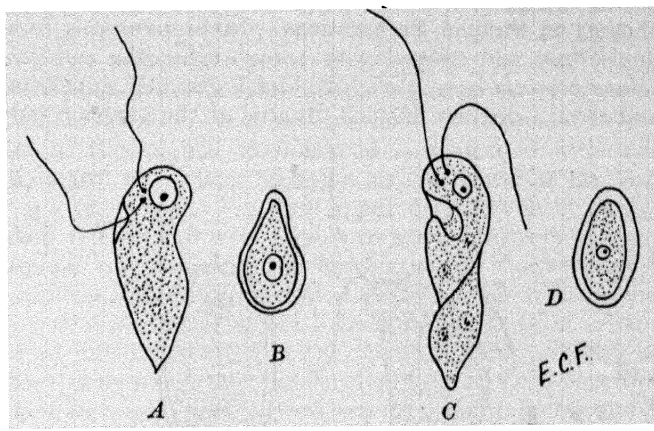


FIG. 19.—*Embadomonas intestinalis* and *E. sinensis*. *A*, Trophozoite of *E. intestinalis*; *B*, cyst of *E. intestinalis*; *C*, trophozoite of *E. sinensis*; *D*, cyst of *E. sinensis*. × 2500. (After Faust, in Jour. Lab. and Clin. Med., courtesy of C. V. Mosby Company.)

Another species of *Embadomonas* has been described by Faust and Wassell (1921), which they named *Embadomonas sinensis*, and which has not been described from any country except China. It differs from *Embadomonas intestinalis* in being larger, measuring on the average 13.4 microns in length and 5.2 microns in breadth; in having two flagella of equal length neither of which is contained within the cytostome, and in having a smaller nucleus situated at some distance from the two blepharoplasts instead of being in contact with them. The cysts are also slightly larger than those of *Embadomonas intestinalis*.

5. MORPHOLOGY OF ENTEROMONAS HOMINIS

This is a rare flagellate of the human intestine and is identical with the flagellate described as *Tricercomonas intestinalis*. It has a motile, or trophozoite, stage and a cystic stage in its life-cycle in man.

Morphology in Unstained Preparations.—The motile *trophozoites* are round, oval or pear-shaped and measure from 4 to 10 microns in length and from 3 to 6 microns in breadth. They are colorless and have a

rapid jerky progressive motion. When the organism is moving actively no structures can be detected within it in the unstained preparation but when motility slows three flagella may be distinguished projecting anteriorly and one flagellum projecting from the posterior end of the body. The body is slightly flattened upon one side and the posterior flagellum runs along this flattened side until it becomes a free flagellum at the posterior extremity.

The *cysts* have a double-outlined cyst-wall and measure from 6 to 8 microns in length and from 3 to 4 microns in breadth. They are colorless and oval in shape.

Morphology in Stained Preparations.—In preparations fixed with Schaudinn's fluid and stained with hematoxylin, the *trophozoites* of *Enteromonas hominis* have a slightly bluish granular appearing cytoplasm and show a distinct nucleus situated at the anterior end of the

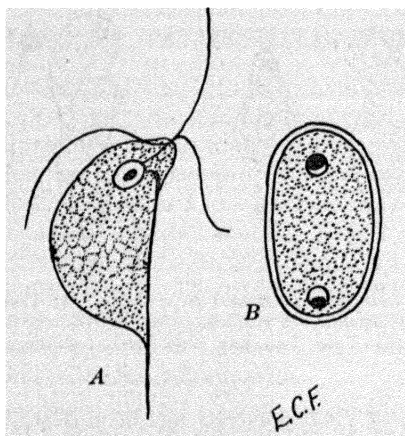


FIG. 20.—*Enteromonas hominis*. *A*, Trophozoite of *E. hominis*, *B*, cyst of *E. hominis*. × 2000. (Modified from Wenyon and O'Connor by Faust, in Jour. Lab. and Clin. Med., courtesy of C. V. Mosby Company.)

body. The nucleus has a well-defined black nuclear membrane arranged in a conelike manner, in some of the organisms. There is a central black stained karyosome and black chromatin granules may sometimes be seen in the space between the nuclear membrane and the karyosome. Near the nucleus is a black, oval or round chromatin mass, which, in very well stained and differentiated specimens, is seen to consist of two black granules, the blepharoplasts, from one of which arise three flagella which project from the anterior end of the body, while from the other there arises the posterior flagellum. The latter is adherent to the flattened side of the body of the parasite until it reaches the posterior extremity when it becomes a free flagellum.

The *cysts*, in stained preparations, have a double-outlined unstained cyst-wall and are rounded or oval in shape. They may contain from

1 to 4 nuclei, each having a delicate black nuclear membrane and a large black karyosome situated centrally or in contact with the nuclear membrane. In the binucleate and quadrinucleate cysts the nuclei are arranged in pairs at opposite poles of the cyst.

TRICHOMONAS TENAX AND TRICHOMONAS VAGINALIS

In addition to the intestinal trichomonad of man, *Trichomonas hominis*, there are two other trichomonads parasitic in human beings, *i.e.*, *Trichomonas tenax* (*T. buccalis*), occurring in the mouth, and *Trichomonas vaginalis*, occurring in the vagina under certain conditions. These two species are apparently identical in morphology with *Trichomonas hominis* although slight differences have been described by some authorities. Neither of these trichomonads have been proven to be pathogenic but as the vaginal trichomonad is associated with a form of vaginitis, many physicians regard it as the cause of the condition. That it is a true species has been apparently proven by Stabler, Feo and Rakoff (1940) who were unable to cause infection of the vaginæ of 50 women volunteers by the inoculation of *Trichomonas hominis* but were successful after inoculation of the vaginæ of 10 women with *Trichomonas vaginalis*, of whom 8 became infected but with no evidence of an inflammatory reaction. More recently, Stabler and Feo (1942) have determined that the survival time of *Trichomonas hominis* in the human vagina did not exceed forty-eight hours and that of *Trichomonas tenax* four hundred and thirty-two hours, most of the organisms perishing within twenty-four hours in the case of *Trichomonas hominis* and forty-eight hours in the case of *Trichomonas tenax*. The latter species survived for a much longer period than *Trichomonas hominis* but a permanent infection could not be established in the human vagina with either species, thus proving that they are distinct species and not identical with *Trichomonas vaginalis* as believed by some authorities.

CHAPTER VII

COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION

STAINING METHODS—CULTIVATION METHODS—CRITIQUE OF DIAGNOSTIC METHODS

COLLECTION OF MATERIAL FOR EXAMINATION

THE directions relating to the collection of material for examination for *Endamœba histolytica* already described (see page 41) are equally applicable to the collection of material for the examination for the intestinal flagellates. The stools should be passed in the laboratory, if possible, and examined at once, as the flagellates soon become motionless at room temperature and degenerate.

Preparation of Material for Examination.—In the examination for the intestinal flagellates both unstained preparations and preparations stained with the iodine stain should be examined except that in the case of *Trichomonas hominis* iodine preparations are not needed as this parasite does not produce cysts. The same rules apply to the preparation of material containing the intestinal flagellates as in preparing material for the examination for *Endamœba histolytica* (see page 41). In the case of *Trichomonas vaginalis* a small amount of the vaginal secretion or any discharge from the vagina is placed upon a microscopic slide, covered with a cover-glass, and examined at once. If staining methods are to be employed the material is smeared upon a microscopic slide and immersed at once in Schaudinn's solution, after which the preparation is stained.

STAINING METHODS FOR FLAGELLATES

It is very difficult, no matter what staining method is used, to secure specimens of any of the flagellates of the human intestine which show all of the morphological features in a single organism and for this reason it is necessary to study several organisms before all of the structural features can be determined. The various staining methods that have already been described for *Endamœba histolytica* may also be used with success in staining the intestinal flagellates. It is essential that any method of staining should be preceded by wet-fixation with the Schaudinn bichloride of mercury solution or some other fixing solution, and the same precautions should be employed against allowing the preparations to dry during any stage of the staining process. The reader is referred to the section upon the staining of *Endamœba histolytica* for information regarding the various stains that may be employed in staining the intestinal flagellates (see pages 45, 49). Any of

the methods of staining described may be used for staining these parasites. The Wright stain, used as in staining the leishmania (see page 164) often gives beautiful pictures of the nuclear chromatin and flagella.

CULTIVATION METHODS

The intestinal flagellates, as well as *Trichomonas tenax* and *Trichomonas vaginalis* may be cultivated upon a variety of culture media. The intestinal flagellates will grow upon most of the media recommended for the cultivation of *Endamœba histolytica*, especially the Boeck and Drbohlav Locke-egg-serum medium (see page 79), but better results have been obtained in some of the species by the use of special media which will be described.

Adler's Medium.—The following medium is recommended by Adler (1942) for the cultivation of *Trichomonas hominis*.

Agar (3 per cent)	1 part
Locke solution (containing 0.1 per cent of glucose)	8 parts
Goat blood serum	1 part

Remarks.—Upon this medium Adler was able to maintain *Trichomonas hominis* for over one year, subculturing every ten days.

Adler and Pulvertaft's Medium.—This medium has been found by Adler and Pulvertaft to be an excellent one for obtaining pure cultures of *Trichomonas vaginalis*. The formula follows:

Locke's solution	4.0 cc.
Goat blood serum, inactivated	0.5 cc.
Septamide (Heyden) 17 per cent sol.	0.1 cc.
Rice starch.	

The material containing the flagellate is inoculated directly into this medium to which sufficient penicillin has been added to give a concentration of 90 units per cc.

Growth is excellent in this medium and all bacteria are killed. Transfers are made into medium containing 230 units of penicillin per cc.

Remarks.—It would appear that this is an excellent medium for obtaining pure cultures of *Trichomonas vaginalis*. Adler had already obtained pure cultures of *Trichomonas hominis* upon a rather complicated medium, (1942) and it is probable that this medium may be successful in securing pure cultures of the latter organism. The use of penicillin to destroy bacteria in other media used in the cultivation of amebæ and flagellates should be investigated.

Boeck's Medium.—The formula for this medium is as follows:

Locke's solution	4 parts
Human blood serum	1 part

The Locke solution is sterilized in the autoclave and the human blood serum is mixed with it in the proportion of 1 part to 4, and then distributed in culture tubes in 5 cc. amounts. The human blood serum should be passed through a bacteria-proof filter before it is

used. After inoculation the tubes are kept in the incubator at 37° C. (98.6° F.).

Remarks.—This medium has been found to be very efficient for the cultivation of *Chilomastix mesnili*. It is also good for the cultivation of the other intestinal flagellates.

Hogue's Medium No. 1:

Locke's solution	200 cc.
Egg, hen's	No. 1

The egg is broken into a sterile flask containing glass beads and the Locke solution is added, the mixture being thoroughly shaken until the egg forms a homogeneous solution with the Locke solution. It is then heated over a water-bath for fifteen minutes, being stirred with a glass rod during the entire time. It is finally filtered through cotton wool with the aid of a suction pump, distributed in tubes in 6 cc. amounts and autoclaved for twenty minutes at 15 pounds pressure.

Remarks.—Hogue found this medium to be an excellent one in the cultivation of *Trichomonas hominis*.

Hogue's Medium No. 2:

Sodium chloride solution, 0.7 per cent	600 cc.
Egg white, hen's eggs	No. 6

The whites of 6 hen's eggs are placed in a flask and thoroughly shaken, after which the salt solution is added and the mixture again shaken. It is then heated over the water-bath for twenty to thirty minutes being stirred with a glass rod during the time it is heated. The mixture is then passed through coarse cheese-cloth and filtered through cotton wool, using a suction pump. The filtrate is then distributed in 5 cc. amounts to culture tubes and autoclaved at 15 pounds pressure for twenty minutes. Inoculated tubes are kept in the incubator at 37° C.

Remarks.—Hogue found this medium an excellent one for the cultivation of *Trichomonas hominis* and *Embadomonas intestinalis* and Hegner and Becker found that it was also useful in the cultivation of *Chilomastix mesnili*, as well as the flagellates already mentioned.

Jirovec and Rodova's Medium.—This medium is prepared as follows:

Blood serum, human or horse	5 cc.
Ringer's solution	95 cc.

The Ringer solution has the following formula:

NaCl	6 00 gm.
$CaCl_2$	0 10 gm.
$MgSO_4$	0 10 gm.
KH_2PO_4	3 00 gm.
n/I NaOH to a pH of 7.4.	18–22 gm.

From 4 to 6 cc. of the serum mixture is distributed into test tubes and the tubes sterilized in the autoclave for two or three successive days, and then stored in the ice-box until used. The initial culture

should be made at room temperature and transfers cultured at 37° C. (98.6° F.). Maximum growth occurs from the fourth to the sixth day and sub-cultures should be made on the seventh day.

If desired a 0.6 per cent sodium chloride solution may be used for diluting the serum instead of Ringer's solution, the required pH of 7.4 being obtained by the use of n/50 HCl or n/60 NaOH, as indicated.

Remarks.—This would appear to be an excellent and very simple method of cultivation and is very similar to the methods of the author employed in the cultivation of *Endamœba histolytica*.

Kofoid and Swezy's Medium:

Locke's fluid	9 parts
Rabbit or guinea-pig blood serum	1 part

The Locke solution is sterilized by filtration or in the autoclave and the blood serum is inactivated by heating it for half an hour at 56° C. (132.8° F.). The two are then mixed, passed through a bacteria-proof filter, and tubed in 5 cc. amounts. After inoculation the tubes are kept in the incubator at 37° C.

Remarks.—This medium was found efficient in the cultivation of *Trichomonas hominis*.

Lynch's Medium:

Sodium chloride solution, 0.5 per cent	10 parts
Human blood serum	1 part

The sodium chloride solution should be sterilized by heating in an autoclave and the blood serum by passing it through a bacteria-proof filter. The two are then mixed in the proportions indicated, passed through a bacteria-proof filter, and tubed in 5 cc. amounts. After inoculation the tubes are kept in the incubator at 37° C.

Remarks.—Lynch cultivated *Trichomonas vaginalis* upon this medium.

Noguchi and Ohira's Medium:

Ascitic fluid	500 cc.
Ringer's fluid (see page 81)	500 cc.

The Ringer solution and ascitic fluid are passed through a bacteria-proof filter separately, mixed, and distributed in tubes in 5 cc. amounts. A small piece of guinea-pig kidney, removed with aseptic precautions, is placed in each tube at the time the tubes are inoculated.

Remarks.—This medium, which is very useful in the cultivation of spirochetes, has also been found to be useful in the cultivation of *Trichomonas tenax, Trichomonas hominis* and *Trichomonas vaginalis*.

Tanabe's Medium.—This simple culture medium has the following formula:

Sodium chloride	0 7 gm.
Sodium citrate	1 .0 gm.
Distilled water	100 0 gm.

The mixture is sterilized by boiling or in the autoclave and when cool 0.5 gm. of Loeffler's dehydrated blood-serum and 2 cc. of whipped egg

white (hen's egg) are added. The medium is then placed in test tubes, 10 cc. to each tube, and when used, is warmed to 37° C. (98.6° F.) and inoculated with the material to be cultured.

This medium is often combined with an agar base, which is prepared as follows:

Agar	15 gm.
Sodium chloride	6 gm.
Distilled water	900 gm.

Steam for one hour to dissolve the agar, filter through cotton wool, and place 5 cc. in test tubes, sterilize in the autoclave and allow to cool in a slanting position, and store in a cool place. Before using, heat the liquid medium described above to 37° C. (98.6° F.) and add the contents of each tube to the top of the agar slants. The liquid portion of the combined media is then inoculated with the material to be cultured and the tubes incubated at 37° C. Examination of the cultures is made by pipetting a small portion of the liquid at the bottom of the agar slants.

Remarks.—This medium is an excellent one for the cultivation of all of the intestinal flagellates with the exception of *Giardia lamblia*, and it is also a good medium for the cultivation of *Balantidium coli*. It is easily prepared and can be kept indefinitely if stored in a cool place.

Wenyon's Medium:

Bacteriologic nutrient agar, 2 per cent pH 7.6	30 cc.
Sodium chloride solution, 0.85 per cent pH 7.6	270 cc.

Add the nutrient agar to the sodium chloride solution, shake well, and tube in 10 cc. amounts. Autoclave for twenty minutes at 15 pounds pressure and when the tubes have cooled to 50° C. (122° F.), add 20 drops of defribrinated rabbit blood to each tube, observing aseptic precautions. The tubes should then be incubated at 37° C. for twenty-four hours to determine sterility and, if found sterile, are then ready for use. Wenyon cultivated *Embadomonas intestinalis* upon this medium and a species of *Trichomonas* from the tortoise. It is probable that the other intestinal flagellates of man could also be cultivated upon the medium.

CRITIQUE OF DIAGNOSTIC METHODS

While the diagnosis of flagellate infections of the human intestine is not of importance from a clinical standpoint, as none of these parasites produce disease, so far as either pathological or experimental evidence shows, it is important from the standpoint of differential diagnosis, as the cysts of some of them have been mistaken for the cysts of *Endamœba histolytica*, the pathogenic ameba of man.

The laboratory diagnosis depends upon the study of the flagellates in both fresh and stained preparations of material which may contain them and upon the study of cultures. Cultivation is not an essential

method of diagnosis but is useful in studying life-cycles and for the purpose of securing material for animal inoculation and for class presentation. The microscopic examination of material containing the flagellates is the most simple and accurate laboratory diagnostic method and it is very rarely that one has to resort to staining in order to make a diagnosis. In fact, it is very difficult to secure well-stained preparations of the intestinal flagellates and, as already stated, one must usually examine many stained flagellates in order to distinguish all of the morphological features, so that the examination of fresh, unstained material containing them is preferable and almost always successful.

It should be remembered that the motile, or trophozoite, forms of the flagellates, like those of the intestinal amebæ, occur most often in fluid or semi-fluid stools, while the cysts occur more frequently in semi-formed or formed stools, although it is more common to find trophozoites in formed stools in flagellate infections than in amebic infections. As in amebic infections, the use of the iodine stain should be reserved for the demonstration of the structure of the cysts, for if motile trophozoites be present alone all that is necessary to demonstrate them is to mix a little of the material containing them with normal saline and examine a drop of the mixture under the microscope. In order to make a differential diagnosis it is necessary to wait until motility almost ceases for when the various flagellates are rapidly moving it is very difficult to distinguish the species.

If staining is resorted to, the best results will be obtained with the Faust stain or James modification of the phosphotungstic acid-hematin stain (see pages 56 and 61).

Of the cultivation methods, those of Hogue and Tanabe are probably the most useful but all of the methods described give satisfactory results after a little practice.

Animal inoculation is of no value in diagnosis of the intestinal flagellates but Trussell (1946) has reported good results with a micro-agglutination test in a number of known infections with *Trichomonas vaginalis* while Trussell and Wilson (1942) obtained positive reactions with a complement fixation test in infections with the same flagellate, employing an antigen prepared from pure cultures of *Trichomonas vaginalis*. (See page 141.) As already stated, the complement fixation test, specific for infection with *Endamœba histolytica*, gives negative results in flagellate infestations of the intestine.

PART II

Laboratory Diagnosis of the Leishmaniases; Kala-azar, Oriental Sore and Espundia

CHAPTER VIII

THE LABORATORY DIAGNOSIS OF THE LEISHMANIASES

MORPHOLOGY OF LEISHMANIA DONOVANI—MORPHOLOGY OF LEISHMANIA TROPICA—MORPHOLOGY OF LEISHMANIA BRASILIENSIS

THERE are three important diseases of man caused by flagellates belonging to the Genus *Leishmania*. These diseases are kala-azar, caused by *Leishmania donovani;* Oriental sore, caused by *Leishmania tropica;* and espundia, caused by *Leishmania brasiliensis*. While all of these diseases present clinical pictures which are more or less characteristic, their diagnosis should be based upon the demonstration of the respective causative leishmania if possible.

The morphology of the leishmania must be studied in stained preparations as in unstained preparations it is impossible to distinguish their structure and such preparations are never used for diagnostic purposes. All of the stains that are used in diagnosis color the chromatin of the nucleus and other structures containing chromatin a brilliant red or violet, while the cytoplasm of the cell stains a pale blue.

1. MORPHOLOGY OF LEISHMANIA DONOVANI

This parasite, the cause of kala-azar, is found in the endothelial cells of the capillaries of the viscera, most frequently in those of the spleen, liver, bone-marrow, intestinal mucosa and the mesenteric glands, but also in those of the kidneys, lungs, suprarenals, meninges and, rarely, in the cerebrospinal fluid. It has also been found in the nasal secretions and urine and stools of infected individuals.

Leishmania donovani has two stages in its life-cycle, an aflagellar stage, occurring in man, and a flagellate stage, occurring in insects, especially sand flies. Both stages of development may be seen in cultures but the flagellate stage is most commonly observed.

The *aflagellar* stage, always observed in man, occurs within the cytoplasm of the endothelial cells, as already mentioned. The organisms occur as round or oval bodies, collected in groups, and measuring usually from 2 to 3 microns in longest diameter. When stained with the Wright or other modification of the Romanowsky stain, two very definite structures may be seen within the organism, both staining a brilliant red or violet, the nucleus and kinetoplast. The cytoplasm

may contain a large unstained vacuole and stains a pale blue, while the entire organism is surrounded by a membrane. The nucleus is spherical in shape and usually lies near or in contact with the membrane surrounding the organism. It is often flattened upon the side in contact

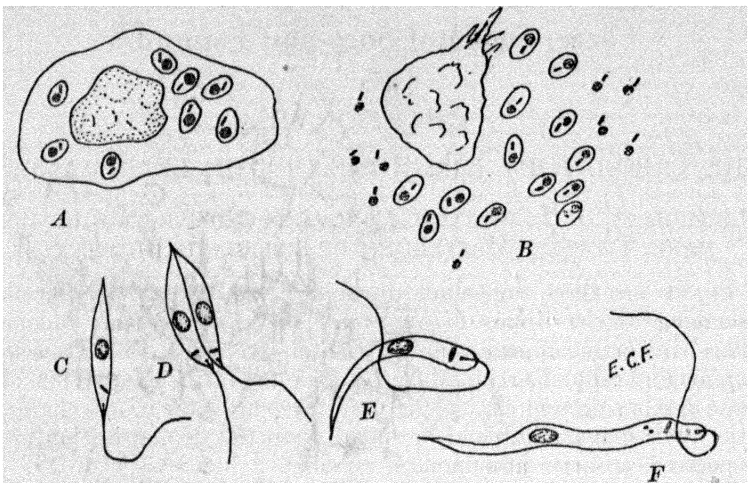


FIG. 21. *Leishmania donovani*. *A*, *B*, Leishmaniform stage of *L. donovani*. From endothelial cells of spleen; *C*, flagellate stage from culture; *D*, dividing flagellate form; *E*, short flagellate form; *F*, long flagellate form. × 2000. (After Faust, in Jour. Lab. and Clin. Med., courtesy of C. V. Mosby Company.)

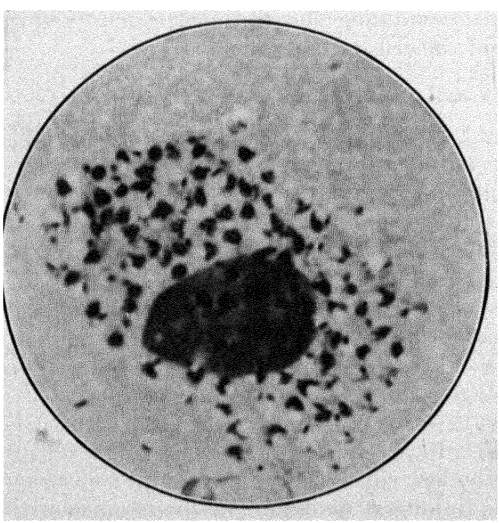


FIG. 22. *Leishmania donovani*. Stained with Wright's stain. Large endothelial cell filled with leishmaniform stage of *L. donovani*. × 1800. (Photomicrograph, Army Medical Museum Collection. Preparation by Craig.)

with the membrane and may appear hemispherical in shape or consist of a narrow band in contact with the membrane. With the Wright stain the nucleus appears to consist of a mass of red granules but if the preparations be wet-fixed and stained with one of the hematoxylin stains, the nucleus shows a central round karyosome and a nuclear membrane. The kinetoplast usually appears as a rodlike, deep red or violet body, situated opposite the nucleus and at right angles to it. Usually the kinetoplast appears as a solid staining body but in

very well stained preparations it is seen to consist of two portions, a rodlike parabasal body and a minute dotlike blepharoplast. Sometimes a delicate filament, staining red, may be seen arising from the blepharoplast and extending to the surface of the body, which is called the axoneme, or rhizoplast, and from which arises the flagellum when the organism reaches an invertebrate host or develops in suitable culture media.

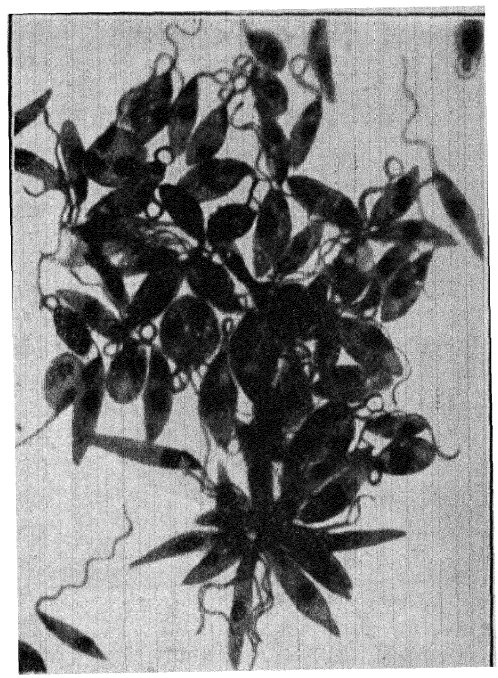


FIG. 23.—*Leishmania donovani*. Stained with Wright's stain. Flagellate forms of *L. donovani* from culture. × 1800. (Photomicrograph from Army Medical Museum Collection. Preparation by Craig.)

Long, spindle or torpedo-shaped forms are sometimes observed in which the nucleus and kinetoplast lie in apposition and may appear as a single body. Large forms, measuring as much as 7 to 9 microns in longest diameter are also rarely observed, as well as many degenerate forms in which the morphology is atypical.

The leishmania may lie in large groups within the endothelial cells or may occur in small numbers in these cells, while in smears many of the leishmania are apparently free in the plasma or tissue juices. Many of these free parasites are undoubtedly produced by rupture of cells containing them by pressure during the making of the smear.

Multiplication occurs by simple fission and multiplying forms are sometimes observed in preparations made from the spleen and liver. The nucleus and kinetoplast divide and forms are observed in Wright stained preparations showing 2 nuclei and 2 kinetoplasts, while 2 axonemes may rarely be seen arising from 2 blepharoplasts.

The *flagellate* forms of *Leishmania donovani* occur only in insects or in cultures and while the aflagellar forms also occur in cultures they do not occur in as large numbers as do the flagellate forms. There is considerable variation in the morphology of the flagellate forms, some being short stumpy spindle-shaped, round or oval bodies while others are slender spindle-shaped bodies varying considerably in length. The flagellate forms measure from 10 to 20 microns in length, when

fully developed, without counting the length of the flagellum which may measure as much again or even more. The smaller round and oval flagellates usually measure from 4 to 8 microns in longest diameter. The flagellates present the same staining reactions with the Wright or other modification of the Romanowsky stain as do the aflagellar form.

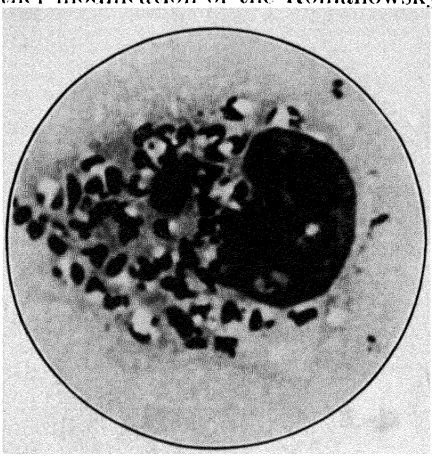


FIG. 24. *Leishmania tropica.* Stained with Wright's stain. Large mononuclear cell filled with leishmaniform stage of *L. tropica*, in smear from tropical ulcer. × 1800. (Photomicrograph, Army Medical Museum Collection. Preparation by J. H. Wright.)

The flagellate forms have a nucleus situated near the flagellate end of the body or near the center and a kinetoplast situated at the anterior end of the body. The nucleus appears as a round or oval red mass of granules while the kinetoplast appears as a rodlike body which is often divided into two portions, a rodlike parabasal body posteriorly, and a dotlike blepharoplast anteriorly, from which arises the single threadlike flagellum. Both the kinetoplast and flagellum stain pink or red, the kinetoplast usually being very deeply stained and sometimes almost black in color unless the preparation has been well decolorized. In cultures many degenerated organisms occur in which the morphology is very variable.

Multiplication of the flagellate forms occurs by binary longitudinal division, the blepharoplasts and parabasal bodies first dividing, followed by the division of the nucleus. Thus, organisms may be observed having 2 nuclei and 2 kinetoplasts, as well as 2 flagella, but the flagellum does not divide, the new flagellum arising from the new blepharoplast produced by division.

In fresh, unstained preparations the forms of *Leishmania donovani* observed in man are immotile but the flagellate forms in insects and cultures are very actively motile. In cultures the flagellate forms are often observed arranged in the shape of a rosette, the flagella being directed towards one another. (See Fig. 23.) Such an arrangement is most frequently observed when the cultures are from three to five days old and in stained preparations present a very characteristic appearance.

2. MORPHOLOGY OF LEISHMANIA TROPICA

Many attempts have been made by numerous observers to demonstrate that the morphology of *Leishmania tropica* differs from that of *Leishmania donovani*, but without success. The writer believes that

if the two organisms are observed side by side when properly prepared and stained, *Leishmania tropica* is larger and coarser than is *Leishmania donovani* in the aflagellar stage of development but the difference is so slight as to be of no diagnostic importance. The description already given of the morphology of *Leishmania donovani* applies equally well to that of *Leishmania tropica* as regards both the forms observed in man and in insects or cultures.

3. MORPHOLOGY OF LEISHMANIA BRASILIENSIS

The morphology of *Leishmania brasiliensis* is identical with that of *Leishmania donovani* and *Leishmania tropica*. It is impossible to differentiate any of these organisms by a study of the morphology alone and the differential diagnosis must rest upon the location of the parasite, the lesions produced by it, and the result of serological reactions.

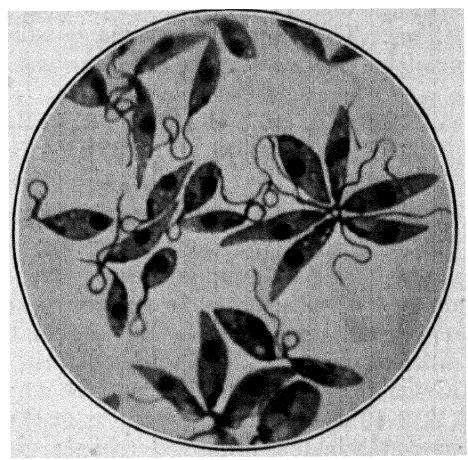


FIG. 25. *Leishmania tropica.* Flagellate forms of *L. tropica.* × 1800. Stained with Wright's stain. (From Army Medical Museum Collection. Preparation by Craig.)

A fourth species of leishmania has been described by Chagas and his colleagues (1937) which has been named *Leishmania chagasi*. This parasite occurs in Brazil in patients presenting symptoms similar to those of kala-azar, but its specific nature has not been demonstrated. The work of Da Cunha (1938) and Adler (1940) has shown that in insects and animals the so-called *Leishmania chagasi* cannot be distinguished from *Leishmania donovani*, while the morphology is identical with the latter species. These observations have been confirmed repeatedly and it has now been demonstrated that *Leishmania donovani* occurs not infrequently in Brazil and probably in other countries in South America. Therefore, *Leishmania chagasi* becomes a synonym of *Leishmania donovani*.

The morphology of the various leishmania occurring in man is very characteristic and a diagnosis of the presence of leishmania based upon morphology alone is possible but not of the particular species of leishmania. Furthermore, one should never make a diagnosis of leishmania unless the bodies so diagnosed possess a definite nucleus, kinetoplast and limiting membrane and occur within endothelial cells in groups numbering several organisms.

CHAPTER IX

METHODS OF COLLECTING AND PREPARING MATERIAL FOR EXAMINATION FOR LEISHMANIA DONOVANI, LEISHMANIA TROPICA AND LEISHMANIA BRASILIENSIS—STAINING METHODS FOR LEISHMANIA

THE diagnosis of infections with *Leishmania donovani* should be based upon the demonstration of this parasite in the peripheral blood or in material obtained by puncture of the spleen, sternum, lymphatic glands or liver, and of *Leishmania tropica* and *Leishmania brasiliensis* upon the demonstration of the parasites in material obtained from the lesions. The preparations must be stained and the various methods available for this purpose are described later in this chapter.

1. THE COLLECTION AND PREPARATION OF MATERIAL FOR THE DIAGNOSIS OF LEISHMANIA DONOVANI, CAUSING KALA-AZAR

A. **Examination of the Peripheral Blood.**—The microscopic examination of stained preparations of the peripheral blood is a valuable method of diagnosis in infections with *Leishmania donovani*. The percentage of patients suffering from kala-azar in whom this parasite may be demonstrated in the peripheral blood has varied greatly with different observers, depending upon the care and amount of time spent in examining the blood films and the number of films examined. Patton (1912–1914) found the organism in the peripheral blood of all of 84 patients whom he examined in Madras but in many instances numerous blood films were examined before it was found. Thus, he found it in 42 cases in the first preparation, in 13 cases in the second, in 12 cases in the third, in 5 cases in the fourth, in 2 cases in the fifth, in 4 cases in the sixth, and in the remaining 6 in a larger number of films. Other observers have not been as successful for Mackie (1914) found it in only 21 per cent of native Assamese and 64 per cent of the tea-garden coolies, while Knowles (1920) could demonstrate *Leishmania donovani* in only 33, or 45 per cent, of 73 cases of kala-azar, averaging the examination of over 9 blood smears to each patient. Napier (1943) states that the careful examination of four properly prepared blood smears should result in a positive diagnosis in from 60 to 70 per cent of cases of kala-azar. These varying results, confirmed by other observers, demonstrates that, while the parasite does occur in the peripheral blood in kala-azar patients, the percentage of positive results that may be expected varies in different localities and that many blood smears may have to be carefully examined before it can be found.

PREPARATION OF BLOOD SMEARS.—In preparing blood smears for the demonstration of *Leishmania donovani* it should be remembered

that the parasites occur within the endothelial cells, large mononuclear leukocytes and, much more rarely, within the neutrophilic leukocytes, and, for this reason, thicker films should be made than for the examination for the malaria plasmodia. Using a clean microscopic slide, a drop of blood obtained from the finger or ear, is placed upon one end of the slide. The edge of the end of another slide is then placed in front of, and in contact, with the drop of blood, at an angle of about 35 degrees. The blood is allowed to spread along the edge of the opposing slide which is then pushed gently toward the end of the under slide and quickly removed near the other end. This procedure results in a fairly thick smear over the surface of the under slide which is considerably thicker at the straight edge of the smear made by quickly raising the spreading slide. The smears should then be allowed to dry at room temperature in a closed Petri dish to protect them from flies and insects and may then be stained and examined, or stored in an insect-proof slide-box for future staining. Most of the leishmania will be found in the leukocytes at the thick end of the smear.

In order to increase the number of leishmania in the peripheral Knowles (1920) has recommended the hypodermic injection of 10 to 20 mm. of a 1 to 1000 solution of adrenalin half an hour before the blood smears are made.

Preparation of Thick Blood Films.—Knowles and Das Gupta recommend the following method of preparing thick blood films for the diagnosis of *Leishmania donovani* and were able to demonstrate the parasite in 67 per cent of kala-azar cases they examined by this method.

Four drops of blood from the patient are placed upon the middle of a microscopic slide and mixed with a needle so as to cover a space about one-quarter of a square inch. The preparation is then allowed to dry at 37° C. (98.6° F.) in a covered Petri dish for two hours, and then immersed in a mixture of glacial acetic acid (2 per cent) 4 parts, crystalline tartaric acid (2 per cent) 1 part, and allowed to remain for from five to ten minutes. The preparation is then removed, allowed to drain, fixed with methyl alcohol and stained.

Preparation of Material from Centrifugalized Blood.—Some authorities report an increased number of positive results in preparations of blood after centrifugalization. Young and van Sant have obtained excellent results by centrifugalizing 5 cc. of blood diluted with a modified Locke solution at a speed of 750 revolutions until the blood is sedimented. The Locke solution is a modified one having the following formula:

Sodium chloride	9.0 gm.
Potassium chloride	0.4 gm.
Calcium chloride	0.2 gm.
Sodium citrate	10.0 gm.
Distilled water	10.0 cc.

The sediment from the bottom of the centrifuge tube should be removed with a pipette and smears of this material be made upon microscopic slides, allowed to dry and stained.

B. **Preparation of Splenic Pulp Smears.**—It appears to be the consensus of opinion of many who have had an extended experience with the diagnosis of *Leishmania donovani* that the microscopic examination of stained preparations of spleen pulp gives the highest percentage of positive results of any method of microscopic examination, with the possible exception of sternal puncture, usually varying between 85 and 95 per cent. Thus Knowles obtained 70 positive results in 79 cases of kala-azar, or 88 per cent and other authorities have reported positive results in a higher percentage of cases.

The number of positive results obtained by the examination of splenic pulp can be increased by culturing such material (see page 170.)

Technique of Splenic Puncture.—Puncture of the spleen is attended with some danger but if carefully performed by an experienced operator, the danger is very slight. Thus, Napier (1936) has reported 5000 splenic punctures without a death and similar results have been obtained by other operators. However, puncture of the spleen should not be attempted unless the organ is enlarged so as to project some distance below the ribs and a careful examination of the blood should be made before puncture in order to rule out any blood dyscrasia that might influence the result of the puncture, as leucocythemia, as well as to determine the presence or absence of *Leishmania donovania*, which, if present, would obviate the necessity of splenic puncture.

Prior to the puncture, the coagulation time of the blood should be ascertained and if it is more than five minutes, using the Wright tube, splenic puncture should not be performed. The night before the day upon which the puncture is to be made the patient is given 30 grains of calcium lactate and the same dose is administered a half hour before the puncture is made, and an hour before the puncture 1/100 grain of atropine should be given. At the time of the puncture the patient should be lying down in bed and an abdominal binder should be ready for application as soon as the puncture is completed to immobilize the abdomen as much as is possible, thus reducing the chances of bleeding. An assistant should be ready to steady the spleen with his hand while the puncture is being made and the patient should be warned not to make any sudden movement during the operation, which might result in tearing the capsule of the spleen and consequent hemorrhage. A glass syringe, of not more than 5 cc. capacity, fitted with a needle measuring $1\frac{1}{2}$ inches in length and having a good sized bore, should be used in making the puncture, and the needle should be connected with the syringe by a short piece of rubber tubing, which allows greater freedom of manipulation. The syringe needle should have been sterilized before use and should be perfectly dry, for if any water remains in the needle it will render the leishmania indistinguish-

able. The skin over the site of puncture is cleaned and sterilized with tincture of iodine and the puncture is made at the middle of the breadth of the spleen and at least 1 inch below the costal margin and that distance away from the lower border of the spleen if possible. Before the puncture is made it is best to infiltrate the site of the puncture with novocaine as passing the needle through the skin is painful and may cause the patient to wince, with possible tearing of the spleen capsule. In making the puncture the needle should be pushed rapidly and firmly into the spleen in a slightly upward direction and suction should then be made slowly until a little blood appears in the barrel of the syringe. If this does not occur, the piston may be drawn back rapidly for about an inch, two or three times, when a drop of blood usually will appear in the syringe. If not, the needle should be withdrawn quickly and the contents blown upon a microscopic slide and smears made upon other slides. Very often no blood will appear in the syringe but this does not mean that the puncture is worthless, as splenic pulp will usually be found in the needle in sufficient amounts to make diagnostic smears. After the smears are prepared they should be stained or may be stored in a slide-box, protected from insects, for future staining.

After the puncture is completed, a sterile gauze pad is placed over the site of the puncture and the abdominal bandage applied tightly. The patient should remain in bed, if possible, for a period of twenty-four hours, during which time he should be kept quiet. In the case of out-patients, rest in bed should be insisted upon for at least one hour and the patient should not be allowed to leave until he has been observed for at least another hour.

In obtaining material from the spleen for examination for *Leishmania donovani* it should be remembered that the parasite is found in the endothelial cells of the pulp and that, in order that the examination be successful, one should obtain such material, rather than blood, for the leishmania are sometimes absent from blood even when it is obtained from the spleen. Very little material is needed for the demonstration of the organism.

C. **Preparation of Bone-Marrow Smears.**—Within recent years some authorities have advocated the examination of smears of the bone-marrow, rather than of the splenic pulp, in the diagnosis of *Leishmania donovani*, claiming that as high a percentage of positive results are obtained by this method and with less danger of serious complications, as hemorrhage. The evidence concerning the percentage of positive results obtained by this method, as reported in the literature, is conflicting, but the consensus of opinion appears to be that it is slightly smaller than when splenic puncture is employed. Usually the bone-marrow is obtained by sternal puncture but it may be obtained by tibial puncture.

TECHNIQUE OF STERNAL PUNCTURE.—The patient should be prepared as for an operation and the operator should wear gloves and a

sterile gown. The patient should be in the recumbent position, with a cushion placed between his shoulders, thus raising the sternum. The skin over the sternum should be scrubbed with alcohol and painted with iodine. The site of puncture should be selected and the best site is the upper portion of the sternum between the second and third rib level or directly opposite either of these ribs, in the middle of the sternum. A short, beveled, 18 gauge spinal puncture needle, from 3 to 4 cm. in length, with a lumen from 1 to 2 mm. in diameter, having an adjustable guard attached to the needle, should be employed. Special needles for sternal puncture may be obtained, the one known as Sharp's needle being especially suitable. The needle should contain a stylet.

The site of puncture having been selected, the skin and subcutaneous tissues are infiltrated with a 1 per cent procaine solution to produce anæsthesia. When this is secured and the needle and stylet sterilized the puncture is made. One should wait for from 5 to 10 minutes after the procaine is injected before attempting the puncture.

In making the puncture, the needle is forced gently into the skin, being held in a nearly vertical position, and pushed onward through the lamina until the marrow cavity is reached, which will be indicated by a sense of loss of resistance or a "give." It should then be passed from 1 to 2 mm. into the marrow cavity, which is normally from 5 to 15 mm. in depth. Care should be taken that the needle be not pushed too far into the marrow cavity.

After the needle has penetrated the marrow cavity, the stylet should be removed, and a sterile 2 cc. glass syringe attached to the needle. Gentle suction is now made and enough marrow will be drawn into the syringe to enable one to make several smears. It is not wise to try to obtain more than this as the dilution with blood will render a positive diagnosis more difficult. If no material is obtained the needle is gently shifted in position and suction again made. If this is unsuccessful 0.5 cc. of sterile normal saline may be injected and removed at once. This procedure may wash out enough of the marrow to enable one to prepare useful smears for diagnosis.

After the marrow is obtained it is ejected in minute amounts upon microscopic slides, smears prepared, and stained at once with a suitable stain. The needle wound is touched with tincture of iodine and sealed with collodion.

Dangers of Sternal Puncture.—This procedure, if properly performed, is practically harmless, although either hemorrhage or infection might result in the absence of proper technique or asepsis. Serious consequences have not been reported following sternal puncture and it can be employed in both children and adults.

Diagnostic Value of Sternal Puncture.—It is the opinion of many recent authorities that sternal puncture is the most accurate and best method of diagnosis for kala-azar. Thus, Snapper (1941) states that

at the Peiping Union Medical College Hospital, sternal puncture has almost completely replaced splenic puncture, the latter being performed only in special cases. It should be preferred to splenic puncture in all cases in the opinion of the writer.

D. **Preparation of Gland Juice Smears.**—The examination of properly prepared smears of material obtained by the puncture of enlarged lymphatic glands in cases of kala-azar gives a very high percentage of positive results and Kirk and Sati (1940) claim that, in their experience, this method of diagnosis gives a higher percentage of positive results than either splenic or sternal puncture. Kirk and Sati (1940), in the Anglo-Egyptian Sudan, demonstrated *Leishmania donovani* in 30 consecutive cases of kala-azar by the examination of smears of gland juice obtained by puncture. The method of gland puncture they employ is as follows, quoted from their report:

"The glands to be punctured are the lower group of the superficial inguinal glands (Gray's Anatomy, 1936), which are disposed vertically along the terminal part of the long saphenous vein, and receive all the superficial lymph vessels of the lower limb except those from the back and lateral side of the calf of the leg. They can usually be readily palpated if a deep grip is taken with the thumb and forefinger. The skin having been sterilized, the glands are identified, pulled up from the underlying tissues and steadied above the thumb and forefinger of the operator's left hand. A dry, sterile hypodermic needle (No. 16 is a convenient size) is pushed through the skin and into the gland by the right hand of the operator. No syringe is attached to the needle, as once the gland has been penetrated the juice runs up into the needle of its own accord. It is usually easy to feel when the needle has entered the gland. Once the needle has entered the gland, it is held there for a second or two, then quickly withdrawn, and attached to a small syringe by means of which the gland 'juice' in the needle can be blown out on a slide in the usual way for examination. With practice, the operation can be carried out rapidly, and the amount of danger or discomfort to which the patient is subjected is negligible."

While the authors above mentioned prefer to puncture the inguinal glands, any enlarged gland may be punctured with approximately the same results, if the patient is infected with *Leishmania donovani.* If possible, it is better to have an assistant hold the gland for puncture, thus giving the operator both hands free for the operation, and after the puncture has been made the site should be painted with tincture of iodine and a sterile compress applied and retained for twenty-four hours. The needle and syringe used should be sterilized and be perfectly dry before the puncture is made. The material obtained from the punctured gland should be carefully smeared over the surface of a microscopic slide, allowed to dry and stained or the smears placed in an insect proof slide-box to be stained later.

E. **Preparation of Liver Pulp Smears.**—Some authorities prefer to puncture the enlarged liver rather than the spleen for the demonstration of *Leishmania donovani,* as the procedure is attended with less danger than splenic puncture. However, the percentage of positive results is considerably smaller than with splenic puncture and the

danger of the latter procedure is practically *nil* in the hands of experienced operators. If the operator has had little or no experience with splenic puncture, puncture of the liver is undoubtedly safer and should be preferred, but sternal puncture should replace both.

Technique of Liver Puncture.—The technique of splenic puncture already described (see page 157) applies equally well to that of liver puncture and need not be repeated.

F. **Examination of the Skin for Leishmania Donovani.**—Recent observations have shown that in a considerable percentage of cases of infection with *Leishmania donovani*, the parasite may be demonstrated in the skin, especially in cases of dermal leishmanoid, or post-kala-azar leishmaniasis. Scrapings may be made from the lesions and the material smeared upon microscopic slides, or sections of the skin may be made and properly stained.

G. **Examination of the Nasal Secretions, Feces and Urine.**—In 1929, Shortt and his co-workers found *Leishmania donovani* in the *feces* of patients suffering from kala-azar, and previously, Shortt, Swaminath and Sen demonstrated its presence in the *urine*. Their observations have been confirmed but the examination of smears prepared from the urine or feces has not proven of much value in diagnosis, as positive results are so seldom obtained and there are so many other and better diagnostic methods available.

In 1934, Forkner and Zia found *Leishmania donovani* in smears prepared from material obtained from swabs passed over the mucous membrane of the *nose* in 9 kala-azar patients and also in material blown from the nose. In a later report (1935) they record finding the parasite in the nasal secretions of 12 of 22 cases of kala-azar, while Archibald (1935) found the parasite in 7 of 22 cases in the nasal secretions. Others have confirmed these findings and this diagnostic method would appear to be well worthy of a careful trial in suspected infections with this parasite.

2. THE COLLECTION AND PREPARATION OF MATERIAL FOR THE DIAGNOSIS OF LEISHMANIA TROPICA

In infections with *Leishmania tropica* the parasite does not occur in the peripheral blood except in the immediate vicinity of the lesions, so that blood examinations are of practically no value in diagnosis, which must depend upon the demonstration of the organism in material removed from the lesion.

TECHNIQUE OF COLLECTING MATERIAL.—Some authorities have recommended removing any crust covering the sore, cleansing it with distilled water, and making microscopic slide smears from material obtained by scraping the tissue under the edge of the ulcer with a small curette. This method should not be used as the leishmania in preparations so procured stain very poorly and are either present in small numbers or, more frequently, are not present even in typical cases of Oriental sore.

The material to be examined should be obtained from the tissue at the base of the ulcer and this is best done by making a small incision through the skin at the edge of the ulcer and then forcing a glass capillary pipette into the tissue at the base of the sore when serum and tissue cells will flow into it by capillary attraction. Care should be taken not to obtain blood but only serum and tissue cells. The skin should have been sterilized before making the tiny incision through which to push the capillary pipette.

Another good method for obtaining material is to puncture the tissue back of, and near the edge of the ulcer, with a hypodermic needle having a good sized bore, attaching the needle to a small glass syringe, and aspirating gently until material is just visible in the syringe. This is ejected upon microscopic slides, allowed to dry, after smearing, at room temperature and protected from insects, and stained at once or stored in an insect proof slide-box for future staining.

If ulceration is not present, the nodule produced by *Leishmania tropica* may be punctured with a hypodermic needle as described above, or a glass capillary pipette may be employed, as already described. The skin over the nodule should be sterilized before the puncture is made and the puncture site should afterwards be treated with tincture of iodine. The material obtained should be smeared upon microscopic slides as already described.

3. THE COLLECTION AND PREPARATION OF MATERIAL FOR THE DIAGNOSIS OF LEISHMANIA BRASILIENSIS

As this species of leishmania causes an infection characterized by cutaneous lesions resembling those of Oriental sore as well as lesions within the mucous membranes of the nose and throat, the parasite may be demonstrated in stained preparations obtained from the skin or mucous membrane lesions. In the early stage of the infection the lesions occur upon the skin in the form of nodules and ulcers very like those of Oriental sore and the methods of collecting material are the same as those described above for the collection and preparation of material for the diagnosis of *Leishmania tropica.*

The collection of material from the lesions in the mouth, nose or throat is more difficult and the best method would appear to be the excision of a small piece of the nodular or fungating masses and squeezing serum from the excised tissue upon a microscopic slide for staining. In some cases puncture of the lesion with a hypodermic needle or glass capillary pipette may be sufficient but if such preparations are negative the excision of tissue from the lesion should be followed before a negative report is accepted.

4. STAINING METHODS FOR THE LEISHMANIA

As already stated, all of the leishmania must be stained in order to demonstrate them for diagnostic purposes and many excellent stain-

ing methods are available, all of them modifications of the original Romanowsky stain which is no longer used. With all of these stains the staining reactions of all the species of leishmania are the same, the limiting membrane and cytoplasm taking a pale blue color, while the chromatin of the nucleus, the kinetoplast, and the flagellum of the flagellated forms stain varying shades of red or violet, differing somewhat in intensity with the stain employed and the length of time of the staining process.

It is sometimes desirable to study the developmental stages of the leishmania in living unstained organisms and for this purpose the following method has been found to give excellent results:

Study of Living Leishmania.—Material obtained by sternal puncture, in the case of *Leishmania donovani*, or from the lesions, in the cases of *Leishmania tropica* and *Leishmania brasiliensis*, is mixed with normal saline solution (0.85 per cent) in a small test tube or upon a microscopic slide and examined at frequent intervals. In this fluid the leishmania gradually elongate and become flagellated and by the end of forty-eight to fifty-five hours fully developed flagellated forms will be found. As the leishmania are colorless it is necessary that the iris diaphragm of the microscope, as well as the condenser, be carefully adjusted, or the organisms will be invisible or poorly defined. For *diagnostic* purposes, however, this method is not very useful and stained preparations should be relied upon for that purpose.

Methods of Staining. There are several staining methods that are valuable in the diagnosis of the leishmaniases and, save for cost and difficulty of preparation of the stains, there is really very little difference between them so far as staining properties are concerned. The writer has found the Wright stain to be the most generally useful but any of the other stains described will be found to give excellent results if properly employed. For convenience the stains are described in alphabetical order but this arrangement does not indicate their value in diagnosis.

Bhattacharji, Singh and Sen Gupta's Stain.—This stain was developed by the authors and described in 1946, as a substitute for the Giemsa stain. It depends upon the fact that efficient chromatin staining may be obtained by oxidizing methylene blue. The formula recommended is the following:

Methylene blue (medicinal)	1 0 gm.
Potassium permanganate (medicinal)	0.3 gm.
Water-soluble yellow eosin	0 4 gm.
Water, distilled or tap	250 0 cc.

Preparation of Stain.—Dissolve 1 gm. methylene blue in 100 cc. of the water, and 0.3 gm. potassium permanganate in another 100 cc. of the water. After the methylene blue is dissolved transfer it to a porcelain dish and allow it to steam, but not boil, for about five minutes, over a bunsen burner or alcohol lamp. Now add slowly the

potassium permanganate solution and continue the heating for ten minutes, being careful not to boil the mixture. In the remaining 50 cc. of water dissolve 0.4 gm. of eosin and add it to the steaming methylene-blue-potassium permanganate solution, mixing thoroughly with a glass rod. Continue heating for an hour or longer until the mixture in the dish has evaporated, being careful not to heat too much or to allow boiling. After evaporation the dish will contain a thick precipitate of a metallic greenish color. Dry thoroughly in an incubator over night and then remove the precipitate which appears as metallic green flakes. These are powdered in a dry glass mortar and the powder kept in a dry glass container.

Use of the Powder in Staining.—If one desires to use this stain as a substitute for Leishman's stain, take 0.1 gm. of the powder and dissolve it by grinding in a glass mortar in 40 cc. methyl alcohol. This is the staining solution and it is used as in staining with the Leishman stain. (See page 163.)

If one desires to use this stain as a substitute for the Giemsa stain, place 0.3 gm. in a glass mortar and grind it slowly in a mixture of glycerin and methyl alcohol, 25 cc. of each. The solution so obtained is placed in a glass bottle and allowed to remain at room temperature over night and then placed in a water-bath up to the level of bottle neck for about two hours.

The staining technique is the same as for the Giemsa stain. (See below.)

Remarks.—It is the consensus of opinion of all who have used this stain that it gives as good results as the Leishman or Giemsa stains and the simplicity of obtaining the chromatin-staining powder recommends it for general use.

Giemsa's Stain.—The Giemsa staining solution has the following formula:

Azur II-eosin (Grubler)	3 0 gm.
Azur II (Grubler)	0 8 gm.
Glycerin (Merck pure)	250 0 cc.
Methyl alcohol (Merck's reagent)	250 0 cc.

Dissolve the Azur II-eosin and Azur II in the glycerin at a temperature of 60° C. (140° F.) in a glass flask, shaking frequently. When dissolved add the alcohol, which has been previously heated at the same temperature, thoroughly shake and allow the mixture to stand at room temperature for twenty-four hours, when it is filtered into a chemically clean, sterile air-tight bottle which can be used as a stock bottle. It is most important, during the filtration, that the filtering funnel be covered as the mixture is hygroscopic.

This stain is employed as follows:

The films of the material to be stained, prepared as already described (see page 152) are immersed in absolute methyl alcohol (Merck's Reagent) for ten minutes, in order to fix them. The staining solution

is diluted in the proportion of 10 drops of the solution to 10 cc. of acid-free distilled water and if intense staining is desired it is well to add to this mixture 1 drop of a 1 per cent bicarbonate of potassium solution in distilled water to each 10 cc. of the staining solution.

The fixed films upon the microscopic slides are either covered with the staining solution or immersed in it in a suitable slide container and stained for from ten to fifteen minutes and if intense staining is desired, for one or more hours. The flagellum of the flagellated leishmania stain with some difficulty and usually require at least one hour's staining and frequently two or three hours before the best results are obtained.

After staining the films are washed in running distilled water for about one minute, or until they have a distinct pink tinge, after which they are allowed to dry at room temperature and are ready for examination. They should not be mounted unless the balsam used is absolutely neutral, owing to the danger of fading, and mounting is not necessary. If an oil-immersion lens is used in examining the preparation, as is usual, the immersion oil is placed directly upon the film and can be washed off after the examination with a little xylol, the preparation dried, and stored, if desired, in a suitable slide-box or cabinet. If a dry lens is used it is necessary to cover the film with a thin layer of immersion oil in order to demonstrate the leishmania.

Remarks.—The Giemsa stain is an excellent one for the diagnosis of the leishmaniases and is preferred to any other by many authorities. It can be obtained already made up from commercial houses dealing with stains and this is recommended owing to the difficulty of preparation which often results in a poor stain. Unless one is experienced in the use of this stain it is very apt to overstain and considerable experience is necessary with it before the best results are obtained.

Jenner's Stain.—This stain is prepared as follows:

Equal parts of a 1.2 per cent solution of water-soluble eosin (Grubler's or National Aniline) in acid-free distilled water, and a 1 per cent aqueous solution of medicinal methylene blue, are mixed together in a glass flask, thoroughly shaken, and allowed to stand at room temperature for twenty-four hours. A coarse precipitate will have formed by the end of this time, of a dark purple color and having a metallic lustre. This is collected by filtration through a small filter paper and the deposit upon the filter paper washed with distilled water until the resulting filtrate comes through the paper almost colorless. The precipitate is then dried upon the filter paper and removed and placed in an air-tight bottle and stored in a dark place at room temperature.

The staining fluid is prepared by dissolving 0.5 gm. of the precipitate in 100 cc. of pure methyl alcohol (Merck's Reagent) and the smears of material containing Leishmania stained with this mixture by covering the preparation with the undiluted stain, allowing it to remain for one or two minutes, in order to fix it, and then adding distilled water,

drop by drop, until there appears a distinct metallic sheen upon the surface, after which the stain should be allowed to act for from five to fifteen minutes, or longer, according to the intensity of staining desired.

Remarks.—This stain frequently gives excellent results but is not as reliable as either the Giemsa, Leishman or Wright stain, although prepared more easily.

Leishman's Stain.—This popular stain is prepared as follows:

1. Make a 1 per cent solution of medicinal methylene blue in distilled water made alkaline by the addition of 0.5 per cent of sodium carbonate. This is heated for twelve hours at 65° C. (149° F.) in a hot-air oven and then allowed to stand for ten days at room temperature before using.

2. Make a 1 to 1000 solution of yellow aqueous eosin (Grubler's or National Aniline).

3. Mix equal parts of the methylene blue solution, after shaking, and the eosin solution in an open dish and allow the mixture to stand for six to twelve hours, stirring at intervals.

4. Collect the precipitate that has formed during this time by filtering the mixture through one small filter paper, and then wash the precipitate upon the paper by passing distilled water through it until the filtrate is colorless or a pale blue in color. The filter paper is then dried carefully in an oven and the precipitate removed or the precipitate may be first scraped from the paper and dried in an oven. The dried precipitate is powdered and placed in a clean dry bottle and kept in a dark closet.

The *staining solution* is made by dissolving 0.15 gm. of the powder in 1000 cc. of Merck's methylic alcohol (Reagent) and the smears of material containing the leishmania are stained with it in the same manner as described for Jenner's stain (see above). After staining the smears are thoroughly washed in distilled water and examined unmounted.

Remarks.—This stain is a favorite one with English authorities for the staining of leishmania, trypanosomes and the malaria plasmodia and when properly made and used gives excellent results. The powder may be obtained already prepared and this is preferable to making it oneself, provided a reliable product is obtainable.

Wilson's Stain.—The preparation of this stain is somewhat complicated and is as follows:

Dissolve 2 gm. of silver nitrate in 50 cc. of water and add a 2 to 5 per cent solution of sodium hydroxide until the resulting silver oxide is entirely precipitated. The precipitate is then washed by decanting the supernatant fluid and pouring distilled water several times into the flask, decanting each time, until the alkali is all removed. After the last washing there is added to the moist silver oxide 200 cc. of a solution containing 2 gm. of methylene blue and 0.5 per cent sodium carbonate. This mixture is then boiled gently in a porcelain dish for

thirty minutes, stirring from time to time. One-third of the mixture is then poured into a 200 cc. graduated cylinder. To the remainder of the mixture in the dish add the same amount of boiling distilled water as placed in the cylinder and boil for thirty minutes. Pour into the same cylinder another third of the contents of the dish, and boil the remaining one-third in the dish for thirty minutes without adding any distilled water, after which the contents of the dish are added to those of the cylinder and enough distilled water added to bring the entire amount to 200 cc. This is now filtered into a 500 cc. beaker and there is added to it 200 cc. of a solution containing 1 gm. of yellow aqueous eosin in distilled water. The mixture is allowed to stand for thirty minutes and the precipitate that forms is then collected upon a single small filter paper and the filtrate discarded. The precipitate is then dried in an incubator or oven at 60° C. (140° F.) and placed in a dry, clean bottle for storage.

The staining solution is made by dissolving 0.2 gm. of the powdered precipitate in 50 cc. of pure methylic alcohol (Merck's Reagent). In using this stain the smears of material containing the leishmania are covered with the staining solution which is allowed to remain for one minute, thus fixing the material. Distilled water is then added drop by drop until a metallic scum appears upon the surface of the staining solution and the smears are then stained for five minutes, after which they are thoroughly washed in distilled water, allowed to dry at room temperature, and examined at once or placed in an insect proof slide-box and stored in a dark place for future examination.

Remarks.—This is an excellent staining method and is highly recommended by several authorities. In the writer's experience it gives no better results than the Wright or Leishman stains and is more difficult of preparation.

Wright's Stain.—The Wright stain is the one most generally used in this country for the staining of leishmania, trypanosomes and the malaria plasmodia. It may be obtained already prepared from commercial houses but, as there are many poor Wright stains upon the market, one should be very careful that it is obtained from a firm known to sell a reliable stain. It is available in powder form and the staining solution is prepared by dissolving the requisite amount of the powder in pure methylic alcohol, preferably Merck's Reagent Methylic Alcohol.

The writer has always prepared his own Wright's stain and it is believed that more consistent results are obtained in this way than by relying upon purchased stains which vary considerably, even when made by reputable firms. If properly made and used the Wright stain always gives excellent results. The method of preparation is as follows:

Place 100 cc. of distilled water in a flask and dissolve in it 0.5 gm. of sodium bicarbonate. Add slowly, shaking continually, 1 gm. of

methylene blue (Grubler's or National Aniline Co.). This mixture is then heated for one hour in an Arnold sterilizer after the steam is up and is then allowed to cool. It will be noted that a considerable amount of the methylene blue is still undissolved at the end of this time but it should be allowed to remain in the solution.

After the solution has cooled there is added to it 1000 cc. of a solution containing 1 gm. of yellow aqueous eosin (Grubler's or National Aniline) in distilled water. This must not be added until the methylene blue solution is cold and has been poured into a white porcelain dish, and should be added very slowly, stirring meanwhile, until a well-marked precipitate occurs and the surface of the mixture becomes coated with a greenish metallic scum. The time for discontinuing the addition of the eosin solution is best ascertained by placing a drop of the mixture upon a piece of white filter paper, from time to time. When enough of the eosin solution has been added the drop of the mixture upon the filter paper should show a center composed of a deep bluish precipitate surrounded by a well-marked pink halo. When this is noted, the mixture should be allowed to stand for fifteen minutes and then is filtered through one small filter paper. The precipitate, which is the staining powder, is then carefully removed from the filter paper, placed in a small Petri dish, and dried in a hot-air oven at 60° C. (140° F.), and then powdered and stored in a clean glass bottle in a dark place. The resulting powder is of a greenish color with more or less of a metallic lustre. The filter paper containing the precipitate may be placed in the hot-air oven and dried before the precipitate is removed, this method usually being more economic as more of the precipitate can be recovered than by scraping it off the paper while it is moist.

The preparation of the staining solution and method of using it is as follows:

Take 0.3 gm. of the powder and add it to 100 cc. of pure methylic alcohol (Merck's Reagent) in a flask. Shake thoroughly and filter, adding to the filtrate enough of the alcohol to bring the entire amount to the original 100 cc. This is the staining solution which should be placed in an amber-colored bottle and kept in a dark place. Under ordinary conditions the solution will keep for many weeks without losing its staining properties.

In using this stain proceed as follows:

The smears of material, upon microscopic slides, are covered with the undiluted stain. In order to save the stain it is well to mark off on the slide with a wax pencil the space to be covered by the staining solution and to add the stain, drop by drop, with an ordinary medicine dropper, being sure to entirely cover the space so outlined. The stain is allowed to remain for not more than three minutes, being careful to prevent evaporation by adding a drop or more of the stain during this period. If evaporation of the staining solution occurs the preparation

will be worthless owing to the heavy precipitate that will be deposited. The undiluted stain fixes the preparation. At the end of the fixing period distilled water is added, drop by drop, to the solution upon the slides with a medicine dropper, until a well-marked greenish metallic scum appears upon the surface of the mixture, after which the preparations should be stained for from five to twenty minutes, or longer, according to the intensity of staining desired. After staining is complete the slides are washed in running distilled water until they appear pinkish in color, dried at room temperature, and examined at once or stored in a light-proof slide-box for future examination. The smears are examined, without mounting, with an immersion lens, the immersion oil being placed directly upon the slide and removed with a little xylol when the examination is ended. Such preparations may be covered with a cover-glass but if so, the balsam used in mounting must be absolutely neutral or the stain will soon fade and the preparation become worthless.

The washing of the stained smears with running distilled water is most important as it not only removes any precipitate that may have formed during the staining process but also greatly aids in the differentiation of the cytoplasm of the cells and the chromatin of the nucleus.

Remarks.—The writer has employed the Wright stain almost exclusively in the staining of leishmania, trypanosomes, and malaria plasmodia for a period of over forty years and has never seen any reason for using any other stain. When properly prepared and used it gives excellent results, coloring the cytoplasm of the flagellates a robin's egg blue or a deep blue and the chromatin of the nucleus and the kinetoplast a brilliant pink, red, or purple, according to the length of time the stain is allowed to act. It is comparatively easy to prepare and simple in application but the dyes used in its preparation should be the best obtainable and only pure methylic alcohol, preferably Merck's Reagent Methylic Alcohol, should be used in making the staining solution. The length of time required for staining varies with different organisms but for the leishmania from five to ten minutes is usually sufficient to secure good diagnostic preparations of the forms occurring in man. In staining the cultural forms or those occurring in insects a longer staining period is usually required to secure good staining of the flagellum, as this structure stains more slowly than the rest of the organism, and from fifteen to twenty minutes staining is usually necessary.

Other Staining Methods.—The leishmania may be stained with any of the hematoxylin methods of staining, after wet fixation, but such methods are never used in routine diagnosis but only for special morphological studies.

Preparation and Staining of Sections of Tissue.—In order to study the distribution of the leishmania in tissues it is necessary to fix pieces of the tissue, as the spleen or liver, in Zenker's fluid, embed in paraffine, and cut and stain serial sections, preferably with hematoxylin and

eosin, following the technique usually employed in pathological studies. The method recommended by Pereira and Medina which follows gives excellent results.

Pereira and Medina's Method of Staining Leishmania in Tissues.—This method was recommended by Pereira and Medina, in 1945. The following staining solutions are employed:

1. Ferric chloride, U. S. P. XII solution, aqueous dilution to 10 per cent.
2. Picric acid, saturated solution in 75 to 76 per cent ethyl alcohol.
3. Sodium thiosulphate, aqueous solution, 2 per cent.
4. Hematin solution, prepared as follows: Hematin, 10 per cent in alcohol-glycerin mixture, 10 ml.; 95 to 96 per cent ethyl alcohol 60 ml.; ammoniacal solution, 1 per cent, 20 ml.; Tannin solution, 20 per cent, in distilled water, 10 ml.
5. Ammoniacal silver, prepared as follows: To a 10 per cent aqueous solution of silver nitrate add ammonia drop by drop until the resultant precipitate is just dissolved while the mixture is being well shaken, and then dilute the solution to 0.5 per cent silver nitrate.

The method of staining the tissues is as follows:

1. Mordant the sections in the ferric chloride solution for one minute or longer.
2. Stain in the hematin solution three minutes or longer.
3. Rinse in water and differentiate in the picric acid solution, one-half minute.
4. Rinse in water and tone in the ammoniacal silver solution five minutes or longer.
5. Rinse in water and fix in the thiosulphate solution, one minute or longer.
6. Rinse in water, dehydrate, clear and mount in balsam.

All of these steps in the staining process should be with the section upon the microscopic slide.

"Results.—Chromatin, nucleoli, centrioles, kinetic apparatus of the flagellates and certain parts of striated muscle fibers are stained black; cytoplasm greyish; red blood cells bluish. The Leishmania are easily visible with the high dry power and sharply defined with the oil immersion lens."

CHAPTER X

CULTIVATION OF THE LEISHMANIA

DIAGNOSTIC VALUE OF CULTURES—TECHNIQUE OF CULTIVATION—MAINTENANCE OF LEISHMANIA IN CULTURES—MORPHOLOGY IN CULTURES—VIRULENCE IN CULTURES—CULTURE METHODS AND MEDIA

ROGERS (1904) was the first to cultivate leishmania upon artificial culture media, being successful in cultivating *Leishmania donovani* in a citrate solution culture medium. His results were confirmed by numerous observers and were followed by the development of other and more efficient culture media and the cultivation of *Leishmania tropica* and *Leishmania brasiliensis*. Using suitable media and aseptic technique it is possible to maintain cultures of these parasites for long periods of time and Manson-Bahr (1940) states that Wenyon has succeeded in keeping a strain of *Leishmania donovani* alive in successive cultures for a period of fifteen years.

DIAGNOSTIC VALUE OF CULTURES

Owing to the scarcity of *Leishmania donovani* in the peripheral blood the culture of this fluid for the parasite has received much attention. The results of blood cultures have varied considerably as reported by different authorities. Thus, Knowles cultured the peripheral blood in 12 cases in Assam and obtained a positive result in but one case, while Young and Van Sant (1923), in China, obtained positive results in over 90 per cent of untreated cases of kala-azar examined by them, and Archibald (1922) states that cultures are more useful in diagnosis than is the examination of the peripheral blood. The difference in the results obtained by different observers in the cultivation of *Leishmania donovani* from the peripheral blood are undoubtedly due to differences in the technique employed, the culture medium used, and the number of culture tubes inoculated. There is no doubt that in cases where the peripheral blood is negative the culture of the blood upon suitable media not infrequently results in the demonstration of the parasite, and that culture methods are useful in the diagnosis of *Leishmania donovani* and should be resorted to before splenic puncture is attempted.

In the diagnosis of infections with *Leishmania tropica* (Oriental sore) and *Leishmania brasiliensis* (Espundia), in patients in whom the parasites cannot be demonstrated in material removed from the lesions, cultural methods are valuable and should not be neglected in any

suspicious case when the causative organism cannot otherwise be demonstrated.

In the routine examination of the peripheral blood for *Leishmania donovani* and of material from the lesions for *Leishmania tropica* or *Leishmania brasiliensis*, it is a good practice to also inoculate tubes of suitable culture media at the same time, provided the parasites are not demonstrated by microscopic examination. While in the case of infections with *Leishmania donovani* we possess serological tests that are now considered as practically diagnostic it is still most desirable to demonstrate the parasite and cultural methods should be given a trial if the peripheral blood is negative for the organism before resorting to puncture of the liver, spleen or bone-marrow. Cultural methods are time consuming but usually the diagnosis of kala-azar is not so urgent that such methods cannot be given a trial.

TECHNIQUE OF CULTIVATION

The technique of the cultivation of the leishmania demands the utmost attention to asepsis in securing and inoculating the material to be examined, and most of the failures to secure positive results have been due to faulty technique in this respect. This is so, because if bacteria of any kind contaminate the material inoculated, or the cultures after inoculation, the leishmania will not develop or will quickly die, as these organisms cannot live in cultures in the presence of bacteria.

If the *peripheral blood* is to be cultured it should be obtained from one of the large veins in the forearm. The syringe used for the purpose should be a 10 cc. glass syringe fitted with a suitable needle and sterilized before use. Before puncturing the vein the skin over the site of the puncture should be sterilized with tincture of iodine and the syringe washed out with citrate solution. Collect 10 cc. of blood and expel it into a flask containing from 50 to 70 cc. of normal saline (0.85 per cent) or Locke's solution, shake, and divide into two 50 cc. centrifuge tubes and centrifugalize at a speed of 750 revolutions per minute for about five minutes, or until the red blood corpuscles are lightly packed at the bottom of the tubes. The cloudy supernatant fluid is then poured into another centrifuge tube and centrifugalized for five minutes at about 1350 revolutions per minute, and the sediment so obtained, which contains most of the Leishmania, is used for inoculating suitable culture media. Everything used in collecting the blood and centrifugalizing it should be sterile.

In order to secure cultures of *Leishmania donovani* it is essential that a considerable amount of blood be collected, as it is practically useless to attempt to secure cultures from a few drops of blood. The use of the centrifuge is also necessary in order to concentrate the leukocytes containing the leishmania and too great speed should be

avoided, only sufficient being used to secure a lightly packed sediment. Too great speed will result in injury to the organisms and failure to develop in cultures.

A more simple method of securing material from the blood for culture consists in placing the blood in centrifuge tubes and after centrifugalizing it, to carefully pipette off the leukocyte layer at the junction of the column of blood and serum, and inoculate tubes of culture media with this material. This method does not give as consistent results as the method described above and is not recommended in preference to the former.

It is very rarely necessary to resort to culture of material obtained by splenic, liver or sternal puncture in the diagnosis of *Leishmania donovani* for the reason that if the patient is really suffering from kala-azar, the leishmania will almost invariably be demonstrable in such material by a microscopic examination. In the rare instances in which the symptoms are very suspicious and a microscopic examination of such material has resulted negatively, culture may be sometimes successful and should not be neglected. The material to be cultured is obtained in the manner already described for puncture of the spleen, liver or sternum (see pages 154 and 156) and expelled from the collecting syringe directly into suitable culture media, strict precautions regarding asepsis being observed during the entire procedure.

If *material from the lesions* of Oriental sore and espundia is to be cultured for *Leishmania tropica* and *Leishmania brasiliensis* respectively, it must be obtained bacteria-free and this is much more difficult than in collecting blood for examination for *Leishmania donovani*. It is useless to try to culture material obtained from scraping the lesions as bacteria are always present in such material and the leishmania will not develop in the cultures. The material should be obtained by running a glass capillary pipette into the tissue immediately beneath the base of the lesion, through a small puncture made in the skin, which has been previously sterilized with tincture of iodine. The material so obtained consists of a mixture of tissue juices and blood cells and this should be immediately inoculated into tubes containing suitable culture media. All apparatus employed in the procedure should be sterile.

If cultures are made upon the N.N.N. medium (see page 175) the blood or other material which is to be cultured for any of the leishmania should be inoculated into the water of condensation at the bottom of the culture tubes as it is in this fluid that the leishmania develop in greatest number. If leishmania are present in the inoculated material in large numbers flagellate forms will usually be found in the cultures within two to three days but usually, especially in the case of *Leishmania donovani*, cultures will not show these forms before seven to ten days have passed, and if the inoculated material has contained few organisms the cultures may not prove positive for several weeks.

Because of this fact, the cultures should be examined at least every other day and not discarded as negative until at least a month has passed from the time of inoculation. The utmost precautions should be taken to prevent bacterial contamination in securing material from the cultures for microscopic examination as such contamination will result in the destruction of any leishmania that may be present. A platinum loop should be used for obtaining material for examination from the cultures as in bacteriological technique. If negative, a small amount of the water of condensation may be secured with a capillary pipette and examined.

The *temperature* at which the cultures should be kept is very important for these parasites grow best at a lower temperature than do the bacteria. Rogers was the first to show that to obtain good cultures of *Leishmania donovani* it was essential that the inoculated cultures be kept in an incubator at a temperature between 22° C. and 25° C. (71.6° F. and 77° F.) and his observations have been confirmed by all subsequent observers. To secure the best results the cultures should be kept at a temperature as near 22° C. (71.6° F.) as is possible and should not be kept at 37° C. (98.6° F.) as in the case of bacterial cultures. Failure to observe this rule in attempting to culture the leishmania has led to many failures.

The two most important factors in the successful cultivation of the leishmania are the observance of aseptic precautions in the manipulation of the cultures and the temperature at which they are kept.

MAINTENANCE OF CULTURES

While the maintenance of cultures of the leishmania is not necessary in the diagnosis of the infections caused by them, it is essential in the differentiation of the three species by serological reactions, for the study of the life-cycle of these organisms, and their pathogenicity for various animals. It is possible to maintain cultures for long periods of time by transfer at suitable intervals and in a suitable manner. It has already been noted that Wenyon was successful in maintaining *Leishmania donovani* in successive cultures for over fifteen years and Nicolle (1925) was able to keep *Leishmania tropica* viable in cultures for over fourteen years, during which time it was subcultured three hundred and eighty-four times, and a strain of *Leishmania donovani* for nearly fourteen years, during which time it was subcultured three hundred and ninety-five times.

A most favorable medium for maintaining the leishmania in cultures is the N.N.N. medium (see page 175) and transfers should be made about every two weeks, although longer periods of time between transfers may be followed, provided the water of condensation in the tubes is not allowed to evaporate and the culture medium to become dry. In making the transfers it is best to use a glass capillary pipette

which has been sterilized. This is inserted into the water of condensation and as much of the material allowed to flow into the pipette by capillary attraction as possible, after which it is expelled into the water of condensation in the uninoculated tube of culture medium. As the leishmania not only develop in the water of condensation but also upon the surface of the medium above the water of condensation, it is well to secure material from this portion of the medium by gently scraping the surface with a large platinum loop and transferring the material so obtained to new culture media, thus increasing the chances of securing a successful transfer.

In making transfers the utmost precautions should be taken to prevent bacterial contamination for if this occurs the leishmania will fail to develop after a few days and the strain will be lost.

MORPHOLOGY OF LEISHMANIA IN CULTURES

It has already been stated (page 149) that the morphology of the various species of leishmania in cultures differs greatly from the morphology of these organisms as observed in the blood or tissues of man in that in cultures flagellate forms develop similar in morphology to those observed in insects, as the sand-flies and bed-bugs. These forms are never observed in man but in cultures the typical leishmania forms that are observed in man may also occur, so that it is possible to study the forms of these parasites which occur in both the vertebrate and invertebrate cycles of development in artificial culture media.

As the morphology of all of the forms in both man and cultures has already been described (page 147) it is unnecessary to repeat the description, to which the reader is referred.

VIRULENCE IN CULTURES

The cultural forms of the various species of leishmania are virulent for susceptible animals for varying periods of time but may eventually become avirulent. The virulence is not increased by repeated passage through cultures but is decreased more or less rapidly, varying with different strains of the same species and with different species. Cultures are useful for the experimental infection of susceptible animals and much of our knowledge of the etiology and epidemiology of kala-azar, Oriental sore and espundia has been acquired through the use of cultures of the respective causative leishmania in producing infection in susceptible animals.

CULTURE METHODS AND MEDIA

Various culture methods and media have been devised for the cultivation of the leishmania and the most valuable of these will be considered. The media will be found arranged alphabetically and will be critically discussed after the description of the method of preparation and use.

Kligler's Medium.—This very simple medium consists of a mixture of dextrose agar and normal salt solution as follows:

Dextrose agar, 1 per cent	10 parts
Normal salt solution (0.85 per cent)	90 parts

The medium is sterilized in the autoclave and should have a reaction between pH 7 and pH 7.6.

After sterilization, from 5 to 10 cc. of the medium is placed in culture tubes, cooled to 48° to 50° C. (118° to 122° F.), and to each tube is added fresh sterile rabbit blood in the proportion of 1 part of blood to 10 parts of the medium. The tubes are rapidly rotated in order to thoroughly mix the agar and blood. They are then slanted upon ice, in order to secure the water of condensation, and tested for sterility by incubation at 37° C. (98.6° F.) for twenty-four hours, after which they are ready for use. Kligler states that flagellate forms of *Leishmania tropica* begin to appear in the cultures in from three to four days but sometimes not before the end of a week.

Remarks.—This medium, which is really a modification of the N.N.N. medium, was found by Kligler (1924), to give very excellent results in the cultivation of *Leishmania tropica* and it would probably give as good results in the cultivation of *Leishmania donovani* and *Leishmania brasiliensis.*

Kriukova's Modification of the N.N.N. Medium.—This medium was introduced by Kriukova (1942) for the cultivation of leishmania. It is prepared as follows:

Into a 250 cc. glass-stoppered bottle are placed 150 cc. normal saline and 6 cc. of chemically pure hydrochloric acid. To this is added gradually 50 cc. of defibrinated rabbit, sheep or human blood, shaking all the time. When the mixture becomes blackish in color add 1 gm. pepsin and place in a water-bath at 50° to 55° C. (122° to 131° F.) for three to four hours, shaking from time to time. The bottle is then placed in an incubator at 37° C. (98.6° F.) for one to two days. When digestion is completed the mixture has a thick consistency and it should then be neutralized by adding 20 per cent NaOH, employing phenol red and cresol red as indicators. When the reaction is pH 7.0 to 7.2 add 0.5 cc. of 0.25 per cent chloroform, stopper with a glass stopper smeared with vaseline and allow to stand for two to three days, the mixture is then stored in ampules and can be thus preserved for more than one year.

In using this medium test tubes containing 1.5 cc. of hypotonic agar are removed from the sterilizer and 0.5 cc. of the above digest is added to each tube after the tube has been cooled to about 95° C. (203° F.). The medium is then slanted, allowed to cool, and 0.5 cc. sterile 1 per cent peptone solution is added to each tube.

Remarks.—The leishmania of all species grow excellently upon this medium, even in primary cultures from animals or lesions, and are

numerous by the fourth day after inoculation. Because this medium is economical and because of the fact that the stored digested blood mixture can be used for several months, it is a very simple and practical method of cultivation.

Lourie's Medium.—This medium has been recommended by Lourie (1946) as a simple and practical one for the cultivation of *Leishmania donovani* and is composed of the following:

Plain powdered agar	0 3 gm.
Defibrinated rabbit blood	15 0 cc.
Normal saline	100 0 cc.

Place the required amount of agar and normal saline in a flask and heat over a Bunsen burner to the boiling point. Allow the solution to boil for about two minutes and then place the flask in a hot water bath containing a thermometer. When the temperature of the water in the bath has fallen to between 45° and 50° C. (113° to 122° F.) the flask is removed and the required amount of defibrinated rabbit blood is added and the flask shaken gently until the blood is distributed evenly. The mixture is then poured into ordinary test tubes in requisite amounts, from 3 to 15 cc. according to the size of the tube.

Remarks.—This medium is simpler than the N.N.N. medium to prepare, the tubes do not need to be sealed and, as the medium is moist, it is not necessary that water of condensation be maintained. It can be kept in the refrigerator for months and then used and it is equally useful in the diagnosis of kala-azar and for the maintenance of cultures of the leishmania. A very rich growth of the organism occurs in the upper portion of the tubes containing 15 cc. of the medium and, for diagnosis, the cultures should be kept at room temperature, *i. e.*, between 15° and 21° C. (59° and 71.6° F.). According to Lourie this medium has been employed for years at the Warrington Yorke Department of Chemotherapy for the diagnosis of *Leishmania donovani* and for the maintenance of cultures of the same. Subcultures can be made every two or three months.

Noguchi and Lindenberg's Medium.—This medium, described by Noguchi and Lindenberg (1925) is identical with the medium elaborated by Noguchi for the cultivation of spirochetes (leptospira). It has the following formula:

Salt solution (0.9 per cent)	800 parts
Fresh rabbit blood serum	100 parts
Nutrient agar, 2 per cent, pH 7.2	100 parts
Rabbit hemoglobin solution, made by laking 1 part of defibrinated rabbit blood in 3 parts of distilled water	10 to 20 parts

The rabbit serum is obtained by bleeding the animals (see page 103), allowing the blood to clot, and passing the serum through a bacteria-proof filter. The serum is then added to the mixture of nutrient agar and saline solution, thoroughly mixed, and the hemoglobin added in

the required amount. The medium is tubed, the tubes plugged with cotton, and tested for sterility by incubating at 37° C. (98.6° F.) for twenty-four hours, when it is ready for use.

Remarks.—Noguchi and Lindenberg obtained excellent results with this medium in the cultivation of *Leishmania brasiliensis* and others have obtained similar results with it in the cultivation of *Leishmania donovani* and *Leishmania tropica*. The leishmania grow upon the surface of this medium as a grayish-white scum, smooth and moist in appearance above, and uneven and indefinite below, extending into the culture medium as deep as 4 mm. in some cultures. Unless bacterial contamination has occurred there is no change in the appearance of the medium and no odor is perceptible. Usually the growth becomes visible in from four to five days as a surface cloudiness which gradually increases in intensity and depth. Examination shows that the growth consists of multitudes of active flagellated forms of the leishmania under cultivation. Sometimes the growth is delayed and will not be visible for two weeks, or even more, so that the cultures should not be discarded as negative until after three weeks. The best results are obtained if the cultures are kept at a temperature of 18° to 20° C. (64.4° to 68° F.) but development will occur at ordinary room temperature or at 26° C. (78.8° F.). This medium may be stored in the ice-box for two or more months and still be serviceable.

Noguchi and Lindenberg claimed for this medium the following advantages: Simplicity of preparation; stability at ice-box temperatures; the luxuriant growth obtained, and the long period of life of the cultures without transfer. As regards the latter claim, subcultures have been successful after a month and transfers do not have to be made more often than every two or three weeks if the cultures are kept at the temperatures mentioned.

The Novy-MacNeal-Nicolle, or N.N.N. Medium.—This medium was first described by Novy and MacNeal (1904) and modified by Nicolle (1908), so that it is usually known as the N.N.N. medium. It consists of a mixture of agar and rabbit's blood prepared in the following manner:

Culture tubes are filled to about one-third capacity with plain agar having the following formula:

Agar	14 grams
Sodium chloride (sea salt)	6 grams
Distilled water	900 cc.

The agar is dissolved in the water which has been brought to the boiling point and is then distributed in the test tubes, plugged, and sterilized in the autoclave as usual. After sterilization, if cultures are not to be made at once, the tubes are placed in storage, and used as needed.

If cultures are to be inoculated at once, the tubes of agar are cooled to 48° to 50° C. (118.4° to 122° F.) and to each tube of agar is added

about one-third of its volume of sterile defibrinated rabbit's blood, which should be thoroughly mixed with the agar by rapidly rotating the tube, after which the tubes are placed in a slanting position and allowed to cool. This is best done by placing the tubes upon ice, as in this way much more water of condensation is obtained in the tubes, which is important, as most of the leishmania develop in the water of condensation. After cooling the tubes are placed in the incubator and kept at a temperature of 37° C. (98.6° F.) for twenty-four hours, in order to determine sterility, before they are inoculated. During this period the tubes should be kept in an upright position as this position is favorable for the collection of the water of condensation at the bottom of the tube.

If the agar slants are not to be inoculated as soon as prepared they should be stored in a dark, moist place until required. At this time the agar should be melted, cooled, the rabbit blood added, the tubes slanted on ice and incubated for sterility, as already described.

There are several *methods of collecting and adding the rabbit's blood* to the agar tubes. The most simple method, and one that is useful if only a few blood-agar tubes are required, is to allow the blood to drop into each tube from the rabbit's ear until from 15 to 20 drops have been added to each tube of melted agar. The animal should be held by an assistant, the ear shaved over the marginal vein, the skin in the same area sterilized with tincture of iodine, and allowed to dry. When dried, the ear in this area, both above and below and upon the margin, is coated with a layer of hot melted paraffin which should be so thin over the marginal vein as to render the vein visible beneath it. A pressure clip is now applied over the vein near the base of the ear and an incision is made in the vein with a scalpel and the blood allowed to drop from the ear into the culture tubes.

If a considerable number of tubes of the N.N.N. medium are required the blood may be obtained directly from the heart of the living rabbit by means of puncturing the heart through the thorax, using a needle with a large bore attached to a sterilized syringe by a short piece of rubber tubing which has also been sterilized. The blood so obtained is then ejected in required amounts directly into the tubes containing the melted agar, before it has time to clot, or placed in a sterilized flask or bottle containing glass beads, shaken to defibrinate it, and then distributed to the agar tubes with a pipette. Clotting may be prevented by rinsing the syringe out with citrate solution before the blood is drawn. This method requires some practice in bleeding the animals through the heart but when properly done has but little effect upon the rabbits and they can be used over and over again as a source of blood for this culture medium.

If there is no objection to destroying the rabbit it may be chloroformed, the heart exposed, and the blood be collected by puncturing the organ with the needle of a large glass syringe, in the same manner

as described for puncture through the thorax. A larger amount of blood is obtained by this method but the animal is sacrificed.

After the blood-agar tubes have been incubated to assure sterility they may be inoculated at once or stored in the ice-box until required. If stored, rubber caps should be placed over the cotton plugs to prevent evaporation anddrying of the medium. Kept in this way the medium is frequently useful for many weeks but if the surface becomes very dry and the water of condensation has evaporated, the medium will be useless and the tubes should be discarded.

Remarks.—The Novy-MacNeal-Nicolle or N.N.N. medium has been more largely used for the cultivation of the species of leishmania infecting man than any other and the consensus of opinion at the present time is that it is one of the best mediums known for diagnostic purposes. When properly prepared and used it gives a very high percentage of positive results in the culture of the peripheral blood or material obtained by splenic, liver or sternal puncture and it is the medium that the writer would recommend for routine diagnostic work.

Rogers Medium.—It was in this medium that Rogers (1904) was successful in being the first to cultivate *Leishmania donovani.*

The medium consists of normal saline (0.85 per cent), to which is added 8 per cent of sodium citrate, dissolving the latter thoroughly. If the resulting solution is not acid in reaction it should be made slightly so by the addition of citric acid, after which it is sterilized in the autoclave and tubed in 1 cc. amounts in small culture tubes.

In inoculating the tubes from 0.5 cc. to 1 cc. of the peripheral blood of the patient or a small amount of splenic or liver pulp, or bone-marrow or material obtained from lesions, is placed in each tube, under strict aseptic precautions. If the examination of the peripheral blood has resulted negatively for *Leishmania donovani* it is best to inoculate each tube with 1 cc. of the patient's blood. The cultures should be kept at a temperature between 20° and 22° C. (68° to 71.6° F.) and should never be allowed to exceed 25° C. (77° F.).

Remarks.—This medium is not as useful as the other media that have been described and is seldom employed for diagnostic purposes. However, its great simplicity warrants its use under circumstances in which the other media cannot be prepared or obtained, and while the results may not be as uniformly successful as with the more complicated methods, positive results are sometimes obtained in cases in which the microscopic examination of material has proven negative.

Senekji(e)'s Media.—The following media have been recommended by Senekji(e) (1939–1941) for the cultivations of the species of leishmania and for *Trypanosoma cruzi.*

1. *Blood Culture Medium.*—Bacto-beef extract, 50 parts, is dissolved in 1000 parts of distilled water and heated at 50° C. for one hour, then at 80° C. (176° F.) for five minutes. The solution is then passed through filter paper and the following added: neo-peptone, 20 parts;

agar (nobel), 20 parts; and NaCl (c.p.), 5 parts. Adjust the reaction to pH 7.2 to 7.4 and autoclave at 15 pounds pressure for twenty minutes. When cool, add defibrinated rabbit's blood to make 10 per cent of the medium.

2. *Egg-Liver Extract Medium.*—Four eggs are emulsified in a sterile flask containing glass beads, and then add 50 cc. of the following solution: 0.02 gm. each of NaCl, $CaCl_2$, KCl and $NaHCO_3$, in 100 cc. distilled water. Emulsify, filter and make into test tube slants or store in a flask. Sterilize at 90° C. (194° F.) at 10 pounds pressure for ten minutes and then reduce the pressure very slowly in order to avoid the formation of bubbles in the medium. Then overlay the solidified medium in the tubes with sterile 0.5 per cent liver extract in normal saline and incubate the tubes for twenty-four hours to test sterility.

Remarks.—According to the author, the leishmanias and *Trypanosoma cruzi* form luxuriant colonies on the blood medium (Medium No. 1) and contaminated cultures may be purified by making frequent transfers from the blood medium to the egg-liver extract medium. The writer has had no experience with these media but they are undoubtedly good media for the cultivation of the organism mentioned.

Young and Van Sant's Method of Cultivation.—This method was found by Young and Van Sant (1923) to be very efficient in culturing *Leishmania donovani.* It is as follows:

1. Collect 10 cc. of blood from a vein in the arm of the patient in a sterile syringe containing 2 cc. of Locke's solution, and expel it immediately into a flask containing 70 cc. of the same solution.

2. Mix the blood and Locke solution thoroughly and divide between two 50 cc. centrifuge tubes.

3. Centrifugalize at about 750 revolutions per minute for five minutes or until the red blood cells are lightly packed at the bottom of the centrifuge tubes.

4. Decant the cloudy supernatant fluid from each tube into another centrifuge tube and centrifugalize for five minutes at about 1375 revolutions per minute.

5. With the sediment inoculate tubes of the N.N.N. medium made with rabbit blood and having a reaction of about pH 7.6, and incubate at 22° C. (71.6° F.). The utmost care should be taken throughout the process to maintain sterility.

Remarks.—As stated, Young and Van Sant found this method to be most efficient in culturing *Leishmania donovani* from the peripheral blood of patients suffering from kala-azar, who had not been treated, recovering the organism in this manner from over 90 per cent of such patients, and others have confirmed their results.

Choice of Culture Media in the Diagnosis of the Leishmania.—All of the culture media that have been described have given good results in the hands of those who have had an extended experience with them and here, as in all laboratory procedures, experience with a particular

method or medium is essential for success. A comparatively poor method or medium, in the hands of one experienced in its use, will give better results than the best method or medium in inexperienced hands, and this applies especially in the cultivation of the leishmania. In the hands of the writer the Novy-MacNeal-Nicolle, or N.N.N. medium, has given the best results but it is apparently not as efficient as some of the more recent methods that have been described. The Noguchi-Lindenberg medium is especially useful for the maintenance of cultures as transfers do not have to be made frequently, the growth obtained is profuse, and the medium is comparatively easy to prepare and keeps well. For diagnostic purposes it is less useful than the N.N.N. medium, as the flagellates do not develop in it as quickly as in the latter medium. The results that have been obtained with Lourie's medium would indicate that it is a most excellent one which has been thoroughly tested for many years both in the diagnosis of *Leishmania donovani* and for the maintenance of cultures of the same.

Whatever method and medium is selected the maintenance of sterility throughout the process of preparation, collection of the material to be cultured, and the inoculation of the medium with such material, is absolutely necessary. If the culture medium becomes contaminated with bacteria or yeasts, the leishmania will not develop and the tubes so contaminated should be at once discarded. In making transfers it is well to employ a capillary pipette rather than a platinum loop, as the amount of material that can be transferred with the latter is sometimes too small to insure success.

agar (nobel), 20 parts; and NaCl (c.p.), 5 parts. Adjust the reaction to pH 7.2 to 7.4 and autoclave at 15 pounds pressure for twenty minutes. When cool, add defibrinated rabbit's blood to make 10 per cent of the medium.

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CHAPTER XI

SEROLOGICAL DIAGNOSIS OF THE LEISHMANIASES

SEROLOGICAL DIAGNOSIS OF KALA-AZAR—SEROLOGICAL DIAGNOSIS OF ORIENTAL SORE AND ESPUNDIA—SEROLOGICAL DIFFERENTIATION OF THE LEISHMANIA

Introduction.—Several serological tests have been evolved for the diagnosis of the infections of man caused by the leishmania in which both specific and non-specific substances are employed and it is rather curious that the tests employing non-specific substances as reagents have given far better results than those in which specific antigens have been used. At the present time serological methods of diagnosis are confined almost entirely to the diagnosis of kala-azar and have been found of practical value only in infections caused by *Leishmania donovani*. For convenience of description the various serological tests which have been used in the diagnosis of kala-azar will be considered under two headings: tests employing specific antigens and tests employing non-specific substances.

In addition to the use of serological tests in the diagnosis of infections caused by *Leishmania donovani* such tests are also employed in the *differentiation* of the various species of *Leishmania* and are of great value and importance in this respect, as *Leishmania donovani, Leishmania tropica* and *Leishmania brasiliensis* are indistinguishable morphologically and can only be differentiated by their serological reactions, when growing in cultures or when dissociated from the clinical picture of which they are respectively the cause.

1. **Tests Employing Specific Antigens.**—The inoculation of susceptible animals with *Leishmania donovani* is followed by the appearance in their blood serum of specific agglutinins, precipitins and complement-fixing bodies but up to the present time it has been impossible to devise a *diagnostic* test with which results of *practical* value can be obtained by taking advantage of this fact. While this is true it does not prove that it is impossible to develop such a specific diagnostic test and the following short review is of value in demonstrating what has already been accomplished in this direction.

Agglutination Reactions.—In 1911, di Cristina obtained specific agglutinins in the blood serum of rabbits immunized to *Leishmania donovani* and in 1912, Caronia demonstrated agglutinins in the blood serum of a child immunized with killed cultures of this leishmania. In 1913, Caronia obtained agglutination reactions in the blood of 4 of 5 children suffering from kala-azar in dilutions varying from 1 to 20 to 1 to 30, but stated that such reactions were of no diagnostic importance

because of the low dilutions. In 1914, Archibald called attention to the fact that normal human blood serum when added to a normal saline solution containing cultural forms of *Leishmania donovani* caused loss of motility and finally degeneration of such forms, and he was also unable to demonstrate agglutinins or precipitins in the blood serum of kala-azar patients. From these observations, as well as those of others, it is evident that the possibility of developing an agglutination or precipitation test for the diagnosis of kala-azar is remote.

Complement Fixation Reactions.—Several investigators have obtained promising results with specific complement fixation tests in the diagnosis of the leishmaniases.

Bramachari's Complement Fixation Test.—In this test Bramachari (1917) employed an antigen consisting of the supernatant fluid obtained after heating 1 part of freshly ground kala-azar spleen and 3 parts of a mixture of equal parts of alcohol (absolute) and normal salt solution (0.85 per cent). Using this antigen he obtained positive results in 6 of 8 cases of kala-azar but others were unable to confirm his results.

Hindle, Hou and Patton's Complement Fixation Test.—These investigators (1926) reported good results with the use of an antigen prepared by triturating the spleen of a heavily infected hamster in 5 cc. of normal saline solution, filtering through muslin, and adding 0.5 per cent phenol and 1 per cent glycerin. The patient's blood serum was inactivated by heating it at 56° C. (132.8° F.) for one-half hour and adding to 0.2 cc. of the inactivated serum, 0.8 cc. of the antigenic solution.

With this antigen the investigators mentioned obtained complement fixation in 19 of 24 patients suffering from kala-azar, and partial fixation in the remaining 5. False positive reactions were obtained in 5 syphilitic patients but negative reactions in 49 patients suffering from other diseases.

Remarks.—The results obtained by Hindle, Hou and Patton are the most favorable that have been reported and it is surprising that others have not repeated their work and endeavored to improve upon the technique of so promising a test.

Complement fixation has not been used to any extent in the diagnosis of infections with *Leishmania tropica* or *Leishmania brasiliensis* although Moses (1919) obtained 80 per cent of positive results in 41 cases of espundia, employing an antigen prepared by extracting cultures of the flagellate form in distilled water.

2. **Tests Employing Non-Specific Substances.**—While diagnostic tests, employing specific antigens, have not as yet proven practicable, the same cannot be said of serological tests employing non-specific substances as antigens. Several tests of this type have been evolved for the diagnosis of kala-azar which are of very great value while others of lesser value are sometimes useful in the diagnosis of this infection. The following are the most important of these tests:

Brahmachari's Serum-Globulin Test.—In 1917, Brahmachari found that the addition of distilled water to the blood serum of patients suffering from kala-azar produced a white precipitate of a globulin-like substance and later he devised the so-called serum-globulin test for the diagnosis of this condition.

This test may be performed in two ways, either by placing 0.5 to 1 cc. of the patient's blood serum in a small test tube and adding two to three times this amount of distilled water, or as a *ring-test,* by first diluting the blood serum ten to twenty times with normal saline solution (0.85 per cent) in a test tube and then carefully placing distilled water upon the top of the diluted serum by means of a 1 cc. glass pipette.

If the test is performed by mixing the blood serum and distilled water in the test tube the reaction consists in the appearance of a profuse whitish precipitate in the mixture, while if it is performed as a ring-test the reaction consists in the appearance of a white ring at the junction of the mixture of blood serum and salt solution and the distilled water.

Remarks.– Brahmachari found this test to be specific for kala-azar, provided the amounts of distilled water recommended were not exceeded but that if larger amounts of distilled water were added the test proved positive in other infections and diseases. He obtained positive results in 20 cases of kala-azar and negative results in 12 controls from patients suffering from other diseases. His results were confirmed by Milio (1922), who obtained positive results with this test in 14 cases of kala-azar and negative results in 14 cases of other conditions, but other investigators have not obtained as favorable results and regard this test as of limited diagnostic value because of the difficulty of interpreting the reactions and because it has given positive results in other infections, especially those accompanied by marked anemia. The test, as modified by Sia (see page 185) is apparently of considerable suggestive value in diagnosis.

Chopra's Antimony Test.—In 1927, Chopra, das Gupta and David called attention to the fact that the blood serum of kala-azar patients, when mixed with solutions containing antimony derivatives, especially those containing urea, was followed by the rapid appearance of a profuse precipitate, and that this precipitate did not occur when the solutions were brought into contact with the blood serum of individuals suffering from other diseases or in normal individuals. They used a solution of urea-stibamine as the antimony solution and, as the result of their observations, suggested that this phenomenon would be useful in the diagnosis of kala-azar. Their work was confirmed by Napier (1927) and the technique of the test, as described by these investigators, has been considerably modified from time to time. Chopra (1936) describes the technique now employed as follows:

"Whole serum and serum diluted 1 in 10 with distilled water are put in miniature test-tubes (2½ to 3 inches long, made by sealing one end of a piece of glass tubing 4 to 5 mm. in diameter) with a capillary pipette. A 4 per cent solution of urea-stibamine made with distilled water is then slowly run along the side of the tubes. A heavy coarsely-flocculent precipitate forms when the antimony solution comes in contact with the serum. Tartar emetic does not give this reaction but stibosan does. During treatment with pentavalent antimony compounds the test becomes uncertain at first and then, after the full course of treatment, it is negative. In very early cases the 1 in 10 dilution of the serum may give negative results and whole serum should be employed."

Remarks.—Chopra claims, that with this test 88.2 per cent of cases of kala-azar give a positive reaction and that a correct diagnosis can be made in this percentage of cases with this test alone. Napier (1928), in comparing the relative value of this test and the aldehyde test (see below) states that the antimony test is the more delicate but is not absolutely accurate. It is undoubtedly a very valuable diagnostic test and has proven of great value in the diagnosis of infections with *Leishmania donovani.*

Chopra's Finger-prick Antimony Test.—Chopra (1936) thus describes this very simple and excellent test for the diagnosis of kala-azar:

"A drop of blood from the cleaned finger is received into a small test-tube (⅜ inch internal diameter and 2 inches long) containing 0.25 cc. of a 2 per cent solution of potassium oxalate. The corpuscles are allowed to settle down and the test is performed with the supernatant fluid. A 4 per cent solution of urea-stibamine is added in exactly the same way as in the serum test. (Described above.) The reading should be taken from five to ten minutes after mixing the solution."

Remarks.—Chopra claims, that with this very simple test no less than 86 per cent of cases of kala-azar may be diagnosed. The positive reaction gradually disappears under treatment and is negative when the patient is cured.

It is important to remember, in using either of the Chopra tests, that the mere appearance of a slight granular precipitate should not be regarded as diagnostic. The precipitate should be flocculent in character and large in amount, except in very early cases of kala-azar, when it may be much smaller in amount but still flocculent in appearance.

Napier's Aldehyde Tests.—This test, also known as the formol-gel test, was first described as a diagnostic test for kala-azar by Colonel Spackman, I.M.S. but Napier (1921) described the technique in detail and the test is usually known by his name.

The technique of this test is exceedingly simple, consisting in adding 1 drop of commercial formalin (40 per cent formaldehyde) to 1 cc. of the patient's blood serum in a small test tube. The mixture is then well shaken and allowed to stand at room temperature. If it becomes solid and opaque within from three to thirty minutes the reaction is regarded as positive and diagnostic of infection with *Leishmania donovani.* A control of normal serum should also be used.

The blood serum for the test is obtained by withdrawing from a vein in the forearm 5 cc. of blood which is immediately ejected into a very small Petri dish and allowed to clot, after which 1 cc. of the clear serum is pipetted off and used in the test.

The aldehyde test has proven of great value in the diagnosis of kala-azar but certain precautions should be observed in the interpretation of the reactions obtained with this test. The *time* in which the reaction occurs is very important as the test may be positive in other diseases if too long a time elapses before solidification of the mixture of blood serum and formalin. In well-advanced and chronic cases of kala-azar the blood serum may become solidified and opaque within three minutes and usually within twenty minutes, while in early cases the characteristic changes never appear or are delayed for hours, thus rendering the test useless, as similar changes may occur in the blood serum of patients suffering from malaria, syphilis, leprosy and some other infections after an hour or more. The *time limit* of a diagnostic reaction has been generally accepted as varying from one minute to half an hour at longest and reactions obtained after that time should be regarded as only suggestive or confirmatory of the results of other diagnostic procedures.

The *character* of the reaction is also of great importance. In cases of kala-azar solidification of the mixture of blood serum and formalin usually commences within one minute and may be complete within three to five minutes, so that the test tube containing it can be inverted without the contents being disturbed. In from three to twenty minutes the mixture begins to show a whitish opalescence which finally becomes opaque and at the end of the diagnostic period the mixture appears as a solid white opaque column in the test tube. Mere solidification is of no diagnostic importance unless accompanied by the changes in the appearance of the mixture. In very early cases of kala-azar solidification does not occur, the mixture consisting of a fluid of milky appearance. The test is not usually positive in early cases of kala-azar before the end of the third month and sometimes not until five months have elapsed after the appearance of symptoms.

The delayed reactions that often occur with this test are a source of diagnostic difficulty and, as already stated, cannot be accepted as conclusive unless other corroborative evidence is present. In 1923, Napier suggested the following classification of the reactions:

\+ + + +, serum solid and completely opaque in 20 minutes, positive.
\+ + +, serum solid and completely opaque in 2 hours, positive.
\+ +, serum solid and completely opaque in 24 hours, positive.

It is the consensus of opinion at the present time that the above classification of the positive results obtained with this test is too liberal and that a diagnosis of kala-azar should not be made upon the results of the test *alone* unless the serum is solidified and opaque within a half hour.

Remarks.—The results that have been obtained with this test in the diagnosis of kala-azar have varied with different observers but all are agreed that this is the most generally useful of the tests that have been described. Napier (1922) tested 150 individuals suspected of suffering from kala-azar and compared the results with those obtained by puncture of the spleen and found that they corresponded in 98 per cent of the individuals. Struthers and Ch'un (1924) tested 141 patients, in China, in 140 of whom *Leishmania donovani* had been demonstrated, and found that the aldehyde test was positive in 98 per cent and the globulin test in 93 per cent. On the other hand, Elwes, Menon and Ramakrishnan (1924), found that this test was negative in no less than 23.4 per cent of 81 cases of kala-azar in which *Leishmania donovani* had been demonstrated. However, despite the divergent results which have been obtained with the test, Rogers and Megaw (1939) state that "The aldehyde test has largely replaced spleen puncture in the large kala-azar clinic of the Calcutta School of Tropical Medicine, where it was worked out by Napier, as it is simpler and safer." Napier (1943) states that the test is positive in 70 per cent of cases at that institution and that a positive reaction is diagnostic of kala-azar in India, as in over 20,000 tests with the blood serum of persons suffering from other infectious and diseases, he obtained only about one dozen positive reactions.

Ray's Test.—In 1921, Ray noted that a clear solution of the blood could not be obtained with the blood of kala-azar patients when tested with the Gower hemoglobinometer and that this was due to some change in the blood serum preventing complete hemolysis of the red blood corpuscles. Based upon this observation he suggested the following diagnostic test for this infection.

Two drops of blood are placed in a small test tube and 20 drops of distilled water are added. If the reaction is positive the mixture becomes turbid after standing and a flocculent precipitate appeared.

Remarks.—Ray obtained positive results with this test in 55 cases of kala-azar and negative results with the blood serum of normal individuals and those suffering from other diseases. The test is really a modification of Brahmachari's test (see page 182), and has been largely replaced in practice by the modification recommended by Sia, which follows:

Sia's Euglobulin Precipitation Test.—This very simple test, devised by Sia (1921–1924) is as follows:

Using a Sahli hemoglobinometer pipette, 20 cu. mm. of the patient's blood is drawn and expelled into a small test tube containing 0.6 cc. of distilled water, and gently shaken until thoroughly mixed. The mixture is then examined at once and at intervals of fifteen minutes up to one hour and the reaction read. An immediate turbidity of the mixture indicates a positive reaction while the appearance of a precipitate within fifteen minutes is called a ++++ reaction, within thirty

minutes, a +++ reaction, within forty-five minutes a ++ reaction, and in one hour or longer, a + reaction. If the blood is negative hemolysis occurs and there is no visible precipitate.

Remarks.—This test has been extensively used in localities where kala-azar is prevalent, as a *presumptive* test of infection with *Leishmania donovani* but it is not as accurate a test as either the aldehyde or antimony tests and cannot be relied upon to the same extent for diagnostic purposes. The results should be confirmed by the use of these tests and the demonstration of the parasite, if possible.

Complement Fixation Test of Dharmendra, Bose and Sen Gupta.—Within recent years a complement fixation test for the diagnosis of kala-azar based upon the researches of Witebsky, Klingenstein and Kuhn; Greval, Sen Gupta and Das (1938); Napier, Sen Gupta and Sen (1942); Sen Gupta (1944) and Dharmendra, Bose and Sen Gupta (1946), in which antigens prepared from pure cultures of the tubercle bacillus or other acid-fast bacilli are used, has given excellent results in the hands of those who have employed it for diagnosis. At the present time an antigen described by Dharmendra, Bose and Sen Gupta (1946), in which the Kedrowsky bacillus is employed instead of the tubercle bacillus would appear to give the best results.

The technique of this test follows:

Preparation of the Antigen.—Kedrowsky's acid-fast bacillus is grown in glycerin broth in flasks each containing 200 cc. the pH of the medium being adjusted to pH 7.3 to 7.4 The growth is collected by filtration through paper, washed free from the medium, using three changes of sterile distilled water and two of alcohol, and then placed in a desiccator and dried *in vacuo.*

The dried bacillary substance is extracted with 90 per cent alcohol in a flask fitted with a reflux condenser for three hours over a water-bath, kept overnight in a refrigerator, and the residue filtered and again dried *in vacuo.* This alcohol insoluble portion is extracted with pyridine for eight hours at 135° C. to 140° C., in a Soxhlet apparatus over a glycerin bath, then transferred to an evaporating dish placed over a hot-air bath at 70° C. and evaporated to dryness. This pyridine soluble fraction is now extracted with acetone in a Soxhlet apparatus for two hours over a water-bath. The fraction insoluble in acetone is dried and this is used as the antigen. It can be stored in the ice-box in ampules for use later, if desired.

Method of Use.—In using this antigen, 0.1 gm. is dissolved in 10 cc. of benzol; prepare a 1 per cent alcoholic solution of lecithin and evaporate 5 cc. of the solution to dryness and take up the residue by adding to it the 10 cc. benzol solution of the antigen. The mixture is then filtered through paper, any loss of the benzol solution being made up by the addition of the amount lost in filtration. This mixture is the lecithinized benzolic solution of the antigen which is used in the test.

The prepared antigenic solution should be kept in an incubator at 37° C. for ten to fifteen days before it is used and just before use it should be titrated for hemolytic, anti-complementary and complement fixation properties.

The following methods of titration of the antigen, complement, and performing the test are given as follows by Sen Gupta. (Indian Medical Gazette, page 396, August, 1945.)

1. *Titration of the Antigen.*—0.1 cc. of the benzolic solution of the antigen is taken up with a 1 cc. pipette and spread on a glass mortar and allowed to dry completely. 0.2 cc. of normal saline is then added to the residue in the mortar and a suspension is made by trituration. This forms the starting point for making the dilutions 1/10, 1/20 upwards.

In a series of test tubes labelled to show the dilutions, 1/20, 1/30, 1/40, etc., up to 1/90, put 0.25 cc. of the corresponding antigen dilution, 0.25 cc. of 1/25 dilution of an inactivated negative serum, and 0.25 cc. of complement dilution containing 1 MHD. Mix by gently shaking the tubes. Keep at room temperature for half an hour, then at 37° C. for half an hour. Add 0.25 cc. of sensitized cells to each tube, mix, incubate at 37° C. for half an hour; read for hemolysis. The strongest dilution of the antigen permitting a complete hemolysis corresponds to the anticomplementary activity short of 1 MHD of complement, and this is the dilution of the antigen to be used in the test proper.

This dilution is then tested for hemolytic activity by incubating 0.75 cc. of this dilution with 0.25 cc. of sensitized cell suspension. The antigen is usually found to be non-hemolytic.

The power of fixation of the antigen is tested by putting up comparative tests with a series of known sera using a known antigen and the new antigen.

Supposing that 1/60 is the working dilution arrived at by titration, in order to make up the dilution needed for the test proper, a total of 12 cc. of saline will be required for the residue left after evaporation of 0.1 cc. of the benzolic solution.

The titration of the antigen is not repeated daily, but the method of titration of the complement and the putting up of an antigen control during the test proper, serves to check the correctness of the antigen titre.

2. *Titration of the Complement.*—A 1 in 10 dilution of the complement in normal saline is first prepared. From this 1/20, 1/30, 1/40, 1/50, . . . 1/120 dilutions are made. Two rows of tubes are set up on a rack to correspond to these dilutions of the complement. Two tubes, one behind the other, correspond to each dilution. In the tubes of the first row are placed 0.25 cc. of the corresponding complement dilution and 0.5 cc. of normal saline. In the tubes of the back row are put 0.25 cc. each of the corresponding complement dilution, 1/25 dilution of inactivated negative serum, and the working dilution of the antigen. The tubes are kept at room temperature for half an hour, then incubated at 37° C. for half an hour. Then 0.25 cc. of sensitized sheep cells is added to each tube and these are incubated at 37° C. for half an hour, after which the minimum hemolytic dose (MHD) of the complement is read. This is taken as the highest dilution at which there is complete lysis of the sheep cells. The tubes in the two rows usually show a similar degree of lysis. If there is a disagreement in the titre as indicated in the two rows, this is usually due to the defect in the antigen dilution, and can be rectified by again titrating the antigen against 1 MHD of complement in the presence of 1/25 dilution of a negative serum, and using the antigen in the new working dilution thus obtained.

3. *The Test Proper.*—The serum to be tested is inactivated to destroy the complement by heating to 55° C. for half an hour in a water-bath. A 1 in 25 dilution is prepared by mixing 0.1 cc. of serum with 2.4 cc. of normal saline.

The antigen dilution is made up as described previously (*vide supra*).

Two strengths of complement dilution are made so as to contain 2 MHD and 5 MHD in a volume. Suppose 1 in 80 is the titre (MHD) of the complement, a 1 in 40 solution of the complement will contain 2 MHD and a 1 in 16 solution 5 MHD in a volume.

Three tubes, placed one behind the other, are required for testing each serum; the first tube is for serum control with 2 MHD of complement, the second and the third for the test with the antigen and 2 and 5 MHD of complement respectively.

Remarks.—According to those who have used this test extensively in the diagnosis of kala-azar it gives a positive reaction in between 90 to 95 per cent of the cases of kala-azar and this reaction is obtained earlier than that with any other method, as the aldehyde and Chopra tests, being positive as early as the third and fourth weeks after infection, thus, this test gives an earlier diagnosis than any other diagnostic method with the exception of sternal puncture. It is undoubtedly a very valuable diagnostic test and will probably, in time, largely replace the other serological tests now in use.

SEROLOGICAL TESTS FOR THE DIFFERENTIATION OF THE SPECIES OF LEISHMANIA

It has already been stated that morphologically, *Leishmania donovani, L. tropica* and *L. brasiliensis* cannot be distinguished from one another and the only methods, aside from animal inoculations, which are available for this purpose are certain serological tests, especially agglutination and complement fixation. The work of Nicolle and Manceaux (1909), Pavoni (1914), Noguchi (1924–1926), Wagener and Koch (1926) and others, has demonstrated that it is possible, by means of immunological tests, to differentiate between the species of leishmania causing disease in man and also to differentiate these species from species of flagellates belonging to the genus *Herpetomonas.*

Agglutination Tests.—By immunizing rabbits by intravenous injections of living cultures of the three species of leishmania, *i. e., Leishmania donovani, L. tropica* and *L. brasiliensis,* and employing their blood sera in agglutination tests, it has been shown that while there is a group-reaction in low dilutions, in higher dilutions it is possible to separate these species in many instances, although different strains of the same species sometimes yield confusing results.

The *technique* of the agglutination tests for the differentiation of the species of leishmania is similar to that employed in bacteriological agglutination tests, suspensions of the cultured leishmania being used as antigens and various dilutions of the immune sera being employed

to determine their strength in agglutinins. The results obtained by Wagener and Koch (1926) in the differentiation of the leishmania by agglutination tests, are apparently the best that have been reported and their technique follows:

Rabbits were immunized by giving them three intravenous injections of the washed living flagellates from cultures, three and five days apart. Five days after the last intravenous injection the animals were bled, the serum allowed to separate, and this immune serum was employed in making the tests.

The *antigen* was made by washing the flagellates obtained from twelve day old cultures of the various leishmania upon the N.N.N. medium and suspending the washed organisms in 0.9 per cent salt solution, adding a drop of toluol. The suspension should be of such a consistency that newsprint may be read through it and should be kept in the ice-box.

In making the tests 0.1 cc. of the antigen is added to 1 cc. of the various dilutions of the anti-serum, the tubes incubated at 56° C. (132.8° F.) for two hours, the results read, and the tubes then placed in the ice-box overnight, when the results are again read.

Remarks.—The authors mentioned obtained clear-cut results in the differentiation of *Leishmania donovani, L. tropica* and *L. brasiliensis* with this method of agglutination and similar results have been obtained by other observers using other methods of performing the test. At the present time cross-agglutination tests are the only available methods of differentiating the various species of leishmania with any degree of certainty and these are frequently unsuccessful.

A slide agglutination test has been described by Senekjie and Lewis (1944) which they state is efficient in a rapid diagnosis of visceral, cutaneous and muco-cutaneous leishmanias. The antigen is obtained by immunization of rabbits with killed cultures of *Leishmania donovani, Leishmania tropica* and *Leishmania brasiliensis* and the usual slide agglutination technique is employed. They state that the homologous organism is agglutinated to a high titer, but that co-agglutinins are present in low titer with the heterologous organisms. They also state that higher titers are obtained with kala-azar than with either cutaneous or mucocutaneous leishmaniasis. It is too early to assess the real value of this method as a practical test for the diagnosis of the various forms of leishmaniasis but the results obtained by the authors certainly indicate that it should be given a thorough trial.

Other Diagnostic Tests.—Wagener's Intradermal Test.—In 1923, Wagener reported the results she obtained with an intradermal test in the diagnosis of kala-azar and Oriental sore. The technique of the test is as follows:

Cultures of *Leishmania donovani* and *Leishmania tropica* are prepared

on the N.N.N. medium, and the material obtained by pipetting off the water of condensation containing the flagellates is washed in normal saline, centrifugalized, and the sediment which contains the organisms diluted with Coca's solution until it contains 2,000,000 leishmania per cubic centimeter, after which the mixture is covered with toluol and allowed to stand at room temperature for three days. At the end of this period the suspensions are centrifugalized and the supernatant fluid is pipetted into sterile tubes and furnishes the test antigen. (The Coca solution used in making the antigen has the following formula: NaCl, 0.5 per cent; $NaHCO_3$, 0.05 per cent and Phenol, 0.4 per cent.)

Rabbits immunized to *Leishmania donovani* and *Leishmania tropica* were tested by injecting 0.2 cc. of the antigen into the skin and it was found that after twenty-four hours a small, red papule developed at the site of the injection which was most marked at the end of forty-eight hours, and persisted for five days before it disappeared. Negative results were obtained in normal rabbits and following the intradermal injection of Coca's fluid used in the dilution.

Montenegro (1926) using a similar antigen prepared from cultures of *Leishmania brasiliensis*, and the same technique, obtained 32 positive reactions in 37 patients suffering from espundia, or South American leishmaniasis (muco-cutaneous leishmaniasis), doubtful results in 3 supposedly normal controls and negative results in 33 of the total 36 normal individuals tested.

Gomes (1939) has recently confirmed Montenegro's results, using as antigen washed flagellates suspended in normal saline to which were added four parts of phenol per thousand of suspension, and kept at 2° to 4° C. (35.6° to 39.2° F.) for ten days before use. He obtained 97.5 per cent of positive reactions in 120 individuals suffering from espundia whom he tested, and 2.5 per cent of weak, but suggestive, reactions. He found the test gave negative reactions in other diseases and in Chagas' disease.

Dostrovsky and Sagher's Intracutaneous Test. — In 1935, Dostrovsky demonstrated positive skin reactions on intracutaneous injections of a vaccine containing 100,000 *Leishmania tropica* per 0.1 c.cm. The intracutaneous test has since been reported upon by Dostrovsky and Sagher (1946), who obtained a high percentage of positive results with it in the diagnosis of cutaneous leishmaniasis. They studied 434 cases of cutaneous leishmaniasis in Palestine and found that while the microscopic demonstration of *Leishmania tropica* was positive in only 66.6 per cent of clinically verified cases the skin test was positive in 91.6 per cent of 251 cases tested while it gave a non-specific result in 6.3 per cent in a control series of 144 cases. If these results are confirmed it would appear that the intracutaneous test is a very valuable diagnostic one especially as it is positive within a week after inoculation as shown by experiments upon human volunteers.

Remarks.—The results so far obtained would indicate that an intradermal diagnostic test for infection with leishmania might be of clinical value but as such tests demand much technical ability and experience in reading the reactions and give no better results than the simple tests of Chopra and of Napier, so far as kala-azar is concerned, it is doubtful if they would be of greater clinical value in the diagnosis of infections with *Leishmania donovani,* although they might prove of much value in the diagnosis of Oriental sore and espundia. Further research is needed before a correct evaluation of intradermal tests is possible.

CHAPTER XII

INOCULATION OF ANIMALS WITH LEISHMANIA—CRITIQUE OF DIAGNOSTIC METHODS FOR THE LEISHMANIASES

THE INOCULATION OF ANIMALS IN THE STUDY OF THE LEISHMANIAS

IT is never necessary to rely upon the results of animal inoculations in the diagnosis of any of the leishmaniases but if it is desired to study the distribution and effect of these parasites upon susceptible animals resort must be had to the experimental use of such animals.

Susceptibility of Animals to Infection.—Various animals differ greatly in their susceptibility to experimental infection with the various species of leishmania and different strains of the same species of leishmania differ in their effects upon certain animals. The only animals that have been found to be *naturally infected* with the leishmania are dogs and cats, and the latter are very rarely infected. In regions where kala-azar, Oriental sore and espundia are endemic, natural infections in dogs are usually common and these animals are believed to be sources of infection for man by most authorities.

Experimental infections with *Leishmania donovani* have been produced in hamsters, dogs, monkeys, white mice, rats, guinea-pigs, rabbits, the jackal, gerboa and gerbil; with *Leishmania tropica* in hamsters, dogs, white mice, rats, guinea-pigs, gerbils and monkeys; and with *Leishmania brasiliensis* in the baboon, hamsters, dogs, cats, guinea-pigs, monkeys, rats, mice and rabbits. For experimental purposes hamsters are now generally employed because of their greater susceptibility to infection but if these animals are not available dogs are most suitable for inoculation.

Methods of Inoculation.—Animals may be inoculated intraperitoneally, subcutaneously, or, in the case of hamsters, by intratesticular inoculation and by feeding infective material. The material inoculated may consist of macerated tissue obtained by crushing infected spleen, liver, or bone-marrow or of cultures of the leishmania, mixed with a suitable amount of normal saline solution (0.85 per cent). Large amounts of the infective material should be inoculated or fed in order to secure good results and even then the animals may not become infected. Much better results are obtained after inoculation with material containing the forms of the leishmania observed in man than after inoculation with cultures and cultures that have been maintained for a long time are usually non-infective. Intravenous inoculation or inoculation directly into the liver may be used in good sized dogs with

success. Subcutaneous inoculation of dogs with *Leishmania donovani*, while generally followed by a general infection, is rarely evidenced by the occurrence of subcutaneous nodules containing the organisms, while subcutaneous inoculation of *Leishmania tropica* and *Leishmania brasiliensis* is always followed by cutaneous lesions. In the case of smaller animals, as mice, rats and guinea-pigs, intraperitoneal inoculation of *Leishmania tropica* has been followed by a general infection. Experimental infections with all of the *leishmania* develop very slowly and are difficult to maintain as the parasites gradually lose their virulence upon passage from animal to animal but Chu and Zia (1940) have shown that hamsters can be readily infected by intratesticular inoculation and that the leishmania may be detected in material obtained by testicular puncture as early as the fourth day after the inoculation. They also found that intratesticular inoculation of rabbits is followed by a local infection which may persist for as long as one month although it is impossible to produce a general infection in these animals.

Until quite recently none of the animals used in experimental research upon the leishmaniases were satisfactory because of their resistance to experimental infection, but in 1924, Smyly and Young showed that the hamster (*Cricetulus griseus*) is very susceptible to infection, and the observations of Young, Smyly and Brown, and of many others have confirmed their findings and today hamsters are used in practically all research upon the leishmania infections which requires the use of animals.

CRITIQUE OF DIAGNOSTIC METHODS

The diagnosis of infections with *Leishmania donovani*, *Leishmania tropica* and *Leishmania brasiliensis* should, if possible, be based upon the demonstration of the respective organism and in Oriental sore and espundia such a demonstration is essential but in the diagnosis of kala-azar the consensus of opinion today appears to be that if the aldehyde, antimony or complement fixation tests are strongly positive a diagnosis may be based upon these tests alone and they have replaced other diagnostic methods in many kala-azar clinics.

The laboratory methods that are available for the *diagnosis* of *kala-azar* are sternal, gland, liver and splenic puncture; examination of the peripheral blood; culture of the blood or material obtained by puncture, and the various serological tests, *i. e.*, complement fixation, the aldehyde and antimony tests and the globulin test.

Sternal puncture has largely replaced splenic, gland and liver puncture and while splenic puncture remains probably the most accurate of the puncture methods it is dangerous in inexperienced hands and is little used at present. In the opinion of many authorities the percentage of positive results obtained by the microscopic examination

of material obtained by sternal puncture is almost as high as when splenic puncture is employed.

Chung (1938) has employed sternal puncture in 300 patients and was able to demonstrate the leishmania in 171 of these by this method. He states that it is the best method for the diagnosis of kala-azar in hospitals, dispensaries and health stations, as it is simple, safe, and can be repeated if necessary.

The value of the *microscopic examination of the peripheral blood* in kala-azar apparently depends upon the patience of the observer. Those who have been willing to examine scores of blood smears have obtained a high percentage of positive results but the examination of only a few smears frequently results negatively. This method of diagnosis is time consuming but should certainly be given a trial before resort is had to splenic, liver or sternal puncture.

Gland puncture is a very efficient method of diagnosing kala-azar and the examination of material so obtained results in a very high percentage of positive results in infected individuals. If enlarged or palpable glands be present this method should always be tried as the technique is simple and the results are as good as are obtained from the puncture of the liver or sternum. Unfortunately, in many cases of kala-azar the glands may not be enlarged at the time of examination of the patient and this method of diagnosis cannot be employed.

The *culture of material* obtained by puncture of the spleen, liver, sternum or glands sometimes results in the demonstration of *Leishmania donovani* when other methods have failed but if the material obtained by puncture of the spleen, sternal puncture or gland puncture has proven negative upon microscopic examination for the leishmania the chances that the cultivation of such material will result in the demonstration of the parasite are practically *nil*. However, culture of the peripheral blood has frequently given positive results when the microscopic examination of this fluid has been negative for the parasite.

The *serological tests* employed in the diagnosis of kala-azar are all useful but the most valuable are the complement fixation test, the aldehyde test of Napier and the antimony tests of Chopra. It has already been stated that the aldehyde test is regarded by many observers as practically diagnostic of kala-azar when strongly positive and the antimony tests are almost as diagnostic when positive in the opinion of most observers. The technique of these tests is so simple that both tests may be employed at the same time and if both give positive reactions the evidence is that much more conclusive in favor of infection with *Leishmania donovani*. If only one of these tests can be employed the aldehyde test should be preferred as, in the opinion of those with experience, it is more reliable than are the antimony tests, but in justice to the latter it may be said that there is but little difference in the results obtained with these various tests as shown by published statistics.

The complement fixation test (see page 186) is even more accurate than either the aldehyde or antimony tests and as a positive reaction is obtained with this test much earlier in the infection than with the aldehyde or antimony tests, it is to be preferred for diagnosis if facilities are available.

The *Sia globulin* test is useful as a presumptive test in the diagnosis of kala-azar, if the reaction is positive, but it has given positive results in other diseases and should not be relied upon alone and the results should be confirmed with the aldehyde or antimony tests.

Procedure for the Diagnosis of Kala-azar.—The following procedure is suggested as a useful one in the diagnosis of kala-azar:

The blood of the suspected individual is first tested with the complement fixation test or with the Napier aldehyde test, and, if convenient, with the Chopra antimony test. If the results are positive and it is impracticable to make other tests, the diagnosis of kala-azar may be made and treatment instituted. If the results are negative it does not prove that infection with *Leishmania donovani* is not present, as these tests are not positive for several weeks after infection and in a very small proportion of cases are negative even after several months. When a negative result is obtained and suspicious symptoms or physical signs are present resort should be had to other laboratory methods of diagnosis.

Whenever possible, the positive results obtained with any of these tests should be confirmed by the demonstration of *Leishmania donovani* in the blood or tissues of the suspected individual, the blood being first examined and cultured, followed by the examination of material obtained from either gland, sternal, liver or splenic puncture if necessary.

Draw 5 or 10 cc. of blood from a vein in the arm and make blood smears directly and after centrifugalization for the microscopic examination, and at the same time inoculate several tubes of the selected media with blood that has not been centrifugalized. Of the culture media that have been recommended it is believed that the Lourie, Kriukova and N.N.N. media are the best. The flagellates usually begin to appear in the cultures in from three to seven days and the cultures should be examined at intervals of two or three days and for a period of one month before a negative result is accepted.

If the results of the complement fixation, aldehyde or antimony tests have been negative and blood examination and cultures have also resulted negatively, sternal puncture should be performed, or, if enlarged glands are present, gland puncture should be done. In many kala-azar diagnostic laboratories sternal puncture alone is relied upon in making a diagnosis as the demonstration of the leishmania in the material so obtained is almost invariably possible if the patient is suffering from the disease.

If the material obtained by puncture is negative some of it should

be cultured upon one of the culture media described but it is seldom that such cultures will be positive if the microscopic examination of the material has given negative results.

Procedure for the Diagnosis of Post-Kala-azar Dermal Leishmaniasis.—In the macular and erythematous lesions of this condition *Leishmania donovani* is present but it is rarely possible to demonstrate them in material obtained from the lesions by puncture. If nodules are present the microscopic examination of material obtained from such lesions is almost invariably successful in demonstrating the parasites.

A small nodule should be excised and the cut surface smeared upon microscopic slides. The smears so obtained should be stained with the Wright, Giemsa, or other suitable stain, and numerous leishmania will usually be seen within endothelial cells or lying free in the preparations. Large nodules may be punctured and the material so obtained smeared upon microscopic slides, stained and examined.

Procedure for the Diagnosis of Oriental Sore and Espundia.—The methods of laboratory diagnosis available for the *diagnosis of Oriental sore and espundia* are the microscopic examination of material obtained from the lesions and cultures of such material. Sometimes the microscopic examination or culture of blood obtained from the immediate vicinity of the lesions will result in the demonstration of the causative parasite but the results of the examination of material obtained from the lesions are so much better that blood examinations are seldom used in diagnosis.

The following procedure is suggested as a routine one in the laboratory diagnosis of these infections:

Material obtained from the base or tumefied border of the lesion by puncture with a glass capillary pipette or a syringe through a small incision in the skin, is spread upon microscopic slides and stained and examined. At the same time some of the material so obtained is ejected into tubes of the selected culture medium and the cultures kept and examined at two- or three-day intervals for a period of at least one month. If the smears of the material show *Leishmania tropica* or *Leishmania brasiliensis* the cultures need not be made but if the smears are negative cultures should always be made before a negative diagnosis is accepted.

PART III

Laboratory Diagnosis of the Trypanosomiases: West African or Gambian Sleeping Sickness. East African or Rhodesian Sleeping Sickness. Chagas' Disease

CHAPTER XIII

THE LABORATORY DIAGNOSIS OF THE TRYPANOSOMIASES

MORPHOLOGY OF TRYPANOSOMA GAMBIENSE—MORPHOLOGY OF TRYPANOSOMA RHODESIENSE—MORPHOLOGY OF TRYPANOSOMA CRUZI

THERE are three important diseases of man caused by flagellate parasites belonging to the Genus *Trypanosoma*. These are West African sleeping sickness, sometimes called Gambian sleeping sickness; East African or Rhodesian sleeping sickness; and Chagas' disease, of South and Central America. The cause of West African sleeping sickness is *Trypanosoma gambiense*, of East African sleeping sickness, *Trypanosoma rhodesiense* and of Chagas' disease, *Trypanosoma cruzi*.

While the clinical pictures presented by these infections are quite characteristic their diagnosis should be based upon the demonstration of the respective trypanosome in the blood or tissues of the infected individual and thus a knowledge of the morphology of these organisms is essential. In the living, unstained condition, the morphology is not characteristic and stained preparations are necessary for diagnosis.

GENERAL MORPHOLOGY OF TRYPANOSOMES

The trypanosomes causing disease in man have two cycles of development in their life history, one in man and the other in an invertebrate host, in each of which there are variations in morphology, which will be discussed in considering the various species.

A typical trypanosome is a colorless, serpentine-like, spindle-shaped body having a more or less rounded posterior end and a sharp pointed anterior end. At or near the center of the body is a large round nucleus, called the trophonucleus, while at the posterior end there is a kinetoplast, consisting of a minute blepharoplast and a parabasal body.

Arising from the blepharoplast is a long, delicate flagellum which runs forward, forming the edge of an undulating membrane, becoming a free flagellum at the anterior end of the body. The undulating membrane originates near the blepharoplast and terminates by merging with the body of the trypanosome at the anterior end. The cytoplasm of trypanosomes usually contains volutin granules and may contain vacuoles.

The variations from the typical morphology noted above which occur in the trypanosomes causing disease in man will be described in the discussion of the morphology of each species.

1. **Morphology of Trypanosoma Gambiense.**—In *unstained preparations Trypanosoma gambiense* is a colorless, spindle-shaped body varying greatly in length and breadth and having a very rapid motion, so rapid that it cannot be distinguished well in freshly drawn blood, the

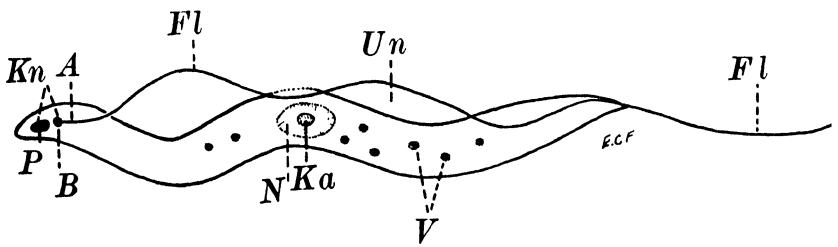


FIG. 26.—Diagram illustrating the morphology of a typical trypanosome. *Kn*, Kinetoplast; *P*, parabasal body; *B*, blepharoplast; *A*, axoneme; *Fl*, flagellum; *Un*, undulating membrane; *N*, nucleus; *Ka*, karyosome; *V*, volutin granules. (After Faust, in Craig and Faust's Clinical Parasitology.)

only evidence of its presence being the spinning motion of the red blood corpuscles caused by its passage between them. After the blood has been drawn for some time the motility of the trypanosome is much reduced and the undulating membrane and flagellum may be distinguished. While a diagnosis of sleeping sickness may be most easily made by the demonstration of the trypanosome in unstained preparations it should be confirmed by stained preparations if possible.

In *stained preparations* three forms of this trypanosome occur in the blood, a long, very slender form having a free flagellum; a shorter, thicker form having a free flagellum; and a short broad form without a flagellum, the long slender form being most numerous in the blood of man and the short broad form least numerous. In staining the trypanosomes some modification of the Romanowsky stain is used and with all of these modifications the staining reactions are the same, the cytoplasm staining a pale blue and the nucleus, kineoplast and flagellum a bright pink to deep violet color, according to the intensity of staining.

In stained preparations *Trypanosoma gambiense* measures from 14 to 33 microns in length and from 1.5 to 3.5 microns in breadth. The cytoplasm stains a blue color, deeper in some places than in others,

and very deep staining, almost black granules are often present, as well as small vacuoles. The nucleus, usually situated at or near the center of the body, is stained a pink or red color and is oval or round in shape. In preparations stained with the hematoxylin stains the nucleus presents a nuclear membrane and a large central karyosome but in preparations stained with Wright's stain, or other modifications of the Romanowsky stain, the nucleus consists of a pink or red, oval or round, mass of a more or less granular structure. At, or near, the posterior end of the body there is a structure known as the kinetoplast, usually appearing as a small oval or rod-shaped deep red or violet body, but which, in very carefully stained preparations is seen to consist of a very minute round granule which stains a deep red or violet, the blepharoplast, and a second oval or spherical body, situated very close to the blepharoplast, also staining a deep red or violet, the parabasal body. Arising from the blepharoplast there is a flagellum, appearing as a delicate pink or red filament which extends toward the anterior end, forming the edge of the undulating membrane, and terminating at the anterior end as a free flagellum of considerable length. Although the blepharoplast and parabasal body are connected by a very delicate filament, this is rarely seen in stained preparations and the blepharoplast and parabasal appear to be separate. The portion of the flagellum arising from the blepharoplast and extending to the root of the undulating membrane is known as the axoneme. In the short, broad forms of *Trypanosoma gambiense* a flagellum is not present but these forms are rarely observed in the peripheral blood, except in experimental animals. In the long, slender forms of this trypanosome the free flagellum is from one-quarter to one-half the total length of the organism, while in the intermediate type the flagellum may terminate at the anterior end or project as a short spinelike structure.

The undulating membrane commences at the posterior end near the blepharoplast on the lateral aspect of the body and stains a pale blue. The membrane is broad and thrown into folds and extends to the anterior, or flagellate, end of the organism, where it merges into the cytoplasm of the body. It is not striated and myonemes are not present. The border of this membrane is colored red because it is formed by the flagellum. The number of folds in the undulating membrane varies with the length of this structure but in the long, slender forms there are three or four folds and sometimes more.

Dividing forms may be observed in the blood and cerebrospinal fluid of cases of sleeping sickness, in which two nuclei and two kinetoplasts are present, and in which two flagella may be distinguished. Division is initiated by the division of the blepharoplast followed by that of the nucleus and finally by the longitudinal splitting of the body into two trypanosomes. The flagellum does not divide but a new flagellum is developed from one of the two blepharoplasts produced by division.

All stages of division may be observed from organisms showing the division of the blepharoplast to those in which division has almost been completed, the two organisms being held together by more or less of the cytoplasm. The dividing organisms may be equal or unequal in size.

Trypanosoma gambiense, besides living in man, has a life-cycle in flies belonging to the Genus *Glossina*, especially *Glossina palpalis*.

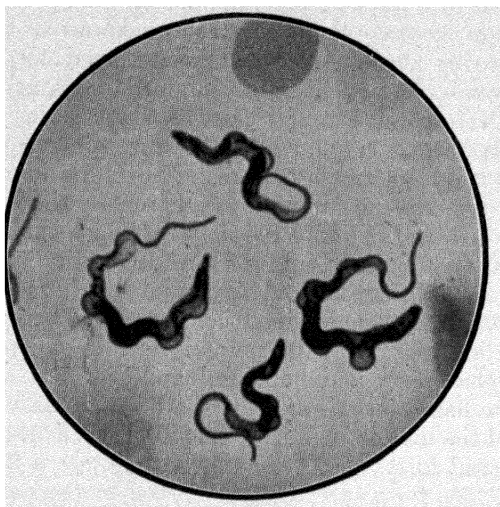


FIG. 27.—*Trypanosoma gambiense*. In blood smear. (Photomicrograph by Novy.)

The trypanosomes multiply in the intestinal tract and finally reach the salivary glands, from which they are ejected into the wound made by the fly in biting, thus initiating the cycle in man. The morphology of the trypanosome in the flies varies greatly from that observed in man and the reader is referred to works upon parasitology for a description of these forms.

2. **Morphology of Trypanosoma Rhodesiense.**—In the blood and cerebrospinal fluid of man the morphology of *Trypanosoma rhodesiense* is identical with that of *Trypanosoma gambiense* and a differential diagnosis based upon morphology alone is impossible. In the blood of experimental animals, however, forms frequently occur which are known as "posterior nuclear forms," in which the nucleus, instead of being centrally located is located at the posterior end of the body, either close to or behind the kinetoplast. For some time these forms were supposed to be diagnostic of this species but later observations have shown that similar forms may occur in experimental animals infected with *Trypanosoma gambiense*. At the present time it is the consensus of opinion that while posterior nuclear forms may occur in animals infected with *Trypanosoma gambiense* their occurrence is so

rare that if such forms are found the diagnosis of *Trypanosoma rhodesiense* is justifiable.

At the present time we have no method of differentiating *Trypanosoma gambiense* and *Trypanosoma rhodesiense*, as observed in man, upon morphology alone and resort must be had to serological methods or the inoculation of susceptible animals.

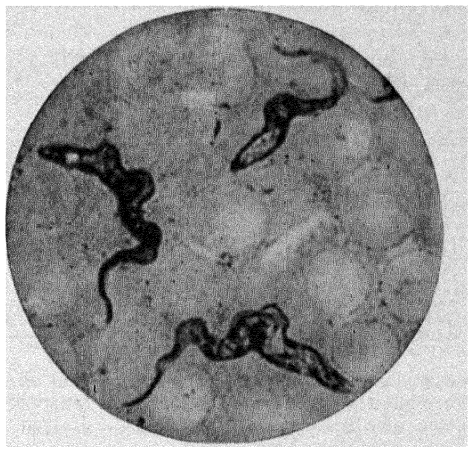


FIG. 28.—*Trypanosoma rhodesiense.* Stained with Wright's stain. Forms in peripheral blood film. × 2000. (Army Medical Museum Collection. After Craig, in Parasitic Protozoa of Man, courtesy of J. B. Lippincott Company.)

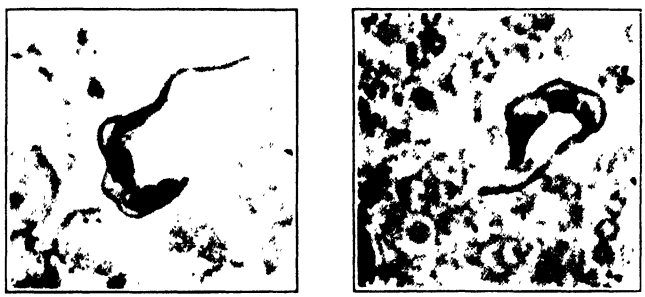
3. **Morphology of Trypanosoma Cruzi.**—This trypanosome, sometimes called *Schizotrypanum cruzi*, differs markedly in its morphology from *Trypanosoma gambiense* and *Trypanosoma rhodesiense* in both man and experimental animals. It lives in the blood and in the endothelial and tissue cells of man and experimental animals and also goes through a life-cycle in several bugs belonging to different genera, most frequently in triatomid bugs belonging to several genera. The reader is referred to Craig and Faust's "Clinical Parasitology" for a discussion of the life-cycle in the transmitting bugs, morphology of the trypanosome in the intestine of the bugs, the species of bugs concerned, and the methods of transmission.

In man, *Trypanosoma cruzi* occurs in the blood as a typical trypanosome but in the endothelial cells and tissue cells it occurs as a leishmania so far as morphology is concerned.

The morphology of *Trypanosoma cruzi* in the blood of man differs considerably from that of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* and a differential diagnosis may be made upon the morphology alone. The staining reactions are the same, the nucleus, kinetoplast and flagellum coloring a bright pink, red or violet, while the cytoplasm stains blue.

In the blood the trypanosomes measure about 20 microns in length

and two forms occur, a long, slender one and a short, broad one. In stained preparations the trypanosomes very frequently assume a C-shape and this is very characteristic of this species. Both the long and broad forms have a centrally placed nucleus and a kinetoplast, situated at the posterior end which stains a very deep violet or almost black color and which, especially in the broad forms, is very large, often causing a distinct bulge in the posterior end of the trypanosome. This



A B

FIG. 29.—*Trypanosoma cruzi*. Note large kinetoplast and short, broad shape. *A*, Form with large kinetoplast; *B*, C-shaped form commonly observed in films of peripheral blood. (Photomicrographs by Mazza in "Pub. No. 22. Universidad de Buenos Aires, 1935.")

large kinetoplast, or blepharoplast, as it is usually called, is very characteristic of *Trypanosoma cruzi*. It is usually oval in shape but may be round, and in the longer forms it may be much smaller, resembling the kinetoplast of *Trypanosoma gambiense* and *Trypanosoma rhodesiense*. In most stained preparations the entire kinetoplast stains as a solid mass but in very well stained preparations it is seen to be composed of a blepharoplast and parabasal body, as in other trypanosomes.

The undulating membrane arises near the kinetoplast and extends forward to the anterior end of the body where it merges into the cytoplasm. The border of this membrane is formed by the flagellum which originates in the blepharoplast and extends forward, becoming a free flagellum at the anterior end of the organism. In this species the undulating membrane is not as well developed as in *Trypanosoma gambiense* and *Trypanosoma rhodesiense*, being narrow and having only one to three convolutions. In the shorter, broad forms the undulating membrane may be almost invisible. The flagellum measures about one-third the total length of the body of the trypanosome.

The nucleus is round or oval in shape and is situated at the center of the body. In preparations stained with modifications of the Romanowsky stain the nucleus appears to be composed of a compact mass of pink or red granules but when the hematoxylin stains are employed it is seen to have a well-defined nuclear membrane and a large central karyosome.

Multiplication forms of this trypanosome are never observed in the blood as in infections with *Trypanosoma gambiense* or *Trypanosoma rhodesiense*, as division of the organism occurs after it has entered the host cells. The absence of dividing forms in the blood is a very important differential feature between this species and the other species of trypanosomes occurring in man.

The morphology of the forms of this trypanosome occurring in the endothelial and tissue cells of the body differs greatly from that of the forms occurring in the blood. In the latter fluid the morphology is that of a typical trypanosome while in the endothelial and tissue cells the morphology is that of a typical leishmania, so that from the morphological standpoint this species is a combination of trypanosome and leishmania, and multiplication occurs only in the leishmania stage of the life-cycle. The trypanosome forms from the blood invade the host cells, round up, lose the undulating membrane and flagellum, and divide by binary fission, thus producing leishmania forms which, in turn, also divide by binary fission until the host cell becomes merely a cystlike body packed with the leishmania forms. Eventually these are liberated by the destruction of the host cell and, in the opinion of some authorities, immediately invade other cells or, in the opinion of others, develop into the typical trypanosome forms and again appear in the peripheral blood, eventually invading new host cells and becoming leishmania forms. In chick embryo cultures Romana and Meyer (1942) were able to follow the complete development of *Trypanosoma cruzi* including binary fission of the leishmania forms, transformation into crithidial forms and finally into trypanosomes. In some instances the trypanosome forms appeared to develop directly from the leishmania forms without passing through a crithidial stage.

The leishmania forms within the host cells appear as small oval or round bodies, measuring from 1.5 to 4 or 5 microns in diameter, the invaded cell having the nucleus pushed aside if the organisms do not entirely fill the cell, or the cell may appear as a limiting membrane surrounding the mass of organisms, all trace of its structure having disappeared. The individual leishmania form consists of a round or oval body, the cytoplasm of which may stain a pale blue and containing a spherical pink or dark red nucleus and a rodlike kinetoplast which stains a deep violet or almost black color. The invaded cells occur in clumps in the viscera or in the striated heart muscles and such collections of invaded cells can be easily seen with low power objectives.

During the development of this trypanosome in the tissue and endothelial cells, or perhaps after the liberation of the leishmania forms from the host cells leptomonas and crithidial forms occur before the development of the typical trypanosome as observed in the peripheral blood. For a description of the morphology of such forms the reader is referred to works upon protozoölogy or parasitology as these forms are of no diagnostic value in the differentiation of *Trypanosoma cruzi* from either *Trypanosoma gambiense* or *Trypansoma rhodesiense*.

The morphology of *Trypanosoma cruzi* in the transmitting insects is similar to that of other trypanosomes in insect hosts, short and long crithidial forms being present as well as the typical trypanosome forms. The reader is referred to works upon parasitology and protozoölogy for a detailed description of the forms observed in the transmitting insects.

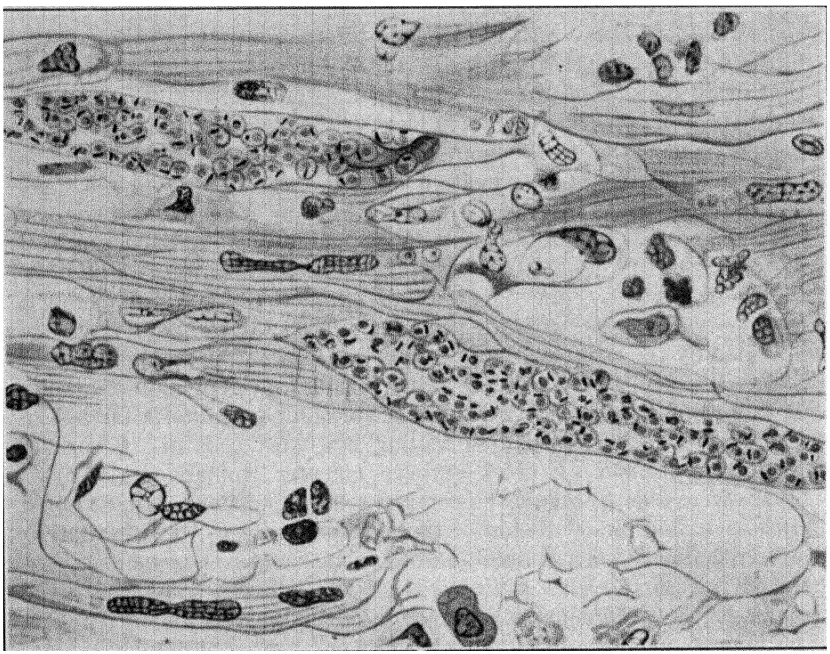


FIG. 30.—*Trypanosoma cruzi.* Leishmania-like forms in the heart muscle. (After Chagas.) (From Wenyon's "Protozoölogy," courtesy of Baillière, Tindall & Cox.)

In the diagnosis of Chagas' disease it should be remembered that the trypanosome is frequently apparently absent from the peripheral blood and that it invades the endothelial cells of the lymphatics and capillaries and, especially, the cells of the cardiac muscle. It also occurs in the striated muscles, the bone-marrow, liver, spleen, lymphatic glands, mesenteric lymph nodes, testes, ovaries, the nervous system, intestinal mucous membrane, the cells of the epidermis and the histiocytes of the cutaneous tissue.

The characteristic features of the morphology of *Trypanosoma cruzi* during the trypanosome stage in the peripheral blood are the smaller size of the organism, the C-shape often assumed, the narrow undulating membrane and the very large kinetoplast (usually called blepharoplast). The absence of dividing forms of the trypanosome in the peripheral blood is also a valuable differential feature.

CHAPTER XIV

METHODS OF COLLECTING AND PREPARING MATERIAL FOR EXAMINATION FOR THE TRYPANOSOMIASES

COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION OF TRYPANOSOMA GAMBIENSE AND TRYPANOSOMA RHODESIENSE—COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION FOR TRYPANOSOMA CRUZI—STAINING METHODS

THE diagnosis of African sleeping sickness and of Chagas' disease should be based, if possible, upon the demonstration of the causative trypanosomes and, except in very rare instances, it is possible to demonstrate these trypanosomes in individuals infected with them. In describing the methods of collecting and preparing material for examination it is convenient to consider the methods available for laboratory diagnosis of sleeping sickness and Chagas' disease separately, as certain methods are available for sleeping sickness that cannot be employed in the diagnosis of Chagas' disease.

1. COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION FOR TRYPANOSOMA GAMBIENSE AND TRYPANOSOMA RHODESIENSE CAUSING SLEEPING SICKNESS OF AFRICA

In the laboratory diagnosis of both Gambian and Rhodesian sleeping sickness certain features of the clinical course of these infections should be considered. In both types of sleeping sickness the trypanosomes are present in the peripheral blood in greatest numbers during the febrile periods and before marked involvement of the central nervous system, while after such involvement has occurred the trypanosomes will be found in the cerebrospinal fluid. They may be demonstrated in gland juice obtained by puncture if enlarged glands are present and this method of demonstration is most successful during the acute, febrile stage of the infections. Thus, the examination of the blood and of gland juice should be the laboratory methods employed during the early acute stage of these infections and examination of the cerebrospinal fluid during the late stage, after the involvement of the central nervous system. From the standpoint of success in treatment it is most essential that an early diagnosis be made in these infections, before the involvement of the central nervous system, as treatment is very successful during the acute stages but very much less so later. It follows that the examination of the blood and of gland juice, if the

lymphatic glands are enlarged, is of first importance, and as the trypanosomes are often few in number in the blood several preparations should be examined before a negative report is accepted.

Collection and Preparation of Blood.—The blood is collected from the finger or lobe of the ear, a drop being placed upon a microscopic slide, covered with a cover-slip, and examined at once. If trypanosomes are present they may be seen swimming about among the corpuscles and thus a rapid and accurate diagnosis can be made without the aid of staining. However, if the examination of several drops of blood results negatively stained preparations should be made in the same manner as recommended in the diagnosis of the leishmaniases (see pages 152 and 157). It is best to examine the unstained preparations a few minutes after they are made as the movements of the trypansomes in freshly drawn blood are so rapid that it is impossible to distinguish them.

In preparing blood smears for staining they should be made a little thicker than in preparing such smears for examination for the malaria plasmodia, should be air-dried, and stained at once or placed in an insect proof slide-box for future staining. If such smears are negative, thick blood smears should be prepared (see page 153).

Owing to the small number of trypanosomes frequently present in the peripheral blood in both types of African sleeping sickness it is sometimes necessary to examine material obtained by centrifugalization of the blood. If the direct examination of the blood is negative, 10 cc. of blood should be drawn from a vein in the arm and discharged into a centrifuge tube large enough to contain the entire amount. This is then placed in the centrifuge and spun at from 900 to 1000 revolutions per minute for three minutes. The supernatant fluid is then pipetted off and spun at about 1500 revolutions per minute for about ten minutes, the supernatant fluid again removed and spun for from fifteen to twenty minutes at 1800 to 2000 revolutions per minute, after which smears are made of the *sediment*, stained, and examined for trypanosomes. Staining is not essential as a minute amount of the sediment can be placed upon a microscopic slide, covered with a cover-slip, and examined for the trypanosomes.

Collection and Preparation of Material from Enlarged Glands.—In the acute febrile stages of sleeping sickness, whether caused by *Trypanosoma gambiense* or *Trypanosoma rhodesiense*, the superficial lymphatic glands, especially those situated in the posterior cervical region, are enlarged and the microscopic examination of material obtained by puncture of an enlarged gland is usually positive for the trypanosomes.

The technique of gland puncture has already been described in the section of this work devoted to the laboratory diagnosis of kala-azar (see page 157) and will not be repeated. The material so obtained is spread upon microscopic slides and stained or examined unstained for the trypanosomes.

In the sleeping sickness stage of African sleeping sickness the glands are usually atrophied and this method of diagnosis is impossible.

Collection and Preparation of Bone-marrow.—If the examination of the blood gives negative results smears of bone-marrow, obtained by sternal puncture (see page 155) may be made and stained with suitable stains (see page 160). This method of diagnosis is seldom employed at present but deserves careful consideration and trial.

Collection and Preparation of the Cerebrospinal Fluid.—With the termination of the acute febrile stage of African sleeping sickness and the advent of symptoms connected with the central nervous system the examination of the blood generally results negatively and the laboratory diagnosis of these infections depends upon the demonstration of the causative trypanosome in the cerebrospinal fluid. The examination of this fluid for the trypansomes is useless unless symptoms connected with the central nervous system are present and the more severe these symptoms the greater are the chances of finding the trypanosome in this fluid.

The cerebrospinal fluid is obtained by lumbar puncture, a procedure which is perfectly safe if properly conducted in suitable patients.

Technique of Lumbar Puncture.—Before undertaking this procedure the possible dangers connected with it should be understood. Sudden death may occur, usually in patients suffering from tumors of the cerebrum or cerebellum; or apoplexy, with a fatal termination, may occur from the sudden removal of the cerebrospinal fluid, in patients having arteriosclerosis. Sudden death following lumbar puncture has been reported in patients suffering from tuberculosis of the brain, uremia, aneurism of the cerebellar vessels, hydrocephalus and hydatid cyst. In patients suffering from any of these conditions lumbar puncture should be done only after the most careful consideration.

If it is possible, the patient should be placed in bed and kept there for twenty-four hours after a lumbar puncture, thus preventing or greatly alleviating the unpleasant symptoms that may follow the puncture, as severe headache, dizziness, nausea and vomiting. Slight headache is often present but if the patient is kept in bed for twenty-four hours is much less apt to be severe than if he is allowed to keep about after the puncture. Some physicians make lumbar punctures as a routine procedure in office practice but the writer would recommend that, whenever possible, lumbar puncture be done at the patient's home or in a hospital and that rest in bed for twenty-four hours be enforced. Of course, in the case of natives, this is often impracticable and one has to make the puncture under the best conditions possible.

In making the puncture the *position* of the patient is important and it can be made with the patient in bed and lying upon his side or sitting up on the edge of the bed or on a stool. It is best to make the puncture with the patient in the reclining position as the unpleasant symptoms that often follow puncture are less apt to be severe and the danger of

sudden death due to a too rapid fall in the pressure of the cerebrospinal fluid is lessened.

The bed should have a hard mattress and the patient should lie upon the edge of the bed with his back parallel with the edge and his thighs and legs flexed upon his abdomen while his head should be brought as far forward as possible and the chin flexed upon the chest. This position results in widening the spaces between the spinous processes of the vertebræ, thus facilitating the puncture. If the mattress is so soft as to cause a flexion in the patient's spinal column, it will be necessary to place a board beneath it so as to support the patient. If the puncture is to be made in a hospital an operating table is more suitable than the bed.

The utmost precautions should be taken to preserve asepsis. The operator should prepare as for any operation and the instruments used should be sterilized. The skin over the lumbar region should be scrubbed with soap and water, washed off with alcohol and the site of the puncture covered with tincture of iodine or other antiseptic used for skin sterilization. The only apparatus usually required is a suitable steel needle of from ½ to 1.5 mm. bore and about 10 to 12 cm. in length, provided with a stylet. The needle should have a comparatively short bevel and a very sharp point. The size of the needle will vary with the age and weight of the patient. In children a needle 7.5 to 8 cm. long should be employed while in heavy individuals the needle should be at least 11.5 to 12.5 cm. in length. Special needles for lumbar puncture are available in the market and special apparatus for this purpose is listed in every instrument dealer's catalogue. In cases where it is necessary to measure the pressure of the fluid during withdrawal special apparatus is necessary but in ordinary routine diagnostic work needles of the Quincke type and of the size indicated are satisfactory.

The *site of the puncture* is ascertained most easily by running the finger along the spines of the vertebræ until the so-called "soft spot" is encountered, which is indicated by a distinct feeling of softness to the finger and is situated between the third and fourth lumbar vertebræ, but it may also be ascertained by drawing a straight line between the iliac crests which passes directly over the third lumbar interspace and the puncture may be made here or in the space below it. In children it is best to make the puncture in the fourth lumbar interspace in order to avoid the possibility of injuring any nerve structures.

Having ascertained the site for puncture and sterilized it and the surrounding area, the puncture is made in the median line into either the third or fourth lumbar interspace, the needle being held perpendicular to the skin and parallel with the surface of the bed or operating table. Some authorities prefer puncturing the interspace a little to one side of the median line but the writer agrees with most authorities that the median puncture is preferable.

The needle should be grasped firmly between the thumb and index finger with the hub resting against the palm of the hand and quickly

pushed through the skin, subcutaneous and muscular tissue, after which it is pushed forward gently until there is a sudden sensation of loss of resistance when the needle enters the subarachnoid space. Care should be taken not to push the needle any further as doing so will result in injuring the anterior wall of the space, with consequent hemorrhage into the space, and this will also occur if too much force is employed in making the puncture. If bony resistance is encountered, the needle should be slightly altered in direction and, if this is unsuccessful in avoiding the obstruction, the needle should be withdrawn and another puncture made. After entering the subarachnoid space the stylet of the needle should be removed, when cerebrospinal fluid will flow from the needle if the puncture has been successful. If no fluid appears it may be that the needle is plugged with a minute piece of tissue or with exudate and this may usually be removed by pushing the stylet gently through the needle or pushing it into the needle and quickly withdrawing it, thus creating enough suction to clear the needle. Sometimes the needle may be occluded by contact with the membrane and in this case withdrawing it a little and again advancing it into the subarachnoid space will result in a successful puncture. The needle should never be forced through an obstruction.

If the puncture is entirely successful the cerebrospinal fluid should come through the needle uncolored by blood but not infrequently it is more or less blood stained but unless blood comes from the needle this does not interfere with the demonstration of the trypanosomes and the blood-stained cerebrospinal fluid should not be discarded but should be prepared for examination. If blood comes from the needle it should be withdrawn and another puncture should be made.

It sometimes happens that after entering the subarachnoid space no fluid is obtained. If this is not due to obstruction in the needle it means that there is insufficient pressure within the canal to force the fluid into the needle or that there is a decreased amount of fluid. This lack of pressure may sometimes be overcome by asking the patient to cough or strain or by pressure on the jugular veins but if these procedures are unsuccessful in forcing fluid through the needle the puncture is a "dry" one and should not be repeated.

Unless there is increased pressure in the spinal canal the cerebrospinal fluid will usually emerge drop by drop from the needle and should be caught in a sterile glass centrifuge tube. Not more than 5 cc. of fluid should be withdrawn unless it flows rapidly from the needle when as much as 10 cc. may be withdrawn.

After the cerebrospinal fluid has been obtained the site of the puncture is brushed with tincture of iodine, covered with a sterile gauze pad held in place by adhesive tape, and the patient placed in bed and made to lie flat on his back for at least three hours, during which time he should be watched for possible symptoms of collapse or other symptoms demanding trained assistance.

The services of an assistant are necessary during lumbar puncture in

seeing that the patient does not make any sudden movement during the operation and that the proper position for puncture is maintained. In cases in which a brain tumor is present sudden collapse may occur during the puncture and the assistant should watch carefully for premonitory symptoms of such a condition. In very nervous individuals syncope may occur and the assistant should be prepared to cope with this emergency. Any marked change in the pulse or respirations should be a signal for stopping the flow of the fluid and persistence in such symptoms calls for the withdrawal of the needle and the employment of such restorative measures as are indicated. Artificial respiration should be resorted to if the breathing does not quickly become normal.

Local or general anesthesia is not usually necessary in performing a lumbar puncture but if the patient is very nervous or suffering from mental derangement, as is common in sleeping sickness, a local or general anesthetic should be used, as indicated. If a local anesthetic is used a 1 per cent solution of novocaine is best, the skin at the site of the puncture being infiltrated with a few drops after which the needle of the injecting syringe is pushed deeper into the tissues and from 2 to 2.5 cc. of the 1 per cent solution is infiltrated, the syringe being gradually withdrawn during the process. If it is necessary to use a general anesthetic it is best to use chloroform and a very little need be employed, just enough to quiet the patient. The writer has very rarely found it necessary to use an anesthetic in making lumbar punctures.

Preparation of the Cerebrospinal Fluid.—After collection the cerebrospinal fluid should be centrifugalized at a speed of 1500 to 2000 revolutions per minute for fifteen to twenty minutes and the sediment examined for trypanosomes by placing a small amount of it upon a microscopic slide and covering it with a cover-glass, or smears of the sediment can be made, air dried, and stained. Both procedures should be employed in routine diagnosis. The trypanosomes can be easily seen with the $\frac{1}{6}$ inch microscopic objective.

If the preparations are negative for the trypanosomes the cell count of the cerebrospinal fluid often gives suggestive results as the cells are usually much increased in number; counts of from 1000 to 1500 leukocytes per cu. mm. being sometimes obtained, the cells consisting mostly of large mononuclears and lymphocytes. Some authorities believe that a cell count of over 50 per cu. mm. in the presence of suggestive symptoms and an increase to 0.03 per cent in the protein content are of diagnostic significance. The amount of albumin in the cerebrospinal fluid is also increased in the later stages of sleeping sickness and the estimation of this is of some diagnostic importance, if an increase is present.

Pandy's Test for Protein Content.—This is a very useful and practical method of ascertaining an increase in the protein content of the

cerebrospinal fluid and it is so simple that it can be used in the field in making surveys. A positive result indicates involvement of the central nervous system and in cases in which trypanosomes have been demonstrated in the blood of gland juice such a result will enable one to make a diagnosis of such involvement long before clinical symptoms are evident. The technique is as follows:

One drop of cerebrospinal fluid is dropped into 2 cc. of a carbolic acid solution composed of 1 part pure carbolic acid in 15 parts of distilled water.

A positive reaction consists in the appearance of a bluish-white cloud in the mixture which should be definite and easily seen.

Collection of Blood for Animal Inoculations.—If examinations of the blood, gland juice and cerebrospinal fluid for the trypanosomes causing African sleeping sickness give a negative result, resort will have to be had to the inoculation of susceptible animals with the blood of the suspected individual. In collecting blood for this purpose extreme care should be taken to prevent bacterial contamination. The blood should be drawn from a vein in the arm after the site of puncture of the vein has been sterilized, and the syringe used should be of glass and it, and the needle, sterilized before use. A small amount of sodium citrate solution should be left in the syringe after it has been rinsed out with it, and from 5 to 20 cc. of blood should be withdrawn, the amount depending upon the animal to be inoculated. The technique of animal inoculation and the laboratory animals used for diagnostic purposes will be mentioned later (see page 236).

2. COLLECTION AND PREPARATION OF MATERIAL FOR EXAMINATION FOR TRYPANOSOMA CRUZI, CAUSING CHAGAS' DISEASE

In the acute forms of Chagas' disease *Trypanosoma cruzi* may usually be demonstrated in the peripheral blood, but, as it occurs only in small numbers in most infections, prolonged search is necessary and several blood films or unstained preparations should be carefully examined before a negative result is accepted. In the chronic forms of the disease the blood is usually negative unless fever be present, and even at this time the trypanosomes are seldom numerous and several preparations may have to be examined before they are found. The examination of the cerebrospinal fluid is sometimes successful in demonstrating the trypanosome and should be followed in cases presenting symptoms connected with the central nervous system. If the blood is negative in the acute stage of the infection the inoculation of susceptible animals is often successful as a diagnostic measure.

The methods of collecting and preparing blood and cerebrospinal fluid for examination for *Trypanosoma cruzi* are identical with those employed in the collection and preparation of material for examination

for the parasites causing African sleeping sickness, *i. e.*, *Trypanosoma gambiense* and *Trypanosoma rhodesiense* already described (see page 205).

Staining Methods.—There are several staining methods available for the diagnosis of the trypanosomes causing disease in man, the routine ones all being based upon the Romanowsky method, and those already described in discussing the leishmaniases are equally valuable in infections caused by trypanosomes. Of the various stains that have been employed the writer believes that the Wright and Giemsa stains give the best results, prepared and used as already described for staining *Leishmania donovani*, *Leishmania tropica* and *Leishmania brasiliensis* (see page 164), the smears of blood, glandular juice or cerebrospinal fluid being air dried and stained immediately. In order to secure good preparations it is usually necessary to allow the Wright stain to act for at least twenty minutes after adding the distilled water and sometimes for longer, according to the thickness of the smear and if the Giemsa stain is used the smears may be stained for several hours with excellent results.

With the Wright, Giemsa or other similar stains the nucleus of the trypanosome stains a pink, red or violet color, the kinetoplast a deep red or violet, the flagellum pink or red, and the cytoplasm various shades of blue according to the intensity of the staining. In those individuals of *Trypanosoma cruzi* in which the kinetoplast is very large, it usually stains an almost black color, or a very deep violet, and there is no distinction between the blepharoplast and the parabasal body. In order to secure good preparations they should be thoroughly washed in gently running distilled water after staining, as this process removes any sediment that may have been deposited from the stain and also increases the brilliance of the pink, red or violet staining of the chromatin of the nucleus, kinetoplast and flagellum. Thick blood films are also useful in diagnosis (see page 153).

Films of the sediment of the cerebrospinal fluid may also be wet-fixed and stained with the hematoxylin stains, as in staining the intestinal amebæ, but much practice is necessary to secure good preparations and this method of staining is never used as a routine diagnostic measure but only for the study of the minute structure of the trypanosomes.

As already stated, it is not essential to prepare stained specimens of the trypanosomes for the diagnosis of African sleeping sickness or Chagas' disease, as the examination of unstained preparations of blood, gland juice or cerebrospinal fluid will demonstrate these parasites if they be present. It is well, however, to prepare stained preparations as sometimes, if the trypanosomes be few in number, they may be found in a stained specimen when the unstained material was apparently negative.

CHAPTER XV

CULTIVATION OF TRYPANOSOMA GAMBIENSE, TRYPANOSOMA RHODESIENSE AND TRYPANOSOMA CRUZI

DIAGNOSTIC VALUE OF CULTURES—TECHNIQUE OF CULTIVATION—MAINTENANCE OF TRYPANOSOMES IN CULTURES—MORPHOLOGY IN CULTURES—VIRULENCE IN CULTURES—CULTURE METHODS AND MEDIA

THE cultivation of the trypanosomes causing disease in man is difficult and the maintenance of these organisms in cultures beyond a few generations is usually impossible. For these reasons the cultivation of the trypanosomes is of little value in diagnosis and is seldom employed. However, for the study of the cultural forms, which are like those occurring in the insect hosts, cultures are valuable and may be used for this purpose.

In 1903, Novy and MacNeal were the first investigators to successfully cultivate a trypanosome *in vitro*. They were successful in cultivating *Trypanosoma brucei*, the cause of a fatal disease in cattle and horses, known as "nagana," upon the N.N.N. medium. In 1905, Thomas and Breinl succeeded in keeping *Trypanosoma gambiense* alive upon a special medium for as long as sixty-eight days but no multiplications occurred in the cultures and subcultures were negative. In 1907, Gray and Tulloch, using the N.N.N. medium, kept this trypanosome alive for twenty days and multiplication occurred in the cultures but subcultures were not obtained. Thomas and Sinton (1912) were able to keep *Trypanosoma gambiense* and *Trypanosoma rhodesiense* alive upon a modified N.N.N. medium for thirty-seven and twenty-one days respectively and multiplication occurred in the cultures, while Nöller (1920), Torres (1922), Bonacci (1934), Kelser (1936) and others have been successful in cultivating *Trypanosoma cruzi* upon several special media, including the N.N.N. medium. In the case of the latter trypanosome multiplication readily occurs in cultures and subcultures may be maintained for long periods of time.

DIAGNOSTIC VALUE OF CULTURES

Cultures are of comparatively little value in the diagnosis of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* infections, *i. e.*, in African sleeping sickness, because of the difficulty of cultivating these trypanosomes, but in infections with *Trypanosoma cruzi*, *i. e.*, Chagas' disease, they possess some diagnostic value as this trypanosome is more

easily cultivated and in patients in whom the blood has proven negative cultures have sometimes been successful in demonstrating the organism, although they have not been used as a routine diagnostic measure to any great extent.

Technique of Cultivation.—The technique employed in the cultivation of the trypanosomes is similar to that already recommended for the leishmania (see page 169), the blood being collected with aseptic precautions from a vein in the arm of the patient and distributed in the culture tubes. At least 10 cc. of blood should be collected and defibrinated by shaking it in a flask containing glass beads and from 0.5 to 1 cc. of the defibrinated blood added to each tube containing the culture media. The inoculated tubes should be examined daily and should not be discarded as negative until the end of a month. The most favorable temperatures for cultivation lie between 22° to 30° C. (71.6° to 86° F.) and, in the case of *Trypanosoma cruzi*, room temperature has been found to be satisfactory. Further details regarding the technique of cultivation will be found in the descriptions of the various culture media which follow.

MAINTENANCE OF TRYPANOSOMES IN CULTURE

Until recently (see page 221) no way of maintaining *Trypanosoma gambiense* and *Trypanosoma rhodesiense* in cultures has been devised, subcultures of these parasites being usually unsuccessful, and if successful, for only a very limited period of time, and varying greatly with different strains of these trypanosomes. This is not true of *Trypanosoma cruzi* for this organism has been maintained in cultures for long periods of time by several authorities and such cultures have been found to be very useful in studying the morphology and life-cycle of this trypanosome and for preparing antigen for the complement fixation test devised by Kelser (1936) for the diagnosis of Chagas' disease. The best results in the cultivation of *Trypanosoma cruzi* have apparently been obtained by Kelser, using the medium he devised, and he recommends that stock cultures be kept in rubber-capped test tubes, to prevent evaporation of the water of condensation, and left at room temperature in a dark closet or cabinet. Subcultures are made at ten day intervals and the strain of the organism that is being cultivated is passed through a guinea-pig now and then to insure its vitality.

The N.N.N. medium is also an excellent one for maintaining *Trypanosoma cruzi.*

MORPHOLOGY OF TRYPANOSOMA GAMBIENSE, TRYPANOSOMA RHODESIENSE AND TRYPANOSMA CRUZI IN CULTURES

In cultures the morphology of both *Trypanosoma gambiense* and *Trypanosoma rhodesiense* differs markedly from the forms observed in the patient's blood in that both short and long crithidial forms, *i. e.*,

forms having the kinetoplast anterior to the nucleus with a short undulating membrane and flagellum, as well as the typical trypanosome form, occur in the cultures, identical in appearance with the forms observed in the flies transmitting these trypanosomes, all belonging to the Genus *Glossina.*

The morphology of *Trypanosoma cruzi* in cultures also differs from that observed in man in that crithidial forms occur as well as leishmania and trypanosome forms and these forms are identical in morphology with those occurring in the transmitting bugs, with the exception of the leishmania forms.

VIRULENCE IN CULTURES

As it is impossible to maintain either *Trypanosoma gambiense* or *Trypanosoma rhodesiense* in cultures nothing is known regarding the effect of cultivation upon virulence but *Trypanosoma cruzi* may be maintained in cultures for long periods of time and it has been found that the virulence of the trypanosome for susceptible animals declines very slowly during cultivation and that cultures are still infective after several months of continuous subculturing. (see page 221)

CULTURE METHODS AND MEDIA

With the exception of cultivation in the living chick embryo (see page 221) no method of cultivation of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* has proven successful as subcultures are usually impossible to obtain, but *Trypanosoma cruzi* can be readily cultivated upon a variety of media. The methods of cultivation that have been employed in the cultivation of the trypanosomes causing disease in man follow in alphabetical order with remarks as to their usefulness.

Bonacci's Media and Methods.—Bonacci, in 1934, described three culture media which gave good results in the cultivation of *Trypanosoma cruzi,* calling the media Nos. 1, 4 and 9.

Bonacci's Medium No. 1.—The formula for this medium is as follows:

Witte's peptone	3 gm.
Sodium chloride	1 gm.
Agar	2 gm.
Nutrient beef broth	200 cc.

The peptone, sodium chloride and agar are dissolved in the hot beef broth, the mixture made neutral to litmus, heated in the autoclave at 115° C. (280° F.) for twenty minutes and filtered through cotton wool. It is then distributed into Erlenmeyer flasks containing 100 cc., sterilized in the autoclave at 110° C. (220° F.) for twenty minutes, and then stored for future use.

When used the medium is melted, cooled, and there is added to each 100 cc. 0.5 gm. of glucose and 5 cc. of young guinea-pig blood.

Bonacci's Media Nos. 4 and 9.—The only difference between these media and the No. 1 medium is in the amount of peptone and sodium chloride that they contain. The No. 4 medium contains 2.5 gm. of Witte's peptone and 0.7 gm. of sodium chloride while the No. 9 medium contains 3 gm. of Witte's peptone and 0.7 gm. of sodium chloride to each 100 cc. of the agar mixture.

Remarks.—These media were found by Bonacci to give good results in the cultivation of *Trypansoma cruzi* and subcultures could be maintained for long periods of time. Whether they would be equally useful in the cultivation of *Trypanosoma gambiense* and *Trypansoma rhodesiense* has not been ascertained.

Brutsaert and Henrard's Medium.—The following medium has been recommended by Brutsaert and Henrard (1938) for the cultivation of *Trypanosoma gambiense* and *Trypanosoma rhodesiense:*

Two solutions are employed, a Ringer solution containing 0.06 per cent of sodium chloride and Tyrode's solution having the following formula:

NaCl	8 00 gm.
KCl	0 20 gm.
$CaCl_2$	0 20 gm.
$MgCl_2$	0 10 gm.
NaH_2PO_4	0 05 gm.
$NaHCO_3$	1 00 gm.
Glucose	1 00 gm.
Distilled water enough to make	1000 00 cc.

Both solutions are sterilized by filtration and are then distributed in culture tubes in the proportion of 2 cc. of the Ringer solution to 2.5 cc. of the Tyrode solution. To each tube is then added 2 cc. of citrated human blood, containing 1 per cent of citrate, and the tubes are then incubated at 37° C. (98.6° F.) for twenty-four hours to determine sterility. The tubes should be kept on ice until inoculated and will remain serviceable for two weeks.

The blood for inoculation is obtained by withdrawing 5 cc. from a vein in a syringe containing 1 cc. of Roche fluid (a 1 per cent solution of polyanethol sulphonate of sodium), which is then distributed in amounts of 0.5 cc., in each of 10 culture tubes and incubated at 25° to 28° C. (77° to 82.4° F.).

Remarks.—Brutsaert and Henrard state that the trypanosomes are demonstrable in this medium between the tenth and twentieth days after inoculation and recommend it as a valuable diagnostic aid in African sleeping sickness.

Davis Medium.—The following medium was recommended by Davis (1943) for the cultivation of *Trypansoma cruzi:*

A blood-agar base is employed which is made by adding to the requisite amount of beef or horse infusion, 2 per cent of proteose peptone No. 3 (Difco); 0.7 per cent sodium chloride; 0.5 per cent dextrose; 2 per cent agar and 10 per cent defibrinated rabbit blood.

The pH is adjusted to 7.6 and the medium slanted in large tubes (25 mm. x 200 mm.). After the slants have solidified they are each covered with 25 cc. of infusion broth containing 2 per cent proteose peptone No. 3, and 0.5 per cent dextrose.

The tubes are then inoculated with 0.5 to 1 cc. of an actively growing culture of *Trypanosoma cruzi* and incubated at 25° to 28° C. (77° to 82° F.).

Remarks.—This medium is especially recommended by Davis for obtaining a good growth of the trypanosome from which to prepare a specific antigen for his complement fixation test used in the diagnosis of infection with this parasite. Its value in diagnosis by culturing blood or gland juice has not been ascertained to date.

Gray and Tulloch's Medium.—The Gray and Tulloch medium consists of the Novy, MacNeal and Nicolle medium (N.N.N. medium) to which defibrinated dog's blood has been added instead of rabbit's blood. For a description of the N.N.N. medium the reader is referred to page 175.

Remarks.—Gray and Tulloch (1907) were successful in cultivating *Trypanosoma gambiense* upon this medium for a limited period of time. The trypanosomes underwent multiplication but subcultures were not obtained.

Kelser's Medium.—In 1936, Kelser described a method of cultivating *Trypanosoma cruzi* upon a medium closely resembling Bonacci's, described above. The formula of this medium follows:

Bacto-peptone (Difco Laboratories, Detroit, Mich.)	12 5 gm.
Sodium chloride	3 5 gm.
Agar (Bacto-Agar, granular)	5 0 gm.
Bacto-Beef, dehydrated	25 0 gm.
Distilled water	500 0 cc.

The medium is prepared as follows: To a flask containing 500 cc. of distilled water there are added 25 gm. of "Bacto-Beef" and the flask is then placed in a water-bath at 55° C. (131° F.) and allowed to remain for one hour. At the expiration of this time there are added 12.5 gm. of "Bacto-Peptone" and 3.5 gm. of sodium chloride. The water-bath temperature is now raised to the boiling point and the mixture allowed to remain in the boiling water for five minutes, being shaken several times in order that the peptone and salt be thoroughly dissolved, after which the mixture is filtered through a thickness of cotton until it comes through clear. It is then rendered neutral to litmus by using a N/1 solution of sodium hydroxide, after which it is measured and 1 per cent of "Bacto-Agar" (approximately 5 grams) is added, the addition being made while the flask containing the mixture is in boiling water. After the agar has dissolved the medium is distributed, without filtering, into test tubes or small Erlenmeyer flasks, 5 cc. being placed in each test tube and 10 cc. in each flask, and then sterilized in the auto-

clave under 12 pounds pressure for one-half an hour. The medium is then allowed to harden and is ready for use.

It is best to inoculate the medium as soon as it has been prepared and allowed to harden. The tubes or flasks containing it are placed in a water-bath in boiling water until the medium is melted, after which the temperature of the bath is lowered to between 50° and 55° C. (122° to 131° F.) and there is added to each tube of the medium 0.25 cc. of a sterile 1 per cent solution of dextrose (C.P.) and 0.25 cc. of fresh, sterile, defibrinated guinea-pig blood, and to each flask 0.50 cc. of the dextrose solution and 0.50 cc. of the guinea-pig blood. The tubes should then be slanted in such a manner as to result in a deep butt and little slanted surface, while the medium in the flasks should be allowed to harden at the bottom of the flask.

If inoculations are to be made upon medium that has been kept and there is very little water of condensation present in either the tubes or flasks, a little of the dextrose broth solution may be added and repeated, if necessary, in order to preserve a small amount of fluid at the bottom of the tubes or in the flasks. The dextrose broth solution is made by adding 1 part of the sterile 1 per cent dextrose solution to 2 parts of peptone broth prepared as described above in the preparation of this medium. If flask cultures are to be inoculated 2 cc. of the dextrose solution should be added to each flask and this amount should not be exceeded as the addition of too much of the dextrose solution hinders the growth of the trypanosomes.

Kelser recommends that stock cultures of *Trypansoma cruzi* be maintained in test tubes, the cotton plugged end of each tube being covered with a rubber cap to prevent evaporation and drying of the medium, and that the flask cultures be inoculated from the tube cultures. The inoculation of culture media is done with sterile glass capillary pipettes fitted with cotton air-filters and rubber nipples. Transfers of stock cultures should be made at ten day intervals but the trypanosomes will remain alive in a stock culture for about one month.

In the initial cultures of the blood leishmania forms of *Trypansoma cruzi* begin to appear in from three to five days, usually in clusters, and in a few more days large numbers of typical trypanosome forms are present. In subcultures large numbers of the flagellated forms are present in from two to three days and within one week the growth is so abundant that it may be seen as a white deposit in the lower part of the water of condensation in the tubes, and in still older cultures it may be seen to extend along the edge of the slated surface which is in contact with the wall of the tube, but it seldom occurs on the surface of the agar slant.

Remarks.—The medium prepared as described by Kelser is apparently the best yet described for the continued culture of *Trypanosoma cruzi*. Enormous numbers of the trypanosomes develop in this medium and the organism may be subcultured indefinitely. Kelser employed

this medium in cultivating *Trypansoma cruzi* used in the preparation of antigen for his complement fixation test for Chagas' disease (see page 229) and states (1936) that "no difficulty whatever has been experienced in maintaining our cultures and regularly obtaining prompt and excellent growth in subcultures."

The excellent results obtained with this medium in the cultivation of *Trypanosoma cruzi* suggests that it might give good results in the cultivation of *Trypansoma gambiense* and *Trypansoma rhodesiense.* The writer is not informed as to whether the medium has been used for the cultivation of these trypanosomes but, if not, it should be given a trial.

Novy, MacNeal and Nicolle's Medium (N.N.N.) Medium.—This medium, the preparation and technique of which is described on page 175, and which is so useful for the cultivation of the leishmania, is also an excellent one for the cultivation of trypanosomes, especially for *Trypanosoma cruzi.* Subcultures of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* can be obtained from primary cultures a month old but such subcultures are very often negative. Subcultures of *Trypanosoma cruzi* can be obtained with this medium from primary cultures several months old and Packchanian (1943) obtained subcultures from primary cultures several years old. In cultures upon this medium kept at from 18° to 30° C. (64.4° to 86° F.), Packchanian found motile forms of *Trypanosoma cruzi* in 13 tubes kept for six years, of 124 subcultures of this parasite, and 68 of 110 subcultures showed motile forms after a period of six months.

Reichenow's Medium.—This medium has been recommended by Reichenow (1934) for the cultivation of pathogenic trypanosomes.

The medium is a mixture of citrated human blood and Ringer's solution. Culture tubes, each containing 1 cc. of Ringer's solution made with 0.6 per cent of sodium chloride, are sterilized, and to each tube is added 1 cc. of citrated blood from a normal individual, collected from a vein in the arm with aseptic precautions. The tubes are then kept in the ice-box for two or three days before they are used, and after inoculation are kept at 24° C. (75.2° F.) and subinoculations are made every two weeks, to maintain the cultures, if positive.

Remarks.—This medium has been recommended by Reichenow as an excellent one for maintaining pathogenic trypanosomes in culture and with it he was able to maintain a strain of *Trypanosoma gambiense* in culture for 111 days. From his experiments with this medium it is evident that it did not give good results in obtaining primary cultures of this trypanosome and that its usefulness, as a diagnostic method, is very limited, unlike that of Brutsaert and Henrard's medium (see page 216), which they claim gives excellent results in this respect and is a valuable diagnostic medium.

Senekjie's Media.—These media, recommended by Senekjie for the cultivation of the leishmania are also excellent for the cultivation of

Trypanosoma cruzi (see page 177). He found that this trypanosome survived in the egg-liver medium for three to four weeks.

Tom's Modification of the N.N.N. Medium.—Tom (1943) recommends the following modification of the N.N.N. medium for the cultivation of *Trypanosoma cruzi:*

Infuse 500 gm. of fat-free beef in 1000 cc. of water overnight in the ice-box at from 4° to 6° C. (39.2° to 42.8° F.). Heat the infusion gradually until the proteins are coagulated, cool, strain through gauze, and add 10 gm. of Bacto-peptone and 5 gm. of sodium chloride C.P. Adjust the reaction to pH 7.4 and boil vigorously for twenty minutes and then filter through filter paper. Make up the volume to 1000 cc. with distilled water, place in culture tubes, and autoclave at 15 pounds pressure for one hour. Store in ice-box.

When the medium is to be used add 0.2 per cent agar and one-third the volume in each tube of fresh, sterile, defibrinated rabbit blood. Inspissate in the autoclave at 15 pounds pressure for twenty minutes, place tubes in ice-box overnight, and then incubate the tubes for twenty-four hours to test sterility. At the end of this time the tubes are inoculated with *Trypansoma cruzi*, rubber capped, and incubated at 25° C. (77° F.).

Remarks.—It was determined by Tom that the maximum growth of the trypanosomes upon the medium was obtained in about one week and that the organism remained viable in the medium for at least six weeks. Tom was successful in cultivating ten generations of the trypanosome upon this medium.

It would appear that this modification of the N.N.N. medium is an excellent one for the cultivation of *Trypanosoma cruzi* and it will probably be found valuable also in the cultivation of other trypanosomes and the leishmania.

Torres' Medium.—This very simple medium was first recommended for the cultivation of *Trypanosoma cruzi* by Torres, in 1915. The formula for the medium is as follows:

Peptone, Witte's .	5 gm.
Sodium chloride .	7 gm.
Beef broth .	100 cc.

The beef broth is made by adding 5 gm. of Bacto-Beef, made by the Difco Laboratories, Detroit, Michigan, to 100 cc. of distilled water and allowed to heat in a water-bath at 55° C. (131° F.) for one hour. The salt and peptone are then added and the mixture allowed to remain in boiling water for long enough to dissolve these substances, after which it is filtered through cotton and the reaction brought between pH 6.55 and 7.18. The medium is then tubed and sterilized in the autoclave. After inoculation the cultures are kept at room temperature in a dark cabinet or in an incubator at temperatures between 22° and 25° C. (71° to 77° F.).

Remarks.—This medium has been found to give good results so far as obtaining an abundant primary growth of *Trypanosoma cruzi* is concerned but it is not recommended for the maintenance of this organism as subcultures are difficult to obtain and maintain.

Tissue Cultures.—The use of living tissue cultures for the cultivation of the viruses causing various diseases in man has suggested the employment of the same in the cultivation of some of the Protozoa, but up to the present time the only successful results so obtained have been in the cultivation of some of the trypansomes in the growing chick embryo. The results of Longley, Clausen and Tatum (1939) in the cultivation of *Trypanosoma rhodesiense, T. brucei, T. equiperdum, T. evansi* and *T. hippicum* in the living chick embryo were so successful that this method of cultivation deserves careful study.

Technique of Culture in Chick Embryos.—Various methods of utilizing the chick embryo for the cultivation of the filterable viruses have been devised but the simpler methods of Longley, Clausen and Tatum and of van Rooyen and Rhodes would seem to be best adapted to the cultivation of the trypanosomes causing disease in man and the lower animals.

Longley, Clausen and Tatum's Method.—Fertile hen's eggs are incubated for eight to ten days and the eggs are then candled and the site of the embryo is located. Using a sterile dissecting needle two small holes are made through the previously alcohol cleaned shell, one into the air-sac and the other directly over the embryo. The material to be inoculated, approximately 0.5 cc. for each egg, is injected into the allantoic cavity by inserting a 26 gauge needle parallel to and immediately under the chorio-allantoic membrane. After injection is completed the holes in the egg shell are sealed with melted paraffin. The eggs are kept in an incubator at 37° C. (98.6° F.).

Remarks.—In the hands of the observers mentioned *Trypanosoma rhodesiense* and other trypanosomes were readily cultivated by this method and the cultures maintained for eight generations over a period of forty-one days. The injection of material containing the trypanosomes was followed by a heavy infection in the blood of the chick embryos and caused their death in from four to five days. Subcultures were made upon the fifth day. Longley, Clausen and Tatum (1939) state that there is apparently no reason why the trypanosomes may not be maintained indefinitely in chick embryo cultures. Their results have been confirmed by Chabaud (1939) who was successful in cultivating *Trypanosoma rhodesiense* by the inoculation of 2 drops of citrated or defibrinated blood containing about 50 of the trypanosomes to the microscopic field into the allantoic membrane of the embryo chick. The embryos became infected and Chabaud was able to pass the infection through 15 eggs, subcultures being made every fifth or sixth day.

Van Rooyen and Rhodes' Method.—This comparatively simple method of inoculating the chicken embryo is recommended by van

Rooyen and Rhodes (1940) in the study of virus infections but it should prove equally applicable to the cultivation of trypanosomes. The following is their description of the method:

"The egg is candled to see if germination has occurred; this is indicated by a dense shadow at one part of the egg, with a clear transparent zone immediately adjacent. The egg is then rotated before the light until this clear zone occupies the top half of the shell, a pencil mark being made at the uppermost part. The position of the air-sac at one end of the egg is also pencilled. The egg is now thoroughly washed and scrubbed in lukewarm soapy water (40° C.) (104° F.) containing some 2 per cent phenol. Immediately prior to inoculation, the surface of the egg is still further sterilized by setting alight a few drops of alcohol on the upper surface and painting with a 4 per cent tincture of iodine."

"The method of choice for inoculation depends upon the bulk of the fluid to be injected. If this does not exceed 0.05 cc., the shell is carefully punctured with the point of a pair of sterile scissors and the material introduced with a syringe and needle to a vertical depth of not more than 3 mm., so that the needle point rests on, but does not penetrate, the chorio-allantoic membrane. Should, however, it be desired to inoculate a larger bulk it is advisable to puncture the shell over the air-sac to make available more space inside the shell. Sometimes a little hemorrhage may accompany the puncture but the mortality resulting from this is negligible. Apertures in the shell are sealed with ovals of tissue paper soaked in melted paraffin, and finally painted over with more paraffin. Thereafter the inoculated egg is incubated at 37° to 39° C. in an incubator containing a trough of water to secure a moist atmosphere."

In employing the above method for the cultivation of trypanosomes the injection of material containing them should be made into the air-sac and the allantoic membrane as recommended in the Longley, Clausen and Tatum method already described.

Kofoid, Wood and McNeil (1935) have successfully cultivated *Trypanosoma cruzi* in tissue cultures prepared from the embryonic heart muscles of rats and mice but their method is not adapted to the diagnosis of Chagas' disease at present.

Weinman's Method.—Weinman (1944) has found the following method gives excellent results in the cultivation of *trypanosomes*. It is prepared as follows:

Solution No. 1

Sodium chloride	8 gm.
Nutrient agar, 1.5 per cent (Difco)	4 gm.
Distilled water	900 cc.

Solution No. 2

Citrated human blood plasma	100 cc.
Human hemoglobin (whole blood, one part, distilled water, five parts)	20 cc.

Solution 1 is autoclaved and Solution 2 is then added and the mixture placed in test tubes.

The medium is inoculated and incubated at 26° to 28° C. (78.8° to 82.4° F.). Trypanosomes appear in the medium in from seven to ten days but tubes should be kept for one month before being discarded as negative.

Remarks.—Weinman states that the trypanosomes lived in this medium for as long as seventy-one days and that cultures have been maintained by transfer for one hundred and twenty-seven days. Apparently this medium is an excellent one for the cultivation of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* and should be equally valuable in the cultivation of *Trypanosoma cruzi*. According to Weinman it is successful in cases in which the blood has proven negative and in many inapparent infections and its use in surveys for the prevalence of these parasites is indicated.

Preservation of Trypanosomes in Blood.—The possibility of sending citrated blood for examination at distant laboratories for trypanosomes has been investigated by Sullivan (1944). She has found that blood collected in early infections with *Trypanosoma cruzi* in citrated blood and stored for as long as two hundred and fifty-seven days revealed that the trypanosomes not only remained viable but may multiply, even when the blood is stored at room temperature. Such blood may be sent to far distant laboratories and will give positive results both by direct microscopic examination and by cultures. Even though no trypanosomes are observed by direct examination cultures upon Kelser's blood agar slants proved positive for as long as two hundred and fifty-seven days.

Whether *Trypanosoma gambiense* or *Trypanosoma rhodesiense* will survive as long in citrated blood has not been ascertained, so far as the writer knows.

CHAPTER XVI

SEROLOGICAL DIAGNOSIS OF THE TRYPANOSOMIASES

Serological Diagnosis of Infections with Trypanosoma Gambiense and Trypanosoma Rhodesiense (African Sleeping Sickness)—Serological Diagnosis of Infections with Trypanosoma Cruzi (Chagas' Disease)

Introduction.—Owing to the identity in morphology of the trypanosomes causing African sleeping sickness, *i. e., Trypanosoma gambiense* and *Trypanosoma rhodesiense*, it is usually impossible to differentiate them without the aid of serological tests, using the blood serum of immunized animals for differential purposes as well as the serum of infected individuals. Rabinowitsch and Kempner (1899) first demonstrated the presence of protective substances in the blood of animals recovered from trypanosomiasis when they found that the blood serum of a rat recovered from an infection with *Trypanosoma lewisi* protected normal rats from infection with this trypanosome, and Laveran and Mesnil (1901) confirmed their observations and showed that the blood serum of animals rendered immune to other species of trypanosome was protective to normal animals when mixed with infective blood and the mixture inoculated. In sleeping sickness of Africa the blood serum of patients during the chronic stage of the disease, whether caused by *Trypanosoma gambiense* or *Trypanosoma rhodesiense*, is protective when inoculated along with the respective trypanosomes into normal animals, but Laveran and Nattan-Larrier (1912) demonstrated that this protection depended upon the particular strain of the trypanosomes, freshly isolated strains being usually killed, while strains that had been passed through animals for some time were resistant and the protective action of the blood serum was lost. However, Mesnil and Blanchard (1916) found this resistance, in the case of *Trypanosoma gambiense*, may be lost after long periods of time, while Jacoby (1909) demonstrated that trypanosomes may acquire a resistance after many passages through susceptible animals. From these various observations it may be seen that the results which have been obtained have been very inconstant and that different strains of the same trypanosome vary greatly in their capacity to resist immune serum. If this were not so it would be comparatively easy to differentiate various trypanosomes by cross-immunity experiments and such experiments have proven useful for this purpose to a certain extent, although the results have been inconclusive, in many instances, because of the confusion caused by the erratic behavior of different strains of the same trypanosome. Agglutinins and complement fixing bodies have also been

demonstrated in the blood serum of individuals infected with *Trypanosoma gambiense, Trypanosoma rhodesiense* and *Trypanosoma cruzi* by various observers and agglutination and complement fixation tests have been used in the diagnosis of African sleeping sickness and Chagas' disease.

1. SEROLOGICAL DIAGNOSIS OF INFECTIONS WITH TRYPANOSOMA GAMBIENSE AND TRYPANOSOMA RHODESIENSE (AFRICAN SLEEPING SICKNESS)

The serological methods that have been tried in the diagnosis of infections with these trypanosomes are agglutination and complement fixation.

(*a*) **Agglutination.**—In 1900, Laveran and Mesnil demonstrated that the blood serum of rats recovered from infections with *Trypanosoma lewisi* when added to blood containing this trypanosome caused an agglutination of the trypanosomes. This agglutination was peculiar in that the trypanosomes became arranged in a rosette-like formation with the anterior, or flagellar, ends directed outward. They found that agglutination of the trypanosomes also occurred after they were killed but that, in this instance, no orientation of the bodies of the trypanosomes occurred but they were arranged in irregular clumps. The same peculiar type of agglutination occurs when blood serum of animals immunized to *Trypanosoma gambiense* and *Trypanosoma rhodesiense* is added to a suspension of these organisms, and several observers have shown that by means of properly conducted agglutination tests it is possible to differentiate certain species of trypanosomes. Thus, Lange (1911), having determined that the blood serum of normal animals usually gave negative agglutination results and never agglutinated trypanosomes in dilutions of more than 1 to 100, showed that the blood serum of animals immunized with dead trypanosomes gave agglutination titers as high as 1 to 1600 and that the titer in infected animals sometimes ran as high as 1 to 12,500. In his work he used as an antigen saline suspensions of washed trypanosomes, 0.2 cc. each of the antigen and the animal blood serum being mixed and then incubated for twelve hours at 37° C. (98.6° F.) and then at room temperature for from twelve to twenty-four hours. Employing this method Lange found that it was possible to differentiate *Trypanosoma gambiense* from *Trypanosoma brucei* and *Trypanosoma equiperdum.*

Although it is possible to differentiate various species of trypanosomes by means of agglutination tests and to demonstrate the presence of infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* by adding the blood serum of the infected individual to a suspension of trypanosomes obtained from infected animals or cultures, no practicable method of diagnosing such infections by means of agglutination tests has been perfected. Of course, such a test would only be of value in those infections of man in which the causative trypanosome could

not be demonstrated in the blood, gland juice, bone-marrow or cerebrospinal fluid but in practice such cases not infrequently occur, especially in the latent stage of sleeping sickness, and a practicable agglutination test would be of much value in diagnosis.

(*b*) **Auto-Agglutination of Erythrocytes.**—In infections with many of the trypanosomes the normal rouleaux formation of the erythrocytes observed in preparations of blood is lost and the red blood corpuscles are arranged in irregular masses instead of in rouleaux. This phenomenon was first observed by Kanthack, Durham and Blandford (1898), who found that auto-agglutination occurred in animals infected with *Trypanosoma brucei* and that the addition of the blood serum of such animals to the blood of normal animals also resulted in the agglutination of the erythrocytes of these animals. Since their observations, which have been repeatedly confirmed, Christy (1904), Thomas and Breinl (1905), Dutton and Todd (1905), Martin, Leboeuf and Roubaud (1909) and many others have described the occurrence of auto-agglutination of the erythrocytes in blood freshly drawn from patients suffering with African sleeping sickness. Dutton and Todd (1910) demonstrated that it is not invariably present while Yorke (1911) found that auto-agglutinins may also occur to a lesser extent in normal blood and that the auto-agglutination was most marked when the preparations were exposed to a temperature of 0° C. (32° F.).

The auto-agglutination test has been used to some extent as a diagnostic method in infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* in the past but at the present time it is no longer so used; if auto-agglutination is present it is considered as very suggestive and as indicating a most careful search for the trypanosome, which, if negative, should be followed by the inoculation of susceptible animals with the blood of the suspected individual.

(*c*) **Complement Fixation.**—Many observers have demonstrated that complement fixing bodies are developed in the blood serum of individuals suffering from African sleeping sickness, but while practicable complement fixation tests have been devised for the diagnosis of infections with *Trypanosoma equiperdum*, causing the diseases known as "dourine" in horses, and *Trypanosoma cruzi*, the cause of Chagas' disease, such tests have not proven successful in the diagnosis of African sleeping sickness, caused by either *Trypanosoma gambiense* or *Trypanosoma rhodesiense*. Various types of antigens have been tried, as extracts of normal organs, extracts of the spleen or liver of infected animals, and extracts of isolated or cultured trypanosomes but an efficient antigen remains to be discovered. The researches of Hartoch and Yakimoff (1908), Winkler and Wyschelessky (1911), Mohler, Eichhorn and Buck (1913), Watson (1914, 1915), Dahmen (1922), Robinson (1926), Kelser (1938) and many others, have shown that the complement fixation test is very useful in the diagnosis of "dourine" and Kelser states that it is also useful in the diagnosis of infections

with *Trypanosoma evansi*, causing "surra" in horses and mules. The type of antigen used has varied but at the present time antigens made by extracting the sediment of centrifugalized blood rich in the trypanosomes obtained from infected animals, especially the white rat, are usually employed. The same type of antigen has been tried in human infections with both *Trypanosoma gambiense* and *Trypanosoma rhodesiense* but the results have not been satisfactory from a diagnostic standpoint. A specific complement fixation test would be useful in the diagnosis of African sleeping sickness in those cases in which the trypanosomes are so few in number that they cannot be demonstrated in the blood, gland juice or cerebrospinal fluid but no such test is now available, as it is for the diagnosis of infections with *Trypanosoma cruzi*, or Chagas' disease.

Other serological methods that have been tried for the diagnosis of African sleeping sickness are the precipitin reactions, trypanolytic reactions and cross-immunity tests but none of these have proven practicable, and, at the present time, we possess no serological test that can be depended upon for the diagnosis of these infections.

The *formol-gel*, or *Napier's aldehyde test* (see page 183) has been suggested as possessing diagnostic value. It is performed in the same manner as for the diagnosis of kala-azar (see page 183). Dye (1926) tried this test in infections with *Trypanosoma rhodesiense* and found that solidification of the mixture of blood serum and formaldehyde usually occurred in about five hours and that the test was negative in this time in malaria, amebiasis, and filariasis. That this test does give positive results within certain time limits in many infections with both *Trypanosoma gambiense* and *Trypanosoma rhodesiense* has been confirmed by other observers but the results are so irregular and so many patients give negative results that, at most, the positive result can be regarded as suggestive only and cannot be relied upon in diagnosis. In some patients the reaction may be positive within one hour and in such cases many authorities regard the result as diagnostic but even in such cases one must eliminate the possibility of the existence of kala-azar, leprosy and yaws, in which a positive result is also obtained. In all but very rare cases these infections can be readily eliminated by attention to the clinical signs and symptoms that are present and, if this is done, a positive reaction occurring within the time mentioned should certainly be regarded as most suggestive and should be followed by intensive efforts to demonstrate the causative organism.

2. SEROLOGICAL DIAGNOSIS OF INFECTIONS WITH TRYPANOSOMA CRUZI (CHAGAS' DISEASE)

In the diagnosis of Chagas' disease complement fixation tests have been devised that are reliable and practicable. This is most fortunate as it is frequently impossible to find *Trypanosoma cruzi* in the blood

even during the acute stage of the infection and it is generally impossible to demonstrate in the peripheral blood during the chronic stage unless fever be present. Before the complement fixation tests that are at present employed in the diagnosis of Chagas' disease were discovered, it was often necessary to inoculate susceptible animals with the blood of the suspected individual and this is still a valuable diagnostic method but, if facilities are available, complement fixation tests offer a rapid and accurate method of diagnosis. The tests that have been found most useful are those of Kelser, Davis, Guerreiro and Machado and Villela and Bicalho.

Davis Complement Fixation Test.—This method of complement fixation is recommended by Davis (1943), who obtained excellent results with it in the diagnosis of Chagas' disease. The antigen is prepared from cultures of *Trypanosoma cruzi* grown upon the Davis medium (see page 216) or the Kelser medium (see page 217.)

The antigen is prepared by carefully drawing off the broth covering the agar slants and centrifuging at 4000 revolutions per minute until the trypanosomes are packed at the bottom of the centrifuge tube. The sediment is now washed three times with normal saline by rapid centrifugation and finally the material at the bottom of the tube is washed and centrifuged in a 15 cc. graduated centrifuge tube using for washing normal saline merthiolate (1 to 10,000). The amount of sediment is measured, the washing solution poured off, and nine volumes of saline with merthiolate (1 to 10,000) added. This suspension is then frozen in dry ice and methyl cellosolve and allowed to thaw slowly. The freezing and thawing is repeated three times when the mixture is ready for titration. It should be thoroughly shaken before use and stored in a tightly stoppered bottle at 4° to 6° C. (39.2° to 41° F.).

A good antigen will fix complement when diluted 1:60 to 1:80, the known positive and negative sera used in titration being diluted 1 to 10. The undiluted antigen is usually anticomplementary but not hemolytic. To secure the best results the dilution of the stock antigen should be about twice the lowest dilution that is not anticomplementary.

The general technique of the test is that of the Kelser test (see page 229) but Davis prefers the Kolmer technique employed for the diagnosis of syphilis.

Remarks.—From the results that have been obtained with it by Davis, this method of complement fixation would appear to be an excellent one and merits thorough trial.

Guerreiro and Machado's Complement Fixation Test The (Machado Test).—In 1913, Guerreiro and Machado were the first to describe a complement fixation test for Chagas' disease and this test, usually known as the Machado test, has been used extensively in the diagnosis of infections with *Trypanosoma cruzi.*

The *antigen* employed by Guerreiro and Machado is an aqueous

extract of the spleens of puppies infected with *Trypanosoma cruzi*, animals being selected that are severely infected. The spleens are ground and triturated in distilled water containing 1 per cent phenol, the resulting mixture filtered, and to the filtrate is added twice its amount of a 1.7 per cent saline solution in distilled water, again filtered, this filtrate used as the antigen. In practice it has been found that antigens prepared in this manner vary very greatly in antigenic strength and are sometimes so weak as to be worthless.

The *technique* of the test is identical with that of complement fixation tests in general.

Remarks.—Several investigators have confirmed the results obtained by Guerreiro and Machado with this test. The latter found the test to be positive in 80 patients in the chronic stage of Chagas' disease and that patients giving a positive Wassermann reaction did not react with this test. While they claimed that their antigen was more reliable than antigens prepared from trypanosomes, the latter observations of Kelser (1936) demonstrate that properly prepared antigens from cultures of *Trypanosoma cruzi* are more specific than the Guerreiro and Machado antigen, in that they give positive results in latent infections and a larger percentage of positive results in known infections with these parasites. A distinct disadvantage of the Guerreiro and Machado antigen is that it contains not only extractives from the trypanosomes present in the splenic tissue but also extractives from the tissue cells. However, the test appears to be a specific one and may be employed when it is impossible to prepare antigens from cultures of *Trypanosoma cruzi*.

Recently, Dussert, Faiguenbaum and Neghme (1939) have published the results that they obtained with this test in 50 suspected individuals as well as in normal and syphilitic individuals. Of the 50 individuals suspected of infection with *Trypanosoma cruzi*, 38 gave a positive reaction, while the sera of all normal individuals living in nonendemic areas of Chagas' disease, and of all syphilitics, gave a negative reaction. These observers regarded the test as of greatest value in the chronic forms of the infection and found that it was seldom positive within one month after infection. They also found that it gave a positive reaction with sera from cases of African sleeping sickness and leishmania infections. Their results suggest the possibility of the diagnostic use of this test, as well as of Kelser's complement fixation test, in patients suffering from infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense*.

Kelser's Complement Fixation Test.—In 1936, Colonel Kelser, of the United States Army Veterinary Corps, described a method of complement fixation for the diagnosis of infections with *Trypanosoma cruzi* which has been proven to be specific and of great value in the diagnosis of Chagas' disease, especially in the identification of latent infections. The technique of this test is as follows:

(*a*) *Preparation of Antigen.*—The antigen employed in Kelser's test is an extract of cultured *Trypanosoma cruzi*, the organism being grown upon the special medium already described (see page 217). The trypanosomes may be cultivated either in test tubes or in flasks. *If in test tubes*, 25 to 50 tubes of the medium are each inoculated with a drop or two of a rich culture and incubated for a week to ten days. At the end of this time 0.75 cc. of sterile distilled water is added to each culture and the growth of trypanosomes is mixed with the water by means of a sterile capillary pipette provided with a rubber nipple. The resulting mixture of trypanosomes and distilled water is then collected from each tube in a 15 cc. conical bottom centrifuge tube and the resulting total mixture is centrifugalized at approximately 4000 revolutions per minute for one-half hour. At the end of this time the trypanosomes are contained in the sediment at the bottom of the centrifuge tube. The supernatant fluid is very carefully pipetted off, so as not to disturb the sediment, and 8 cc. of sterile normal salt solution (0.85 per cent) is added and the centrifugalization repeated at the same speed for one-half hour, for the purpose of washing the trypanosomes. At the termination of this period the salt solution is carefully pipetted off and two volumes of 50 per cent glycerin (C.P.) in normal salt solution are added and the sediment, containing the trypanosomes, is gently, but thoroughly, mixed with this solution by means of a platinum loop. This mixture is the stock antigen employed in the test, and should be kept in the ice-box.

If the cultures of trypanosomes are made upon medium *in flasks* they are incubated for eight to ten days. being gently shaken at intervals, and then a small drop of the culture from each flask is examined microscopically to determine its purity. The fluid portion of the culture in each flask is then pipetted off with a sterile rubber nipple pipette and placed in the centrifuge tube, care being taken not to get any of the agar from the medium in the material so removed. One cubic centimeter of sterile distilled water is then added to each flask and gently shaken over the agar surface to remove the remaining trypanosomes. This is added to the material previously obtained and the centrifugalization carried out as described for tube cultures. Two parts of the glycerin-saline solution is then added and the antigen placed in the ice-box.

The antigen for this complement fixation test should be kept in an ice-box and, if so kept, has been found to be efficient for as long as one month, but it gradually loses its antigenic strength during this time and should be retitrated before each series of tests.

(*b*) *Titration of the Antigen.*—The titration of the antigen is very important and should be made, as stated, before each series of tests. A metal test tube rack, like those employed in the Wassermann test, and test tubes measuring 100 by 12 mm., are used in making the titration. Twenty tubes, 10 in the anterior row and 10 in the posterior

row of the rack, each containing 0.5 cc. of normal salt solution (0.85 per cent) are set up, and to each of the tubes in the anterior row, except the last, there is added 0.1 cc. of a known positive serum, preferably obtained from a patient suffering from Chagas' disease, but, if this is not possible, the serum of a guinea-pig which has been infected with *Trypanosoma cruzi* may be used. In the posterior row of tubes there is added to each tube, except the last, 0.1 cc. of a known negative serum. The rack is now placed in a water-bath and kept at 57.5° C. (135° F.) for one-half hour, in order to inactivate the sera.

The antigen is now diluted with normal salt solution in the proportion of 1 part of antigen to 20 or 25 parts of salt solution and the following amounts of the dilution placed in the first 8 tubes in the anterior row, after inactivation is completed: 0.025, 0.05, 0.075, 0.10, 0.125, 0.15, 0.20 and 0.25 cc. respectively, and the same amounts are placed in the corresponding tubes in the posterior row. In the ninth tube, the anterior and posterior row will contain only the positive and negative serum respectively, together with the hemolytic system, thus acting as a control of the hemolytic system and serum. The tenth tube in the anterior row will contain only the hemolytic system, acting as a control of it, and the tenth tube in the posterior row will contain only the antigen and the hemolytic system, this tube containing 0.1 cc. of the diluted antigen. This is the antigen control.

The *complement* employed is fresh guinea-pig blood serum and to every tube in the rack there are added $1\frac{1}{2}$ units of complement so diluted that this amount is contained in 0.1 cc. of the mixture of guinea-pig serum and normal saline solution. The tubes are then shaken gently and placed in the water-bath at 37° C. (98.6° F.) and allowed to remain for one-half an hour. At the end of this time there are added to each tube 2 units of anti-sheep cell amboceptor, contained in 0.1 cc. of the normal saline solution, and 0.1 cc. of a 5 per cent suspension of washed red blood cells from a sheep. The tubes are again incubated in the water-bath at 37° C., for one-half an hour and the results of the titration are then read. The tube containing the smallest amount of the positive serum and antigen in which no hemolysis occurs, is noted, and this amount is considered as one unit of antigen. The tubes in the posterior row containing negative serum should show hemolysis as should tubes 9 and 10 in the anterior row and tubes 9 and 10 in the posterior row.

The unit of complement is determined before each series of tests by titrating a 10 per cent solution, in normal saline, of freshly obtained guinea-pig serum against 2 units of sheep cell hemolytic amboceptor and 0.1 cc. of a 5 per cent suspension of washed red blood cells from a sheep, the tubes being heated in a water-bath at 37° C. (98.6° F.) for fifteen minutes and the result of the titration then read, the smallest amount of complement producing complete hemolysis in combination with the amboceptor being considered as one unit of complement.

Kelser recommends a glycerin-preserved hemolytic amboceptor diluted so that 2 units of amboceptor are contained in 0.1 cc. of the mixture of complement and amboceptor serum. There would appear to be no reason why the sheep cell amboceptor used in this test should not be preserved upon filter paper in the manner described for the complement fixation test for ambeiassis (see page 102).

The unit of amboceptor serum is ascertained by titrating it with an arbitrary amount of diluted complement, as 0.1 cc., of a 1 to 10 dilution in normal saline. The amount of amboceptor contained in the first tube showing complete hemolysis is considered as one unit of amboceptor.

Technique of the Test.—If a large number of tests are to be made, as in making a survey, two tubes are employed for each serum to be tested but if only a few tests are to be made it is preferably to use three tubes for each serum. If three tubes are used two are placed in the front row of the test tube rack and one in the back row, while if only two tubes are used one is placed in the front row and one in the back row. In each of the tubes there is placed 0.5 cc. of normal salt solution and in the front row tubes, if three tubes are used, 0.1 and 0.05 cc. of the serum to be tested, while if only two tubes are used, 0.1 cc. of the serum to be tested is used. In the tubes in the back row is placed 0.1 cc. of the serum to be tested. In addition, for each series of tests an anterior and posterior tube is used for a control of a known positive and a known negative serum, 0.1 cc. of such serum being placed in each tube. The tubes are now placed in a water-bath at 57.5° C. (135° F.) for one-half hour in order to inactivate the sera.

After inactivation has been completed there is added to every tube 1½ units of complement and to the tubes in the front row of the rack 1½ to 2 units of the antigen, and the tubes incubated in the water-bath at 37° C. (98.6° F.) for one-half an hour, after which there is added to each tube 2 units of the hemolytic amboceptor and 0.1 cc. of a 5 per cent suspension of washed sheep's corpuscles. After incubating again at the same temperature for one-half an hour the results of the tests are read.

In *reading the tests* it should be found that complete hemolysis has occurred in all of the tubes in the posterior row and in both tubes containing the negative control serum. Complete inhibition of hemolysis should be present in the anterior tube containing the positive control serum and in all anterior tubes containing sera from individuals infected with *Trypanosoma cruzi* in which case the reaction is called a 4-plus reaction. Varying degrees of inhibition of hemolysis may be observed in tubes containing serum from infected individuals or in uninfected individuals whose blood serum may contain inhibiting substances of non-specific nature. Complete hemolysis should be observed in normal individuals. A 4-plus and 3-plus reaction is

regarded as positive and diagnostic, while 2-plus and 1-plus reactions are regarded as suspicious.

Remarks.—In his original paper, describing this test, Kelser (1936) stated that it had been used in testing over 400 sera, including sera from known cases of Chagas' disease, and that it had given positive results in all infected cases and negative results in individuals in whom there were no evidences of this infection. In a later paper, Johnson and Kelser (1937) detail the results obtained with the test in a survey for Chagas' disease in Panama. Of 1251 individuals tested, 37 gave a positive reaction and 11 gave suspicious reactions, a combined rate of 3.83 per cent. The individuals tested who gave positive reactions presented no symptoms of the infection and were evidently carriers, thus demonstrating the value of the test in ascertaining the real prevalence of this infection. Individuals positive with the Wassermann test were invariably negative with this test and no cross-reactions were obtained with antigens prepared from *Trypanosoma equiperdum* or *Trypanosoma hippicum*.

The Kelser complement fixation test for Chagas' disease is apparently a most useful addition to our diagnostic methods and, because of the ease with which the antigen is prepared, and the fact that it is not contaminated by extractives from tissue cells, as are the antigens previously used in complement fixation tests for this infection, it is the test that should be employed in diagnosis, in the opinion of the writer. In addition, this test would appear to be especially valuable for the detection of symptomless infections (carriers), thus enabling one to ascertain the real incidence of the infection in any locality.

Villela and Bicalho's Complement Fixation Test.—This complement fixation test, described by Villela and Bicalho, in 1923, is a modification of the Guerreiro and Machado test.

The *antigen* is prepared from the heart and spleen of severely infected puppies. These organs are removed aseptically, blood clots removed, and the tissue washed thoroughly in normal saline solution. They are then cut into very small pieces and triturated in a mortar, after which to 1 part of the triturated pulp there is added 2 parts of distilled water, 1 part of glycerin, and enough phenol to make a 1 per cent concentration. The mixture is then allowed to remain at room temperature for forty-eight hours, shaking it at intervals, and is then filtered through gauze and placed in the ice-box, until a sediment appears. It is then filtered and the filtrate is used as the antigen. It should be kept on ice and retains its antigenic properties for as long as four months.

The *technique* of this test is similar to that of other complement fixation tests.

Remarks.—Using this test, Villela and Bicalho obtained positive results in 34 of 39 patients suffering from Chagas' disease and consistently negative results in normal individuals, and individuals giving

a positive Wassermann reaction did not react with this test. Lacorte (1927), using an antigen prepared from the spleen of an infected puppy after the method of Villela and Bicalho, tested 200 cases of suspected Chagas' disease and obtained positive results in 159, or 79 per cent and others have obtained similar results. There is apparently little choice between the Guerreiro and Machado and Villela and Bicalho tests so far as the results are concerned but here, as in the Guerreiro and Machado test, the antigen contains extractives of tissue cells as well as trypanosomes and is not as truly a specific antigen as the one devised by Kelser.

There can be no question but that complement fixation is a valuable serological method of diagnosing Chagas' disease and that, of the tests so far elaborated, that of Kelser appears to be the most accurate and delicate.

Packchanian's Agglutination Test.—Recently Packchanian (1940) has described an agglutination test for the diagnosis of Chagas' disease which gives a high degree of agglutination with the serum of animals infected with *Trypanosoma cruzi*, the immune serum being obtained by inoculating rabbits with washed cultures of this organism. The antigen for this test is prepared as follows:

Trypanosoma cruzi is cultivated for from one to three weeks on N.N.N. medium (see page 175), the tubes being sealed with rubber caps or stoppers and kept at 25° C. (77° F.). After incubation the growth in the tubes is suspended in Tyrode's solution and shaken in a flask with glass beads, centrifugalized at low speed to remove coarse particles, and then at high speed for one hour. The resultant deposit of trypanosomes is resuspended in Tyrode's solution and washed several times by centrifugalization at high speed, after which a suspension of the washed trypanosomes is made in the Tyrode solution of such concentration that 1 cc. contains about 2,000,000 trypanosomes.

To *immunize rabbits* the trypanosomes are killed by adding 0.04 per cent of formalin and the animals inoculated intravenously every two or three days with 1 cc. of the antigen. The agglutination titre of their sera is tested at intervals and after it reaches 1 to 1000, from 18 to 24 subsequent inoculations are made with a suspension of living trypanosomes which have been washed in Tyrode solution. Animals so immunized show a very high agglutination titre, in one case as high as 1 to 260,000.

The agglutination test for infections with *Trypanosoma cruzi* is performed by adding to each testing tube 0.5 cc. of the serum to be tested and an equal amount of freshly prepared suspension of the organism containing over 2,000,000 per cc. The tubes are then shaken and kept at 37° C. (98.6° F.) for thirty minutes. Agglutinations are obtained in high titre with the immunized rabbit's sera.

Remarks.—While good agglutination is obtained in immunized rabbit sera, the diagnostic value of this test has not been ascertained in

man. It presents certain difficulties that would apparently make it of uncertain value as a practical method of diagnosis, in the writer's opinion.

Mayer and Pifano's Intradermal Test.—Mayer and Pifano (1941) have described an intradermal reaction for the diagnosis of Chagas' disease, employing an antigen prepared from cultures of *Trypanosoma cruzi*, as follows:

The trypanosome is cultivated upon the N.N.N. medium (see page 175), the resultant growth drawn from the bottom of the tubes with a glass pipette, washed three times with normal saline, and the sediment emulsified, in normal saline containing 0.5 per cent phenol. The antigen is called "cruzin" and was tested in patients suffering from known Chagas' disease, by intradermal injection.

The diagnostic reaction consists in a marked inflammatory reaction accompanied by a local swelling at the site of injection. The reaction reaches its maximum at the end of forty-eight hours. The reaction was not positive in malaria, hook-worm infection, or phagedenic ulcers. More recently Mazza, Basso and Basso (1942) have recommended this test as highly valuable in the practical diagnosis of Chagas' disease.

Remarks.—From the reports that are available it would appear that in this intradermal test we possess a valuable method of diagnosis of comparative simplicity.

CHAPTER XVII

INOCULATION OF ANIMALS WITH TRYPANOSOMES

INOCULATION OF ANIMALS WITH TRYPANOSOMA GAMBIENSE—WITH TRYPANOSOMA RHODESIENSE—WITH TRYPANOSOMA CRUZI—OTHER METHODS OF DIAGNOSIS OF THE TRYPANOSOMIASES—CRITIQUE OF DIAGNOSTIC METHODS FOR THE TRYPANOSOMIASES

Introduction.—In both African sleeping sickness and in Chagas' disease, especially in the latter, the causative trypanosomes may be so scarce in the blood, gland juice or cerebrospinal fluid, that one must resort to some other method of demonstrating their presence than the examination of these fluids. In the diagnosis of Chagas' disease animal inoculation is more valuable, as a diagnostic measure, than in African sleeping sickness, for even in the acute stage of Chagas' disease *Trypanosoma cruzi* may occur in such small numbers in the peripheral blood as to render a microscopic examination useless, and the inoculation of susceptible animals necessary in order to demonstrate the parasite. Animal inoculation is also very important in the differentiation of the various species of trypansomes by means of immune serum reactions and in the study of the lesions of diagnostic importance produced by the trypanosomes in the tissues of such animals.

Susceptibility of Animals to Infection.—Natural infections with all of the trypanosomes causing disease in man have been found in certain animals, and some of these animals act as reservoirs of infection for man. *Trypanosoma gambiense* has been reported as occurring in naturally infected antelope (Duke, 1912); in dogs (Gray and Tulloch, 1907); in cattle (Bruce, Hamerton, Bateman and Mackie, 1910); in monkeys (Koch, Beck and Kleine, 1909); in cows (Kleine and Eckard, 1913) (Yorke and Blacklock, 1915) and in a sheep and a goat, by Kleine and Eckard. The domestic pig has been found naturally infected by Van Hoof, Heurard and Peel (1937) and these observers believe this animal to be a common reservoir of infection for man. Unfortunately, the impossibility of distinguishing between this trypanosome and *Trypanosoma brucei*, which commonly causes infection in most of the animals mentioned, renders the reports mentioned of doubtful value in most instances, and while domestic animals, as cattle, sheep and dogs, may sometimes act as reservoirs of infection for man it has not been definitely proven that wild animals, as the various species of antelope, do not act as reservoirs in the case of *Trypanosoma gambiense*.

Natural infections with *Trypanosoma rhodesiense* have been found to

exist in most of the big game animals of Africa, especially in various species of antelope, and these animals act as reservoirs of infection for man. While there is not sufficient evidence to establish any of the species of antelope as reservoirs of infection of man with *Trypanosoma gambiense* it is believed by practically all authorities that these animals play a very important part in the epidemiology of sleeping sickness caused by *Trypanosoma rhodesiense*, acting as reservoirs from which the transmitting flies, belonging to the Genus *Glossina* derive the trypanosome, which, after a cycle of development within them is, in turn, transmitted to man by the bites of infective flies. This trypanosome has also been reported in a naturally infected dog (Kinghorn and Yorke, 1912).

Natural infections with *Trypanosoma cruzi* have been found in the dog (Pinto, 1923) (Clark and Dunn, 1932); the cat (Chagas, 1909); various species of armadillo (Chagas, 1912) (Torres, 1915) (Chagas, 1918); various species of bats (Clark and Dunn, 1932); the opossum (Clark and Dunn, 1932) (Kofoid and Donat, 1933); the squirrel (Clark and Dunn, 1932); the wood rat (Kofoid and Donat, 1933), and monkeys (Chagas, 1909). All of these animals probably act as reservoirs of infection for man.

Experimental infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* have been produced in cats, chimpanzees, dogs, guinea-pigs, lemurs, mice, monkeys, rabbits, rats and several other animals but those most useful in the laboratory diagnosis of these infections and for maintaining strains of these trypanosomes in animals are white rats, white mice and guinea-pigs. Young animals are most easily infected and the trypanosomes are much more numerous as heavy infections are more frequently obtained. The trypanosomes multiply in the infected animals and finally become so numerous that the death of the animal results. Usually the virulence of both trypanosomes is increased by passage from animal to animal until a peak is reached, after which the virulence gradually decreases and by rotating different species of animals for inoculation the virulence may be much diminished. When the virulence of a strain is at its highest mice usually survive after infection for about five days, rats for about ten days and guinea-pigs for about twenty days. *Trypanosoma gambiense* is much less virulent to experimental animals than is *Trypanosoma rhodesiense*, rats surviving for several months after inoculation with the former, the infection being mild in type and trypanosomes scarce in the blood, while inoculation with the latter usually results in a lethal infection within a comparatively short time, accompanied by many trypanosomes in the peripheral blood.

Experimental infections with *Trypanosoma cruzi* have been produced in cats, chimpanzees, dogs, guinea-pigs, lemurs, monkeys, rats, mice, and some other animals but the most useful animal for diagnostic purposes is the guinea-pig, while for maintaining strains of this trypan-

osome white rats and guinea-pigs are usually employed. Young animals are most easily infected and the trypanosome loses its virulence after repeated passages through an animal, so that in order to maintain the organism in animals it is best to inoculate only young guinea-pigs or rats and to subinoculate as soon as trypanosomes appear in the blood. It is also well to change from guinea-pigs to rats at intervals as this practice appears to favorably affect the transmission of the infection. Unless prompt subinoculations and change in the animal host is followed a strain may lose its virulence and become non-infective.

In the case of *Trypanosoma cruzi*, the experimental infection of the invertebrates that transmit Chagas' disease is possible and may be used in diagnosis (see Xenodiagnosis, page 241) or to maintain the organism for experimental purposes. The various species of bugs belonging to the Triatomidæ in which *Trypanosoma cruzi* undergoes a cycle of development can be easily infected by allowing them to bite an infected individual or animal and, as these bugs may live for long periods of time and still remain infected, this method of diagnosis and of maintaining a strain of the organism is sometimes useful. In inoculating animals from infected bugs the feces or the macerated intestine of the bug should be mixed with normal saline and used as the inoculum.

Methods of Inoculation.—Animals may be inoculated subcutaneously or intraperitoneally, the latter being preferable. Blood should be drawn from the vein of the suspected individual, the amount varying with the animal to be inoculated. If guinea-pigs are used about 2 cc. of blood is drawn by means of a glass syringe which has been sterilized and rinsed out with citrate solution. This amount is at once injected into the peritoneal cavity, under aseptic precautions, and if trypanosomes are present, the animal will usually become infected and the trypanosomes will appear in its peripheral blood in from one to two weeks. If smaller animals, as white rats or mice are used, from 0.05 to 1 cc. of blood will be sufficient. It has been found by Culbertson and Kessler (1942) that if mice are used it is best to inoculate young animals less than twenty-five days old, as in such animals an intense and fatal infection develops and trypanosomes are numerous in the blood, if they be inoculated intraperitoneally. Mice less than nine days old may be infected orally but the infection is mild and few trypanosomes occur in the blood. If rats are employed a careful examination should be made of the blood before inoculation for the possible presence of *Trypanosoma lewisi*, a common trypanosome infecting these animals, for if this parasite be present it is impossible to differentiate it from the trypanosomes causing disease in man upon morphological grounds, with the exception of *Trypanosoma cruzi*.

In transferring infections from animal to animal a few drops of blood from the ear or tail of the infected animal are mixed with a little

normal salt solution (0.85 per cent) in a watch-glass and the mixture then inoculated intraperitoneally by means of a small glass syringe, the entire procedure being performed under strict aseptic precautions. In the case of guinea-pigs, blood may be removed directly from the heart without sacrificing the animal and this procedure is especially useful in maintaining infections in animals with *Trypanosoma cruzi* as the guinea-pig is the best animal to use in maintaining such infections. In their work in Panama, upon Chagas' disease, Clark and Dunn (1932) were able to maintain infections with *Trypanosoma cruzi* indefinitely in the guinea-pig by intraperitoneal injections of 0.5 cc. to 1 cc. of the heart's blood of infected guinea-pigs. The incubation period, *i. e.*, the period from the time of inoculation to the first appearance of trypanosomes in the peripheral blood, varied from seven to twenty-seven days, with an average period of about ten days in their guinea-pigs. The number of trypanosomes occurring in a microscopic field also varied greatly, some guinea-pigs never showing more than 1 per field while in others as many as 500 trypanosomes were counted in a single microscopic field. Thick blood smears were used in their experiments. The number of days elapsing between the first appearance of trypanosomes in the blood and the highest count varied greatly, from ten days to as long as forty days, and, in one case, in which infection was produced by the inoculation of macerated infected *Triatoma*, this period was not reached until one hundred and fifteen days after the organisms were first seen in the blood. Similar results have been obtained by other observers in experimental infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* in guinea-pigs.

OTHER DIAGNOSTIC METHODS

Besides the various diagnostic methods that have been described for infections with the trypanosomes causing disease in man there are other methods that have been employed to a considerable extent and are recommended by several authorities. These are cisternal puncture, to replace lumbar puncture, puncture of enlarged lymphatic glands, biopsy of lymphatic glands, section of Chagomas, sternal and splenic puncture and xenodiagnosis.

Cisternal Puncture.—This method of obtaining cerebrospinal fluid for diagnostic purposes was first advocated by Wegeforth, Ayer and Essick (1920) and by Eskuchen (1923). Ayer, in 1923, described his method for puncture of the cisterna magna and since these papers appeared numerous authorities have used the method for both diagnostic and remedial purposes in cerebrospinal meningitis infections and for the relief of cerebral pressure. French physicians have strongly recommended the procedure in the diagnosis of African sleeping sickness and Tejera prefers it to lumbar puncture for obtaining cerebrospinal fluid in cases of Chagas' disease. The advantages claimed for

the method are its greater safety, the avoidance of severe headaches and other symptoms after puncture, and its technical simplicity.

TECHNIQUE OF CISTERNAL PUNCTURE.—All instruments and dressings used in the procedure must be sterilized, the occipital region shaved, the skin thoroughly cleansed and painted with iodine, and the area selected for puncture surrounded by sterilized gauze. The needle employed should have a stylet and should be of 18 or 19 gauge, having a guard located not more than 6 cm. from the tip, so as to prevent its being passed more than this distance during the puncture. A lumbar puncture needle is satisfactory and the guard may be provided by passing the needle through a small cork to the required distance from the tip of the needle.

The patient is placed upon his side with the head flexed upon the chest and the knees against the abdomen. Care should be taken to maintain the alignment of the vertebral column, the cervical and thoracic spine being level and not rotated. A pillow should be placed beneath the head and so arranged that the vertebral column is perfectly in alignment with the center of the occipital region. Although not absolutely necessary it is best to infiltrate the skin where the puncture is to be made with a 1 per cent solution of novocaine, as this will make the operation painless. The needle is introduced in the depression beneath the occiput exactly in the mid-line, above the spine of the atlas and close to the base of the skull. It is pushed very gently and slowly forward and obliquely upward toward the normal position of the hair-line. After the needle has penetrated to about $3\frac{1}{2}$ cm. the stylet is removed and at a depth of from 4 to 5 cm., in an adult, a sensation of loss of resistance, sometimes accompanied by a faint snap, will be felt as the needle punctures the dura and enters the cisterna magna. If the needle comes into contact with the occiput during the procedure the point should be depressed sufficiently to clear the obstruction. The needle should never be introduced for more than 6 cm., and fluid should flow from the needle before that depth is reached. The same precautions to avoid accidents and the same contraindications to cisternal puncture obtain as in lumbar punctures (see page 207).

Remarks.—Cisternal puncture, while recommended for the purpose of obtaining cerebrospinal fluid in the diagnosis of the trypanosomiases, cannot be considered as a perfectly safe operation and should never be attempted by anyone who has not performed it on the cadaver several times. There is always danger of penetration of the medulla or cerebellum and Dandy (1935) has demonstrated that in some individuals the cerebellum may extend into or below the foramen magnum, making the cistern so small that it would be impossible to enter it with a needle without injuring the brain tissues. Hemorrhage, requiring operative measures, may occur, and compression of the medulla, if there is a marked increase in the intracranial pressure, through the herniation of the brain stem through the foramen magnum. In the hands of

trained surgeons cisternal puncture is probably as safe, if not safer, than lumbar puncture, but it should not be employed as a routine diagnostic procedure in preference to the latter in the diagnosis of the tryanosomiases except by an experienced operator.

Biopsy of Enlarged Lymphatic Glands.—Mazza, Basso and Basso (1942) have recommended the sectioning of enlarged lymphatic glands occurring in Chagas' disease as a diagnostic method. According to these investigators leishmania forms of *Trypanosoma cruzi* are present in stained preparations, occurring in histiocytes in enormous numbers. As enlarged glands are usually present early in the disease this method of diagnosis is especially valuable at this time when the peripheral blood may show very few or no parasites. Later in the disease enlarged glands may also be present and, if the blood is negative for trypanosomes, biopsy of a gland is often diagnostic.

Puncture of Enlarged Lymphatic Glands.—According to Cersiani and Junqueira (1943) the most efficient diagnostic method, if the blood is negative for *Trypanosoma cruzi*, is the microscopic examination of the juice obtained by puncture of enlarged lymphatic glands, if such are present, as they usually are in acute infections. The technique of gland puncture and of the examinations of gland juice are described on page 206.

Section of Chagomas.—Chagomas are tumor formations following cutaneous inoculation of *Trypanosoma cruzi*. These are caused by the dispersion of the trypanosomes through blood stream infection and the occurrence of metastatic swellings. Section of these chagomas show the presence of the organism in the leishmania stage within histiocytes and this method of diagnosis is positive in some cases in which the blood is negative, as well as the Machado reaction (see page 228) and xenodiagnosis. (See page 228.)

Sternal and Splenic Puncture.—The puncture of the sternum or the spleen has been employed to some extent in the diagnosis of the trypanosomiases but is not as useful as in the diagnosis of kala-azar. The technique is the same (see pages 154 and 155). Robin and Brochen (1939) have proven that sternal puncture does not give as good results as either gland puncture or the examination of the blood but Linhard (1943) claims that the examination of the bone-marrow obtained by sternal puncture is much more successful in demonstrating trypanosomes than the examination of thick blood films.

Xenodiagnosis.—This ingenious, and often successful, diagnostic method for Chagas' disease was introduced by Brumpt, in 1914. It consists in allowing laboratory bred triatomid bugs to bite the suspected individual. If infection with *Trypanosoma cruzi* be present, even though the trypanosomes are in such small numbers in the peripheral blood as to be undemonstrable, the bugs will become infected, the organisms multiplying rapidly in the intestine, and an examination of the intestinal contents will disclose their presence, thus establishing

the diagnosis. This method of diagnosis has proven useful where it could be properly applied but it necessitates the raising of the triatomid bugs in the laboratory, as bugs caught in nature may be infected with the trypanosome and cannot be used in the test. The method probably deserves a much more extended use than it has had in the past but if the complement fixation test proves as accurate as it apparently has to date, xenodiagnosis would very rarely be useful.

CRITIQUE OF DIAGNOSTIC METHODS

The diagnosis of African sleeping sickness and Chagas' disease should, if possible, be based upon the demonstration of the causative trypanosome in the blood, tissues or cerebrospinal fluid of the suspected individual, and this is almost always possible in one way or another.

The laboratory methods that are available for diagnosis in infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense*, *i. e.*, in African sleeping sickness, are the microscopic examination of the blood, gland juice, bone-marrow and cerebrospinal fluid, the cultivation of the trypanosomes, and animal inoculations, while those available for the diagnosis of infections with *Trypanosoma cruzi*, *i. e.*, in Chagas' disease, are the microscopic examination of the blood and cerebrospinal fluid, gland juice, bone-marrow, the cultivation of the trypanosome, the complement fixation test, animal inoculations and xenodiagnosis.

The *microscopic examination of the blood* in all three infections is very important and, if carefully and patiently conducted, will result in the demonstration of the trypanosomes in a goodly percentage of the cases, when acute symptoms are present. As an early diagnosis is most important, from a remedial standpoint, in African sleeping sickness, the examination of the blood should never be neglected but it must be remembered that the trypanosomes are frequently small in number and are only demonstrable during the febrile periods of the acute diseases and disappear from the blood after the chronic, or central nervous stage of these infections is reached. The percentage of positive results that have been obtained from microscopic examination of the blood has varied greatly with different observers, due to the technique employed, the time spent in the examination, the number of blood smears examined, and whether thin or thick films were examined. Ross and Thomas (1909) were the first to demonstrate that in sleeping sickness of Africa, the trypanosomes occur in the peripheral blood in much greater numbers at one time than at another, during the acute stage of these infections. They found that in one patient they studied there were 19 distinct rises in the number of trypanosomes demonstrable in the peripheral blood, there being an average of six and one-half days between each rise, the shortest period being four

days and the longest eight days, and that with each rise in the number of trypanosomes there occurred a rise in the temperature and an increase in the acute symptoms of the infections. The rise in the number of trypanosomes in the peripheral blood was not associated with any cyclical development of the trypanosomes but rather appeared to be caused by variations in the resistance of the patient to the infection. Numerous investigators have confirmed these observations and they partly explain the great variation in the percentage of positive results obtained by different observers in the microscopic examination of the blood in infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense*. Centrifugalization of the blood greatly increases the percentage of positive results. The percentage of positive results obtained by several observers in the microscopic examination of the blood in African sleeping sickness are of interest in showing what may be expected from this method of diagnosis and also in showing the variations obtained by trained investigators, for one reason or another.

Dutton, Todd and Tobey (1906) examined the peripheral blood of 250 cases of sleeping sickness and in 220 cases, in which a direct examination was made, they obtained positive results in only 30, or 13.6 per cent. In 17 cases in which the blood was centrifugalized, positive results were obtained on 8, or 47 per cent. Martin and Leboeuf (1908) examined 217 cases of sleeping sickness and found trypanosomes present in the peripheral blood in 37 per cent, after a direct examination of the blood, while in the centrifugalized blood of 75 cases, trypanosomes were demonstrated in 92 per cent. In 517 cases of sleeping sickness they examined, the causative trypanosome was found in the blood in 96.8 per cent when the results obtained by direct examination and the examination of centrifugalized blood were added together. Clapier (1921) obtained positive results in only 19 of 93 cases of sleeping sickness by direct blood examination, while Broden, in 1920, examining centrifugalized blood from 336 cases of sleeping sickness obtained positive results in 80.7 per cent.

From these observations it is evident that centrifugalization of the peripheral blood greatly increases the chances of demonstrating the trypanosomes before the development of cerebral symptoms and that, using this method, one may expect positive results in from 80 to 95 per cent of the cases. The low percentage obtained after direct examination may be explained by the time when the blood examinations were made, the number of blood films examined, and the failure to use thick blood smears. It is believed that a much higher percentage of positive results may be expected from a direct blood examination, if the blood be examined when there is a decided rise in the patient's temperature, if thick blood smears be examined, and if several such preparations be examined before a negative report is accepted.

In Chagas' disease, the microscopic examination of the peripheral blood is only positive for *Trypanosoma cruzi* during the acute, febrile

stage of the disease and, more rarely, during febrile exacerbations occurring in the chronic stage. The trypanosomes usually are present in small numbers in the peripheral blood even in the acute stage of Chagas' disease and negative results are frequent even after repeated microscopic examination and resort must be had to other laboratory methods of diagnosis. Thick blood preparations should be used and several should be examined before a negative result is accepted. If such preparations are negative, the blood should be centrifugalized and specimens of the sediment examined for the trypanosome.

The percentage of positive results that may be expected from the microscopic examination of the blood during the acute febrile stage of Chagas' disease, if several thick drop preparations are carefully examined, should closely approach 80 per cent, and if centrifugalized blood be examined, a still higher percentage may be expected. The trypanosomes are usually present in very small numbers but if enough preparations be examined, can usually be demonstrated if fever be present. In the chronic stage of this infection, it is useless to examine the blood unless fever be present and, even then, it is usually negative for *Trypanosoma cruzi*.

The *examination of the cerebrospinal fluid*, as a diagnostic method in African sleeping sickness, is useful only after symptoms indicating involvement of the central nervous system appear. In infections with *Trypanosoma gambiense* such symptoms often do not appear until several months after the acute symptoms and, as treatment is sometimes effective even at this stage of the disease, this method of diagnosis is very useful, but in infections with *Trypanosoma rhodesiense* this method of diagnosis is of little value, as treatment is useless at this stage of the infection.

The trypanosomes are most numerous in the cerebrospinal fluid in those patients presenting marked symptoms of central nervous system involvement, especially in those showing profound lethargy. In the acute febrile stage of African sleeping sickness examination of the cerebrospinal fluid for trypanosomes is useless as they cannot be demonstrated in the fluid at that time.

The percentage of positive results that may be expected from the examination of the cerebrospinal fluid in African sleeping sickness depends upon the care with which the examination is made and the severity of the involvement of the central nervous system. The percentage of positive results is very much lower than that obtained from blood examinations in the acute stage of the infections and seldom has exceeded 20 per cent, while percentages between 5 and 10 per cent have been recorded by some observers.

The *cell count* of the cerebrospinal fluid is of considerable value in diagnosis and Broden and Rodhain (1908) found that during the early stage of involvement of the central nervous system there was an increase in the number of lymphocytes while later there appeared

large mononuclear leukocytes. There is a marked increase in the number of leukocytes, the normal 3 to 5 cells of the cerebrospinal fluid being increased to several hundred in advanced infections and varying between 15 to 30 cells in the early stage of involvement of the central nervous system.

In cases of Chagas' disease, in which symptoms indicating central nervous system involvement occur, the examination of the cerebrospinal fluid has sometimes resulted in the demonstration of *Trypanosoma cruzi* but this method of diagnosis is of very limited application in this infection and there are no statistics available showing what percentage of positive findings may be expected from the procedure.

The *examination of glandular juice* is a most valuable diagnostic method in African sleeping sickness and it appears to be the consensus of expert opinion that it gives the highest percentage of positive results of any of the diagnostic measures that are available for infections with *Trypanosoma gambiense* and *Trypansoma rhodesiense.*

This method of diagnosis was first recommended by Greig and Gray (1904) who, following a suggestion made by Mott, examined the lymphatic glands in 15 cases of sleeping sickness and found the trypanosomes in all in much larger numbers than in the blood or cerebrospinal fluid. Later observers demonstrated that they could be demonstrated in the glands in the earliest stages of infection with either *Trypanosoma gambiense* or *Trypanosoma rhodesiense.* Greig and Gray's first observations were made upon excised lymphatic glands but they later found that puncture of the enlarged glands and the examination of fluid so obtained gave similar results.

The percentage of positive results that may be expected from the examination of glandular juice depends upon the stage of the infection, the highest percentage being obtained during the acute febrile stage and the lowest during the sleeping sickness stage. During the acute stage the glands are soft and enlarged while during the chronic, or sleeping sickness stage, when the glands are hard and shrunken, puncture is usually impossible and the glandular juice is negative for trypanosomes.

The percentage of positive results that may be expected from the examination of glandular juice in African sleeping sickness is very high, if the procedure is properly performed and several smears of the juice be examined. The results obtained by Martin, Leboeuf and Roubaud (1909) and by Broden (1920) are illustrative of those which may be expected. Martin, Leboeuf and Roubaud examined the glandular juice of 400 individuals suffering from African sleeping sickness and found trypanosomes in 353, or 88.25 per cent, while Broden examined the 336 patients and found the glandular juice positive for trypanosomes in 294, or 87.7 per cent. In 12 per cent of 459 cases of African sleeping sickness, Martin, Leboeuf and Roubaud found that gland puncture was impossible because the glands were too small or were not

palpable. They found that the highest percentage of positive results were obtained from puncture of the posterior cervical glands.

Some authorities have advocated the microscopic examination of stained preparations of glandular juice but the recent observations of Crozafon (1939) have shown that there is no advantage gained by staining and it simply adds to the labor involved in such examinations. From his experience he concludes that, next to the examinations of centrifugalized blood preparations, the examination of fresh, unstained glandular juice is the best method of diagnosis of African sleeping sickness and this opinion is shared by all authorities who have had an extended experience with the method. Taking into consideration the results that have been obtained by many investigators it is believed that between 90 and 95 per cent of positive results may be obtained by the examination of glandular juice during the acute febrile stage of these infections.

Recent writers have stressed the diagnostic value of *glandular punctures* in Chagas' disease. Usually this is possible only early in the infection during the acute febrile symptoms when the cervical and inguinal glands are enlarged.

The *cultivation of the trypanosomes* causing African sleeping sickness and Chagas' disease is a diagnostic method that may be employed in those cases in which the blood and glandular juice examinations have given negative results. As already stated, the cultivation of *Trypanosoma gambiense* and *Trypanosoma rhodesiense* is attended with great difficulty and for this reason the method is seldom resorted to in the diagnosis of African sleeping sickness and the percentage of positive results that may be expected from culture methods is very small. The more recent work upon the cultivation of these trypanosomes in living chick embryos (see page 221) gives promise of far better results than have been obtained with other culture media and this method of cultivation may prove useful in diagnosis.

The cultivation of *Trypanosoma cruzi*, following the methods described by Kelser (1936) (see page 217) and others, should prove useful as a diagnostic method for Chagas' disease but there are little data available showing the percentage of positive results that may be expected from the use of this method.

The *inoculation of susceptible animals* is an important diagnostic method, especially in the diagnosis of Chagas' disease, in which it has been extensively employed with excellent results. In infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense*, owing to the excellent results obtained from the microscopic examination of the blood and glandular juice in the early stage of these infections, animal inoculations are rarely employed as a diagnostic measure but in Chagas' disease, owing to the small number of trypanosomes often present in the peripheral blood, the inoculation of animals has proven a most useful diagnostic method. The inoculation of from 0.5 to 0.1 cc. of

blood from the suspected individual intraperitoneally into a guinea-pig will result in the animal becoming infected in close to 90 per cent of those so inoculated, even though the patient's blood be negative upon microscopic examination, if infection with *Trypanosoma cruzi* be present.

Complement fixation as a method of diagnosis in African sleeping sickness is, at present, of little, if any, practical value, but in the diagnosis of Chagas' disease it is of great value, as shown by Guerreiro and Machado (1913), Villela and Bicalho (1923), Kelser (1936) and Davis (1943) and it is probable that it will largely replace animal inoculation and culture as a method of diagnosis of infections with *Trypanosoma cruzi.*

The percentage of positive reactions that may be expected with the complement fixation test in Chagas' disease, as perfected by Kelser, is very high. This investigator obtained practically 100 per cent of positive results in known cases of Chagas' disease and a similar percentage of negative results in normal individuals and in individuals suffering from other diseases. These results warrant the use of this test as a routine procedure in all cases in which *Trypanosoma cruzi* cannot be demonstrated in the peripheral blood and the test is especially valuable in latent and chronic infections with this parasite.

Xenodiagnosis, as suggested by Brumpt (1914) is a useful procedure but its practical value is limited to its use in laboratories where bugs belonging to the Triatomidæ can be propagated. The percentage of positive results that may be expected from this test has not been accurately ascertained but, in all probability, is considerable, according to the data available.

The *examination of sections* of the enlarged glands and of bone-marrow obtained by sternal puncture are valuable diagnostic methods but should be employed only if other methods have failed.

Procedure for the Diagnosis of African Sleeping Sickness.—The following procedure is suggested as routine in the diagnosis of African sleeping sickness, whether caused by *Trypanosoma gambiense* or *Trypanosoma rhodesiense.*

During the *acute febrile stage* of these infections a microscopic examination of several preparations of unstained peripheral blood should be made and, if these prove negative, several thick blood preparations should be made, stained with the Wright stain, and examined. If these are negative the blood is centrifugalized and several preparations of the sediment examined (see page 153).

If these are negative for the trypanosomes and enlarged lymphatic glands are present, gland puncture should be performed and several preparations of gland juice examined, either unstained or stained with the Wright or some other modification of the Romanowsky stain. If these are also negative it is altogether probable that the patient is not suffering from African sleeping sickness, but it would be wise, if very

suggestive symptoms are present, to inoculate a white rat or guinea-pig intraperitoneally with from 1 to 5 cc. of the patient's blood the amount varying with the size of the animal used for inoculations. At the same time, cultures of the patient's blood should be made upon the N.N.N. medium or other suitable culture media, and, if facilities are available, the blood should be cultured in living chick embryos. The examination of the bone-marrow obtained by sternal puncture may also be made following the blood examinations, if gland puncture is not practicable.

In the *chronic*, or *sleeping sickness stage* of African sleeping sickness, lumbar or cisternal puncture, and the microscopic examination of the cerebrospinal fluid, after centrifugalization, should be followed, several preparations of the sediment being examined either stained or unstained. If these are negative for the trypanosomes a rat may be injected intraperitoneally with the sediment from the cerebrospinal fluid after centrifugalization.

Procedure for the Diagnosis of Chagas' Disease.—In the acute stage of this infection several blood smears should first be examined unstained for *Trypanosoma cruzi* and, if negative, several preparations should be stained and examined. In making stained preparations the thick drop method should be used as recommended for the leishmania (see page 153). If these are also negative guinea-pigs should be inoculated intraperitoneally with 0.05 to 1 cc. of the patient's blood. Before resorting to animal inoculation the blood serum of the suspected individual should be tested for complement fixation according to the method recommended by Kelser (see page 229) and, if this is negative, gland juice should be examined. Culture of the blood should also be tried, if necessary.

In the *chronic stage* of Chagas' disease blood examinations are useless unless fever is present and then centrifugalized preparations should be examined as well as thick blood films. If nervous symptoms are present the cerebrospinal fluid should be examined as in infections with *Trypanosoma gambiense* and *Trypanosoma rhodesiense* and if both blood and cerebrospinal fluid give negative results the complement fixation test of Kelser or Davis and guinea-pig inoculations should be performed, using both the blood and cerebrospinal fluid for the inoculations in cases showing nervous symptoms but the blood only in other cases.

In laboratories in which the necessary facilities are available the xenodiagnostic test of Brumpt (see page 241) should be employed if other diagnostic methods have failed to demonstrate the presence of *Trypanosoma cruzi* while the examination of bone-marrow obtained by sternal puncture may also be employed to if other methods give negative results.

Considerable assistance in the diagnosis of African sleeping sickness is afforded by the aldehyde or formol-gel test (see page 183) and the

cell count of the cerebrospinal fluid (see page 244). If the aldehyde test shows *complete solidification* within a period of one hour, and kala-azar can be eliminated, many authorities regard the result as diagnostic of African sleeping sickness, but most regard it as only very suggestive and as indicating a very careful search for the causative trypanosome in the blood or cerebrospinal fluid. An *increase in the cell count* of the cerebrospinal fluid is also suggestive but not diagnostic.

Utilizing the various diagnostic laboratory methods now available it is believed that most cases of African sleeping sickness and of Chagas' disease may be diagnosed but much will depend upon the skill and patience of the individual using these methods. The literature is filled with discordant statistics regarding practically all of the methods that have been described and these can only be explained by variations in technique, by lack of experience in employing the methods, and by hasty and insufficient observations. When one author obtains close to 90 per cent of positive results with a certain method and another only from 10 to 20 per cent of positive results with the same method it is very evident that something must be wrong, either with the method or the operator, and it will usually be found that the fault lies with the latter.

PART IV

Laboratory Diagnosis of Coccidiosis

CHAPTER XVIII

LABORATORY DIAGNOSIS OF COCCIDIOSIS

LIFE-CYCLE OF THE COCCIDIA—MORPHOLOGY OF ISOSPORA HOMINIS—MORPHOLOGY OF EIMERIA SARDINÆ—MORPHOLOGY OF EIMERIA CLUPEARUM—PREPARATION OF MATERIAL FOR EXAMINATION FOR ISOSPORA HOMINIS

Introduction.—The Coccidia belong to the Class Sporozoa, are parasitic in nature, and live in the tissues and body fluids of vertebrates and invertebrates. They have an asexual and a sexual cycle of development, both of which may be passed in the same host or alternate hosts may be required. So far as man is concerned both cycles of development are probably completed in the human host. The only species of coccidium usually occurring in man is *Isospora hominis*, although another species, *Eimeria gubleri*, first described by Gubler, in 1858, has been reported as occurring in 5 individuals since that time.

In 1919, Dobell described three species of Eimeria as parasites of man, *i. e.*, *Eimeria oxyspora*, *Eimeria wenyoni* and *Eimeria snijdersi*, but Thomson and Robertson, in 1926, proved that all these were common coccidia of certain fish, and had been previously described by Thelohan (1890–1892). Thomson and Robertson proved that *Eimeria oxyspora* is identical with *Eimeria sardinæ*, parasitic in sprats, and herring; that *Eimeria wenyoni* is identical with *Eimeria clupearum*, parasitic in sprats, herring and mackerel; and that *Eimeria snijdersi* is a degenerate form of *Eimeria sardinæ*. It is important to remember the possible occurrence of these coccidia in the intestine of man after eating infected fish and to differentiate them from *Isospora hominis*, with which they may be confused by untrained observers. Only the oöcysts of these species have been found in the stools, as they simply pass through the human intestine without further development.

MORPHOLOGY AND LIFE-CYCLE OF THE COCCIDIA

The morphology and life-cycles of several species of coccidia have been studied by numerous observers but, as only the oöcysts of *Isospora hominis* are known, it is unnecessary here to describe the morphology of the other stages of development in the species of this genus. How-

ever, it is necessary to briefly sketch the life-cycles of the coccidia that have been demonstrated as it is believed that the life-cycle of *Isospora hominis* is similar in all essential respects.

Schaudinn and Siedlecki (1897) and Schaudinn (1900) were the first to demonstrate that the coccidia have an *asexual* and a *sexual* cycle of development, both of which may be passed in the same host, or the asexual in one host and the sexual in another. In the *asexual cycle* the coccidium penetrates a tissue cell of its host and is then called a *schizont.* The schizont enlarges until it fills the invaded cell, when it divides into several motile fusiform bodies which are called *merozoites.* These are finally liberated by the rupture of the host cell, and the merozoites penetrate other cells and repeat the cycle, which is known as *schizogony.*

The *sexual cycle* is initiated by the differentiation of some of the merozoites into male and female organisms, the male being called *microgametocytes,* and the female, *macrogametocytes.* The nucleus of the *microgametocyte* divides and several flagella-like forms are finally liberated, called *microgametes.* Maturation changes occur within the *macrogametocyte,* preparing it for fertilization, which occurs when a microgamete penetrates it, the resulting organism being known as a *zygote* or *oöcyst.* After maturation, and before fertilization, the macrogametocyte is called a *macrogamete.* Fertilization may occur within or outside the host cell but usually outside the cell.

Nuclear division occurs within the oöcyst resulting in the formation of several small bodies called *sporoblasts.* Each of these sporoblasts secretes a cystic membrane about itself, the entire body being known as a *sporocyst* and the sporoblast being known as a *spore.* The nucleus of each spore divides into several daughter nuclei, followed by the division of the cytoplasm, thus producing a number of sickle-shaped vermicular forms which are called *sporozoites,* a small portion of the cytoplasm of each spore remaining as a granular residual body.

Under favorable conditions the spores rupture and liberate the sporozoites which invade the host cells of the infected individual and become schizonts, thus initiating the asexual cycle, or schizogony. The sporozoites are the only forms of the coccidia that are able to produce infection in a new host.

MORPHOLOGY OF ISOSPORA HOMINIS

As already stated, *Isospora hominis* is the only species of coccidium that has been accepted as a parasite of man. According to the exhaustive contribution of Magath (1935), about 200 authentic infections with this parasite have been described since its original description by Rivolta, in 1878, so that it must be regarded as a very rare parasite. Magath states that, at the Mayo Clinic, he has found but one infection with *Isospora hominis* in the examination of the stools of 60,000 patients and the writer, in the examination of many thousands of

patients has never encountered an infection with this parasite. In view of the fact that no one has, as yet, demonstrated this organism in the tissues of man and that only the oöcysts are known, as well as the known rarity of the infection in man, it is quite probable that it will eventually be found that *Isospora hominis* is really a parasite of a lower animal rather than of man, and that, like *Eimeria oxyspora* and *Eimeria wenyoni*, the oöcysts are simply passengers through the human intestine. However, the occurrence of the oöcysts of this and other species of coccidia in the stools of man, and the liability of confusing them with the eggs of helminths, renders a knowledge of their morphology of importance in diagnosis. Since Magath's publication seven more cases of infection with this parasite have been recorded.

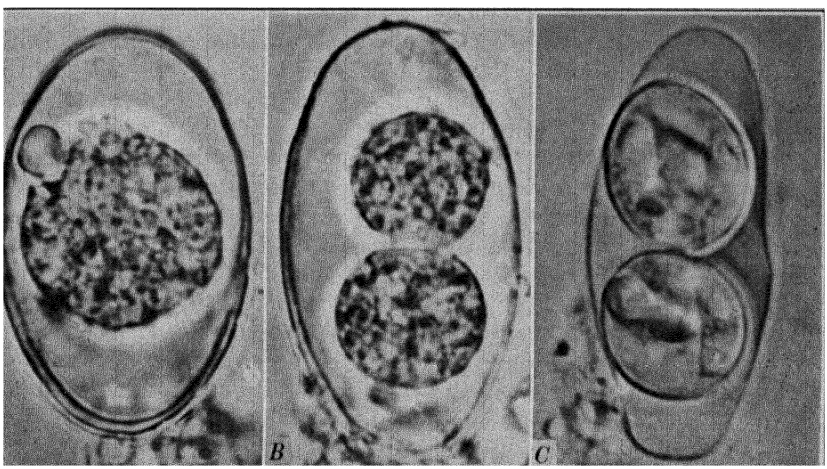


FIG. 31.—*Isospora hominis*. *A*, Cysts from freshly passed feces; *B*, maturing oöcyst with two sporoblasts; *C*, mature oöcyst, having four sporocysts within each sporoblast. × ca. 2000. (Photomicrographs by Mr. J. E. Gullberg, courtesy of Dr. Harold Kirby, from material furnished by Lt. E. K. Markell, U.S.N.)

The oöcysts of *Isospora hominis* are found in the stools in an unsegmented condition and it is in this condition that they are most frequently mistaken for the eggs of worms, the double-outlined capsule and the collection of granules, representing the zygote, closely resembling structures found in the eggs of certain worms. The complete development of the oöcysts does not occur until they have left the body for from one to three or four days, so that it is necessary, in order to differentiate them, to keep the material containing them for that period of time.

The oöcysts are elongate-ovoidal in shape and measure from 20 to 33 microns in length by 10 to 19 microns in breadth. There is considerable variation in size and Wenyon believes that two different coccidia are

involved, the smaller cysts being those of *Isospora hominis* while the larger are those of a species which he has named *Isospora belli*. It is the consensus of opinion, at present, that the latter should be considered as synonymous with *Isospora hominis*.

The oöcysts are hyaline appearing, colorless bodies, having a cyst-wall composed of two layers, the inner thin and membranous and the outer hard and impermeable to fluids, the entire wall being smooth, thin and colorless. The shape varies, some of the oöcysts being long and slender while others are shorter and more spheroidal in shape. One end of the oöcyst is more narrow than the other, so marked that in some this portion has a distinct neck-like appearance. At the narrow end of the cyst there may be a minute micropyl and, in some specimens, a small mass of granular material may lie in contact with the inner side of the microphyl. In most of the cysts, when first passed in the stools, there is a round mass of granular material situated centrally, but in rare instances the entire cyst may be filled with granular material, or this material may occur in two round masses within the cyst, the sporoblasts. If the stool containing the cysts is distributed into Petri dishes, kept at room temperature, and examined at intervals, it will be seen that the central granular mass divides into two granular sporoblasts, which are somewhat ovoid in shape, and which eventually surround themselves with a double-outlined cyst-wall, hyaline in appearance, thus becoming sporocysts, measuring from 12 to 14 microns in length and 7 to 9 microns in breadth, although smaller and larger sporocysts have been observed. Division now occurs within each sporocyst and four sporozoites are eventually produced within each sporocyst, lying in and around a large residual granular mass containing reserve food material.

The sporozoites, four in number in each of the two sporocysts, are falciform in shape and hyaline and refractile in appearance. They are rounded at the anterior end and more pointed at the posterior. They lie within the sporocyst with the rounded ends in the same direction and curved about the residual mass of granular food material. Each sporozoite is finely granular in appearance, has a vacuole at the rounded end containing refractile material, and a nucleus at the junction of the anterior and middle third of the body, which is usually invisible.

The complete development of the oöcyst at ordinary room temperatures occupies from three to four days but a much shorter time in tropical regions. Thus, Wenyon and O'Connor (1917) found that complete development occurred in as short a period as twenty-four hours in Egypt.

MORPHOLOGY OF EIMERIA SARDINÆ

While this coccidium and *Eimeria clupearum*, the description of which follows that of *Eimeria sardinæ* are not parasites of man, the

fact that they may be found in the stools of persons who have eaten fish, as sprats, herring and mackerel, which were infected with these coccidia, renders a description of the oöcysts necessary from the standpoint of differential diagnosis, to avoid their confusion with *Isospora hominis*.

The only stage in the life-cycle of *Eimeria sardinæ* which occurs in the stools of man is the oöcyst. This is spherical in shape and measures about 36 microns in diameter. It has a double-outlined wall, slightly yellowish in color, the inner wall being smooth while the outer may be

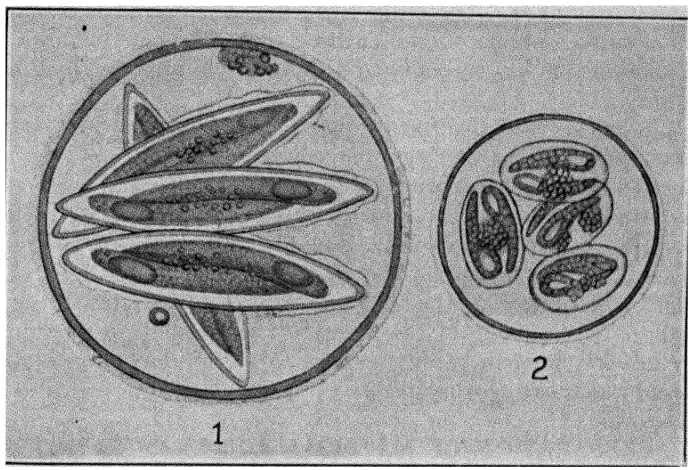


FIG. 32.—*1*, *Eimeria sardinæ* (*E. oxyspora*) (after Dobell). *2*, *Eimeria clupearum* (*E. wenyoni*) (after Wenyon).

incrusted with particles from the feces. Within each oöcyst there are 4 long, sharply pointed whetstone-shaped sporoblasts, measuring from 30 to 32 microns in length by about 7.5 microns in breadth. The spores are surrounded by a double-outlined wall, composed of a uniform inner coat and a thinner slightly uneven outer coat. In addition to the spores the oöcyst contains a very small amount of residual material in the form of an irregular mass of refractile granules.

Within each of the spores there are 2 sporozoites which almost fill the spore, having an anterior pointed end and a posterior rounded extremity. The sporozoites are so arranged that the anterior end of each sporozoite is wrapped about the posterior end of the other sporozoite. The posterior end of each sporozoite contains a nucleus and between the nucleus and the posterior end are two or three bright crystal-like bodies which Dobell (1919) regarded as characteristic of this species. Situated between the nucleus and the anterior end of

each sporozoite the cytoplasm contains a small number of minute refractile granules.

MORPHOLOGY OF EIMERIA CLUPEARUM

Only the oöcysts of *Eimeria clupearum* have been observed in the feces of man, and the following description is compiled from those of Wenyon (1915) and Dobell (1919).

The oöcysts of *Eimeria clupearum*, as seen in human feces have, unlike those of *Isospora hominis* or *Eimeria sardinæ*, been segmented when passed. They are spherical in shape and measure about 20 microns in diameter. The outer surface of the oöcyst is rough while the inner surface is smooth and covered with a delicate membrane, the entire oöcyst having a double outline which appears hyaline and refractile.

Within each oöcyst there are 4 oval spores, having a double outline, the outer surface appearing somewhat roughened. These spores measure 10 microns in length by 7 microns in width and each contains 2 falciform sporozoites, one end of which is more rounded than the other. The rounded end contains a nucleus which appears as a spherical or oval hyaline body and the sporozoites are so arranged that the rounded ends, containing the nucleus, are at opposite poles of the spore. The sporocystic residue in this species is very small in amount, consisting of one or two minute, refractile, granular masses situated between or near the sporozoites.

PREPARATION OF MATERIAL FOR EXAMINATION

The diagnosis of coccidiosis in man must rest upon the demonstration of the oöcysts of *Isospora hominis* in the stools of the suspected individual. The oöcyst must be studied in the unstained condition as they do not stain well with any of the aniline dyes, owing to the impermeability of the cyst-wall.

In preparing material for examination, a small portion of the feces of the suspected individual should be mixed with normal saline or distilled water, placed upon a microscopic slide, and gently flattened out with a cover-glass. The preparation should not be too thick, a good preparation being one that will show print through the film of feces. The oöcysts may be distinguished with the low power dry objective (16 mm.), but the various structures within the cysts require study with the high-dry 4 mm. objective and the 1.9 mm. immersion oil objective. As the oöcysts are transparent, hyaline and colorless, very careful focussing and lighting are necessary in order to define their structure, and as little light should be used as is possible. It is very easy to overlook these objects if the microscopic field is too much illuminated.

As the oöcysts of *Isospora hominis* are passed in the unsegmented

condition it is necessary to keep the fecal material for from three to four days, in temperate climates, in order to demonstrate the spores and sporozoites. This can be done by placing small amounts of the feces in small Petri dishes and keeping them in a dark place at room temperature, making microscopic preparations at intervals. Usually complete development of the oöcysts will occur within this period of time and the characteristic morphology of this species can be determined. In view of the possible occurrence of *Eimeria oxyspora* and *Eimeria clupearum* in the human intestine and the necessity of differentiating these organisms from *Isospora hominis*, it is always well to examine the fecal material in this manner, even though the primary microscopic examination may have been apparently successful in demonstrating *Isospora hominis*.

Remarks Regarding Diagnosis.—No cultural or serological methods have been devised for the diagnosis of *Isospora hominis* and animal inoculations have proven unsuccessful, so that the diagnosis depends upon the demonstration of the oöcysts by a simple microscopic examination. The oöcysts usually occur in small numbers in the stools of the infected individuals and in most cases of infection it has been noted that they did not persist for more than two to four days and, sometimes, for not more than one day; thus indicating that the infection must be self-limited in man and strongly suggesting that *Isospora hominis* may, after all, be only a passenger through the human intestine, as are *Eimeria sardinæ* and *Eimeria clupearum*.

The technique of examination of the feces for coccidia is identical with that employed for the demonstration of *Endamœba histolytica* in unstained preparations (see page 41).

The possibility of the confusion of the oöcysts of *Isospora hominis* with certain helminth eggs should not be forgotten and it is very probable that many infections with this organism have been so confused. It is useful to remember that the oöcysts are considerably smaller than any helminth egg with which they might be confused and much more transparent. Such mistakes would not be made by a trained observer but might easily occur if the observer had never seen the oöcysts of this, or other species of coccidia.

In describing the morphology of the coccidia that have been observed in man, it will be noted that Dobell's "*Eimeria snijdersi*" was not considered. This omission is intentional as it is now accepted that this supposed species was described from degenerate specimens of *Eimeria oxyspora* and that previous descriptions of the morphology are of no diagnostic value, beyond demonstrating the degenerative changes that may occur in the oöcysts of the latter species.

It is a curious fact that, in those cases in which clinical symptoms, as colic and diarrhea have accompanied infection with *Isospora hominis*, the oöcysts have not been found in the feces for some time after the appearance of such symptoms and, in some cases, not until all symp-

toms have ceased. It is, therefore, important to inquire as to the previous presence of such symptoms in apparently normal individuals in whose feces the oöcysts of this organism have been found. The fact that the oöcysts may not be present until some time after the subsidence of symptoms may account, to some extent, for the small number of infections that have been observed, as the examination of the stools in the absence of symptoms would not be suggested. If any pathological lesions are produced in man by *Isospora hominis* they are unknown, as it has never been observed at autopsy. If this organism does develop in the human intestine it must invade the intestinal epithelial cells during its asexual stage, or schizogony, and one would expect that long before this such invasion would have been demonstrated by the presence of anatomical lesions but no one has described such lesions or demonstrated the asexual stage of development in man. The only record we have of the possible pathogenicity of *Isospora hominis* is that of Connal (1922) who observed an infection in a laboratory worker who accidentally ingested the fully developed oöcysts and six days later suffered from diarrhea which persisted for four weeks, the oöcysts being found in the stools three weeks after the appearance of the diarrhea, and persisting for twelve days, after which they disappeared. At the time of the disappearance of the oöcysts the diarrhea had ceased and the stools had become normal in number and appearance. While this case is very suggestive it by no means proves that *Isospora hominis* is normally a parasite of man, and the majority of the infections that have been reported were not accompanied by clinical symptoms, either before or during the time that the oöcysts were found in the stools.

PART V

Laboratory Diagnosis of the Malaria Plasmodia

CHAPTER XIX

THE LABORATORY DIAGNOSIS OF THE MALARIA PLASMODIA

LIFE-CYCLE OF THE MALARIA PLASMODIA—MORPHOLOGY OF PLASMODIUM VIVAX—MORPHOLOGY OF PLASMODIUM OVALE—MORPHOLOGY OF PLASMODIUM MALARIÆ—MORPHOLOGY OF PLASMODIUM FALCIPARUM—DIFFERENTIAL DIAGNOSIS OF THE MALARIA PLASMODIA—OBJECTS MISTAKEN FOR PLASMODIA—BLOOD CHANGES DUE TO MALARIAL INFECTION—MALARIA PLASMODIA OF UNCERTAIN STATUS

Introduction.—The malaria plasmodia, the causative agents of the malarial fevers, belong to several species of animal parasites included in the Protozoa, Class Sporozoa and Genus Plasmodium. At present four species of malaria plasmodia are recognized by both protozoölogists and physicians as responsible for certain clinical types of malaria in man. These species are: *Plasmodium vivax,* the cause of vivax or benign tertian malaria; *Plasmodium ovale,* a recently accepted species, causing a fever with tertian paroxysms or ovale malaria; *Plasmodium malariæ,* the cause of malariæ or quartan malaria, and *Plasmodium falciparum,* the cause of falciparum or malignant tertian, or estivo-autumnal, malaria. All of these plasmodia differ in morphology and their differential diagnosis is essential in the prevention and treatment of malarial infections. Other species of plasmodia have been described as occurring in man but have not been generally accepted.

All of the malaria plasmodia live upon and within the red blood corpuscles of man and eventually destroy them, thus causing the severe anemia so characteristic of these infections. In order to differentiate the various species it is absolutely necessary to be acquainted with their morphology during the various stages of growth within these cells and also, from the standpoint of prevention, to be familiar with the forms of these parasites that are destined to develop in the mosquitoes that transmit malarial infections, so that a knowledge of the life-cycle in the mosquito is essential as well as of the cycle in man.

The observations of Ross, Grassi, Bignami and Bastianelli, Marchia-

fava and Celli and others have proven that the malaria plasmodia have two cycles of development, one within man and one within some species of *Anopheline* mosquito. The cycle in man is *asexual* in type and the process of development is known as *schizogony*, man being the *intermediate* host of the parasite, while the cycle in the mosquito is *sexual* in type and the process of development is known as *sporogony*, the mosquito being the *definitive* host of the parasite. It is necessary to describe these cycles of development as the morphology of the plasmodia during the various stages of development is characteristic and a knowledge of the morphology is of primary importance in diagnosis.

LIFE CYCLE OF THE MALARIA PLASMODIA

A. **The Life-cycle in Man.**—The life-cycle of all of the species of malaria in man is initiated by the injection of *sporozoites* which have been developed in suitable mosquitoes and are inoculated when the insect bites. It is still undecided just what changes occur in the sporozoites after they are inoculated by the mosquito although many authorities believe that they immediately penetrate the red blood corpuscles, becoming small ringlike bodies, known as *trophozoites*.

At the present time most authorities believe that the sporozoites undergo a cycle of development in cells of the reticulo-endothelial system, the so-called *exo-erythrocytic cycle*, before invading the red blood cells and producing trophozoites.

The trophozoites gradually enlarge and develop pigment, while the nuclear chromatin increases in amount and divides, at which time the trophozoites are called *schizonts*. The schizonts continue to grow and eventually completely or partially fill the invaded corpuscle, while the nuclear chromatin divides followed by the division of the cytoplasm, thus producing a number of segments which are known as *merozoites*. These bodies are liberated into the blood stream by the breaking up of the invaded corpuscles and, invading normal red blood corpuscles, repeat the cycle of development in man.

The time consumed in the cycle in man varies with the different species of plasmodia, being approximately forty-eight hours in *Plasmodium vivax;* the same, in *Plasmodium ovale;* seventy-two hours in *Plasmodium malariæ;* and between thirty-six and forty-eight hours in *Plasmodium falciparum*.

B. **The Life-cycle in the Mosquito.**—After a malarial infection has existed for some time in man, certain of the merozoites liberated at the time of segmentation of the schizonts become differentiated into male and female forms which invade the red blood corpuscles and are intended to eventually undergo a life-cycle within the transmitting mosquitoes. These forms are collectively known as *gametocytes*, the male being called a *microgametocyte* and the female, a *macrogametocyte*. Both of these forms enlarge within the invaded cells and finally almost

fill the latter but they do not segment, as do the schizonts, but are liberated from the invaded cell and undergo no further development unless the blood containing them is ingested by a suitable mosquito. If this occurs and the blood reaches the mid-gut, erroneously called the "stomach" of the mosquito, the microgametocyte extrudes long delicate filaments which are liberated from the parent organism and which are rapidly motile. These filaments are known as *microgametes* and usually from four to six are liberated from each microgametocyte.

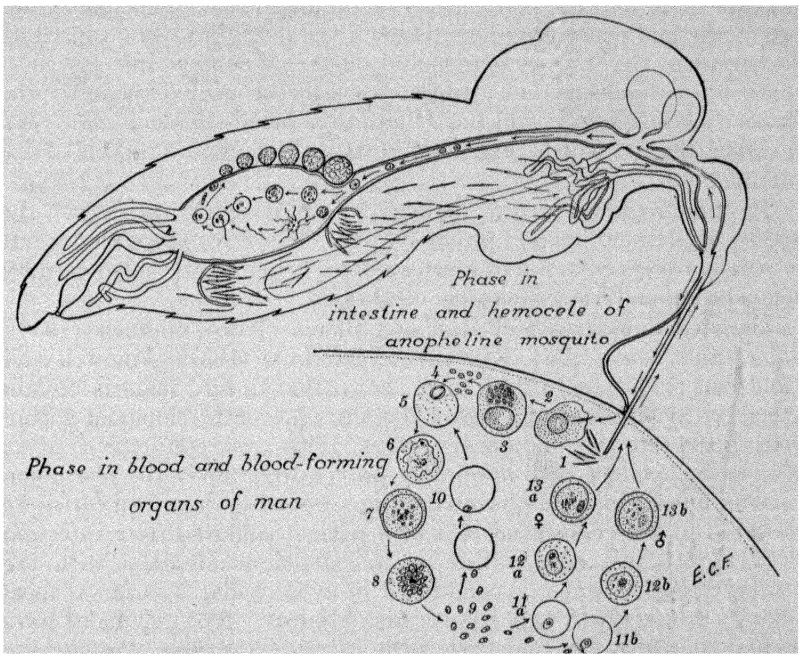


FIG. 33.—The life cycle of the malaria parasite showing both intrinsic phase (in man) and extrinsic phase (in the mosquito). *1*, Sporozoites injected into the peripheral blood of man; *2*, entry into an exo-erythrocytic cell (macrophage) to initiate the exo-erythrocytic stage; *3*, multiplication in exo-erythrocytic cell (schizogony); *4*, escape of exo-erythrocytic daughter parasites (merozoites); *5*, early trophozoite (ring stage) in erythrocyte; *6*, maturing trophozoite; *7*, early schizont; *8*, mature schizont; *9*, merozoites, ready to invade uninfected erythrocyte; *10*, invasion of erythrocyte to repeat asexual phase in red blood cells; *11a*, *11b*–*13a*, *13b*, development respectively of female and male mother sex cells (gametocytes). (Original, Faust.)

Coincident with these changes in the microgametocyte certain maturation phenomena occur within the macrogametocyte preparing it for fertilization and, when this process is completed, it is known as a *macrogamete*, the microgametes and the macrogametes being collectively known as *gametes*. After liberation the microgamete swims about until it comes in contact with a macrogamete, when it penetrates and fertilizes the latter, the resulting product being called a *zygote*.

The latter elongates, acquires a vermicular-like motility and penetrates the epithelial lining of the "stomach." This form, which is called an *oökinete,* then attaches itself to the elastic membrane just beneath the epithelial lining, becomes spherical in shape and forms a cyst which is known as the *oöcyst.* This enlarges and within it are developed multitudes of minute, slender, spindle-shaped bodies, called *sporozoites,* which radiate from centers called *sporoblasts* by some authorities. When the sporozoites are fully developed, the oöcysts rupture and liberate them into the body cavity of the mosquito. The sporozoites are motile and penetrate every tissue of the mosquito being especially numerous in the salivary glands and ducts. When the infected mosquito bites it injects these sporozoites into the wound made by the insect's proboscis, from which they enter the blood stream of man, and, invading the red blood corpuscles, initiate the human life-cycle of the plasmodium.

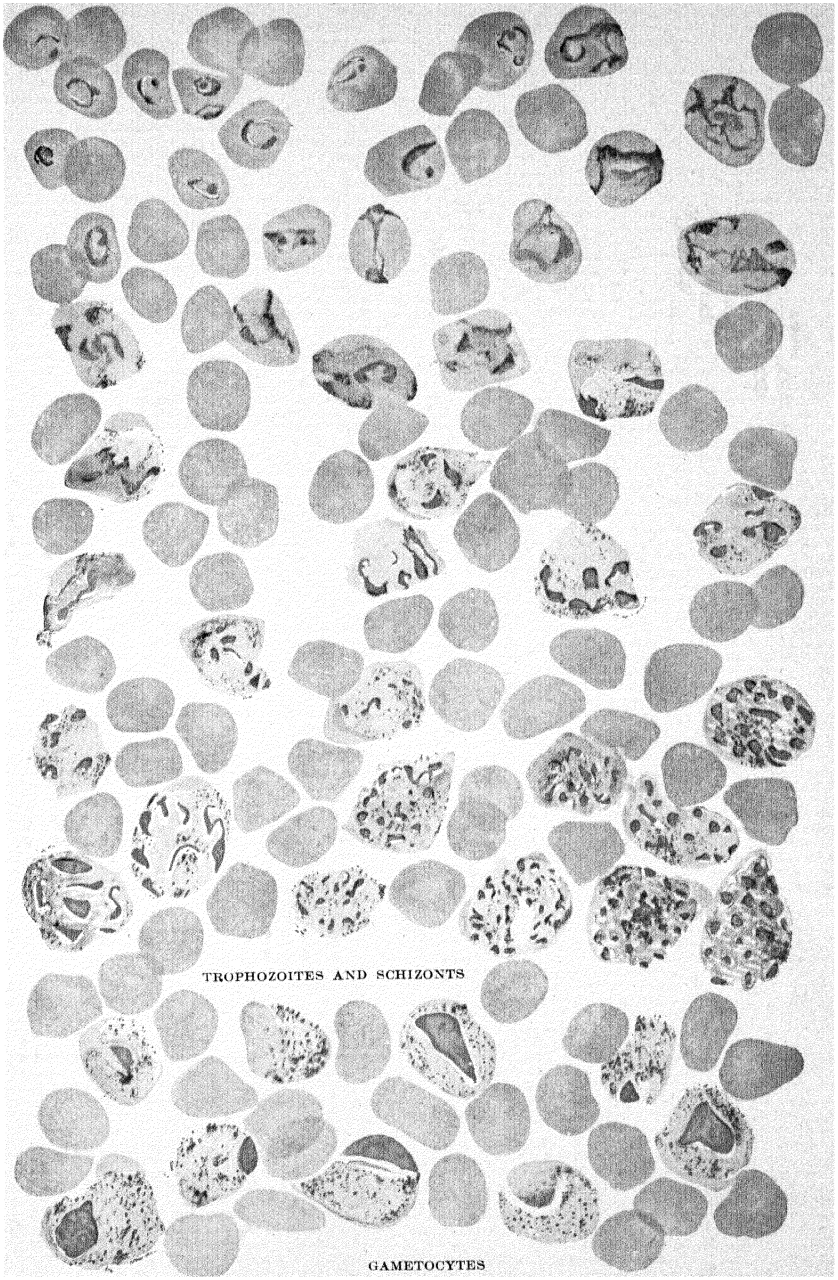
The time required for the life-cycle in the mosquito varies with the species of plasmodium and probably, with the species of mosquito, but it usually covers from ten to fourteen days, being greatly influenced by temperature and other biological conditions.

The recent researches of Huff and Bloom (1935), Raffaele (1936), James and Tate (1937), Kikuth and Mudrow (1937), Manwell and Goldstein (1939), and others have shown that in bird malaria certain species of avian plasmodia have a definite cycle of development within cells of the reticulo-endothelial system. This *exo-erythrocytic cycle,* as it is called, has not been absolutely proven to occur in the plasmodia causing human malaria although Brug (1940) has described forms he observed in the endothelial cells of a patient suffering from infection with *Plasmodium vivax* which he considered as identical with the exo-erythrocytic forms demonstrated in avian malaria so far as morphology is concerned and Kikuth and Mudrow (1941) claim to have seen similar forms in infections with *Plasmodium vivax, Plasmodium malariæ* and *Plasmodium falciparum.* That such an exo-erythrocytic cycle of development may occur in human malarial infections is probable and would explain some of the puzzling features of such infections, as relapses after long intervals of time. Porter and Huff (1940) have published an excellent review to which the reader is referred.

The forms concerned in the exo-erythrocytic cycle of development of the avian plasmodia are characterized by being devoid of pigment but they undergo schizogony within the invaded endothelial cell in a manner similar to schizogony within the red blood corpuscles.

MORPHOLOGY OF THE MALARIA PLASMODIA

Each of the accepted species of the malaria plasmodia differs in its morphology from the other species, and these differences are all im-



TERTIAN MALARIA PLASMODIA.

(*Plasmodium vivax.*)

Various stages in the development of *Plasmodium vivax.* (Preparations from cultures by Bass and Johns. Wright stain.) *From Bass and Johns, with permission from Tice's Practice of Medicine, Vol. III, courtesy of W. F. Prior Company.*

portant in the differentiation of the species without which no malaria diagnosis can be said to be complete or satisfactory.

In practically all texts upon clinical diagnosis the descriptions of the morphology of the various species of malaria plasmodia are confined to the morphology observed in stained preparations and nothing is said of the morphology of these parasites in the living, unstained condition. The writer believes that the morphology of the living organisms is important from a diagnostic standpoint, as it is not always convenient or possible to have staining solutions available in making blood examinations and, for this reason, the morphology of each of the species of plasmodium will be described in both the living, unstained condition and in stained preparations. It is well to remember that all of the basic work upon the differentiation of the species of plasmodia infecting man and the elucidation of the life-cycle of the plasmodium in the mosquito was made with unstained, living organisms, and that, until the development of the various modifications of the Romanowsky stain, all diagnostic and research work upon the plasmodia was accomplished with unstained living plasmodia.

MORPHOLOGY OF PLASMODIUM VIVAX (THE BENIGN TERTIAN PLASMODIUM)

A. **The Living Plasmodium.**—The tertian malaria plasmodium, or *Plasmodium vivax*, appears at first apparently within the invaded red blood corpuscle as a small, non-motile, colorless hyaline disk or "ring," the *trophozoite*, measuring about 2 microns in diameter. It has a rather indistinct veil-like outline and the center of the ring is greenish in color being composed of the unchanged cytoplasm of the invaded erythrocyte. At this stage of development the organism is very apt to be overlooked in unstained preparations because of its delicacy and its hyaline appearance. Just before pigment is developed the ring-form may be lost and the organism is observed to possess ameboid motility, constantly changing its shape by sending out very delicate pseudopodia and withdrawing them, without changing its position in the invaded corpuscle.

In the course of from six to eight hours pigment begins to be visible within the cytoplasm of the organism in the form of minute reddish-brown granules and the outline of the organism becomes more distinct, and it is now known as a *schizont*. The pigment is arranged in an irregular manner throughout the cytoplasm of the parasite and is in very active dancing motion, the cytoplasm being colorless and somewhat hyaline in appearance. At this time the plasmodium does not fill more than a sixth of the invaded red blood corpuscle which is slightly enlarged and slightly paler than normal. The organism is constantly changing its shape and is difficult to distinguish unless as little light as possible is employed in the examination.

At the end of twenty-four hours the plasmodium fills more than half of the invaded red blood corpuscle, contains much actively dancing pigment, of a reddish-brown color in the form of very fine granules, and varies greatly in shape due to the marked ameboid motility of the organism. Very often at this stage of development multiple infection of the cell may be suspected by reason of the appearance of two or more spherical pigmented bodies in the same cell, but careful examination will show that these are but portions of the ameboid pseudopodium of a single plasmodium, the remainder of the organism being situated deeper within the cell and invisible unless focussed upon. The cytoplasm of the plasmodium is colorless and the infected red blood corpuscle is much enlarged and lighter green in color than normal.

At the end of about thirty-six hours the plasmodium has attained its greatest size and almost fills the invaded red corpuscle which is almost twice or three times the normal size and very pale in color, and only visible as a light green border surrounding the large colorless plas-

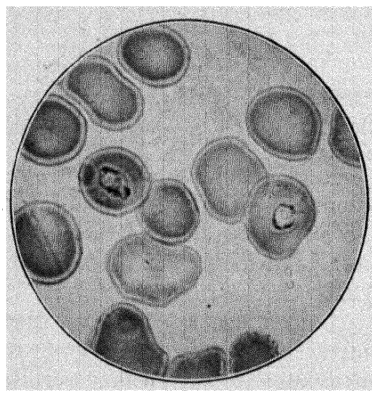


FIG. 34.—*Plasmodium vivax.* Stained with Wright's stain. Trophozoites or "ring forms" of *P. vivax.* × 1200. (Photomicrograph, Army Medical Museum.)

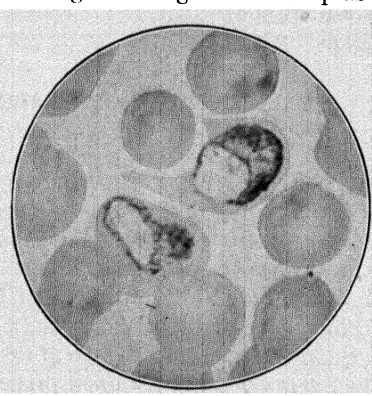


FIG. 35.—*Plasmodium vivax.* Stained with Wright's stain. Schizonts of *P. vivax.* × 1600. (Photomicrograph, Army Medical Museum.)

modium. At this time ameboid activity is almost lost but the pigment is in active dancing movement and is greatly increased in amount. The organism is very easily differentiated at this stage of development, appearing as a more or less spherical colorless body filled with dancing pigment and surrounded by a light greenish border, the remains of the invaded erythrocyte.

At the end of about forty-eight hours the schizont divides into a number of segments, or *merozoites.* At this time the pigment is observed to be collected at or near the center of the organism in a compact, more or less regular mass, of a dark brownish color, and careful focussing will reveal that the cytoplasm of the parasite is divided into

minute oval segments, arranged in two rows, one surrounding the pigment mass and the other surrounding the first row, or the segments may be irregularly arranged. These segments are the *merozoites* and, in *Plasmodium vivax*, are usually from 12 to 24 in number. They are devoid of pigment and, when liberated into the blood stream, are oval in shape and measure from 1.5 to 2 microns in diameter. The center is refractile and the entire merozoite is colorless and hyaline in appearance.

B. **The Stained Plasmodium.**—The various stains that are employed for staining the malaria plasmodia are all modifications of the Romanowsky stain and will be found described in the section of this work treating of the preparation and staining of the malaria plasmodia (see page 308). The staining reactions of all species of the plasmodia are similar when these stains are used and in the following descriptions of the morphology of the plasmodia when stained, the reactions observed after staining with Wright's stain (see page 164) will be described. With this stain the cytoplasm of all of the species of malaria plasmodia stains a robin's-egg blue or a darker blue, the chromatin of the nucleus pink, red or violet, according to the intensity of the staining, while the vesicular portion of the nucleus remains unstained, appearing as a white area near, or surrounding the nuclear chromatin, during certain stages in the development of the plasmodia. The invaded red blood corpuscles should stain a salmon or pink color in well-stained preparations.

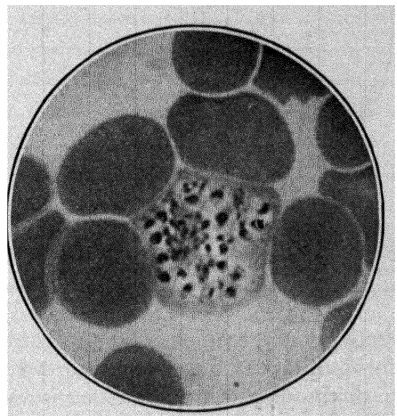


FIG. 36.—*Plasmodium vivax*. Stained with Wright's stain. Presegmenting schizont. (Photomicrograph, Army Medical Museum.)

The trophozoites of *Plasmodium vivax* appear within the salmon or pink colored erythrocytes as blue ringlike bodies having a bright red or purple dot of chromatin situated at one portion of the periphery, giving the organism a "signet-ring" appearance, or attached to the periphery of the red blood corpuscles, in which case the organism appears as a narrow semi-lunar mass of cytoplasm, stained blue and having at the center a red dot of chromatin. Rarely, in infections with this plasmodium, multiple infection of the erythrocytes may occur, from 2 to 3 "rings" being present in the invaded corpuscle. The vesicular portion of the nucleus appears as an unstained area near the chromatin dot.

It will sometimes be observed that the "ring-forms" contain two red

dots of chromatin, either separated or close to one another, and some authorities, as Le Dantec (1929), Alessandrini (1932), Hingst (1934), Beach (1936) and others have interpreted such forms as dividing trophozoites. As the trophozoites enlarge and become *schizonts* the "ring-form" is lost and, because of the intense ameboid activity of the plasmodia at this stage of development, they appear within the invaded corpuscle as very irregular, bizarre-shaped blue bodies containing fine granules or delicate short filaments of pink stained chromatin arranged irregularly throughout the cytoplasm, mingled with which are fine

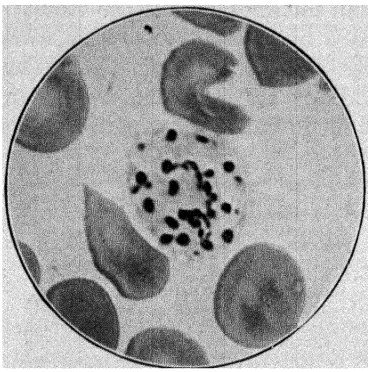


FIG. 37.—*Plasmodium vivax.* Stained with Wright's stain. Segmenting schizont of *P. vivax.* × 1800. (Photomicrograph, Army Medical Museum.)

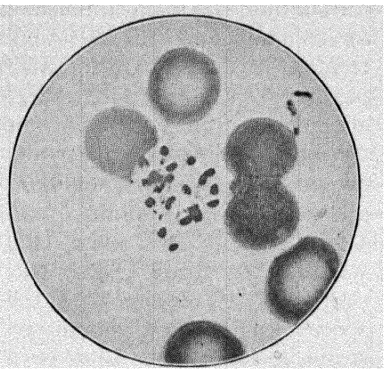


FIG. 38.—*Plasmodium vivax.* Stained with Wright's stain. Free merozoites of *P. vivax.* × 1200. (Photomicrograph, Army Medical Museum.)

granules of greenish-brown pigment. In some of the schizonts the chromatin is so finely divided and stains so faintly pink that it is hard to distinguish but as the time for segmentation approaches it becomes collected in more or less regular masses and stains a bright red or purple color. As soon as division of the chromatin occurs the trophozoite becomes a schizont. The vesicular portion of the nucleus may sometimes be seen as a white, unstained area lying within the cytoplasm.

At the end of about thirty-six hours the stained schizont has become almost spherical in shape and almost fills the invaded red blood corpuscle. The unstained vesicular portion of the nucleus is no longer visible and the cytoplasm of the parasite stains a definite blue color while the chromatin stains a bright red or purplish-red and is beginning to occur in irregular masses throughout the cytoplasm. The pigment is seen as greenish-brown granules scattered throughout the cytoplasm or in small, irregular aggregations, most numerous toward the center of the plasmodium.

The invaded red blood corpuscles present very characteristic appearances in stained preparations. In the cells containing the trophozoites there may be little change in the appearance of the invaded red blood

corpuscle beyond a slight enlargement but as the chromatin of the nucleus begins to divide the cytoplasm of the invaded corpuscle may show a few pink, eosin-staining dots which are known as Schüffner's dots. With the continued growth of the schizont the invaded red blood cell becomes larger and larger and the cytoplasm becomes crowded with Schüffner's dots, which stain pink or red, the normal cytoplasm having been apparently entirely replaced by these dots. The enlargement of the invaded red blood corpuscles and the occurrence of Schüffner's dots are very characteristic of infections with *Plasmodium vivax* but Schüffner's dots do not always occur in infections with this plasmodium in the experience of the writer.

Some hours before segmentation the schizont is seen to practically fill the invaded corpuscle, is spherical or somewhat irregular in shape and the cytoplasm stains blue. The pigment is collected in a more or less regular mass toward the center of the organism and is greenish-brown in color, while the chromatin is collected in irregular masses, stained a bright red or purplish-red, distributed throughout the cytoplasm. The invaded corpuscle is greatly enlarged and appears as a narrow rim of pink-stained Schüffner granules surrounding the plasmodium. The enlarged corpuscles containing the plasmodia may be irregular in shape, thus causing the plasmodia to also present an irregular contour.

At the time of segmentation the plasmodium entirely fills the red blood corpuscle which may appear as a narrow band of pinkish material surrounding the plasmodium or may be entirely indistinguishable. The pigment is collected at or near the center of the organism in a compact more or less spherical mass of a dark greenish-brown color and the blue-stained cytoplasm is divided into from 12 to 24 small oval or round bodies each containing a red chromatin dot. After liberation from the invaded corpuscles these bodies, or merozoites, may be observed free in the blood plasma or just invading other red blood corpuscles at which time they appear as oval blue-stained bodies having a red dot of chromatin at some portion of the periphery or centrally situated, located upon or near the edge of the corpuscle.

The Gametocytes of Plasmodium Vivax.—A knowledge of the morphology of the gametocytes of the malaria plasmodia is important, not only from the standpoint of the differentiation of the various species of plasmodia but especially so from the standpoint of the prevention of malarial infections, for individuals harboring the gametocytes are infective to mosquitoes and are essential in the transmission of the plasmodia from man to man. The gametocytes of the various species of plasmodia differ in their morphology and attention to these differences enables one to differentiate the species of plasmodium producing them.

The Unstained, Living Gametocytes.—In unstained preparations of blood the gametocytes cannot be distinguished with certainty during

early stages of development and it is not until they are almost fully developed or liberated from the host cell that they can be differentiated clearly from the schizonts. In examining the blood of patients suffering from infections with *Plasmodium vivax* at the time of the segmentation of the schizonts it will be noted, if the infection has existed for some days, that certain of the hyaline, pigmented plasmodia almost filling the invaded corpuscles, show no evidences of segmentation and that the pigment instead of being collected at or near the center of the parasite is either distributed throughout the cytoplasm or collected in small masses near the periphery of the organism. These bodies are the gametocytes and may be differentiated into male and female forms, the microgametocytes and the macrogametocytes.

The *microgametocyte* or male, is a colorless, hyaline body, measuring from 8 to 10 microns in diameter, and ovoid or spherical in shape. The pigment is large in amount, of a reddish-brown color, and is distributed throughout the cytoplasm, or collected in small, irregular clumps within the cytoplasm. Usually the pigment is only sluggishly motile and is often immotile.

The *macrogametocyte* of *Plasmodium vivax*, when unstained, is a large, pigmented body, measuring from 9 to 11 microns in diameter, and is spherical in shape. The cytoplasm is colorless and finely granular in appearance and the pigment, of a dark brownish color, and immotile, is arranged in the form of large granules or clumps about the periphery of the organism, or, commonly, in a wreath-like manner at some distance from the periphery.

The gametocytes are differentiated from the schizonts by the absence of any evidences of segmentation after they entirely fill the invaded red blood corpuscles, so that it is possible to state that any plasmodium filling the invaded corpuscle and showing no evidences of segmentation is a gametocyte, while the microgametocytes and macrogametocytes may be distinguished by the arrangement of the pigment within the cytoplasm, and the fact that in the macrogametocyte the process of exflagellation, to be described, never occurs.

If preparations of the blood be properly made (see page 272) it is possible to follow the formation of the microgametes and their liberation from the parent microgametocyte under the microscope and, in rare instances, to observe the penetration of the macrogamete by a microgamete, phenomena which normally occur within the "stomach" of the transmitting mosquito.

In those microgametocytes in which microgametes are forming it will be noted that the pigment becomes very actively motile while the entire cytoplasm appears to be in motion, marked undulations of the periphery of the plasmodium being visible. If such microgametocytes be carefully watched it will be noted that the pigment begins to collect near the center of the organism and suddenly and explosively, there appear at certain portions of the periphery long, threadlike, colorless,

actively moving filaments, varying in number from 2 to 6, which undulate rapidly, lashing about among the red blood corpuscles, to which they often impart a peculiar spinning motion. These filaments are the microgametes.

There may now occur one of several things; either the microgametes may break away from the parent gametocyte, which is the normal procedure, or unable to do so, may become motionless and degenerate, or the microgametocyte may undergo degeneration before the microgametes have succeeded in freeing themselves. In the first instance, after the microgametes have lashed about among the red blood corpuscles for a variable length of time, trying to free themselves from the microgametocyte, they at length succeed, and swim off in a serpentine manner among the erythrocytes. In some instances the efforts of the microgametes to free themselves are so vigorous as to result in the microgametocyte being pulled about for appreciable distances in the microscopic field.

In those instances in which the microgametes are unable to free themselves from the microgametocyte motility is gradually lost and the entire plasmodium shrinks, becomes vacuolated and finally disintegrates. In some instances the microgametocyte degenerates before the microgametes are liberated and when this occurs the organism breaks up into small portions of cytoplasm, each containing pigment. Such fragments may show motile pigment for a long time after degeneration has occurred and in one instance the writer observed motile pigment is such a fragment for a period of eight hours at room temperature.

The microgametes, after liberation from the microgametocyte, are very slender threadlike bodies, perfectly colorless, and having a serpentine, undulating motility, enabling them to progress among the red blood corpuscles. If one is fortunate the actual penetration of a macrogamete by one of these microgametes may be witnessed under the microscope, a process first described for the human malaria plasmodia by the writer, in 1899, in studying infections with *Plasmodium vivax*. A macrogamete may be observed having attached to it one or more of the microgametes and if such a plasmodium is watched carefully it will be noted that the microgametes appear to be trying to penetrate it. They will straighten out and then relax and may sometimes pull themselves loose from the macrogamete and again become attached to it. All of these movements are evidently made for the purpose of penetrating the macrogamete and that this actually occurs has been observed by numerous authorities. Under normal conditions the process of exflagellation of the microgametocyte and the liberation of the microgametes, as well as the fertilization of the macrogamete, occur in the mid-gut, or "stomach" of the mosquito and no such development of either the microgametocyte or the macrogametocyte occurs in the blood of man unless it is removed from the peripheral circulation under certain conditions (see page 272).

The Stained Gametocytes.—The staining reactions of the gametocytes of *Plasmodium vivax* are similar to those of the schizonts when Wright's or other modifications of the Romanowsky stain are used. The cytoplasm stains varying shades of blue and the chromatin of the nucleus a pink, bright red or reddish-purple color. In their earliest stage of development the gametocytes appear as oval or round, blue stained bodies within the invaded red blood corpuscle, measuring from 1.5 to 2 microns in diameter, and may be distinguished from the trophozoites by the fact that they are never ring-shaped but consist of a blue-stained mass of cytoplasm lying in the center of which is a red dot of nuclear chromatin. As the gametocytes enlarge certain staining

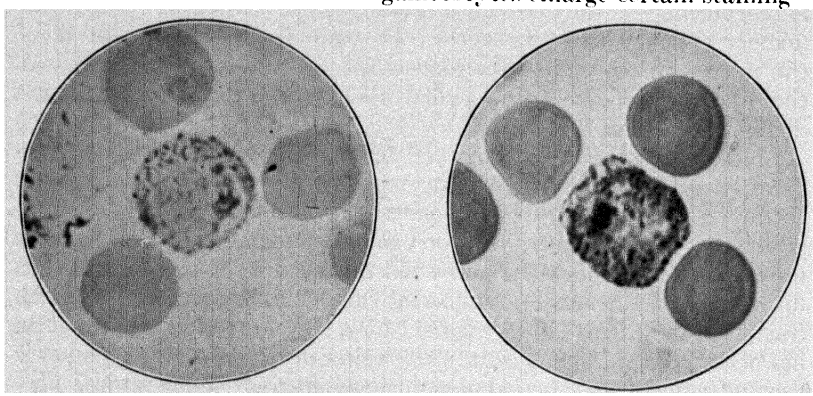


FIG. 39.—*Plasmodium vivax*. Stained with Wright's stain. Microgametocyte of *P. vivax*. × 1800. (Photomicrograph, Army Medical Museum.)

FIG. 40.—*Plasmodium vivax*. Stained with Wright's stain. Macrogametocyte of *P. vivax*. × 1800. (Photomicrograph, Army Medical Museum.)

reactions appear, which, in conjunction with the arrangement of the nuclear chromatin, serve to distinguish the microgametocytes from the macrogametocytes.

The *microgametocytes* stain less intensely blue than do the macrogametocytes, the cytoplasm staining a rather light greenish-blue color, while the chromatin of the nucleus stains a pink or light red color. After the development of pigment it is noted that there is no evidence of great ameboid activity as in the schizonts, the outline of the microgametocytes being regular and the entire body oval or round in contour. As the microgametocytes grow they fill more and more of the invaded red blood corpuscle, until, when fully developed the entire cell is filled. At all stages of growth the chromatin is collected in an oval unstained area in the form of a loose skein or spindle-shaped mass lying within an unstained area, the entire structure representing the nucleus of the microgametocyte. The chromatin is in the form of grains and fibrils which stain a pink or pale red color and is usually arranged in a more or less spindle-shaped skein which is very character-

istic. The pigment is greenish-brown in color, in the form of fine granules, which are distributed throughout the cytoplasm but not within the nuclear area. There is no evidence of division and distribution of the chromatin throughout the cytoplasm as in the schizonts and when the microgametocytes are fully developed and fill the invaded red blood corpuscle there is likewise no evidence of segmentation or division of the nuclear chromatin, the latter being still present as a spindle-shaped skeinlike mass lying in the unstained vesicular portion of the nucleus.

The *macrogametocytes* of *Plasmodium vivax* stain much more intensely than do the microgametocytes, the cytoplasm staining a deep blue color and the chromatin of the nucleus a deep red or almost violet color. The nuclear area is smaller than that of the microgametocyte and the chromatin, instead of having a loose, skeinlike arrangement is collected in a dense red or purplish mass at the center of a small unstained area, the vesicular portion of the nucleus. The pigment is smaller in amount than in the microgametocyte, in larger granules, of a somewhat deeper greenish-brown color, and arranged in a wreathlike manner near the periphery of the organism or in minute clumps close to the periphery. When fully developed the macrogametocytes fill the invaded red blood corpuscle but never show any evidence of segmentation or distribution of the chromatin outside of the nuclear area.

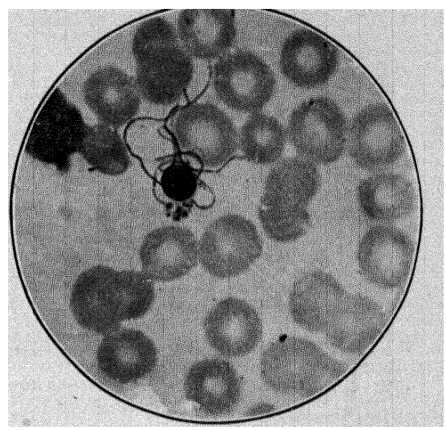


FIG. 41.—*Plasmodium vivax*. Stained with Wright's stain. Flagellated microgametocyte of *P. vivax*. × 1000. (Photomicrograph, Army Medical Museum, Preparation by Craig.)

Sometimes, in stained preparations, *microgametocytes* may be observed in which the nuclear chromatin is seen to be divided into several irregular pink or red masses which are usually collected near the periphery of the plasmodium. Such forms are microgametocytes which are about to undergo exflagellation and produce microgametes and they may be mistaken for presegmenting schizonts.

The invaded red blood corpuscles in which gametocytes are developing are much larger than normal and the cytoplasm is generally filled with pink granules, the Schüffner dots.

Microgametocytes which are undergoing exflagellation present a very characteristic appearance in stained preparations. They are seen as oval, round or irregular bodies, with pale blue staining cytoplasm, containing large, irregular masses of red-stained chromatin, while pro-

jecting from the periphery of the organisms are long, threadlike reddish or violet filaments which are more or less curved in outline, and measure from three to four times the diameter of the parent gametocyte. In some, the filaments apparently originate from a mass of chromatin while in others there is no connection between the chromatin masses within the microgametocyte and the filaments, or microgametes.

The *microgametes*, after liberation from the microgametocyte, stain a reddish or purplish color and are apparently composed almost entirely

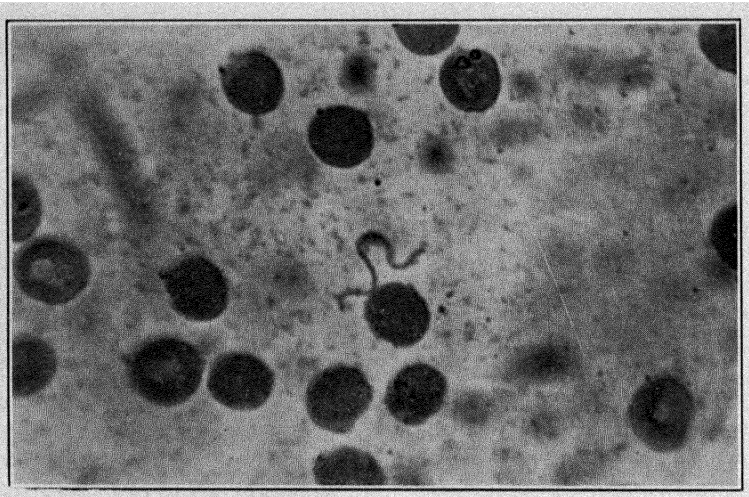


FIG. 42.—Free microgamete of *Plasmodium vivax*. Stained with Wright's stain. × 1800. (Photomicrograph, Army Medical Museum.)

of chromatin. They are slightly thicker at the middle and have pointed extremities. They are very slender, seldom much over a line in thickness, except at the center, and usually a very minute amount of blue-stained cytoplasm is visible at the thickest portion of the microgamete.

In routine blood preparations the exflagellating microgametocytes and the microgametes are never observed but may be frequently seen if the blood is collected upon a microscopic slide which has been moistened by breathing upon it and the blood examined in from five to ten minutes after placing a cover-glass over it, provided unstained material is to be examined, or, if stained preparations are desired, the blood is smeared across the slide after a preliminary examination of the unstained preparation has shown that flagellating microgametocytes are present.

In infections with *Plasmodium vivax* a segmenting schizont may sometimes be observed within an erythrocyte which also contains a macrogamete, giving the impression that the macrogamete is segmenting. Schaudinn (1902) interpreted such organisms as segmenting

macrogametes and claimed that parthenogenesis occurred in the malaria plasmodia and probably explained the relapses of malarial infections. It has been demonstrated, beyond question, that parthenogenesis does not occur in any species of malaria plasmodium and that Schaudinn mistook the forms mentioned as parthenogenetic macrogametes.

MORPHOLOGY OF PLASMODIUM OVALE

This plasmodium was described by the writer, in 1900, who regarded it as probably a variety of *Plasmodium vivax*, because of its resemblance to the latter organism. It was afterwards described by Stephens (1922), who regarded it as a new species and named it *Plasmodium ovale*. That it is identical with the plasmodium described by the writer is beyond question, as he has compared preparations of both organisms and found them to be identical in morphology. The validity of this species has since been confirmed by Stephens and Owen (1927), Yorke and Owen (1930), James, Nicol and Shute (1932), Mühlens (1934), DeMeillon and Gear (1940), Garcia (1945) and others and it is now accepted as a distinct species of malaria plasmodium.

A. **The Living, Unstained Plasmodium.**—The human life-cycle, or schizogony, of *Plasmodium ovale* is completed in approximately forty-eight hours.

In unstained, living preparations of this plasmodium it is seen in its earliest stage of development as a very refractive hyaline ring or disk within the invaded erythrocyte. It is sharply outlined and has no ameboid motility. In a few hours the plasmodium fills about one-third of the invaded erythrocyte and fine, reddish-brown granules of pigment are present, distributed throughout the cytoplasm and actively motile. At this stage of development the schizont is usually round or oval in shape and the invaded red blood cell is somewhat enlarged.

In about twelve hours the plasmodium appears as a colorless, refractile oval or round body, filling about one-half of the invaded erythrocyte and containing considerable dark brown pigment in the form of rather coarse granules, which are sluggishly motile. The plasmodium is generally round in contour but the invaded erythrocyte is usually oval in shape and considerably enlarged. There is no evidence of ameboid motility, the outline of the organism always remaining unchanged.

In from thirty-six to forty hours the plasmodium fills almost two-thirds of the invaded erythrocyte, is usually round in shape, and the pigment has increased in amount, is dark brown in color and arranged in irregular masses in the cytoplasm or in one or two masses at or near the center of the plasmodium. In approximately forty-eight hours the plasmodium fills about three-quarters of the invaded erythrocyte, is circular or, more rarely, oval, in shape, and the pigment is collected near or at the center of the body. If carefully focussed, using reduced

illumination, it will be noted that the cytoplasm of the plasmodium is divided into from 6 to 12 oval or round segments, the merozoites. The cytoplasm of the invaded erythrocyte appears somewhat paler than normal and the cell is considerably enlarged and frequently oval, rather than round in shape.

B. **The Stained Plasmodium.**—The similarity of the morphology of *Plasmodium ovale* to that of both *Plasmodium vivax* and *Plasmodium malariæ* in stained preparations has probably led to its confusion with these organisms in the past in many instances.

In preparations stained with the Wright, or other modifications of the Romanowsky stain, the *trophozoites* of *Plasmodium ovale* appear as deep blue "rings" within the cytoplasm of the invaded erythrocytes, each ring having at its periphery a rather large red dot of chromatin. The cytoplasm of the invaded cells stains an orange or pink color and rarely a few red staining Schüffner dots may be observed in the cytoplasm. The *schizonts* of this species stain a deep blue and are usually round or oval in shape, and the irregular, bizarre-shaped schizonts, always seen in infections with *Plasmodium vivax,* are never observed in this species. The chromatin of the nucleus stains a reddish-pink or bright red and is distributed throughout the cytoplasm of the schizont during the early stages of growth but prior to segmentation it collects into definite clumps, most often situated toward the periphery of the organism. The invaded erythrocytes are enlarged but not as much so as in infections with *Plasmodium vivax* and pink or red Schüffner granules are almost invariably present throughout the cytoplasm of the cell. The cytoplasm stains poorly and the shape of the invaded erythrocytes is usually oval although they may be round in contour. A most characteristic change in the morphology of the invaded erythrocytes is the fimbriated or ragged appearance of the periphery of the cells, an appearance not present in the erythrocytes invaded by any of the other species of malaria plasmodia.

The *segmenting schizonts* of *Plasmodium ovale* very closely resemble those of *Plasmodium malariæ* in morphology. At the time of segmentation the stained plasmodium fills about three-quarters or slightly more of the invaded erythrocyte and the pigment, which heretofore has been more or less distributed throughout the cytoplasm in the form of greenish-brown granules, is now collected in a solid dark greenish-brown mass at or near the center of the plasmodium. Grouped about this pigment mass there are from 6 to 12 oval or round, blue-stained merozoites, each containing a red dot of chromatin. The usual number of merozoites is 8 in the writer's experience. A few hours before segmentation the nuclear chromatin may be arranged in irregular clumps scattered throughout the cytoplasm of the parasite. The cytoplasm of the invaded erythrocytes contains numerous pink Schüffner granules and the edges of the infected cells show the same ragged appearance already described as characteristic.

The *gametocytes* of *Plasmodium ovale* closely resemble those of *Plasmodium malariæ* (see page 279), but may be distinguished by their occurrence in enlarged, oval-shaped red blood corpuscles, the cytoplasm of which contains Schüffner's dots, while from those of *Plasmodium vivax* they may be distinguished by their much smaller size and their

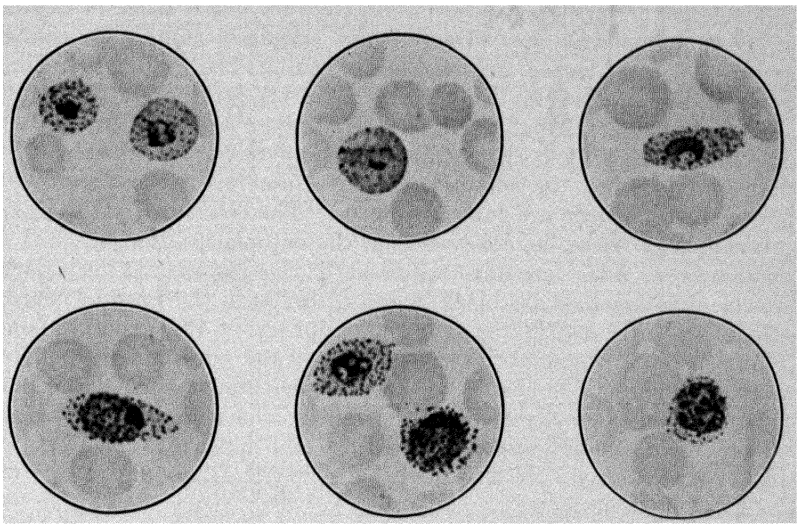


FIG. 43.—*Plasmodium ovale.* Developmental forms of schizonts of *P. ovale.* Note oval shape of infected erythrocytes, Schüffner's granules, and general resemblance of plasmodium to *P. malariæ.* × 1000. (After Mühlens, in Archiv. f. Schiffs- u. Tropen-Hyg., etc., courtesy of Johann Ambrosius Barth, Leipzig.)

situation in oval erythrocytes having ragged fimbriated edges. The *microgametocytes* and the *macrogametocytes* are differentiated by the same variations in their staining reactions and the arrangement of the nuclear chromatin as already described in considering the morphology of the same stages in the development of *Plasmodium vivax* (see page 265).

MORPHOLOGY OF PLASMODIUM MALARIÆ—THE QUARTAN PLASMODIUM

A. **The Living, Unstained Plasmodium.**—The morphology of *Plasmodium malariæ*, the cause of quartan malarial fever, is distinctive and this plasmodium can be easily differentiated from the other malaria plasmodia of man. All forms of the human life-cycle occur in the peripheral blood of the infected individual, as well as gametocytes, and schizogony is completed within approximately seventy-two hours.

The earliest stage in the development of *Plasmodium malariæ* that is visible in the peripheral blood is the *trophozoite*, appearing as a rather refractile "ring" or disk within the invaded red blood corpuscle. It is

more sharply outlined than is the trophozoite of *Plasmodium vivax* and is finely granular and colorless. Ameboid motility is very slight as compared with that of the latter organism. Pigment is developed within a few hours in the form of rather coarse very dark brown granules which are very sluggishly motile.

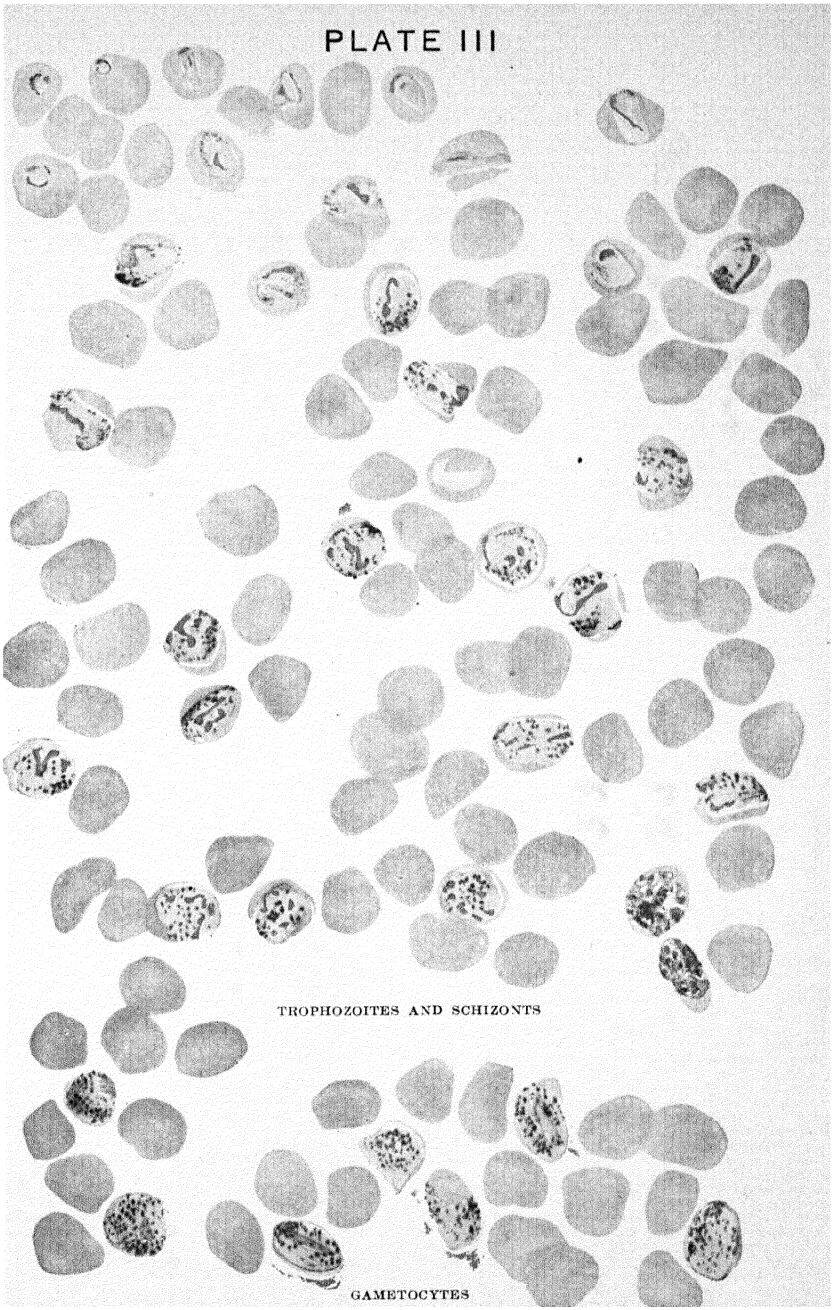
The larger pigmented forms, or *schizonts*, vary in size with the degree of development and are refractile and colorless, containing coarse granules of dark brown, almost black pigment, the cytoplasm being granular in appearance. The young schizonts, at the end of about sixteen hours, are usually spherical in shape, ameboid motility is practically absent, and the invaded erythrocyte is not enlarged. In twenty-four hours the characteristic features of the schizonts of *Plasmodium malariæ* are fully developed. The schizonts are sharply outlined, very refractile, colorless, and the cytoplasm often presents a peculiar, very finely granular appearance; the pigment is very dark brown, sometimes almost black in color, occurs in the form of coarse grains frequently distributed near the periphery of the organism and is immotile. Ameboid motility is absent and the schizonts are usually ovoidal or spherical in shape, lying within erythrocytes which are not enlarged and have a slightly deeper greenish color than the uninvaded erythrocytes. Sometimes the invaded cells appear to be slightly smaller than normal. At this time the schizonts fill about one-half of the invaded erythrocyte.

By the end of thirty-six hours the schizonts fill nearly two-thirds of the corpuscles in which they are developing and the pigment is increased in amount and arranged in irregular masses near the periphery of the organisms. The pigment is motionless and ameboid motility is absent. The schizonts are sharply outlined, refractile and colorless, and the cytoplasm appears finely granular. The invaded erythrocytes are not enlarged and do not appear anemic, as in infections with *Plasmodium vivax*.

After thirty-six hours the development and growth of the schizonts appears to be very slow and it is not until about eight hours before segmentation that presegmenting forms begin to appear in the peripheral blood. Such forms are usually spherical in shape, sharply defined within the invaded erythrocytes, and are bordered by a thin greenish membrane, representing the remains of the invaded erythrocyte, the entire body being about the size of the normal erythrocytes instead of being very much larger, as in infections with *Plasmodium vivax*. Numerous clumps of dark brown, almost black, pigment are distributed in the cytoplasm which appears finely granular, colorless and refractile.

At the end of approximately seventy-two hours the schizonts of this species segment, dividing into from 6 to 12 merozoites, the average number being 8 to 10. Rarely segmenting forms have been observed containing as many as 14 merozoites. At the time of segmentation the

PLATE III



QUARTAN MALARIA PLASMODIA.

(*Plasmodium malariæ.*)

Various stages in the development of *Plasmodium malariæ*. (Preparations from cultures by Bass and Johns. Wright's stain.) *From Bass and Johns, with permission from Tice's Practice of Medicine, Vol. III, courtesy of W. F. Prior Company.*

schizonts usually entirely fill the invaded erythrocytes and the pigment is collected at the center of the organism in a compact almost black, round mass, or in a starlike arrangement, the pigment radiating from a central mass into the cytoplasm of the organism.

At the time of segmentation the colorless cytoplasm is observed to be divided into from 6 to 12 oval segments, or merozoites, each having a well defined outline. These merozoites are arranged in a regular manner about the central pigment mass, like the petals of a flower, forming the so-called daisy or "Marguerite" appearance, so characteristic of the segmenting forms of this species of plasmodium. More rarely the merozoites are irregularly arranged but the usual symmetrical arrangement of the merozoites is characteristic of *Plasmodium malariæ*.

The merozoites of this species are larger than those of the other species of malaria plasmodia and are more clearly defined and refractile in unstained preparations. The red blood corpuscles containing the segmenting plasmodia are not enlarged and are usually so completely filled that little, if any, of the remains of the invaded cells can be distinguished. Sometime segmenting organisms may be seen which do not entirely fill the invaded corpuscle but this is unusual. The segmenting plasmodia vary very little in size.

A most important point in the differential diagnosis between this plasmodium and *Plasmodium vivax* and *Plasmodium ovale* is the lack of enlargement of the invaded red blood corpuscles. In fact, the invaded cells sometimes appear to be a little smaller than normal. The cytoplasm of the invaded corpuscle is a slightly deeper green than that of the normal red blood corpuscle while in infections with both *Plasmodium vivax* and *Plasmodium ovale* the color of the cytoplasm is much paler than that of the uninvaded corpuscle.

B. **The Stained Plasmodium.**—The *trophozoites* of *Plasmodium malariæ* in preparations stained with the Wright stain are observed as ring-shaped bodies the cytoplasm of which is stained blue, and having a red dot of chromatin at some point on the periphery in contact with a minute unstained area, the vesicular portion of the nucleus. The cytoplasm of the invaded corpuscles stains an orange pink or pink color and the ringlike trophozoites stand out very clearly within the cytoplasm as they stain more intensely blue than the trophozoites of other species of plasmodia and the amount of blue-stained cytoplasm is greater, the "rings" appearing thicker than those of *Plasmodium vivax* or *Plasmodium ovale*. The trophozoites vary somewhat in size and the red dot of chromatin is comparatively large in this species.

The *schizonts* stain a deep blue and are usually oval or circular in shape but in this species a very characteristic form is often observed, known as the "band" or "ribbon" form, which stretches across the invaded corpuscle like a band or ribbon of blue-stained cytoplasm, containing granules or clumps of greenish-black pigment. In the

early development of the schizonts the chromatin is finely divided, stains a pinkish color and is distributed in the cytoplasm. Later the chromatin begins to collect in irregular clumps and stains a more intense pink or red, while the greenish black pigment is distributed in minute collections of coarse granules in the blue-stained cytoplasm. In the presegmenting schizonts the cytoplasm stains deep blue and contains large granules or clumps of greenish-black pigment, while the chromatin stains a ruby-red color and is distributed in the cytoplasm in irregular clumps. In the segmenting schizonts the cytoplasm is divided into from 6 to 12 oval, blue-stained segments, the merozoites, which are arranged in a regular manner, like the petals of a flower, about a central mass of greenish-black pigment. Each merozoite consists of a mass of blue-stained cytoplasm containing a bright red dot of chromatin.

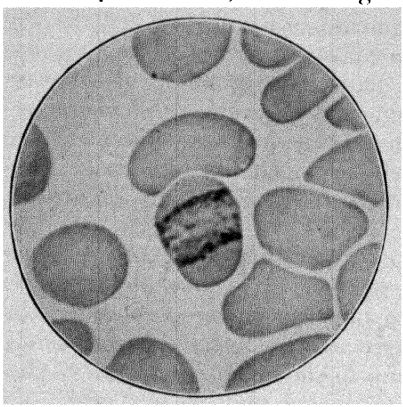


FIG. 44.—*Plasmodium malariæ*. Stained with Wright's stain. So-called "band" form schizont of *P. malariæ*. × 1800. (Photomicrograph, Army Medical Museum.)

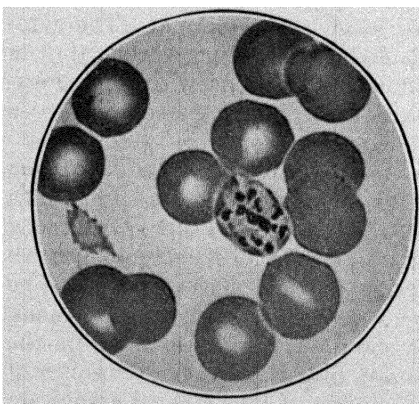


FIG. 45.—*Plasmodium malariæ*. Presegmenting schizont. Stained with Wright's stain. × 1200. (Photomicrograph, Army Medical Museum.)

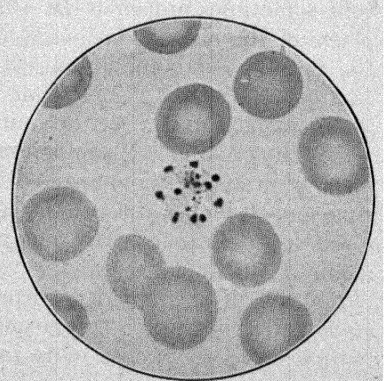


FIG. 46.—*Plasmodium malariæ*. Stained with Wright's stain. Fully segmented schizont of *P. malariæ*. × 1200. (Photomicrograph, Army Medical Museum.)

The invaded red blood corpuscles are not enlarged and the cytoplasm does not contain Schüffner's dots, as in infections with *Plasmodium vivax* and *Plasmodium ovale*. If special methods of staining be employed pinkish dots, much smaller than the Schüffner dots, may some-

times be observed in the cytoplasm of the invaded erythrocytes which are referred to by James as Ziemann's dots.

The Gametocytes of Plasmodium Malariæ.—The gametocytes of *Plasmodium malariæ* are present in the peripheral blood after the infection has existed for a few days and can be distinguished from the human life-cycle forms in both unstained and stained preparations.

The Living, Unstained Gametocytes.—In the earliest stage of development of the gametocytes they cannot be distinguished from the trophozoites, both appearing as colorless, hyaline appearing rings or disks within the invaded erythrocytes, but as pigment develops and the organisms enlarge it will be noted that the pigment is not collected in irregular masses but is either distributed in the cytoplasm or in a wreathlike formation near the periphery of the gametocytes. It will also be noted that, even when the gametocytes almost fill the invaded erythrocytes, there is no collection of the pigment in a central, compact mass and no evidence of the division of the cytoplasm into segments or merozoites. Thus, any organism almost filling the invaded erythrocyte and showing no central pigment mass and no evidence of segmentation, must be a gametocyte.

The description of the morphology of the *microgametocytes* and *macrogametocytes* of *Plasmodium vivax* (see page 268) applies equally well to the *microgametocytes* and *macrogametocytes* of *Plasmodium malariæ* with the exceptions that they are smaller, are not situated in enlarged erythrocytes, and the pigment is darker brown in color and in the form of larger granules. In properly prepared specimens of blood the same phenomena observed in the exflagellation of the microgametocytes and the production of microgametes may be observed as already described in the case of *Plasmodium vivax* (see page 268).

The Stained Gametocytes.—As in *Plasmodium vivax* and *Plasmodium ovale* the gametocytes of this species present staining reactions which differentiate the microgametocytes and the macrogametocytes. The cytoplasm of the microgametocytes stains a much paler blue than does that of the macrogametocytes, which stains a deep blue in well-prepared specimens of blood. The chromatin of the nucleus in the microgametocyte is arranged in a loose skein of fibrils or dots lying in an unstained area, the chromatin staining a pink or pinkish-red unless it is overstained, when it may appear a deep purple or almost black color. In the macrogametocytes the nuclear chromatin is collected in a compact mass of granules within an unstained area of the deep blue cytoplasm and stains a bright red or purplish color. The pigment in both types of gametocytes is a dark greenish-brown color, distributed in the cytoplasm in granules in the microgametocyte and in larger granules and minute clumps near the periphery in the macrogametocytes. Both the microgametocytes and macrogametocytes are situated in unenlarged erythrocytes the cytoplasm of which is pinkish or orange colored and free from Schüffner, or eosinophilic, granules.

In infections with *Plasmodium malariæ* all forms of the parasite concerned in the human life-cycle, *i. e.*, trophozoites, schizonts and merozoites, may be demonstrated in the peripheral blood, as well as the gametocytes. In single infections, if the blood be examined at regular intervals during the seventy-two hours consumed in schizogony, it is possible to study every stage of the process, from the appearance of the ringlike trophozites, through the growth of the schizonts, to the segmentation of the latter, with the liberation of the merozoites. This is most easily done in stained preparations, blood smears being made at intervals of six or eight hours.

MORPHOLOGY OF PLASMODIUM FALCIPARUM (THE ESTIVO-AUTUMNAL PLASMODIUM)

The recognition of *Plasmodium falciparum* is especially important for it is this species that is responsible for most of the pernicious malarial infections and it may be truthfully stated that practically 95 per cent of deaths from malaria are due to infection with this plasmodium.

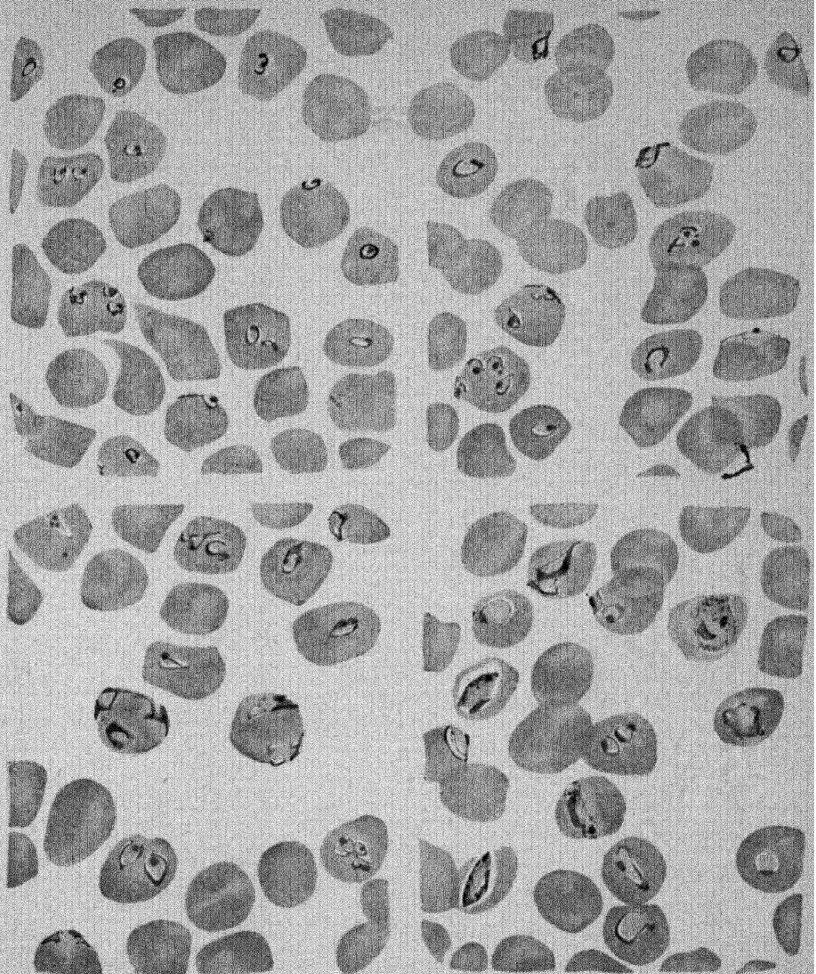
Unlike the other species of malaria plasmodia only the trophozoites and gametocytes are usually observed in the peripheral blood, for the growth and segmentation of the schizonts occurs in the erythrocytes of the visceral circulation, especially in the spleen and bone-marrow. In pernicious infections with this plasmodium the pigmented schizonts and segmenting forms may sometimes be seen in preparations of the peripheral blood but in the usual estivo-autumnal infection only the ring-form trophozoites and the crescentic gametocytes are encountered.

The life-cycle of *Plasmodium falciparum* is completed in man in from thirty-six to forty-eight hours.

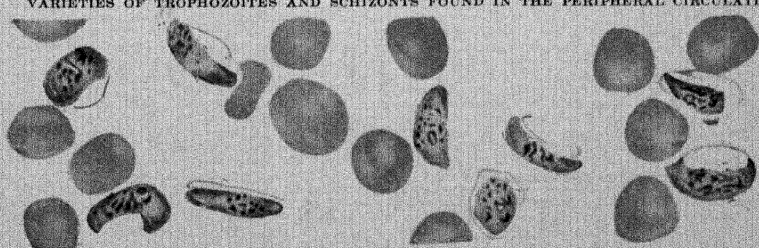
A. **The Living, Unstained Plasmodium.**—The trophozoites of *Plasmodium falciparum* are seen as round or oval, colorless, hyaline disks or rings within the invaded erythrocyte. The ring-forms are very sharply defined, rather refractile, and the cytoplasm appears opaque and very finely granular. Ameboid motility is absent and the cytoplasm of the invaded erythrocytes is a darker green in color than that of the normal erythrocytes and the periphery of the invaded cell may appear crenated or ragged in contour, in cells containing the more fully developed trophozoites. The invaded erythrocytes are usually slightly smaller than the surrounding normal corpuscles.

The larger ring-forms are generally irregular in shape, one portion of the cytoplasm being more expanded than the rest, which gives rise to a signet-ring appearance very characteristic of this species. Ameboid motility, of a very sluggish nature, is present in the larger ring-forms, the periphery of the ring expanding and contracting or sending out very minute prolongations which are instantly withdrawn. In the expanded portion of the ring-forms a few grains of almost black pigment may be sometimes observed and may be very sluggishly motile.

PLATE IV



VARIETIES OF TROPHOZOITES AND SCHIZONTS FOUND IN THE PERIPHERAL CIRCULATION



GAMETOCYTES

AESTIVO AUTUMNAL MALARIA PLASMODIA.

(*Plasmodium falciparum.*)

Various stages in the development of *Plasmodium falciparum.* (Preparations from cultures by Bass and Johns. Wright's stain.) *From Bass and Johns, with permission from Tice's Practice of Medicine, Vol. III, courtesy of W. F. Prior Company.*

Multiple infection of the erythrocytes is frequently observed, corpuscles being present containing from 2 to as many as 6 trophozoites, especially in pernicious infections. In such cases the ring-forms may vary considerably in size although cells are seen in which they are uniform in size. Multiple infections of erythrocytes are so much more common in infections with *Plasmodium falciparum* than with any of the other species of malaria plasmodia that they may almost be considered as a diagnostic feature of such infections.

The *schizonts*, as stated, are not usually present in the peripheral blood but in blood obtained by splenic or bone-marrow puncture, such forms are very numerous, and in severe infections they may also be present in small numbers in the peripheral blood. The schizonts vary in size with the growth of the plasmodia, and may fill from one-third to one-half of the invaded erythrocyte before segmentation begins. Ameboid motility is absent and the schizonts are round or oval in shape and clearly defined. They are colorless, somewhat refractile and have an opaque, finely granular cytoplasm. The pigment is usually aggregated into an irregular clump lying within the cytoplasm or in the form of fine granules, and is of a very dark brown or almost black color and may be apparently motile. The invaded erythrocytes are not enlarged and are usually a little smaller than the uninvaded cells, are darker green in color than normal, and their periphery may appear crenated or ragged.

As the time for segmentation approaches the pigment collects into a dense, almost black mass of granules at or near the center of the organism, and the cytoplasm appears more opaque and granular, while fine radial striations may be observed within the cytoplasm dividing it into irregular segments, which, when fully developed become oval or round in shape and number usually from 18 to 24 but as many as 36 and as few as 8 have been reported as occurring in this species, by different authorities, thus suggesting that perhaps more than one species of plasmodium is included under the name, *Plasmodium falciparum*. The segmenting plasmodium fills from two-thirds to three-quarters of the invaded erythrocytes, which are usually slightly smaller in size than normal and of a much darker green color, often appearing to have a distinct "brassy" tinge, the so-called "brassy corpuscles." The outline of the invaded erythrocyte is often crenated and the entire cell appears shrunken about the body of the plasmodium in some instances, especially in pernicious infections.

In some infections with what is supposedly *Plasmodium falciparum* the ring-forms, or trophozoites, instead of being thicker than those of other species of plasmodia, and having an enlarged portion of cytoplasm at some portion of the periphery, are very thin and delicate and difficult to demonstrate in unstained preparations. Such trophozoites appear as very delicate, threadlike hyaline rings within the invaded erythrocytes.

B. **The Stained Plasmodium.**—The staining reactions of this plasmodium with the Wright, or other modification of the Romanowsky stain, are like those of the other malaria plasmodia, the cytoplasm staining blue while the chromatin of the nucleus stains a ruby-red, dark red or purplish color, varying with the intensity of the stain.

The *trophozoites* appear within the invaded erythrocytes as blue rings having at one portion of the periphery a bright red or purplish dot of chromatin surrounded by a minute unstained area, the vesicular portion of the nucleus. In many of the trophozoites of this species, two chromatin dots may be present in the ring-form, either close together or at varying distances apart, not infrequently at opposite sides of the ring. In some instances the rings appear to be dividing, there being

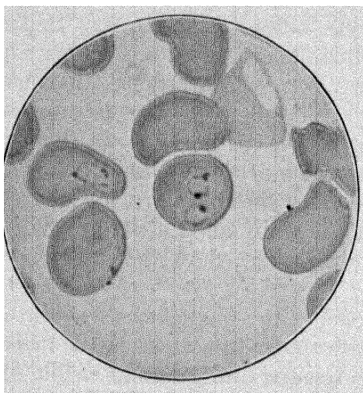


FIG. 47.—*Plasmodium falciparum.* Stained with Wright's stain. "Ring-form" trophozoites of *P. falciparum.* × 1500. (Photomicrograph, Army Medical Museum.)

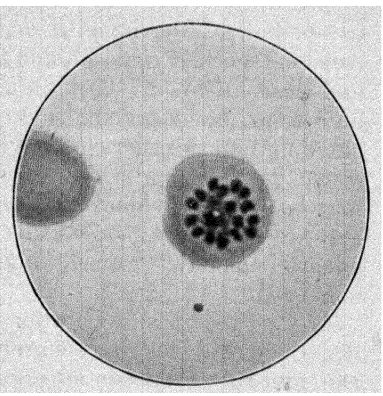


FIG. 48.—*Plasmodium falciparum.* Stained with Wright's stain. Segmenting schizont of *P. falciparum.* × 1800. (Photomicrograph, Army Medical Museum.)

two distinct ring-forms, each containing a chromatin dot and apparently merging into one another at a portion of their periphery. As already stated, some malariologists regard these as dividing forms and thus explain the very frequent multiple infection of the erythrocytes with ring-forms which occurs in estivo-autumnal malaria. It is very common to see from 2 to 4 ring-forms in an invaded erythrocyte, and as many as 8 such forms have been observed within a single invaded erythrocyte.

The larger trophozoites present an expansion of the cytoplasm at some portion of the periphery of the ring, usually opposite the chromatin dot, which stains blue and which may contain a few grains of dark greenish-black pigment.

In some infections with *Plasmodium falciparum*, the trophozoites, or ring-forms, consist of a threadlike perfect ring of blue stained cytoplasm containing a single chromatin dot. These ring-forms are so

delicate that they may be easily overlooked by a careless observer and differ markedly in their appearance from the ring-forms having an expanded portion of cytoplasm which is so characteristic of the trophozoites of this species. These two types of ring-forms do not occur in the same patient, in the experience of the writer, except in very rare instances.

In infections with *Plasmodium falciparum* the so-called "applique" forms are very frequently observed in stained preparations. These are trophozoites situated upon the periphery of the invaded erythrocytes and appearing as short, blue-stained threadlike bodies having at the center a red dot of chromatin. While such forms occur in infections with the other malaria plasmodia they are much more numerous in infections with *Plasmodium falciparum* and often two or more are seen attached to the periphery of a single erythrocyte.

In preparations of peripheral blood from patients suffering from estivo-autumnal malaria the trophozoites and youngest schizonts, when present, are sometimes very irregular in shape, somewhat resembling those of *Plasmodium vivax*. Such forms may be flame-shaped, filamentous or apparently spread out in the cytoplasm of the infected erythrocyte in a netlike arrangement. Stephens (1914) believed that such forms belonged to a hitherto undescribed species and named it *Plasmodium tenue* but most authorities do not accept this species and regard the *tenue* forms as artefacts, being produced by pressure upon the plasmodia during the preparation of the blood films.

The *schizonts* of *Plasmodium falciparum*, as stated, are not usually present in the peripheral blood except in very severe or pernicious infections. When present they are round or oval in shape and vary in size with the age of the schizont. The cytoplasm stains a deep blue and the chromatin is small in amount and stains pink or red and is either distributed in minute grains or in small masses in the cytoplasm, if segmentation is approaching. The pigment in the schizonts is comparatively small in amount and in the form of rather large granules, and is greenish-black in color. In the majority of the stained schizonts the pigment, even in the young organisms, is collected in a loose mass near the center rather than distributed throughout the cytoplasm. In blood obtained from the spleen or bone-marrow many of the schizonts appear as small oval or round, deep blue bodies having at, or near, the center, a small clump of almost black pigment.

The segmenting schizonts usually fill from two-thirds to three-quarters of the invaded erythrocyte and appear as blue, oval or round bodies, the *merozoites*, each containing a bright red dot of chromatin, and arranged in a more or less symmetrical manner around a central mass of dark green pigment. The merozoites are very minute, measuring about 1.5 microns in diameter and usually number from 18 to 24, although this number may be exceeded in rare instances and still more rarely, less than 18 merozoites may sometimes be present.

The invaded erythrocytes present characteristic changes in the staining reactions of the cytoplasm. Instead of staining an orange pink or pink color, as in infections with the other malaria plasmodia, the cytoplasm stains a bluish color, in many instances, and is stippled with minute blue dots, the basophilic stained dots being often improperly called "Maurer's dots." These dots were first described by Stephens and Christophers (1900–1903) and should be known by their names. The true "Maurer's dots" less frequently occur in the cytoplasm of erythrocytes invaded by *Plasmodium falciparum* and consist of minute clefts in the cytoplasm which stain a brick-red color with the Wright or other modifications of the Romanowsky stain. The periphery of the invaded erythrocytes frequently appears crenated and the invaded cells are not enlarged but usually a little smaller than the normal erythrocytes.

The Gametocytes of Plasmodium Falciparum.—The gametocytes of this species of malaria plasmodium, when fully developed, are most characteristic and can be easily differentiated in both unstained and stained preparations of the peripheral blood. They are crescentic or lima bean-like in shape which alone distinguishes them from the gametocytes of the other species of malaria plasmodia which are always spherical in shape. They appear in the peripheral blood some days after the occurrence of symptoms and the male and female gametocytes are easily differentiated.

The Unstained Gametocytes.—In unstained preparations of blood the early stages of the gametocytes are ovoid or spherical in shape and cannot be distinguished from the trophozoites and schizonts until the crescentic shape is developed, which occurs when the gametocytes fill from two-thirds to three-quarters of the invaded erythrocytes. At this time they are very easily recognized because of their peculiar shape, the presence frequently of a distinct double outline and the absence of any evidences of segmentation. The cytoplasm is finely granular and opaque in appearance, sometimes having a distinct greenish hue, due to the enveloping membrane formed by the red blood corpuscle in which they have developed. The shape varies from that of a true crescent to a lima or kidney bean and the pigment is dark brown or almost black in color and may be distributed throughout the cytoplasm or collected at or near the center of the gametocytes. The remains of the erythrocyte in which the gametocyte has developed may be seen as a greenish, serrated border to the crescent or as a "bib-like" greenish mass situated in the concavity of the crescentic gametocyte connecting its two poles. The male and female gametocytes can be distinguished in unstained preparations after they are fully developed.

The *male* gametocytes, or microgametocytes, are not crescentic in shape but resemble in shape a kidney or lima bean, the ends being much rounded, the body plump, and the concavity comparatively slight while the outline opposite the concave portion of the body is

much rounded. They measure from 7 to 10 microns in length by 3 to 5 microns in breadth and may have a double or single outline, those showing a double outline being younger than those having a single outline, the double outline being caused by the remains of the invaded erythrocyte which forms a membrane-like sheath covering the gametocyte. In the older microgametocytes the double outline has disappeared due to the disintegration of the cytoplasm of the invaded erythrocytes and the final liberation of the microgametocyte from the invaded erythrocyte. The cytoplasm is less granular and opaque than that of the macrogametocyte, or female gametocyte, and the pigment is in finer granules and is distributed throughout the cytoplasm. There is no trace of ameboid activity at any stage in the development of the macrogametocyte.

In properly collected specimens of peripheral blood the microgametocytes exflagellate and produce *microgametes* as in the other species of malaria plasmodia. They lose their kidney bean shape and become oval or round, the pigment becomes very actively motile due to currents within the organism and suddenly several slender filaments are projected from the periphery, the *microgametes*. These resemble in their morphology the microgametes of other species of malaria plasmodia, in unstained preparations being colorless, hyaline threadlike filaments, several times the diameter of a red blood corpuscle in length and having an active serpentine motility.

The *female* gametocytes, or *macrogametocytes* are typically crescentic in shape in unstained preparations, the crescent being slender with somewhat pointed ends, very easily distinguished from the plump kidney-shaped microgametocyte with its rounded extremities. The macrogametocytes may have a single or double outline, the cytoplasm is colorless or of a slight greenish tint, granular and opaque. The pigment is dark brown or almost black in color and instead of being distributed throughout the cytoplasm, as in the microgametocytes, is collected at the center of the body as a single or double compact clump, or, more rarely, arranged in a wreathlike manner at the center of the parasite. The pigment is immotile at every stage in the development of the macrogametocyte. They measure from 11 to 15 microns in length by 2 to 3 microns in breadth.

The Stained Gametocytes.—The gametocytes of *Plasmodium falciparum* when fully developed are very characteristic morphologically, appearing as crescentic or kidney bean-shaped bodies, staining blue and containing red-stained chromatin and dark greenish pigment. They occur in most infections in which treatment has not been administered after the infection has existed for approximately twelve to fifteen days, although they may not be present in the peripheral blood until after this period of time, and they are always more numerous in the capillaries of the spleen, bone-marrow and internal organs than in the peripheral circulation. The morphology of the male and female

gametocytes differs markedly and it is easy to distinguish them in properly stained preparations.

The *microgametocytes*, or male gametocytes, stain less intensely than the macrogametocytes, the cytoplasm staining a greenish-blue, or more rarely, a violet-red color, when fully developed, while the chromatin of the nucleus appears as delicate pink or red fibrils or granules arranged in a loose network within the cytoplasm. The pigment is scattered throughout the cytoplasm in the form of dark brown or black granules or minute rods. The amount of stained cytoplasm may be very small

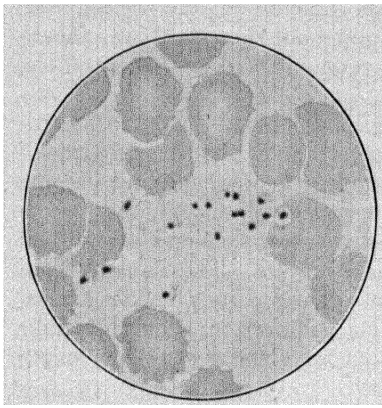


FIG. 49. — *Plasmodium falciparum.* Stained with Wright's stain. Free merozoites of *P. falciparum.* × 1800. (Photomicrograph, Army Medical Museum.)

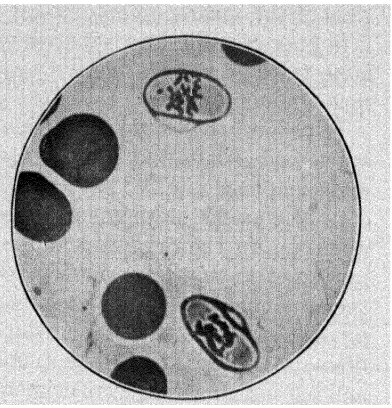


FIG. 50. — *Plasmodium falciparum.* Stained with Wright's stain. Microgametocytes of *P. falciparum.* Note plump bean-shaped "crescents" or microgametocytes. × 1200. (Photomicrograph, Army Medical Museum.)

and the chromatin may stain so poorly as to be almost invisible. Surrounding many of the microgametocytes is a deep red border representing the remains of the erythrocytes in which they have developed and a more faintly stained red mass may be seen connecting the poles of the gametocyte, the so-called "bib" which also consists of the remains of the invaded corpuscle. Very frequently the red or pink stained border appears irregular or crenated. In the microgametocytes which are apparently free in the blood plasma a deep red, narrow band may be detected surrounding the entire body of the organism. The shape of the microgametocytes in stained preparations resembles that of a kidney bean or a lima bean, rather than a crescent, a very important differentiating feature of the microgametocyte.

The *macrogametocytes*, or female gametocytes, stain much more intensely than do the microgametocytes, the cytoplasm staining a deep blue instead of a light greenish-blue, commonly observed in the latter. The staining is usually most intense at the poles of the crescent, the center sometimes appearing almost unstained. The chromatin of the

nucleus stains a ruby red or violet and is collected in a compact mass at or near, the center of the body. The pigment is very dark brown or almost black in color and is collected in minute masses of granules situated in the cytoplasm near the chromatin mass or arranged in a

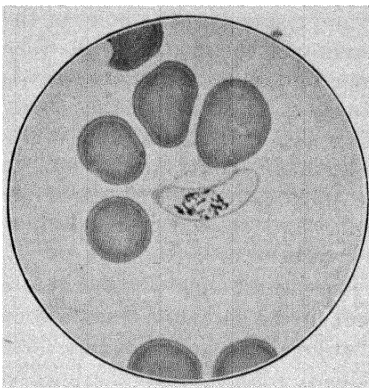


FIG. 51 — *Plasmodium falciparum.* Stained with Wright's stain. Macrogametocyte of *P. falciparum.* × 1200. (Photomicrograph, Army Medical Museum.)

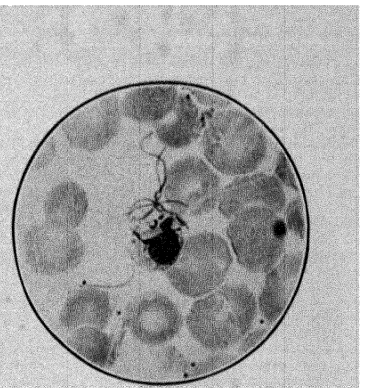


FIG. 52. — *Plasmodium falciparum.* Stained with Wright's stain. Flagellated microgametocyte of *P. falciparum.* × 1000. (Photomicrograph, Army Medical Museum. Preparation by Craig.)

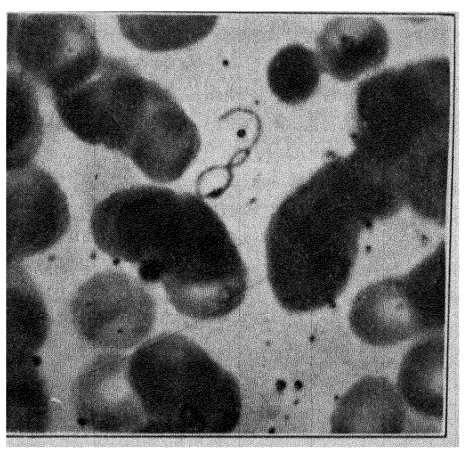


FIG. 53.—*Plasmodium falciparum.* Free microgamete, folded upon itself. Note central chromatin mass. × 1500. (Photomicrograph, Army Medical Museum. Preparation by Craig.)

wreathlike manner around the chromatin. Very frequently the pigment appears to be more or less mixed with the chromatin but this is an optical illusion caused by the pigment overlying the chromatin

mass in such organisms. A deep red band may sometimes be seen surrounding the macrogametocytes or there may be a pink or light red, crenated border formed by the remains of the erythrocyte in which the macrogametocyte has developed. In addition, there may be a hemispherical, pink stained mass of cytoplasm, called the "bib," lying in the concavity of the crescentic body and connecting the poles of the crescent, which also represents the remains of the erythrocyte. The shape of the microgametocytes is typically crescentic, the body being slender and the ends quite pointed, in contradistinction to the microgametocytes, which are plump and shaped like a kidney or lima bean.

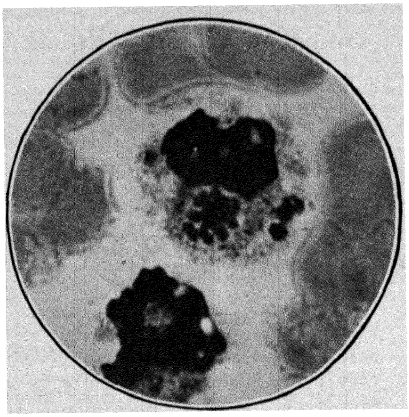


FIG. 54.—*Plasmodium falciparum.* Phagocytosis by a macrophage of a segmenting form and young schizonts of *Plasmodium falciparum.* × 1800. (Photomicrograph, Army Medical Museum. Preparation by Craig.)

The earliest stage in the development of the gametocytes observed in stained preparations are blue stained, round or oval rings of cytoplasm, each containing a red dot of chromatin at the center, instead of upon the periphery as in the trophozoites. As these bodies enlarge they assume the characteristics of the microgametocytes and macrogametocytes but the sex cannot be accurately distinguished until development is practically complete.

In properly prepared blood films (see page 272) the same changes may be observed in the micro- and macrogametocytes prior to fertilization as those previously described for the micro- and macrogametocytes of *Plasmodium vivax* (see page 268). These changes normally occur in the "stomach" of the transmitting mosquitoes. The microgametocyte loses its kidney bean shape, becomes spherical, and several threadlike actively moving filaments are projected from it, which are the microgametes. These eventually are liberated from the parent body and swim off in a serpentine manner among the red blood corpuscles. At the same time the macrogametocytes lose their crescentic shape and become round or oval bodies in which the pigment is arranged in a wreathlike manner near the periphery or in granular clumps, minute in size, between the periphery and the center of the organism. In stained preparations the flagellating microgametocyte stains a pale blue, the chromatin is collected in irregular masses, stained a bright red or reddish-violet, while the threadlike microgametes are stained pink or red and appear to be composed almost entirely of chromatin,

The macrogametocytes stain a deep blue and the chromatin is usually collected in a compact mass within the organism.

THE DIFFERENTIAL DIAGNOSIS OF THE SPECIES OF MALARIA PLASMODIA

In the diagnosis of the malarial infections it is of the utmost importance that it be based not only upon the demonstration of a plasmodium in the patient's blood but that the species of plasmodium present be accurately diagnosed. This is so because of the variations in the virulence of the different species which necessitates variations in the methods of treatment and because no reliable data can be collected regarding the prevalence of the different types of malaria unless the species of plasmodia causing these infections in different localities are recognized and differentiated. The differential diagnosis of the four generally accepted species of malaria plasmodia is not difficult after a little practice when properly stained preparations are examined and stained preparations should be employed in differential diagnosis. The important differential features of the morphology of the different species of malaria plasmodia may be briefly summarized as follows:

PLASMODIUM VIVAX (The Tertian Plasmodium).—(1) Larger size after the development of pigment. This is the largest of the malaria plasmodia. (2) Increased size of the invaded erythrocyte and great distortion in its shape. (3) The presence of Schüffner's eosinophilic granules in the cytoplasm of the invaded erythrocyte. (4) Number of merozoites, 12 to 24. (5) Presence of all stages of the development of the schizont in the peripheral blood. (6) Segmentation and the production of merozoites occurs in approximately forty-eight hours. (7) Gametocytes spherical in shape.

PLASMODIUM OVALE (Ovale Tertian Plasmodium).—(1) Medium size, being slightly larger than *Plasmodium malariæ* and smaller than *Plasmodium vivax*. (2) Invaded erythrocyte larger than normal, usually oval in shape and having a ragged, frayed-out periphery, most noticeable in cells containing nearly fully grown schizonts. (3) Presence of Schüffner's granules even in the cytoplasm of erythrocytes containing young trophozoites, or "ring-forms." (4) Number of segments, or merozoites, varies from 6 to 12, usually 8 in number. (5) Presence of all stages of schizogony in the peripheral blood. (6) Segmenting forms present in the blood in forty-eight hours. (7) Gametocytes spherical in shape.

PLASMODIUM MALARIÆ (The Quartan Plasmodium).—(1) Medium size of plasmodium when fully developed. (2) No increase in the size of the invaded erythrocyte. (3) Schüffner granules are not present in the cytoplasm of the invaded erythrocyte. (4) The presence of the so-called "band" or "ribbon" forms, stretching across the invaded

TABLE 10. THE DIFFERENTIAL DIAGNOSIS OF THE MALARIA PLASMODIA OF MAN. (WRIGHT'S STAIN.)*

	Plasmodium vivax	*Plasmodium malariæ*	*Plasmodium falciparum*	*Plasmodium ovale*
Duration of schizogony	48 hours	72 hours	36 to 48 hours	48 hours
Motility	Active ameboid until about half-grown	Slightly ameboid during trophozoite stage	Active ameboid during trophozoite stage	Slightly ameboid during trophozoite stage
Pigment (Hematin)	Yellowish-brown, in fine grains and minute rodlets	Dark brown or almost black in coarse grains, rods, or irregular small clumps	Very dark brown or black in coarse granules or small masses	Dark brown in coarse granules or irregular masses
Infected red blood corpuscle	Much enlarged, pale with eosinophilic stippling (Schüffner's dots)	Not enlarged. Normal color. No granular stippling	Not enlarged or smaller than normal. Darker green (brassy). Basophilic dots and brick-red clefts in cytoplasm (Maurer's clefts or dots)	Somewhat enlarged, oval or irregular in shape, with eosinophilic stippling (Schüffner's dots)
Stages of development seen in peripheral blood	Trophozoites, schizonts and gametocytes	Trophozoites, schizonts and gametocytes	Usually only trophozoites and gametoyctes. In pernicious infections rarely schizonts may be seen	Trophozoites, schizonts and gametocytes
Multiple infection of red blood corpuscle	Quite common	Very rare	Very common	Rare

Area of red blood corpuscle occupied by fully developed schizont	Entire red blood corpuscle which is enlarged	Almost entire red blood corpuscle which is not enlarged	From two-thirds to three-quarters of red blood corpuscle which is not enlarged	About three-quarters of red blood corpuscle which is enlarged
Trophozoites (ring-forms)	Small and large rings with vacuole and usually one chromatin dot. Ameboid	Small and large rings with vacuole and usually one chromatin dot; or early "band" forms	Very small and larger rings with vacuole and frequently with 2 chromatin dots. Peripheral forms common. (Forms appliqué.) Ameboid	Small and large rings with vacuole. Ameboid
Segmenting schizonts	Irregular, bizarre forms. Vacuole present in early stage. Chromatin in fine grains or small irregular clumps	Oval or round, with vacuole in early stage. Chromatin in coarse granules or irregular clumps. Band forms often seen	Not usually seen in peripheral blood. Oval or round with chromatin in large granules and in small clumps	Round and oval with vacuole in early stage. Chromatin in irregular clumps or filamentous masses
Segmented schizonts	Fill greatly enlarged red blood corpuscle. 12 to 24 merozoites (usually 18 to 20) irregularly arranged about a mass of pigment	Almost fill a normal sized red blood corpuscle. 6 to 12 merozoites (usually 8 to 10) arranged like the petals of a flower surrounding a central pigment mass	Not usually seen in peripheral blood. Fill two-thirds to three-quarters of red blood corpuscle. 8 to 36 merozoites (usually 18 to 24) arranged about a central pigment mass	Fill about three-quarters of red blood corpuscle. 6 to 12 merozoites arranged about a central or eccentric pigment mass
Gametocytes	Round and fill the enlarged red blood corpuscle. Chromatin undistributed in cytoplasm	Round and fill the normal sized red blood corpuscle. Chromatin undistributed in cytoplasm	Crescentic or kidney-bean in shape. Usually appear free in blood. Chromatin undistributed in cytoplasm	Round and fill about three-quarters of the enlarged red blood corpuscle. Chromatin undistributed in cytoplasm

* From Craig and Faust's "Clinical Parasitology." Lea and Febiger, Philadelphia, Pa.

erythrocyte. (5) The segments, or merozoites, number from 6 to 12, usually about 8. (6) Presence of all stages of schizogony in the peripheral blood. (7) Segmentation forms present in the peripheral blood in approximately seventy-two hours. (8) Gametocytes spherical in shape.

PLASMODIUM FALCIPARUM (The Estivo-Autumnal or Malignant Tertian Plasmodium).—(1) Small size when fully developed, filling only a part of the infected erythrocyte. (2) Invaded erythrocyte never enlarged and usually slightly smaller than normal. (3) Presence of Stephens and Christopher's dots (basophilic) and of Maurer's dots or clefts in the cytoplasm of the invaded erythrocyte. (4) Absence of Schüffner's dots in the cytoplasm of the invaded erythrocyte. (5) Number of segments, or merozoites, usually 18 to 24, but may be smaller or greater in number. (6) Only the trophozoites (ring-forms) and gametocytes usually observed in the peripheral blood. (7) Gametocytes crescentic or kidney bean shaped.

THE DIFFERENTIAL DIAGNOSIS OF THE GAMETOCYTES, MICROGAMETOCYTES AND MACROGAMETOCYTES

From the standpoints of the epidemiology and prevention of the malarial infections it is of fundamental importance that one be able to recognize the gametocytes of the various species of plasmodia and to differentiate them from the forms occurring in the human life-cycle, *i. e.*, the trophozoites and schizonts. The gametocytes as well as the microgametocytes and macrogametocytes, can be easily differentiated if attention is paid to certain morphological features which are characteristic.

The following simple rule will serve to distinguish the gametocytes of *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariæ* from the schizonts of these species: Any round or oval plasmodium of these species which almost entirely fills the invaded erythrocyte and which does not show any division of the nuclear chromatin into distinct masses scattered throughout its cytoplasm but in which the chromatin is in a single mass or skein, is a gametocyte. The shape of the gametocytes of *Plasmodium falciparum*, *i. e.*, crescentic or kidney bean, easily distinguishes them from the schizonts and from the gametocytes of the other species of malaria plasmodia.

The *microgametocytes* of all of the species of malaria plasmodia may be identified by the pale blue or, if stained too intensely, the dark violet-red staining of the cytoplasm, while the nuclear chromatin in those of *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariæ* is arranged in a loose pink mass, or skein, of fibrils or granules, spindle-like in shape, lying in an unstained area which may stretch across the body of the gametocyte. The kidney bean shape of the microgameto-

cytes of *Plasmodium falciparum* distinguishes them from the macrogametocytes of this species, which are typically crescentic in shape.

The *macrogametocytes* of all of the species of malaria plasmodia may be identified by the deep blue staining of the cytoplasm while those of *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariæ* may be distinguished by the collection of the nuclear chromatin in a compact red mass lying in an unstained area which is situated at one side of the organism near the periphery or near the center of the body. The macrogametocytes of *Plasmodium falciparum* are crescentic in shape, the crescent being slender with sharply rounded or almost pointed ends.

The most important differential morphological and other features of the four generally accepted species of malaria plasmodia causing disease in man are given in Table 10, from Craig and Faust's "Clinical Parasitology."

Objects in Blood Films Which May Be Mistaken for Malaria Plasmodia.—There are several objects which may be observed in both unstained and stained preparations of blood which may be mistaken for malaria plasmodia. This is especially apt to occur if the observer has had little experience in examining blood for these parasites but such mistakes may occur even when one has had a considerable experience unless care is taken and one is familiar with such bodies. Some of these confusing bodies may be originally present in the blood or are present upon unclean microscopic slides or cover-glasses, or in the staining materials.

Unstained Preparations.—The following may be mistaken for malaria plasmodia in unstained preparations of blood:

Crenations of the Erythrocyte.—In preparations of blood exposed too long to the air before the application of the cover-glass, or made upon slides, or covered with cover-glasses which are not clean, the periphery of the erythrocytes may become crenated and such crenations, when looked directly down upon through the microscope, often appear as round, slightly greenish, hyaline bodies within the cytoplasm of the erythrocyte, somewhat resembling the youngest trophozoites of the malaria plasmodia, from which they may be readily distinguished by the absence of a "ring-like" appearance and the fact that if focused upon they appear as alternately light and dark areas within the erythrocytes, which is not true of the trophozoites, or ring-forms of the malaria plasmodia.

Vacuoles in the Cytoplasm of the Erythrocytes.—In blood from patients suffering from any of the types of malarial infection the uninvaded erythrocytes frequently present vacuoles within their cytoplasm which may be readily confused with the trophozoites of the plasmodia. These vacuoles are small, round or oval areas, having a hyaline appearance, and often seem to present slight undulations of their periphery, resembling ameboid motility, undoubtedly an optical illusion. The

vacuoles may be distinguished from the trophozoites of the malaria plasmodia by their greater refraction and that, when focused upon, they "open out," or grow larger and smaller with the changes in focus.

Hemoglobin-free Areas Within the Erythrocytes.—In many malarial infections, especially in those caused by *Plasmodium falciparum*, there occur small oval areas within the cytoplasm of the normal erythrocytes which are devoid of hemoglobin and which are sometimes called "eye-spots" because of their shape and general appearance. Such areas occur in the erythrocytes in many disease conditions as well as in apparently healthy individuals, and may be easily mistaken for the "ring-form" trophozoites by an untrained observer. The "eye-spots" may be situated at any portion of the erythrocyte and may be spindle-shaped, oval, round or ring-shaped, and are refractile and hyaline in appearance. Many of these areas contain a darker center or what appears to be a minute pigment mass centrally situated, which increases their resemblance to the ring-forms of the malaria plasmodia. These hemoglobin free areas may be distinguished from the trophozoites of the malaria plasmodia by their greater refraction and lack of ameboid motility.

Other objects which may be mistaken for malaria plasmodia in unstained preparations are granules of dirt, which may be mistaken for malarial pigment, the normal pale center of the erythrocytes which is accentuated in all diseases accompanied by anemia, and fragments of degenerated leukocytes which may be superimposed upon the erythrocytes. None of these objects should cause any difficulty if one is well trained in the morphology of the blood in health and disease.

Stained Preparations.—In properly stained specimens of blood there is little chance of confusing other objects with the malaria plasmodia if one is experienced in the recognition of these parasites but certain objects do occur in such preparations that are very apt to be mistaken for them by one who has had little experience. The most important are the following:

Blood Platelets.—The blood platelets are more often mistaken for malaria plasmodia than any other object occurring in stained preparations, and this mistake is not confined to inexperienced observers, for the writer has often had blood platelets shown him as malaria plasmodia by observers who, from their experience in blood work, should have known better. This confusion is caused by the similarity of the staining reactions of the blood platelets to those of the plasmodia, the cytoplasm of the platelets staining a dim blue in which chromatin material is arranged as a more or less regular clump of fine ruby-red granules. The individual blood platelet is about the size of the ring-forms of the trophozoites but they may be easily distinguished from such forms by the fact that the chromatin is not in a solid dot, or two dots, but in the form of fine grains heaped together at some portion of the cytoplasm and by the further fact that the blood platelet is not ring-shaped.

Blood platelets are very frequently superimposed upon erythrocytes and such platelets are the ones that may be confused most often with the young malaria plasmodia. Sometimes the platelets are massed together in groups resembling in shape the schizonts of the malaria plasmodia or the crescentic gametocytes of *Plasmodia falciparum* but such forms are distinguished by the absence of any pigment and the fine grains of chromatin arranged in little heaps within the platelets.

Vacuoles.—The vacuoles that may be present in the erythrocytes may retain the stain and will appear as pinkish or reddish bodies which may be confused with malaria trophozoites. The absence of any blue-stained cytoplasm and pigment, as well as a chromatin dot, or dots, should distinguish such bodies from plasmodia.

Flaws in the Microscopic Slide.—Circular flaws are not infrequently encountered and in these flaws the stain may be so deposited as to cause the appearance of alternate bands of red and blue arranged in a circular manner. Such flaws, when superimposed upon an erythrocyte, have been mistaken for trophozoites and young gametocytes, but the peculiar color arrangement, the absence of pigment, and of definite chromatin dots easily distinguish these artefacts from plasmodia. In addition, if carefully focused upon the flaws will be seen to lie at a different level from the erythrocyte within which they are apparently situated.

There are many other objects occurring in stained blood preparations that have been mistaken for malaria plasmodia, as nucleated erythrocytes, degenerated leukocytes, yeast cells, etc., but such mistakes are not possible if one is trained in the morphology of the blood and no one should undertake the diagnosis of the malaria plasmodia who is not thoroughly acquainted with the morphology of the blood in both health and in disease.

CHANGES IN THE BLOOD CAUSED BY MALARIAL INFECTIONS

The development in the erythrocytes of the malaria plasmodia results in the production of certain changes in the cellular elements that are of great value in the diagnosis of these infections. These may be considered under the following headings: Changes in the erythrocytes and changes in the leukocytes.

Changes in the Erythrocytes.—(*a*) CHANGES IN SIZE.—The invasion of erythrocytes by *Plasmodium vivax* is followed by the enlargement of the invaded cells which is progressive with the growth of the plasmodium, until, at the time of segmentation of the schizont, the invaded erythrocyte may be from two to four times the size of uninvaded cells. A similar enlargement occurs in erythrocytes invaded by *Plasmodium ovale* but to a lesser extent, while erythrocytes invaded by *Plasmodium malariæ* are normal in size, and with *Plasmodium falciparum* are smaller in most infections.

(*b*) CHANGES IN SHAPE. The invaded erythrocytes in infections with *Plasmodium vivax* are much distorted in shape frequently being rhomboidal or irregular, especially after the plasmodium is fully developed. The erythrocytes invaded by *Plasmodium ovale* are usually oval in shape and have a ragged, fimbriated outline, while those invaded by *Plasmodium malariæ* are usually normal in shape, as are those invaded by *Plasmodium falciparum* but the latter often show a serrated periphery due to crenation.

(*c*) CHANGES IN COLOR.—After the plasmodium is half grown the erythrocyte invaded by *Plasmodium vivax*, in unstained preparations, is a pale yellow in color, much paler than the normal erythrocytes, and when the parasite is fully developed the small amount of the cytoplasm of the invaded erythrocyte surrounding it is practically colorless. In infections with *Plasmodium ovale* the invaded erythrocytes are paler than normal in color while in infections with *Plasmodium malariæ* they appear normal in color. Erythrocytes invaded by *Plasmodium falciparum* are darker green than normal in unstained preparations and often have a brass-like hue, the so-called "brassy corpuscles" of some writers upon the malarial fevers.

(*d*) CHANGES IN STAINING REACTIONS.—The staining reactions of the red blood corpuscles invaded by the various species of malaria plasmodia when the Wright or other modification of the Romanowsky stain is employed are different from those observed in the uninvaded corpuscles and are characteristic for the species of plasmodium. In the uninvaded corpuscles the cytoplasm stains a uniform orange-pink or pink color and no granules are present in the cytoplasm. In corpuscles invaded by *Plasmodium vivax* and *Plasmodium ovale* the cytoplasm is filled with eosinophilic granules of considerable size, appearing as pink or red dots distributed throughout the cytoplasm, the so-called "Schüffner's dots." These dots appear earlier in the corpuscles invaded by *Plasmodium ovale*, sometimes being present in corpuscles containing the ring-forms of the trophozoites, while such dots are seldom seen in infections with *Plasmodium vivax* until the plasmodium has become a schizont. The presence of these eosin-staining granules or dots are practically diagnostic of infection with either *Plasmodium vivax* or *Plasmodium ovale*, for while similar granules may be demonstrated in the corpuscles invaded by *Plasmodium malariæ* by special staining methods, they do not occur in preparations stained with the Wright or Giemsa stains.

In erythrocytes invaded by *Plasmodium malariæ* the staining reactions of the invaded corpuscles are normal, the cytoplasm staining a uniform orange-pink or pink color. In infections with *Plasmodium falciparum* the cytoplasm of the invaded erythrocytes frequently stains a bluish color and blue-staining, basophilic dots may be present which are properly called Stevens and Christopher's dots, although generally referred to as Maurer's dots. In some invaded erythrocytes there occur clefts or dots which stain a brick-red color and these are the

so-called "Maurer's dots" but they are very rarely observed, in the writer's experience, while the basophilic stippling is very frequently present, not only in the cells invaded by the parasite but also in uninvaded cells.

(*e*) CHANGES IN NUMBER OF THE ERYTHROCYTES.—Every malarial infection is accompanied by a reduction in the number of the erythrocytes caused by the invasion and destruction of these cells by the malaria plasmodia and probably, to some extent, by toxins produced during the growth and segmentation of these parasites. The destruction of the erythrocytes may be so rapid that in a few days, in a moderate infection, the red cell count may be below 3,000,000 cells per cu. mm. while in very severe infections a count of 2,000,000 cells per cu. mm. may be observed within two or three days following the appearance of clinical symptoms. The degree of the anemia present depends upon the severity of the infection and infections with *Plasmodium falciparum* are usually accompanied by greater erythrocyte destruction than infections with the other species of plasmodia. In fatal cases the red cell count may be below 1,000,000 cells per cu. mm.

During the first few days of an attack of clinical malaria the loss in erythrocytes is usually rapid but if the infection continues it will be noted that there is a compensatory gain and the red cell count, while remaining much below normal, tends to become stabilized. During the first few paroxysms there is a marked decrease in the number of these cells after each paroxysm but if the infection continues the number lost tends to become less and less, even following distinct paroxysms, and in chronic malarial infections the anemia is always less than in acute infections.

The presence of a normal erythrocyte count is incompatible with the presence of a malarial infection and this should be remembered in those cases in which supposed malaria plasmodia are thought to have been demonstrated in a suspected individual. In such cases, if the erythrocyte count is normal, it is extremely improbable that a malarial infection exists, unless the supposed plasmodium was found in the blood during the first day or two of definite clinical symptoms.

(*f*) CHANGES IN THE LEUKOCYTES.—During a malarial paroxysm there may be an increase in the number of leukocytes but this is followed by a reduction in these cells and in most malarial infections which are uncomplicated, and which have existed for some days or longer, there is a leukopenia, the leukocytes numbering between 3500 to 4500 cells per cu. mm. Relatively, the neutrophilic leukocytes are reduced in number and there is an increase in the mononuclear leukocytes, especially in the large mononuclear leukocytes and the transitional leukocytes. In recent infections the leukocyte count may not show a marked relative increase in mononuclear leukocytes but in infections that have existed for some time these cells usually number from 15 to 25 per cent of the total count.

(*g*) PIGMENTED LEUKOCYTES.—In all malarial infections the leukocytes frequently contain pigment derived from segmenting plasmodia and in many instances malaria plasmodia, in various stages of degeneration, may be seen within these cells. The leukocytes containing pigment or plasmodia are known as phagocytes and phagocytosis is a characteristic finding in blood from malarial patients. The phagocytic cells are mostly large mononuclear leukocytes and the reticulo-endothelial cells of the spleen, bone-marrow, liver and other internal organs, while the cells of Küpffer, in the liver, are also phagocytic. While pigment is a common finding within the leukocytes in the peripheral blood, it is much more rare to observe plasmodia within leukocytes in such blood, but in blood obtained from the spleen or bone-marrow very large cells, known as macrophages, and of reticulo-endothelial origin, are observed containing large amounts of pigment and plasmodia in various stages of development and, especially in infections with *Plasmodia falciparum*, these cells may contain segmenting schizonts. In fatal infections with this plasmodium the splenic capillaries, as well as those of the brain, liver and other organs, may be crowded with phagocytic cells containing pigment and parasites. Phagocytosis is usually most marked during or after the malaria paroxysm in comparatively recent infections but in recurrent infections, especially if there is double infection with more than one species of plasmodium or with a single species, phagocytosis may be present at all times.

(*h*) FREE PIGMENT.—The occurrence of pigment in the blood derived from degenerating and segmenting malaria plasmodia, is a characteristic finding in all malarial infections and the more severe the infection the greater the amount of pigment which may be present. Most of the pigment originates at the time of the segmentation of the plasmodia, being liberated into the blood stream with the final liberation of the merozoites. Most of this pigment is taken up by phagocytic leukocytes but a considerable amount is deposited in the internal organs, especially the brain, spleen, and liver, giving rise to the pigmentation of these organs so characteristic of malarial infection. In the peripheral blood the pigment may be seen within leukocytes, as already described, or in the form of dark brown granules, or blackish irregular collections of such granules, free in the blood plasma.

As regards the *diagnostic significance of pigmented leukocytes and free pigment*, it may be said that pigmented leukocytes are diagnostic of a past or present malarial infection but a diagnosis of malaria based upon free pigment alone is untrustworthy, as malaria pigment may be simulated by granules of dirt which may be present in the preparation.

MALARIA PLASMODIA OF UNCERTAIN SPECIFIC STATUS

From time to time other species of malaria plasmodia than the four now generally accepted species have been described by different author-

ities. While none of these have been accepted as good species by the majority of malariologists and protozoölogists, it is probable that some of them may eventually be found to be good species. Our experience, as regards the species of avian malaria plasmodia, should lead us to be cautious in denying the existence of species of human malaria plasmodia other than those now accepted, for not so very many years ago only one species of avian malaria plasmodium was accepted by protozoologists whereas today at least ten species are generally accepted and several more which have been described are probably true species. The following species of malaria plasmodia causing disease in man have been described but are not yet generally accepted as true species:

1. *Plasmodium falciparum quotidianum.*—In 1890, Grassi and Feletti, and in 1891, Marchiafava and Bignami, described a plasmodium causing malaria in man, which they called the "quotidian estivo-autumnal plasmodium." In 1909, the writer, after the study of several infections with a plasmodium answering to their description, recognized it as a subspecies of *Plasmodium falciparum* and named it *Plasmodium falciparum quotidianum.* In its general morphology in both unstained and stained preparations it resembles *Plasmodium falciparum,* but differs markedly from the latter species in being much smaller at every stage in the development of the trophozoites, schizonts and gametocytes, in the much greater quantity of chromatin in the ring-forms, or trophozoites; in segmenting every twenty-four hours instead of every thirty-six to forty-eight hours, and in the smaller number of merozoites, which vary from 6 to 18 in number. The gametocytes are crescentic and kidney-bean in shape but are smaller than those of *Plasmodium falciparum.* The arrangement of the chromatin in the ring-forms is very characteristic, it being spread along one side of the ring as a red band instead of being in a single dot at some portion of the periphery, sometimes almost the entire ring appearing to be composed of chromatin, very little blue-staining cytoplasm being visible. This plasmodium is associated with a clinical type of malaria in which paroxysms occur every twenty-four hours and pernicious infections due to it are comparatively common. It is probable that further observations will prove that this is really a distinct species of plasmodium and, if so, the proper name should be *Plasmodium quotidianum.*

2. *Plasmodium vivax* var. *minuta.*—In 1914, Ahmed Emin described a presumably new species of malaria plasmodium occurring in man which he regarded as a variety of *Plasmodium vivax.* He claimed that it differed from other malaria plasmodia by the lack of enlargement of the invaded erythrocytes, the very marked ameboid activity, the small size of the segmenting schizont and the number of merozoites, varying from 6 to 10. The gametocytes are round in shape and occupy about three-quarters of the invaded erythrocyte when fully developed.

Plasmodium tenue.—This supposed species was first described by Stephens, in 1914, and is characterized by the occurrence in the per-

ipheral blood of trophozoites and schizonts which are markedly ameboid and, in stained preparations, by the tenuous and bizarre shapes which are produced by such ameboid motility. The nuclear chromatin is large in amount and the invaded erythrocytes are not enlarged. Schüffner's dots are absent and the gametocytes are crescentic or kidney-bean shaped but smaller than those of *Plasmodium falciparum*. The trophozoites and schizonts, because of their bizarre shape, resemble those of *Plasmodium vivax*, but the lack of enlargement of the invaded erythrocytes, the absence of Schüffner's dots, and the crescentic and kidney-bean gametocytes serve to distinguish it from the latter plasmodium. Many authorities regard *Plasmodium tenue* as identical with *Plasmodium falciparum*, the peculiar "tenue forms" being produced by pressure in preparing the specimens of blood or in some other manner.

Plasmodium perniciosum.—In 1915, Ziemann described the malaria plasmodium that is responsible for pernicious malaria in West Africa, and regarded it as a distinct species, naming it *Plasmodium perniciosum*. The morphological features that are said to differentiate it from *Plasmodium falciparum* are the complete disappearance of the plasmodium from the peripheral blood after the appearance of the "ring-forms;" the much smaller amount of pigment; the very small size of the schizonts, which, when fully developed, do not occupy more than one-third to one-half of the invaded erythrocytes, and by the number of merozoites, which varies between 12 and 16. The gametocytes are crescentic in shape.

Plasmodium falciparum var. *æthiopicum.*—Raffaele and Lega, in 1937, described a plasmodium occurring in malarial patients in Ethiopia, which they regard as a variety of *Plasmodium falciparum*. It is said to differ from the latter in having larger "ring-forms," or trophozoites, and by a larger amount of chromatin at all stages of development, in the form of much coarser granules. The gametocytes are crescentic in shape but are shorter and broader than those of *Plasmodium falciparum*.

Plasmodium wilsoni.—Roberts (1940) has described as a new species of malaria plasmodium an organism first observed by Wilson in natives of East Africa, and has named it *Plasmodium wilsoni*. The schizonts are compact, resembling those of *Plasmodium ovale*, and the erythrocytes containing them are greatly enlarged, even more so than when invaded by *Plasmodium vivax*. Schüffner dots were absent but there was a much finer stippling of the invaded erythrocytes with eosinophilic granules of minute size. The merozoites number from 10 to 12 and the gametocytes are round in shape and both the microgametocytes and the macrogametocytes stain dimly with Romanowsky stains.

Plasmodium Sp.—James and Kauntze (1930) in studying the so-called estivo-autumnal plasmodium occurring in man in Kenya, Africa, observed differences in morphology which caused them to doubt its identity with *Plasmodium falciparum* and recommended a careful

study of the morphology of the plasmodium. Later, Garuliam (1933), after such a study, stated that it differed from the latter species in that the "ring-forms," which are always unpigmented, are followed or accompanied in the peripheral blood by solid or vacuolated pigmented plasmodia. In addition, segmenting schizonts occur in the peripheral blood and the pigment, instead of being almost black in color is in the form of yellowish grains.

It is quite evident from this list of described new species of the malaria plasmodia, that a more careful study of the morphology of the plasmodia is essential before the question of the existence of species other than the four now generally accepted can be settled. The species mentioned in the above list were not described by beginners in the study of the malaria plasmodia but by trained observers of many years experience in malariology and their conclusions should not be dismissed without careful consideration. There can be no question that marked differences in morphology are encountered in the study of the plasmodia associated with malarial infections in different parts of the world, differences which would apparently be sufficient upon which to base new species, and the writer believes that some of the species mentioned above will be eventually proven to be good species. It is certainly true that students of malaria have given comparatively little study to differences which may exist in the morphology of the plasmodia observed in different localities and have been content to accept the dictum of the older writers that only certain species exist and that other described species are simply variations of the accepted ones. For many years *Plasmodium ovale* was regarded as a variety of *Plasmodium vivax* although now it is accepted by all good authorities as a distinct species and the same history may be true of some of the plasmodia of uncertain status which have been described. It is also well to remember that the morphological differences between species among the malaria plasmodia may be very slight and yet may be sufficient upon which to base a new species.

The criteria for the recognition of a new species of malaria plasmodium should be the study of the entire life-cycle in both man and the mosquito and proof that the specific characters remain the same after many passages through man by mosquito transmission.

CHAPTER XX

METHODS OF PREPARING AND STAINING BLOOD FILMS FOR MALARIA PLASMODIA

PREPARATION OF UNSTAINED BLOOD FILMS—PREPARATION OF STAINED BLOOD FILMS—PREPARATION AND STAINING OF THICK BLOOD FILMS

Introduction.—The diagnosis of malarial infections depends upon the demonstration of the causative plasmodium and this may usually be accomplished by the examination of either unstained or stained blood films. The examination of the blood is the most rapid and accurate method of diagnosing these infections and there has been no other method devised which has replaced this method of diagnosis. No diagnosis of malaria should be accepted unless the causative plasmodium has been demonstrated except in those instances in which quinine or other antimalarial drugs have been administered with favorable results, and even then, the diagnosis of malaria must be accepted with reserve as many febrile conditions are influenced favorably by antimalarial drugs and many others disappear without any treatment whatever. While other methods of diagnosis have been devised none of them can be regarded as accurate as the demonstration of the plasmodia by microscopic examination of the blood, with the exception, of course, of the demonstration of the plasmodia in bone-marrow by sternal puncture or in material obtained by splenic puncture.

The importance of the microscopic examination of the blood for the malaria plasmodia in any febrile condition, especially in regions where the malarial infections are known to exist, cannot be overestimated and the writer has observed so many instances in which the neglect of this procedure led to disastrous and even fatal consequences that he cannot too strongly urge the routine blood examination for these parasites of every patient suffering from clinical symptoms in malarial regions or who has lived in such regions for any length of time.

In symptomatic malarial infections, provided quinine or other antimalarial drugs have not been administered, the causative plasmodia may almost invariably be demonstrated in the peripheral blood, provided repeated examinations be made if necessary, and the statement so often seen in text-books that malaria plasmodia may be absent from the peripheral blood, even when symptoms caused by them are present, has not been true in the writer's experience, for he has never observed a case in which the symptoms were caused by malaria in which the plasmodia could not be demonstrated. In latent infections, or in the periods between relapses, the plasmodia are often

so few in number as not to be demonstrable in the peripheral blood, but even in such cases a large proportion will show the plasmodia in the peripheral blood if thick blood films are carefully examined.

The number of blood films which should be examined before a negative result is accepted will vary with the severity of the infection. In most acute infections with *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariæ* the examination of a single blood film usually will result in the demonstration of the causative plasmodium, but in infections with *Plasmodium falciparum,* several may have to be examined before a positive one is found, and sometimes, in mild infections with this parasite, plasmodia cannot be demonstrated in the peripheral blood several hours after a paroxysm unless thick films be prepared. The importance of repeated examinations of the blood at three- or four-hour intervals in infections with this plasmodium should be recognized, for only the ring-form trophozoites are usually present in the peripheral circulation, and, as segmentation occurs in the internal organs, the trophozoites may be present in the peripheral blood only at, and for a few hours, after segmentation and the occurrence of the symptoms of the malarial paroxysm. However, if such infections have persisted for several days, the crescentic gametocytes may be present and the diagnosis thus established.

The employment of thick blood preparations is essential if ordinary thin blood films are negative for the plasmodia.

The examination of the blood films should be made in a systematic manner in such a way as to cover the entire film and at least 200 microscopic fields should be examined before the preparation is regarded as negative. In the ordinary thin blood films malaria plasmodia are most numerous along the lower and upper margins of the film and less numerous toward and at the center and this is also true of pigmented leukocytes. Experienced observers may complete the examination of an entire blood film within ten to fifteen minutes but the average observer should be willing to spend at least half an hour in such an examination before turning in a negative report. If the examination of a blood film results negatively another should be taken in from three to six hours and examined, and still another in twenty-four hours if the second film is also negative. Sometimes, the examination of a thick blood film made at the same time as the thin film will be positive for plasmodia, thus obviating repeated examinations of thin films, but the writer has very rarely found it necessary to examine thick films in cases in which definite clinical symptoms of malaria were present or to resort to provocative measures to increase the number of plasmodia in the peripheral blood, as recommended by some malariologists.

In latent infections and in making malaria surveys thick blood preparations should always be used as well as in investigations of the curative action of drugs in malaria.

The laboratory equipment essential for the diagnosis of the malarial

fevers by blood examination consists of a good compound microscope furnished with a substage condenser, a mechanical stage, and a 16 mm. ($\frac{2}{3}$ inch), a 4 mm. ($\frac{1}{6}$ inch) and a 2 mm. ($\frac{1}{12}$ inch) oil immersion objective and oculars Nos. 1 and 4; cover-glasses and microscopic slides; a bottle of Wright's or Giemsa's stain; a couple of medicine droppers and distilled water. This equipment can be easily transported to the bedside of the patient, if necessary, and the blood films be made and examined there. This procedure often saves valuable time in pernicious infections or if the patient is located a long distance from a laboratory and transportation facilities are poor. Physicians living in localities where laboratory assistance is not available, should possess the above equipment and be capable of recognizing the various species of malaria plasmodia. The recognition of the gametocytes is also of great importance from the standpoint of preventive medicine, as their recognition enables one to not only demonstrate the presence of a past or present malarial infection but, by taking proper precautions, to prevent gametocyte carriers from being bitten by mosquitoes, thus reducing the incidence of malaria.

Blood from suspected cases of malarial infection may be examined either unstained or stained. Before the discovery of the Romanowsky method of staining and its various modifications, as the Wright and Giemsa stains, most diagnoses of malaria based upon blood examinations were made by the study of unstained blood films but today unstained preparations are very rarely used for diagnostic purposes because the plasmodia are much more easily seen in stained preparations and the staining methods available are so simple and so excellent. However, if one desires to study certain biological activities of the malaria plasmodia, as segmentation, exflagellation and the production of microgametes, and phagocytosis, unstained preparations must be employed, and the writer believes that it is good practice for the diagnostician to use both unstained and stained preparations routinely, when possible, for the recognition of the plasmodia in unstained blood may prove most useful under circumstances in which staining materials may not be available. Furthermore, the study of the plasmodia in unstained preparations is always most interesting and instructive.

Preparation of Unstained Blood Films.—In securing blood for unstained preparations the finger or ear should be punctured with a needle or blood lancet, the first drop of blood allowed to flow away, and the second drop brought into contact with the surface of a thoroughly clean microscopic slide at its center. Only a very small drop of blood should be collected upon the slide and it should immediately be covered with a cover-glass, the latter being allowed to spread the drop of blood upon the slide by its own weight. If the blood does not spread beneath the cover-glass, very gentle pressure may be exerted upon the cover-glass with the nail of the finger or a needle to expedite the spreading of the blood. The preparation should be so thin that

print can be easily read through it and when it is examined under the microscope the red blood corpuscles should be arranged in a uniform layer of single cells and not in clumps or rouleaux. If the blood is in clumps or rouleaux the preparation is unsuitable for examination and should be discarded.

In making the microscopic examination of unstained blood preparations for the malaria plasmodia the 2 mm. ($\frac{1}{12}$ inch) oil immersion lens and a low ocular should be employed and the examination should be made in a systematic manner, beginning at the top of the left side of the preparation and proceeding either vertically or horizontally until the entire surface is covered, if necessary. If the blood cells are much crenated, which may occur if the blood was exposed to the air for some time before the cover-glass was adjusted, the preparation should be discarded unless gametocytes are present and one desires to study the process of exflagellation and merozoite production.

In examining unstained blood for the malaria plasmodia it should be remembered that these parasites are colorless and hyaline and cannot be seen if too much light floods the preparation. The sub-stage condenser and the iris diaphragm should be so adjusted that as little light is allowed as is compatible with a clear differentiation of the organisms and many preparations, in the experience of the writer, have been reported as negative, because of too great an illumination of the microscopic field, a mistake that is frequently made by the beginner in malaria diagnosis.

Method of Preparing Blood for the Study of Microgamete Production and Phagocytosis.—If it is desired to study the exflagellation of the microgametocytes with the production of microgametes, the possible fertilization of the macrogametes, and phagocytosis, unstained preparations of the blood are essential, and it is also essential that the blood be collected in a special manner. The ear or finger should be punctured as usual but the microscopic slide used to collect the droplet of blood should be breathed upon just before touching it to the blood, thus moistening it, the moisture apparently being necessary to stimulate the process mentioned. In addition, after the blood has been collected upon the moistened slide it should be exposed to the air for half a minute or so before it is covered with a cover-glass. A warm-stage for the microscope is not essential for the study of such preparations, as some authorities have recommended, and the writer has found that, with the exception of phagocytosis, ordinary room temperature is far preferable to the use of a warm-stage. For the study of phagocytosis the warm-stage, keeping the preparations at body temperature, is most useful, or the entire microscope may be placed in a specially constructed incubator, several models of which may be secured from laboratory equipment houses.

Preparation of Stained Blood Films.—Preparations of blood for staining are prepared in the same manner as already described for

making blood films for examination for the leishmania and the trypanosomes (see page 152, 206). In making blood films for staining the malaria plasmodia, however, they should be made thinner than for leishmania or trypanosomes, the ideal film being one that contains only one layer of red blood corpuscles over the surface covered by the film. In order to secure such a preparation the drop of blood should be small and it should be spread at once, so that the cells may not be crenated.

In making preparations of both unstained and stained blood for examination for the malaria plasmodia care should be taken to preserve the utmost cleanliness in regard to the apparatus used in collecting the blood and the procedure itself. Microscopic slides and cover-glasses should be perfectly clean and the site of puncture should be washed with alcohol and ether. The puncture should be made with aseptic precautions and the site of the puncture should be painted with iodine *after* the blood preparations have been secured. In infants and children the lobe of the ear is the preferable site for puncture and the writer prefers this location for all patients, as the finger, while commonly used, is apt to be a little sore after the operation and there is also a greater chance for infection if the finger be punctured.

If thin blood films are negative the thick blood preparations should be employed. (See page 316.)

Splenic and *sternal puncture* have been recommended by some authorities in cases where the plasmodia cannot be demonstrated in either thin or thick blood films and there is a history of malarial infection or suspicious symptoms are present. The methods of performing sternal and splenic puncture have already been described (see pages 155 and 159) in discussing the diagnosis of leishmaniasis and trypanosomiasis and the same methods are equally applicable in malarial infections.

Yu and Ying (1943) performed sternal puncture in 100 cases of malaria and obtained positive results in 90 per cent, while only 54 per cent gave positive results when ordinary blood films were used and only 70 per cent after the ephedrin provocative test. However, the writer believes that if thick blood preparations be employed it will be rarely necessary to resort to sternal puncture, especially if any acute symptoms be present. In event such examinations are negative sternal puncture may be successful but puncture of the spleen should never be performed.

Methods of Increasing the Number of Malaria Plasmodia in the Peripheral Blood.—In cases of suspected malarial infection in which it has been found impossible to demonstrate the plasmodia in the peripheral blood by examination of thin and thick blood films, certain measures are available for forcing the plasmodia from the capillaries of the internal organs, where they are always the most numerous, into the peripheral circulation. The injection of certain drugs has given the best results although exposure of the spleen to the Roentgen-ray

or the application of ice to the abdomen over the splenic area has been found useful by some observers. Of several drugs that have been employed for this purpose, the hypodermic injection of strychnine, ergotin or adrenalin have been most successful. Sarsen (1919) reviewed the literature upon this subject and concluded from the results obtained by himself and others, that the subcutaneous injection of 1 mg. of adrenalin, exposure of the splenic area to ultra-violet rays, and physical exercise were the best methods for increasing the number of plasmodia in the peripheral blood, while Dazzi (1919) considered that the subcutaneous administration of 1 mg. of adrenalin was by far the best method of increasing the number of plasmodia in the peripheral blood. He found that the plasmodia began to appear in the peripheral blood within half an hour after the injection and that the maximum number was reached in about one hour after the injection. In twenty-four hours after the injection he found that the plasmodia had disappeared from the blood and could not be demonstrated even in thick film preparations. Di Pace (1923) found that in many patients whose peripheral blood contained few, if any, plasmodia, the subcutaneous injection of from 2 to 3 mg. of strychnine nitrate was followed by the appearance of numerous organisms and he regarded this as the most efficient method of producing such an increase in the number of plasmodia.

Since the results of the investigators mentioned have been published the methods they recommended have been further investigated and other methods have been studied, but at the present time, the consensus of opinion is that of the various provocative measures which have been employed, the subcutaneous injection of 1 mg. of adrenalin is the most generally useful and as efficient as any. It is but rarely that any provocative method is necessary in order to demonstrate the plasmodia in the peripheral blood, provided one is willing to spend the time in the examination of thin or thick blood films which may be essential for the demonstration of the plasmodia when they are few in number and the writer has very rarely found it necessary to resort to such measures.

Yu and Ying (1943) have obtained excellent results following the injection subcutaneously of 0.03 gm. of ephedrin, the blood being examined for the plasmodia fifteen, thirty and sixty minutes after the injection, thick films being examined if necessary.

Concentration Methods.—In cases in which the routine examination of thin blood films has resulted negatively the concentration of the invaded red blood corpuscles by centrifugalization is useful and to be preferred to the thick blood film if saving of time is not essential. Besides the greater chance of finding the plasmodia this method has the advantage that the red blood corpuscles and the plasmodia stain normally and there is not the distortion of the plasmodia so frequently

present in thick blood films. The best of the concentration methods that have been proposed is that of Bass and Johns.

Bass and Johns' Concentration Method.—From 5 to 10 cc. of blood are drawn from a vein in the arm into a glass syringe containing a small amount of sodium citrate solution. The blood is ejected into a suitable centrifuge tube and rotated at 2500 revolutions per minute until the erythrocytes are deposited at the bottom of the tube. The upper portion of the column of erythrocytes is carefully pipetted off and transferred to a smaller centrifuge tube and again rotated at the same speed until the cells are deposited at the bottom of the tube. Thin film preparations are made from the upper portion of the column of erythrocytes by placing a small drop of the mixture of cells obtained in a capillary pipette upon one end of a microscopic slide, placing the end of another slide in apposition to the drop of sediment and gently pushing it toward the other end of the slide, care being taken that the film is not too thick. After the films so prepared are dry they should be stained at once in order to secure the best results.

Halawani and Sobky's Concentration Method.—Halawani and Sobky (1939) have recommended the following method for the concentration of malaria plasmodia:

Take 0.5 cc. of blood from the infected individual and add to it 1 cc. of a 1 per cent acetic acid (or formalin) solution. The mixture is centrifugalized at 3500 revolutions per minute for ten minutes, the supernatant fluid pipetted off, and a drop of the sediment smeared upon a microscopic slide, stained, and examined.

Remarks.—Employing this method the authors examined 100 cases of suspected malaria and demonstrated plasmodia in 80, while the examination of two thick film preparations of blood from the same cases showed plasmodia in only 52 cases. Gametocyte carriers were demonstrated in 31 of these cases with the concentration method and in only 11 with the thick film method. The authors therefore recommend this method for use in malaria surveys as well as in ordinary diagnosis.

The writer has had no experience with this method but its simplicity and the results obtained by the authors would indicate it to be very valuable and one that should be tried when negative results are otherwise obtained in suspected individuals and during surveys for malaria.

STAINING METHODS FOR MALARIA PLASMODIA

All of the staining methods used at present for staining the malaria plasmodia, with but few exceptions, are modifications of the Romanowsky method and all of them have their enthusiastic advocates, and all give excellent results in the hands of experienced workers. The following list of stains and methods includes all of the most important ones and is arranged alphabetically for ease of reference, without

regard to their relative value. Many of these stains have already been described in the chapters relating to the staining of leishmania and trypanosomes and where this is true, the reader is referred to the description of the stain where it is first mentioned, this avoiding useless repetitions. All of the stains usually employed require a certain amount of practice before the best results are obtained and all give variable results unless care is taken in their application.

The staining reactions of the malaria plasmodia have already been described and the original Romanowsky stain and all of its modifications stain the cytoplasm of the plasmodia various shades of blue while the nuclear chromatin stains a ruby red or reddish-violet if the stain is an efficient one. The success of these stains depends upon the fact that when a solution of methylene blue is acted upon by sodium bicarbonate or certain other alkaline reagents, methylene azure and methylene violet result, being produced in the solution, and when these in turn are acted upon by a solution of eosin, certain compounds result which stain the nuclear chromatin an intense red, hence the name "chromatin stains" often applied to these staining mixtures. The same staining bodies are present in old polychrome methylene blue solutions but they are quickly produced in the preparation of the Romanowsky stain and its various modifications.

Bhattacharji, Singh and Sen Gupta Stain.—This method of staining is described on page 160 and is an excellent one, not only for leishmania and trypanosomes but also for the malaria plasmodia. It is a simple methylene blue-eosin substitute for the Wright, Leishman and Giemsa stains and was described by Bhattacharji, Singh and Sen Gupta, in 1946.

Boye's Stain.—The staining solution employed in this method of staining is Stevenel's blue which has the following formula:

Methylene blue (medicinal)	1.0 gm.
Potassium permanganate	1 5 gm.
Distilled water	150 0 cc.

To prepare the staining solution proceed as follows: Dissolve the methylene blue in 75 cc. of distilled water and the potassium permanganate in 75 cc. of distilled water, separately, and when solution is complete mix the two in a flask, shaking gently. A large amount of precipitate should form at once and the fluid should become almost decolorized. The flask is then placed in a water-bath and gently heated for one-half hour, during which time the precipitate should redissolve and the liquid should become a deep violet color. The mixture is then filtered and the filtrate used for staining.

Boye (1940) used this staining solution in the staining of thick blood films but it can also be used for thin films in the following manner: The films are flooded with a 1 to 1000 solution of aqueous eosin for fifteen to thirty seconds and then rinsed in running water until no red

color comes from the preparations, after which the films are stained with the Stevenel blue solution for forty-five seconds to one minute, and again rinsed in water, allowed to dry and examined. A second staining with the eosin solution and subsequent rinsing in distilled water is useful in intensifying the chromatin staining properties of the solution. The water employed both in making the staining solution and in washing the films does not need to be distilled but better results are obtained if distilled water is used.

Remarks.—The writer has had no experience with this stain but Boye recommends it as a good substitute for the Romanowsky stain and its modifications and has found it to give excellent results in the staining of thick film preparations under field conditions, as in military operations.

Chen's Wet Film Method of Staining.—Hewitt (1940) recommends this method for the staining of the plasmodia of bird malaria. Proceed as follows:

1. Make blood films quickly on microscopic slides and fix at once before drying with osmic acid vapor (2 per cent solution) for twenty minutes.
2. Stain with Giemsa's stain for forty minutes. (Two per cent solution of stock Giemsa in buffered water, pH 6.8.)
3. Place films in 100 per cent dioxan for five minutes.
4. Pour off dioxan and place films in xylol for ten minutes.
5. Mount the films in neutral balsam.

Remarks.—While Hewitt recommended this method for the plasmodia of bird malaria only it will probably be just as useful in staining the plasmodia of human malaria.

Giemsa's Stain.—The method of preparing and using this stain is described on page 161 of this work.

Remarks.—Giemsa's stain is very largely employed for the staining of both thin and thick blood films for the malaria plasmodia, especially for thick blood films. Previous fixation of the films with absolute alcohol is not necessary. In the case of thin films staining for from fifteen to twenty minutes is sufficient but in thick films the staining solution should be allowed to act for about thirty minutes. With the Giemsa stain the malaria plasmodia are stained intensely and preparations so stained keep better than those stained with other modifications of the Romanowsky stain although the writer believes that in preparations properly stained with the Wright stain the colors are preserved just as long as in Giemsa stained preparations, and he now has Wright stained blood films which were stained as long as thirty years ago and in which the malaria plasmodia still retain their distinctive coloration. Good Giemsa staining solutions are obtainable from laboratory supply houses and the directions regarding their use, when furnished, should be strictly followed, as overstaining is very apt to occur with some of the commercial preparations.

Jenner's Stain.—This stain is described on page 162 of this work.

Remarks.—Jenner's stain is useful for staining the malaria plasmodia but does not give as consistent results as does the Giemsa, Leishman or Wright stains.

Leishman's Stain.—This stain is described on page 163 of this work.

Remarks.—Leishman's stain is an excellent stain for the malaria plasmodia and is a favorite with English authorities. When properly prepared and used the results are as good as are obtained with any of the Romanowsky modifications in the opinion of those who have had an extended experience with the stain.

Mansell and Gilbert's Stain.—This rapid method of staining the malaria plasmodia is recommended by Chernoff (1943) as an excellent diagnostic stain. It is prepared and used as follows:

1. Stevenal blue is prepared as follows: Dissolve in one flask 1 gm. methylene blue in 75 cc. of distilled water, and in another flask 1 gm. permanganate of potassium in 75 cc. of distilled water.

After each has been dissolved mix together, place in water-bath, boil for one half hour and filter. Let the mixture stand at room temperature for forty-eight hours before using.

2. Prepare a 1 to 200 aqueous solution of water soluble eosin. (If the eosin being used stains intensely the dilution with distilled water should be greater and sometimes a 1 to 2000 dilution will be efficient.)

Thin blood films are prepared and fixed with a solution of equal parts of alcohol and acetone, allowing the mixture to act for a few seconds. The films are then covered with the eosin solution and allowed to stain for fifteen seconds, after which they are washed in distilled water and covered with the Stevenal blue solution and allowed to stain for forty seconds, washed in distilled water, dried and examined. The red blood corpuscles are stained a faint blue, the cytoplasm of the malaria plasmodia a dark blue and the chromation a brilliant red.

Remarks.—The writer has had no experience with this stain but its simplicity and rapidity recommend it for diagnostic purposes.

Marion's Stain.—In this method of staining, two solutions are employed to obtain the staining material as follows:

Solution 1

Methylene blue (Grübler's)	0 5 gm.
Azure blue	0 5 gm.
Sodium bicarbonate, 0 5% sol.	10 0 cc.
Distilled water	100 0 cc.

The methylene blue, azure blue and sodium bicarbonate are mixed with the distilled water and the resulting mixture is kept in the incubator at 37° C. (98.6° F.) for from two to four days or heated at 60° C. (140° F.) for six hours.

Solution 2.—A 0.2 per cent solution of yellow aqueous eosin (Grübler's).

After the methylene blue solution is ready the eosin solution is added to it until a well-marked precipitate occurs and this precipitate should be collected by filtration through a small filter paper, dried, and used as stock material for preparing the methylene blue staining solution. This is prepared by adding 0.4 gm. of the powder obtained by filtration to 20 cc. of pure methylic alcohol (Merck's Reagent) and is used as follows:

The blood films are covered with the staining solution, the number of drops required to do so being recorded. After the stain has been allowed to act for from three to four minutes, double the number of drops of an aqueous solution of eosin, containing 0.05 gm. of yellow aqueous eosin (Grübler's) to 1000 cc. of distilled water are added, and the mixture allowed to remain on the films for from two to three minutes. The films are then thoroughly washed in distilled water and examined at once, after drying.

Remarks.—This staining method gives excellent results but is more complicated than some of the other methods, as Wright's, and for this reason has been little used in routine blood examinations for the malaria plasmodia.

Miyahara's Quick Staining Method.—This method of staining is recommended by Miyahara (1939) as a rapid method of using Giemsa's stain. It is as follows:

The blood films are not fixed with alcohol but the dried film is hemolyzed in a beaker containing water, the side of the slide containing the film having been marked with a wax pencil. The film is then covered with the warm staining solution consisting of 1 drop of Giemsa stain added to each cc. of a solvent composed of 0.003 to 0.005 per cent K_2CO_3 or Na_2CO_3 solution which has been heated to 60° C. (140° F.). The stain is allowed to act for five to ten minutes, after which the films are washed in distilled water and allowed to dry.

Remarks.—Miyahara states that the blood cells and plasmodia are well stained and there is no deposition of staining granules upon the films if care is taken to prevent evaporating of the staining solution during the staining process.

Nocht's Stain.—This staining method was recommended by Nocht (1898–1899) for the staining of the malaria plasmodia and gives excellent results. The following solutions are employed:

1. To 30 cc. of polychrome methylene blue (Grübler) add 5 drops of a 3 per cent solution of acetic acid (U. S. P. 33 per cent).
2. Make a saturated solution of methylene blue (Grübler), dissolving the powder, by gentle heat, in water. This solution should be kept for at least a week before it is used.
3. Make a 1 per cent solution in water of yellow aqueous eosin (Grübler).

The staining solution is prepared as follows:

To 10 cc. of distilled water add 4 drops of the eosin solution, 6 drops

of the polychrome methylene blue solution, and 2 drops of the 1 per cent methylene blue solution, thoroughly mixing.

The staining solution is used as follows:

The blood films are fixed with absolute alcohol for five minutes, covered with the staining solution and stained, in a moist chamber, for two hours or more, the best results being obtained after six or more hours of staining.

According to Nocht, this method of staining may be modified by replacing the two methylene blue solutions by a 1 per cent solution of Ehrlich's rectified methylene blue to which has been added a few drops of ½ per cent solution of sodium hydrate, the mixture being heated for a few days in the incubator at 50° C. (122° F.). When ready for use it is added, drop by drop, to 2 cc. of distilled water containing 2 or 3 drops of the eosin solution, until the red color of the latter has almost disappeared.

Remarks.—Nocht's stain is an excellent one for the malaria plasmodia but it is not as satisfactory a stain as is Wright's stain and is much more time consuming. The modification of the stain described above does not give as good results as the original stain and often fails to color the nuclear chromatin properly.

Plehn's Stain.—This stain has the following formula:

Solution of sodium hydroxide in distilled water, 20 per cent	12 drops
Eosin, ½ per cent solution, in 75 per cent alcohol	20 parts
Methylene blue, concentrated aqueous solution	60 parts
Distilled water	40 parts

In using this stain the blood films are fixed in absolute alcohol for five minutes, flooded with the staining solution which is allowed to act for from five to ten minutes, thoroughly rinsed in distilled water, dried and examined.

Remarks.—The Plehn stain is uncertain in its action and the nuclear chromatin is frequently stained very dimly or not at all. It is not recommended if the other modifications of the Romanowsky stain are available.

Romanowsky's Stain.—Romanowsky, in 1890, described the minute morphology of the malaria plasmodia as shown in preparations stained by a new staining method which later was called the Romanowsky stain. Two solutions are employed in this method of staining, as follows:

Solution 1

Methylene blue (Grübler)	1 part
Sodium carbonate	1½ parts
Distilled water	100 parts

After mixing, this solution should be kept in the incubator at 37° C. (98.6° F.) for several days, or in the sunlight, until a distinct purple color develops, after which it is ready for use.

Solution 2

Eosin, yellow aqueous (Grübler)	1 part
Distilled water	1000 parts

This solution should be kept in the dark until used.

The staining solution is prepared by mixing 5 cc. of each solution with 95 cc. of distilled water, and then adding the methylene blue solution to the eosin solution in the proportion of one part of the former to two parts of the latter. Staining is accomplished by flooding the blood films with this mixture and allowing the stain to act for at least fifteen minutes, but the best results are obtained after from one to two hours staining. After staining, the films are thoroughly rinsed in running distilled water, dried and examined.

Remarks.—This was the first stain devised in which the differentiation into blue stained cytoplasm and red stained nuclear chromatin was obtained and it has formed the basis for all other stains in which a similar staining reaction is obtained. When properly used it gives excellent results but it is more time consuming than either the Wright or Giemsa stains and the results obtained with it are no better. It is very seldom used at present.

Singh and Bhattacharjie's Stain.—(The J. S. B. Stain).—This stain, recommended in 1944 by the authors for staining blood parasites has been extensively used in this country by Manwell at the Army Medical School and elsewhere and the following description of the method is the latter's, as published in the Journal of Laboratory and Clinical Medicine. (See References, page 368.)

Two solutions are used. Solution I is made up from the following ingredients:

Medicinal methylene blue	0 5 gm.
Potassium dichromate	0 5 gm.
Sulfuric acid (1 per cent)	3 0 cc.
Water	500 0 cc.

"Disolve the methylene blue thoroughly in 500 cc. of water. Add the 1 per cent sulfuric acid, mix thoroughly, and then add the chrome salt. A heavy amorphous purple colored precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100° to 109° C. and a pressure of 0 to 5 pounds for three hours. At the end of this period, the solution turns blue which indicates almost complete polychroming. If the color remains greenish, further heating for another hour or so is required. If the temperature is allowed to rise above 110° C., the oxidation of methylene blue may be carried too far and the solution will turn a violet purple.

"When the solution has turned deep blue after three hours' boiling, allow it to cool at room temperature. Then add 10 cc. of 1 per cent potassium or sodium hydroxide solution, drop by drop, very gradually while constantly shaking the flask. After the total amount of alkali has been added, transfer half of the contents of the flask into another of the same capacity and continue shaking for fifteen minutes more. Transfer the contents of the flasks into each other. In this way the precipitate will gradually get dissolved and the solution will turn deep blue with a violet iridescence. Leave it at room temperature for forty-eight hours for the solution to mature; afterward filter through a soft filter paper. . . . The solution will improve in staining qualities with age.

"Solution II. This is readily prepared by dissolving 1 gm. of water-soluble eosin in 500 cc. of tap water. A freshly prepared eosin solution may not yield as satisfactory a stain as one which has turned deep red after some use.

"Solutions I and II should be kept in wide-mouth stoppered jars, 1½ inches in diameter by 3½ inches in height, and set aside for forty-eight hours to mature. These keep well for several months, but in Solution I a thin golden yellow scum is likely to form on its surface due to a slight precipitation of the dye. This does not, however, interfere with staining, and the staining power of the solutions does not deteriorate with age."

The method of using this stain is as follows:

1. Fix the blood smear in methyl alcohol for a minute or two and dry.
2. Immerse the smear in Solution I for thirty seconds.
3. Wash in a jar containing acidulated tap water (pH 6.2 to 6.6).
4. Stain with Solution II for one second.
5. Wash in same acidulated water for four seconds.
6. Immerse in Solution I again for thirty seconds.
7. Wash as above for ten seconds or until the smears show a pink background.
8. Dry and examine.

Remarks.—The method given above can be used with a thin and thick blood film upon the same slide, according to Manwell. He claims for this method of staining the following advantages: Easy preparation from ordinary medicinal methylene blue and eosin; relative inexpensiveness; excellent keeping qualities; extreme rapidity, for thin smears, eighty seconds, thick drops, thirty seconds, and equally efficient for both thin and thick blood preparations. This stain also gives excellent results in staining leishmania and trypanosomes.

Wilson's Stain.—This stain and the method of using it will be found described on page 163 of this work.

Remarks.—This stain gives excellent results in staining the malaria plasmodia and is recommended by many authorities. It is more difficult of preparation than the Wright stain and, as the results are no better when it is employed, the writer prefers the latter stain.

Wright's Stain.—The method of preparation and manner of using this stain will be found described on page 164 of this work, to which the reader is referred. After preliminary fixation with the stain, from three to five minutes staining gives excellent results.

Remarks.—As already stated, in the discussion of the staining of the leishmania and trypanosomes, the writer prefers the Wright stain to any of the modifications of the Romanowsky that have been described but the results obtained with it depends upon the use of chemically pure reagents and experience in the technique of staining. It has also been the writer's experience that better results are obtained if the staining solutions are prepared by the one using the stain rather than by depending upon stains prepared by commercial houses, some of which are reliable and some of which are worthless. The method of preparation is not difficult and, in the writer's experience, personal preparation of the stain is well worthwhile because of the certainty of

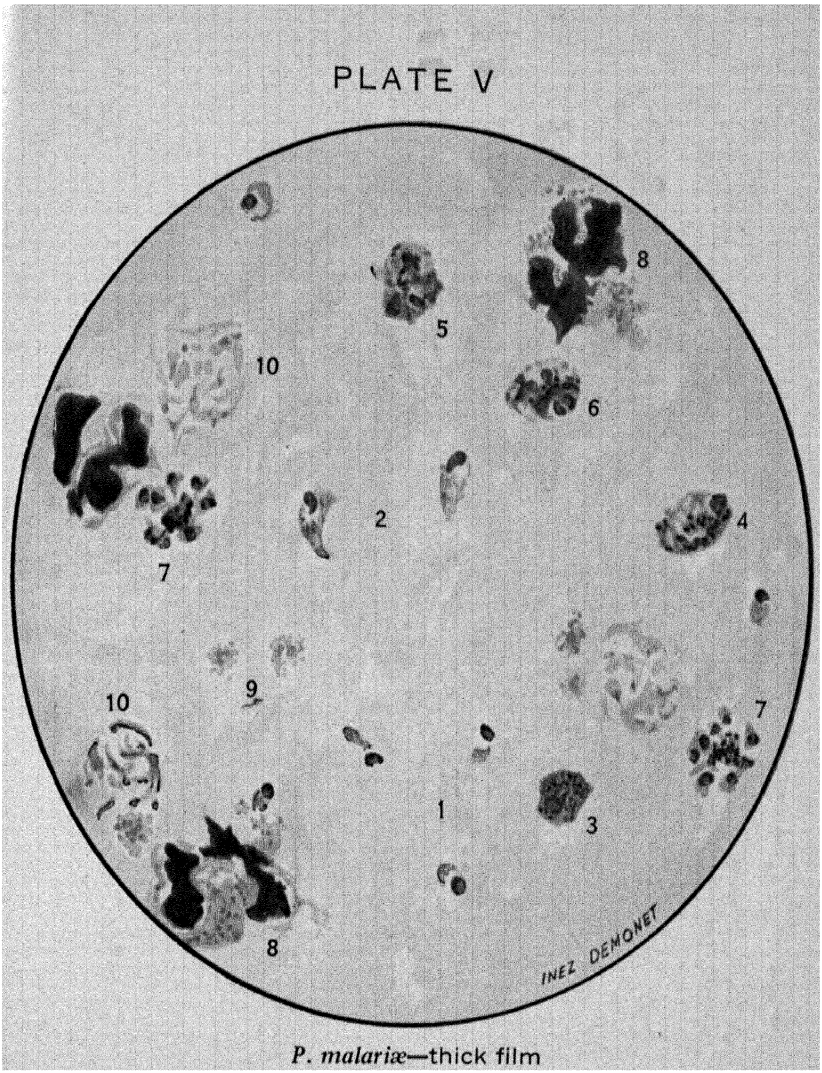
obtaining an efficient product. In over forty years experience with this stain, as prepared in the writer's laboratories, it has never failed to give satisfactory results in the staining of the malaria plasmodia and other protozoan parasites. The differential coloration obtained with this stain between the blue cytoplasm and the red nuclear chromatin of the plasmodia is brilliant and no other stain gives a better picture of the morphological characteristics of the various species of plasmodia.

PREPARATION AND STAINING OF THICK BLOOD FILMS

For routine examinations of blood for the malaria plasmodia in the case of patient's suffering from symptoms caused by infection with the plasmodia it is seldom necessary to resort to the use of thick blood films in order to demonstrate the parasites but in latent infections and in making surveys of malarial localities the thick blood film has proven of great value in diagnosis and is really essential. While in many latent malarial infections the plasmodia may be demonstrated in ordinary thin blood films it is undoubtedly true that a much higher percentage of infections will show plasmodia in thick films and, for this reason, such preparations should be employed in the examination of patients during the period between relapses of malarial infection and in surveys of the population of malarial regions in order to determine the incidence of malarial infections. It is not often necessary to employ thick blood films for the demonstration of the plasmodia in the blood of patients presenting clinical symptoms, unless quinine, chloroquine or atabrine have been administered. In the latter case, small doses of either may so reduce the number of plasmodia in the peripheral blood as to render their demonstration in thin blood films impossible and, when this is true, thick films should be prepared and examined, and will sometimes be found positive for the organisms. Thick films are also essential in testing the specific value of drugs prepared for the suppression or treatment of malaria.

In using thick blood films for the diagnosis of malaria a considerable degree of practice is necessary in order to differentiate the plasmodia from other objects and to differentiate the species of plasmodia. As the hemoglobin of the erythrocytes is usually dissolved in staining thick blood films, thus leaving the plasmodia apparently free, their relationship to the invaded erythrocytes is obscured and the plasmodia are also more or less distorted, so that their recognition requires experience with the use of this method of diagnosis. A diagnosis of malaria based upon the results of the examination of thick blood films is not reliable unless it is reported by one who is thoroughly trained in the use and interpretation of the findings of such examinations.

Ronald Ross was the first to describe a thick film method for the diagnosis of malaria and since his description many modifications of the method have been advocated, the most important of which will be considered in alphabetical order.



1. Small trophozoites.
2. Growing trophozoites.
3. Mature trophozoites.
4, 5, 6. Schizonts (presegmenting) with varying numbers of divisions of the chromatin.
7. Mature schizonts.
8. Nucleus of leukocyte.
9. Blood platelets.
10. Cellular remains of young erythrocytes.

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Archibald's Thick Film Method.—In 1921, Archibald recommended the following method of preparing and staining thick blood films for the diagnosis of malaria plasmodia:

1. Make thick blood smears by collecting a drop of blood upon a clean microscopic slide and spreading it so that an opaque layer of blood is obtained. The preparations should then be allowed to dry thoroughly, preferably in the incubator.
2. Immerse the films, after drying, in 100 cc. methylic alcohol containing 0.5 cc. concentrated fuming hydrochloric acid, until hemolysis is complete.
3. Soak the films for ten minutes in methylic alcohol or for a longer time if they are very thick.
4. Place films in gently running water for two hours.
5. Dry thoroughly and stain with Leishman's stain (see page 163). If Giemsa's stain be employed the films should be stained while wet.
6. Wash films in absolute acetone.
7. Rinse in distilled water and air dry.

Remarks.—While this method may give good results it is very time consuming and is not, for that reason, suitable for making large numbers of examinations, as in malaria surveys.

Barber and Komp's Thick Film Method.—This excellent thick film method was described by Barber and Komp, in 1929. The technique is as follows:

1. Upon a clean microscopic slide collect a large drop of blood and smear it with a needle over an area about one-half as large as is covered by the usual thin blood smear.
2. Allow the blood films to dry by placing them in an incubator at 37° C. (98.6° F.) and allowing them to remain for from one to one and a half hours, or in a covered slide-box overnight.
3. Dilute 1 part of a good Giemsa stain with 6 parts of neutral or slightly alkaline distilled water and place films in this mixture for about one-half hour. Previous fixation with alcohol and dehemoglobinization are not necessary.
4. Place films in distilled water for five minutes for partial decolorization. If the films show a deep blue background and the leukocytes appear almost black, they are overstained and probably worthless, although leaving them in distilled water for a longer time is sometimes successful in decolorizing them enough so that the plasmodia may be detected.
5. Drain the films thoroughly and allow them to dry, after which they should be examined.

Remarks.—It is probable that Barber and Komp's method of thick film preparation and staining has been more extensively used than any other and the results have been most satisfactory. The writer has found it to be a better method than others he has tried as the results are more uniform and reliable. Wright's stain may be used in place of the Giemsa stain, the stock solution being diluted 30 to 1 with distilled

water but better results are obtained with the Giemsa stain. This method is especially useful in making malaria surveys and has been extensively employed for this purpose.

Field's Thick Film Methods.—Field, in 1940, described two thick film methods for the diagnosis of the malaria plasmodia based upon the observations of Pampano and of Simons that distortion of protozoan organisms in thick blood films may be prevented by using basic stains in an isotonic solution. In 1941, Field described a third method which he regards as superior to the others he has described. The technique of Field's methods follow:

Method 1.—The staining solution has the following formula:

Methylene blue	0 5 gm.
Disodium hydrogen phosphate ($Na_2HPO_4\ 12H_2O$)	3 7 gm.*
Monopotassium dihydrogen phosphate (KH_2PO_4)	0 5 gm.
Distilled water	100 0 cc.

* If the anhydrous salt is used the amount of disodium hydrogen phosphate should be 1.48 gm.

The methylene blue solution is isotonic and should have a reaction of pH 7.2.

The procedure for staining thick blood films is as follows:

1. Dip the films for one second into a staining jar containing the methylene blue solution.
2. Wash the films for a few seconds by gently waving them in a deep dish containing tap water. Care should be taken not to wash the film off by too energetic rinsing in the water.
3. Place the films in diluted Giemsa stain, 1 drop of the stain to 4 cc. of distilled water and allow them to remain for from one to two hours.
4. Float off the scum which has accumulated by adding distilled water slowly and then dry the films in the air.

Remarks.—The advantages of this stain are said to be the preservation of the outlines of the malaria plasmodia and the leukocytes and the fact that the thick blood films may be stained immediately after drying instead of allowing them to dry for several hours in order to avoid distortion of the leukocytes and plasmodia.

Method 2.—The staining solution has the following formula:

Brilliant cresyl blue	1 00 gm.
Disodium hydrogen phosphate (anhydrous)	1 00 gm.
Potassium hydrogen phosphate (anhydrous)	1 25 gm.
Distilled water	100 00 cc.

The phosphate salts are first dissolved giving an isotonic solution having a reaction of pH 6.6. The cresyl blue is then added and the mixture is filtered after which it is ready for use.

Instead of the stain recommended above the following staining solution may be used:

Methylene blue	0 4 gm.
Eosin (water soluble)	0 4 gm.
Isotonic phosphate solution (same as in first formula)	100 0 cc.

The methylene blue is dissolved in 80 cc. of the isotonic phosphate solution and the eosin in the remaining 20 cc. of the solution. The eosin solution is then added to the methylene blue solution and the mixture is then filtered. If a scum forms on either of these staining solutions refiltration is necessary.

In using these stains the blood films should not be more than 50 microns thick and one should be able to see print dimly through the films. The films should be dried quickly by waving them through the air and fixed by rapid passage through a flame and care should be taken that the slides be not heated to a degree that they cannot be held against the back of the hand.

The films are then dipped into the staining solution for one second, then rinsed for five seconds in clean tap water and dried in the vertical position. The rinsing should be done very gently in order not to wash the film from the slide.

Remarks.—Field states that if the films are properly stained there will be four distinct staining zones, *i. e.*, at the upper edge there will be little hemoglobin and small forms of the plasmodia, as ring-forms of *Plasmodium falciparum*, are most numerous; next to this area the background will be pale yellow in color and most of the plasmodia will be found here; a central area in which there is too much hemoglobin and few plasmodia can be distinguished; and the lower edge of the film in which the plasmodia can be well differentiated. The great advantage of this method of thick film diagnosis, according to Field, is that the films may be stained immediately after drying and that a diagnosis of the species of plasmodium may be made within two minutes of the preparation of the films, there being approximately a sixteen-fold concentration of the parasites in such films as compared with the ordinary thin film.

Method 3.—With this method two staining solutions are employed, Solution 1 and 2.

Solution 1

Methylene blue	0 80 gm.
Azure 1	0 50 gm.
Disodium hydrogen phosphate (anhydrous)	5.00 gm.
Potassium dihydrogen phosphate (anhydrous	6.25 gm.
Distilled water	500 00 cc.

Solution 2

Eosin	1 00 gm.
Disodium hydrogen phosphate (anhydrous)	5 00 gm.
Potassium dihydrogen phosphate (anhydrous)	6 25 gm.
Distilled water	500 00 cc.

Dissolve the phosphate salts in the water first and then add the stains. Set aside the mixtures after the stains have been dissolved, for twenty-four hours, and then filter. The staining solutions are then ready for use. If a precipitate forms or a scum appears on the surface

of the solutions they should be filtered again and the eosin solution (Solution 2) should be renewed if it becomes greenish in color.

In using the stains the thick blood film, unfixed, is dipped for one second in Solution 1, rinsed gently in distilled water for a few seconds, and then dipped in Solution 2 for one second. It is then rinsed in water once more and allowed to dry, when it is ready for examination.

Remarks.—The chromatin and cytoplasmic staining by this method is excellent and its simplicity and rapidity recommend it, especially in making malaria surveys. *It is said to give better results than either Field's methods 1 or 2.* The films should be thoroughly dried before staining and should not be too thick.

Michelson and Wilcox' Thick Film Method.—The staining mixture employed in this method is a combination of the Wright and Giemsa staining solutions and is prepared as follows, according to the authors:

A. Preparation of Wright's Staining Solution from Powder (National Aniline Dye Co.):

"Place 1,000 cc. of methyl alcohol, acetone free, neutral, and preferably redistilled, in a bottle of 1 liter capacity, which has a tightly fitted screw cap or stopper. Weigh accurately 2 gm. Wright's powder and dissolve in the methyl alcohol. Wrap the bottle in paper and store in a dark place, protected from ammonia fumes, for at least one month. At frequent intervals while stain is aging shake bottle vigorously. At the end of one month, test for staining properties. The Wright's Stain Solution must give satisfactory blood-cell staining before it can be used in the preparation of this Wright-Giemsa stain."

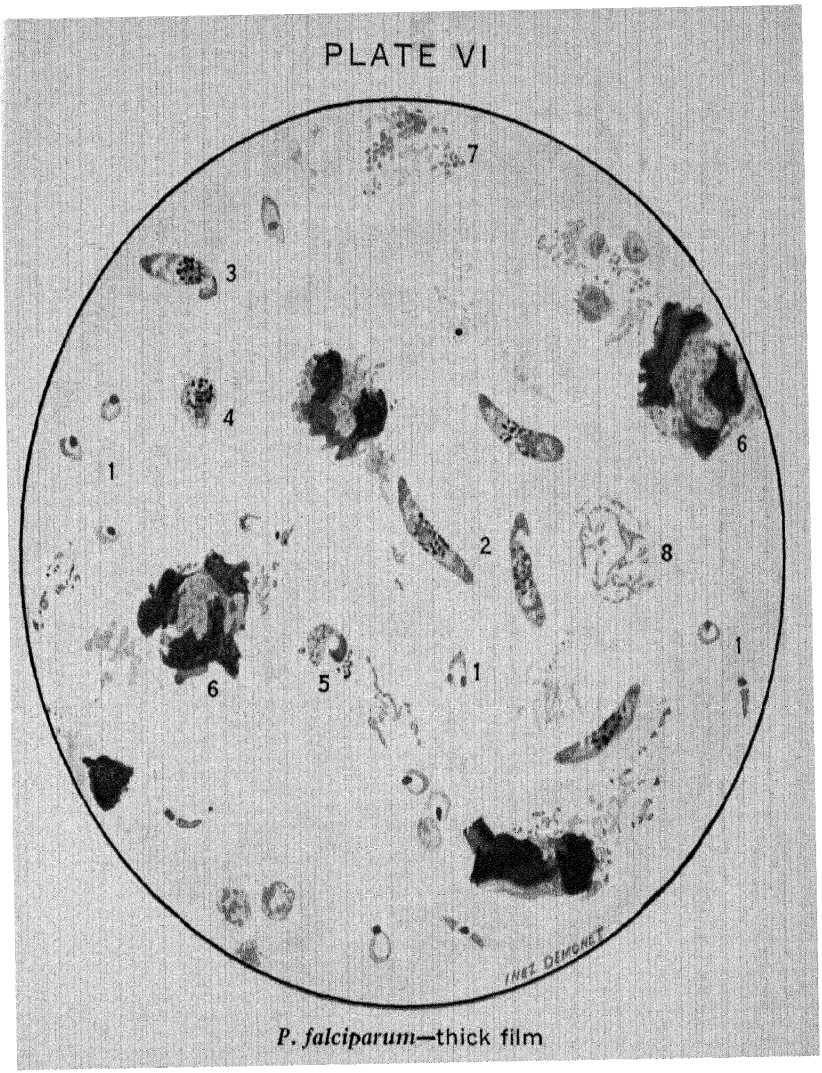
B. Preparation of Wright-Giemsa Solution from Giemsa Powder and Wright's Staining Solution:

"Place 100 cc. of glycerine C.P. anhydrous, in a bottle of 1 liter capacity which has a tightly-fitted screw cap or stopper. Weigh accurately 1.515 gm. Giemsa powder (National Aniline Dye Co., N Ge-3) and suspend in glycerine. Fit stopper tightly, cover entire bottle neck with a double thickness of wrapping paper, and secure with large elastic bands. (These precautions are taken to prevent moisture from being absorbed by the Giemsa-glycerine solution during the heating period in the water-bath.)

"Heat the bottle of Giemsa-glycerine mixture in the water-bath at 55 to 60° C. (131 to 140° F.), for two hours, mixing well with a glass stirring rod at half-hour intervals. At each stirring, remove bottle from water-bath. After two hours remove from water-bath and allow to cool. Then add 100 cc. of unfiltered Wright Stain Solution (*A*) to the bottle of Giemsa-glycerine Solution. Mix well by vigorous shaking and let stand overnight. On the next morning add 800 cc. of Wright's Stain Solution (*A*) to the above mixture. Shake vigorously. Filter into a small bottle the amount of stain needed for a few days' staining. Stain requires no aging and can be used immediately."

In using this stain it should be diluted 1 in 10 with distilled water buffered to pH 7 and thick films are stained with it for ten minutes and washed in distilled water for one minute and dried.

Remarks.—This is a rapid method of staining thick blood films and the results are said to be excellent. The preparation of the staining solution is somewhat complicated and time consuming but, after it is



P. falciparum—thick film

1. Small trophozoites.
2. Gametocytes—normal.
3. Slightly distorted gametocyte.
4. "Rounded-up" gametocyte.
5. Disintegrated gametocyte.
6. Nucleus of leukocyte.
7. Blood platelets.
8. Cellular remains of young erythrocyte.

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once prepared the stock solution keeps well and the method of staining is simple and fairly rapid.

Ross' Thick Film Method.—This is the original method of staining thick blood films for the malaria plasmodia and while it has been largely replaced today by the Barber and Komp, Field and other methods it is still preferred by some authorities.

A large drop of blood from the finger or ear lobe is collected upon the center of a clean microscopic slide and is then spread over an area about $\frac{1}{2}$ inch in diameter with a platinum loop or needle. The spreading should be done carefully and the blood film should be made as evenly as is possible. The preparation should then be allowed to dry in the air, which will usually require about one-half hour or it may be placed in the incubator at 37° C. (98.6° F.) which will hasten the drying.

After the blood films are thoroughly dry they are placed in a staining jar containing a mixture composed of 50 cc. of commercial ethyl alcohol to which 10 drops of chemically pure hydrochloric acid has been added, and allowed to remain in this mixture until the hemoglobin is completely dissolved, which will occur in from ten minutes to half an hour, depending upon the thickness of the blood film to be dehemoglobinized.

After dehemoglobinization the preparations should be washed in running water until all traces of the acid have been removed, a process usually requiring from one-quarter to half an hour. If the acid is not removed the staining will be imperfect or almost prevented. After washing the films are dried and then stained, in a staining jar, with Wright's, Giemsa's or Leishman's stain, in the usual manner, washed thoroughly in distilled water, and dried, after which they should be examined, using an oil-immersion lens and no cover-glass. The erythrocytes are not stained and the plasmodia appear to be free in the blood plasma.

Remarks.—This method gives excellent results if properly applied but, in the experience of the writer, is not as satisfactory as the Barber and Komp method, already described, which obviates the necessity of dehemoglobinizing the preparations.

Singh and Bhattacharjie's Method.—This method is described on page 314, to which the reader is referred.

Remarks.—This is an excellent thick film method.

Wolman's Modification of the Field Method No. 3.—In this modification of the Field Method No. 3, Unna's polychrome methylene blue is substituted in Solution 1 for the methylene blue and azure 1, and erythrosin for the eosin in Solution 2. Otherwise the formula of both solutions is the same. Five gm. of Unna's polychrome methylene blue is used and 1 gm. of erythrosin.

Unna's polychrome methylene blue has the following formula:

Methylene blue (medicinal)	1 gm.
Potassium carbonate	1 gm.
Distilled water	100 cc.

Allow the mixture to stand for two weeks at room temperature before using.

The method of staining is as follows:

1. Stain the thick film in Solution 1, containing the polychrome methylene blue, for one second.
2. Rinse in tap water for two to three seconds.
3. Stain for one second in Solution 2, containing the erythrosin.
4. Rinse in tap water again and dry.

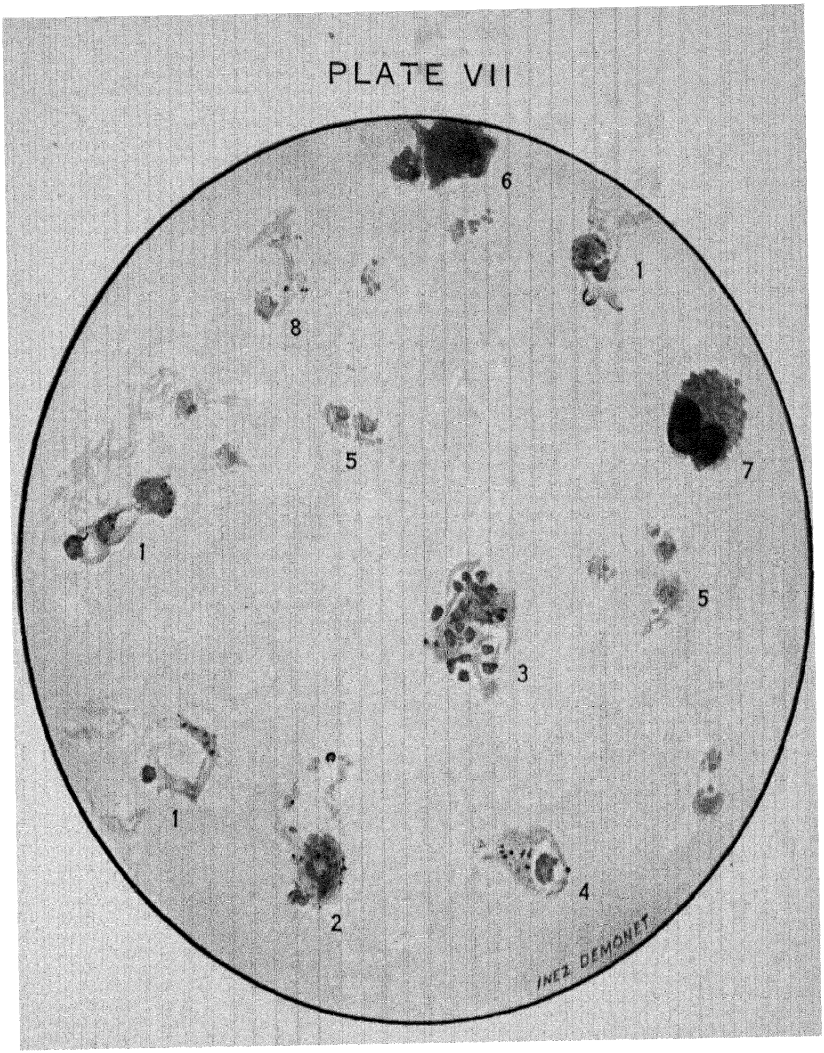
Remarks.—Wolman states that the crimson or purple staining of the chromatin and clear blue staining of the cytoplasms of the malaria plasmodia are invariably obtained with this method of staining. It would appear to be an excellent method of staining thick blood films.

Staining of Old Blood Films.—If blood films have been kept for several days or weeks they will not stain well with any of the modifications of the Romanowsky stain, as the Wright, Giemsa or Leishman stains, the cytoplasm of the erythrocytes staining a dark blue while the nuclear chromatin remains unstained or stains bluish in color. Sometimes it is impossible to stain blood films promptly and important and valuable material may thus be lost. In the tropics blood films will stain poorly even a few days after preparation, so that they should be stained as promptly as possible. The following method is recommended by Daniels (1907) for the staining of old blood films and it will be found to give good results in most instances.

Prior to staining, the blood films are placed in a staining jar containing a mixture of absolute ethyl alcohol and glacial acetic acid, 30 cc. of the alcohol to which have been added 3 to 5 drops of glacial acetic acid. The preparations should be left in this mixture for five or ten minutes and should then be washed thoroughly in distilled water, after which they are stained in the usual manner. The staining reactions, after this treatment, should be normal, *i. e.*, the cytoplasm of the plasmodia should stain blue and the nuclear chromatin red.

THE TIME OF OCCURRENCE OF THE VARIOUS FORMS OF THE MALARIA PLASMODIA IN THE PERIPHERAL BLOOD

In single infections with *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariæ*, the trophozoites, schizonts, merozoites and gametocytes are all present in the peripheral blood at regular intervals during the infection, and the examination of the peripheral blood at suitable periods will demonstrate the presence of all of the forms concerned in the entire life-cycle in man, *i. e.*, in schizogony, as well as the gametocytes which are intended to develop in the mosquito. In infections with *Plasmodium falciparum* only the hyaline ring-forms, or trophozoites, and the gametocytes usually occur in the peripheral blood except in severe and pernicious infections when schizonts, in all stages of development, may also be found in small numbers.



P. vivax—thick film

1. Ameboid trophozoites.
2. Schizont—2 divisions of chromatin.
3. Mature schizont.
4. Microgametocyte.
5. Blood platelets.
6. Nucleus of neutrophile.
7. Eosinophile.
8. Blood platelet associated with cellular remains of young erythrocytes.

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In the diagnosis of malaria by means of the microscopic examination of the patient's blood it is useful to know just what forms in the life-cycle of the various plasmodia may be expected to be present in the peripheral blood during the clinical stages of a malarial infection and the following summary may be used as a guide in this respect.

In the case of *single* infections with *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariæ, i. e.*, when only one generation of the plasmodia is present, the following forms of the plasmodia will be found in the peripheral blood at the time of the malarial paroxysm: Schizonts which are almost mature, filling most of the invaded erythrocyte; mature, segmenting schizonts; free merozoites; and, if the infection has lasted for some time, gametocytes. In addition, merozoites may be found upon the periphery of the erythrocytes or within them, as well as ring-forms, or trophozoites. In infections with *Plasmodium falciparum*, at the time of the paroxysm, only ring-form trophozoites are usually present in the peripheral blood but in very severe or pernicious cases full grown and segmenting schizonts may occur, while, if the infection has persisted for a few days, the crescentic gametocytes may also be present in both mild and severe infections.

A few hours after the paroxysm in infections with *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariæ*, ring-form trophozoites and young gametocytes are present in the peripheral blood and a few young pigmented schizonts. Twenty-four hours after the paroxysm, in infections with *Plasmodium vivax* and *Plasmodium ovale* half-grown schizonts and young and mature gametocytes will be present and at this time, in many infections, a very few almost mature and segmenting schizonts may occur due to irregularity in segmentation, but such forms are rare in infections with a single generation of the plasmodia. In infections with *Plasmodium malariæ* one-quarter to one-third grown schizonts will be present at the end of twenty-four hours, as well as gametocytes. In infections with *Plasmodium falciparum*, in most infections only the ring-form trophozoites and gametocytes occur in the peripheral blood at this time, although in very severe infections a few pigmented schizonts may sometimes be seen.

At the end of thirty-six hours, in infections with *Plasmodium vivax* and *Plasmodium ovale* three-quarter grown schizonts are most numerous while pre-segmenting schizonts may also occur. In addition a few ring-form trophozoites may be found and gametocytes, in various stages of development are common in infections that have lasted for several days. In infections with *Plasmodium malariæ* the peripheral blood at this time will contain half-grown schizonts, especially the "band-forms," a few less mature schizonts, and young or mature gametocytes. In infections with *Plasmodium falciparum*, in the usual case, only the ring-form trophozoites, a few pigmented ring-forms and young and mature gametocytes will be found, except in severe and pernicious infections, when three-quarter grown schizonts and pre-

segmenting schizonts may be present, together with a few segmenting schizonts, in some infections.

At the end of from forty to forty-four hours, in infections with *Plasmodium vivax* and *Plasmodium ovale*, the peripheral blood will show mature schizonts, pre-segmenting schizonts, and segmenting schizonts, a few ring-form trophozoites, and gametocytes in various stages of development. In infections with *Plasmodium malariæ* three-quarter grown schizonts, "band-forms," and gametocytes will be found in the peripheral blood, while in infections with *Plasmodium falciparum* a few ring-form trophozoites may be present and gametocytes, while in severe infections with this plasmodium three-quarter grown, pre-segmenting and segmenting schizonts may occur in small numbers together with ring-form trophozoites.

At the end of seventy-two hours in infections with *Plasmodium malariæ* the peripheral blood will contain mature, pre-segmenting and segmenting schizonts, a few ring-form trophozoites and gametocytes, if the infection has lasted long enough for the latter to develop.

In double infections with any of the malaria plasmodia, with the exception of *Plasmodium falciparum*, at the time of the paroxysm there will be present in the peripheral circulation the forms already noted as occurring in single infections and, in addition, numerous half grown schizonts, while if the blood is examined at later intervals, all of the forms observed in schizogony may be present. In double infections with *Plasmodium falciparum* the blood, examined at any time, will usually show many ring-form trophozoites and, in severe and pernicious infections with this parasite numerous schizonts in various stages of development may be present, as well as young and mature gametocytes.

It should be remembered that even in single infections with any of the malaria plasmodia, segmentation does not always occur in all of the schizonts at the same time, so that one may see an occasional parasite which does not correspond in its stage of development with the rest of the plasmodia that may be present. Both an acceleration and a retardation in segmentation may occur in single organisms which may appear in the peripheral blood and tend to obscure the cyclical picture. Thus, ring-form trophozoites, larger pigmented trophozoites, and partially grown and fully mature schizonts may be found in small numbers in the peripheral blood at any stage of the clinical infection, but only in double infections do we encounter all stages of schizogony in the peripheral blood in large numbers.

Staining of Malaria Plasmodia in Sections of Tissue.—It is often desirable to demonstrate the malaria plasmodia in the organs of fatal cases of malaria or to study the relation of the location of the plasmodia to pathological lesions that may be present. In most instances, when it is desired to simply prove or disprove the existence of a malarial infection in a disputed case, all that is necessary is to make a few

segmenting schizonts may be present, together with a few segmenting schizonts, in some infections.

At the end of from forty to forty-four hours, in infections with *Plasmodium vivax* and *Plasmodium ovale*, the peripheral blood will show mature schizonts, pre-segmenting schizonts, and segmenting schizonts, a few ring-form trophozoites, and gametocytes in various stages of development. In infections with *Plasmodium malariæ* three-quarter grown schizonts, "band-forms," and gametocytes will be found in the peripheral blood, while in infections with *Plasmodium falciparum* a few ring-form trophozoites may be present and gametocytes, while in severe infections with this plasmodium three-quarter grown, pre-segmenting and segmenting schizonts may occur in small numbers together with ring-form trophozoites.

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Staining of Malaria Plasmodia in Sections of Tissue.—It is often desirable to demonstrate the malaria plasmodia in the organs of fatal cases of malaria or to study the relation of the location of the plasmodia to pathological lesions that may be present. In most instances, when it is desired to simply prove or disprove the existence of a malarial infection in a disputed case, all that is necessary is to make a few

smears of the splenic pulp and stain them with Wright's stain, or some other modification of the Romanowsky stain. When it is desired to demonstrate the exact situation of the plasmodia in the tissues it will be necessary to section the organs, especially the brain, spleen and liver, and to stain the sections with a suitable staining solution. The writer has found the following method of sectioning and staining tissues to be satisfactory for this purpose:

The tissue to be examined is cut into small blocks not over 3 mm. in diameter and are then placed in 50, 65, 75 and 90 per cent alcohol, and finally in absolute alcohol, allowing not less than three hours in each alcohol. The blocks of tissue should then be washed and left for two hours in chloroform, after which they are embedded in paraffin at a temperature just sufficient to keep the paraffin in a fluid state. The blocks of tissue should remain in the paraffin bath for about six hours, the paraffin allowed to harden around them, after which they are cut into serial sections with a microtome, the sections being cut as thin as is possible, as only in such sections can the plasmodia be well differentiated.

The sections may be stained with Delafield's hematoxylin, methylene blue, or the Wright or Giemsa stain, but the best results are obtained with hematoxylin and eosin staining. The technique of staining is as follows:

The sections are removed from the microtome and placed upon microscopic slides, the surface of which has been smeared with a little egg-albumin water. The paraffin is dissolved by flooding the sections with xylol and following with absolute alcohol and water, after which the stain selected should be allowed to act for from ten minutes to an hour, then removed by thoroughly rinsing in alcohol. The sections are then cleared in oil of cloves and mounted in neutral Canada balsam.

Great care should be taken in dehydrating with alcohol that all water is removed from the sections and if it is found that in order to do so, too much of the stain is being washed from the tissue, the alcohol should be blotted off and the sections covered with oil of cloves which will complete the dehydration without removing so much of the stain. Care should also be taken that during no stage of the staining process the sections be allowed to dry or they will be worthless.

In properly stained sections the plasmodia show well but appear much smaller than in fresh blood preparations or stained blood films, due to shrinkage during the process of hardening and embedding in paraffin. In sections the chromatin staining of the nucleus is very rarely well defined, while the infected erythrocytes do not always show clearly, the plasmodia often appearing to be lying free in the capillaries, being most numerous in the smaller capillaries and frequently very scarce in the larger vessels. In the capillaries the plasmodia frequently occur in masses which often occlude them, especially in pernicious infections with *Plasmodium falciparum*, but in the smaller

vessels the plasmodia occur most frequently along the vessel walls, few being observed toward the center of the vessel. Usually the plasmodia in the sections are at different stages of development, one or more stages predominating, according to the stage of the infection, or they may be practically all in one stage of schizogony. In infections with *Plasmodium falciparum* it is a common finding for the capillaries of the brain and spleen to be filled with spherical pigmented schizonts in approximately the same stage of development.

In addition to the presence of the malaria plasmodia the capillaries in sections show much free pigment within them and pigmented leukocytes, especially the large endothelial cells, or macrophages, filled with pigment or containing plasmodia in various stages of development, are present in sections made from the brain, spleen or bone-marrow.

A considerable amount of practice is required before one is successful in obtaining well-stained sections of tissue containing the malaria plasmodia and the first few attempts are almost certain to be followed by unsatisfactory results but continued practice will always result in success. Fortunately, the preparation of sections of tissue are never required for the *diagnosis* of malarial infections.

In concluding this chapter, the writer would emphasize the importance of selecting the best dyes for the preparations of all of the stains that have been described. In his opinion, the Grübler dyes have never been excelled and excellent results can always be expected when these dyes are used, but it is impossible to obtain them at present, and the writer has found, that in his hands, the dyes made by the National Aniline Dye Co. give the best results of any of the dyes made in the United States. All other chemicals employed in the preparation of stains or in the staining process should also be chemically pure or of the best quality, especially methylic alcohol, which should be acetone free when used in the preparation of any of the modifications of the Romanowsky stain, as Giemsa, Wright or the Leishman stain. For the preparation of the Wright stain the writer has obtained uniformly excellent results with Merck's Methylic Alcohol (Reagent), which has been used in his laboratories for many years for this purpose.

CHAPTER XXI

THE CULTIVATION OF THE MALARIA PLASMODIA

Diagnostic Value of Cultures—The Value of Cultures as a Control of Treatment—Morphology of the Plasmodia in Cultures—Culture Methods and Media

Introduction.—The cultivation of the malaria plasmodia was first accomplished by Bass and Johns, in 1912, and their success stimulated others to continue research in this direction, in the hope that some method of cultivation would be found by which the various species of plasmodia could be maintained indefinitely in artificial culture media. It is unnecessary to stress the value of such a culture method in the study of the biology of the malaria plasmodia and in diagnosis but, to date, no method has been devised by which more than three or four generations of the plasmodia can be maintained in cultures. In fact, it is more accurate, in speaking of the cultivation of the malaria plasmodia, to speak of methods of preserving the organisms outside of the human body for a few generations rather than to call such methods true culture methods as this term is usually employed in bacteriological or protozoölogical researches. While the culture methods that have been described have proven of little value in diagnosis, they have been used to some extent in ascertaining whether an infection has been eliminated by treatment, as will appear later (see page 347).

DIAGNOSTIC VALUE OF CULTURES

Cultivation of the malaria plasmodia has not been employed as a diagnostic measure for the reason that more simple and more accurate methods are available. It is conceivable that in latent malarial infections, in which the plasmodia are so few in number that they cannot be demonstrated in either thin or thick blood films, the employment of one of the methods of cultivation might result in the demonstration of the presence of the parasites but, in actual practice, it has not been found necessary to resort to cultures for diagnostic purposes. The exact value of cultures in the diagnosis of malaria has never been ascertained but a thorough investigation of this subject would appear to be well worth while, as it has been shown by Sinton and Knowles and Das Gupta that plasmodia may be demonstrated by cultural methods when other methods have failed.

THE VALUE OF CULTURES AS A CONTROL OF TREATMENT

The possible value of cultures of the malaria plasmodia as a test of the curative action of various drugs or in order to ascertain whether

or not a malarial infection has been eliminated after treatment, has not been thoroughly investigated. According to Chopra (1936) it has been demonstrated by Sinton that malaria plasmodia may be present in less than 1 c.mm. of blood and yet may be impossible of detection except by a cultural method, while Knowles and Das Gupta (1932) found that in cases of malaria treated with quinine and apparently cured, as judged by the examination of both thick and thin blood films, plasmodia were still present as shown by the cultivation of the blood from such patients, and at the School of Tropical Medicine, in Calcutta, such cultures were routinely employed with success in order to ascertain whether a malarial infection has been eliminated by treatment.

It would appear from these observations that culture methods are useful in controlling the treatment of malarial infections and that such methods should be more largely employed than they are at the present time. The technique of cultivation, while it demands great care, is not difficult, although somewhat time consuming.

MORPHOLOGY OF THE PLASMODIA IN CULTURES

In cultures all stages of the various species of malaria plasmodia observed during the cycle in man, *i. e.*, schizogony, are observed, as well as gametocytes if the latter are present in the blood of the patient. The morphology of the trophozoites, schizonts and merozoites is the same as of these forms as seen in blood films, but degenerative forms of all of the stages of development also occur, especially as the cultures age. If the cultures are inoculated with blood containing the ring-form trophozoites, and then examined at regular intervals, it will be found that the trophozoites develop into schizonts and that the latter segment and produce merozoites, which penetrate uninvaded erythrocytes and repeat the process for from one to three or four cycles, usually for one to three complete cycles. If gametocytes are present they undergo no further development but remain viable for several days. Most authorities state that gametocytes never develop in the cultures although some have described gametocyte production.

As stated, the morphology of the various species of plasmodia in cultures is similar to that observed in the peripheral blood and the same morphological distinctions are observed between the different species, as well as the changes caused by the plasmodia in the invaded erythrocytes. Thus, in infections with *Plasmodium vivax* there occurs marked enlargement of the invaded erythrocytes, distortion in their shape, and the appearance of Schüffner's granules in their cytoplasm, as in infections in man. The number of merozoites characteristic of the different species of plasmodia are similar in cultures to the number occurring in the peripheral blood of man. *Plasmodium vivax*, *Plasmodium malariæ* and *Plasmodium falciparum* have all been cultivated by numerous observers but none have been able to maintain them in

cultures for more than a few generations. In one instance, Bass and Johns were once able to secure five generations of plasmodia but usually not more than three can be maintained.

Joukoff (1913) and Perekropoff (1914) have described forms of the malaria plasmodia occurring in cultures which they interpreted as similar to those occurring in the transmitting mosquitoes and stated that the mosquito cycle of development occurred also in cultures, but their observations were undoubtedly based upon schizogonic forms altered in their morphology by the conditions prevailing in the cultures. As already stated, degenerative forms of the plasmodia are present in all cultures after a few hours of cultivation and these forms may resemble certain stages in the development of the plasmodia in mosquitoes.

CULTURE METHODS AND MEDIA

The observations of Bass and Johns concerning the cultivation of the malaria plasmodia were published in 1912, and were confirmed by Lavinder (1913), Thomson and Thomson (1913), Ziemann (1913), Row (1917), Sinton (1922) and others, while modifications of the technique advocated by Bass and Johns have been devised. The methods of cultivation that have been found to give the best results are the following:

Bass and Johns' Method.—The following technique of Bass and Johns' method of cultivating the malaria plasmodia is that published by Bass, in 1914, in the American Journal of Tropical Diseases and Preventive Medicine, Volume 1, page 546. (Now out of print.)

For cultivating one generation of the plasmodia the technique is as follows:

"Blood is collected from the patient's vein at the bend of the elbow. If drawn with the syringe it is expelled directly into a defibrinating tube. The latter should be tilted to one side and care should be taken to avoid unnecessary exposure of the blood to the air. One-tenth of a cubic centimeter of a 50 per cent solution of dextrose, which has been sterilized at 100° C. (212° F.) on three successive days, for each 10 cubic centimeters of blood to be taken, is placed in the defibrinating tube before the blood is drawn. Defibrination is effected by gently stirring or whipping with a glass rod or tube which extends through the cotton plug closing the tube. The whipping in of air, causing bubbles, must be avoided. The plug and rod may now be replaced by a plug from another tube of the same size.

"This defibrinated dextrose blood containing malaria plasmodia may be transferred to other tubes or incubated in the original tube. In any event the column of blood must be 2.5 cm. deep. This gives a column of serum 1.25 to 2.5 cm. deep above the cells and parasites, when the latter have settled. Supernatant serum more than 2.5 cm. deep has no advantage. When this is less than 1.25 cm. deep the parasites often die before segmentation occurs.

"The parasites live and develop at the top of the column of precipitated cells in a layer varying in thickness from 0.05 to 0.1 cm. The parasites in the thin layer at the top of the column of cells develop and may be examined at any time by drawing a small quantity of cells from this layer by means of a capillary pipette. Some considerable practice is required to do this

without drawing cells and dead parasites from just beneath this layer. . . . Great care should be taken in handling tubes containing cultures to keep them in an upright position. Tilting to the side results in burying and killing the living parasites in the thin layer at the top of the column of cells."

For cultivating more than one generation of plasmodia they recommend the following method:

"The infected blood from the patient is centrifugalized sufficiently to force the leukocytes to the surface of the column of cells. . . . The supernatant fluid is drawn off and placed in culture tubes. The column of serum in each should be 1.25 to 2.5 cm. deep. Cells and plasmodia are carefully drawn from about the middle of the centrifugalized cells and planted at the bottom of the serum in the culture tubes. One to two-tenths of a cubic centimeter of cells in a ½-inch tube makes the thickest layer in which it is possible to get a homogeneous growth of parasites.

"Parasites in such leukocyte-free cultures develop, segment, and most of the merozoites enter new red blood cells. The young parasites develop in the same manner as the first generation and sometimes reach the stage of segmentation. In fact, we have in one instance observed the development of three successive generations in such a culture. More often, however, the parasites begin to die out after the first segmentation and especially after the second. . . . In order to perpetuate the culture it is necessary to transfer a portion of the cells and parasites to a recently prepared tube containing fresh cells and serum. It is convenient to place the fresh serum in the culture tube and to take up in a large capillary pipette a portion of the cells and parasites of the culture and then about five times the amount of fresh cells. They are mixed in the pipette (avoid air) and then carefully spread on the bottom of the tube. The transplantation should be done within four or five hours of the time of maximum segmentation and therefore approximately every forty-eight hours for the tertian and æstivo-autumnal parasites."

The usual incubation temperature for the cultures should be 37° C. (98.6° F.), but the plasmodia will develop well at 39° C. (102.2° F.) and less well at from 40° to 41° C. (104° to 105.8° F.). Throughout the technique the greatest precautions should be taken to preserve sterility as contamination of the cultures with bacteria will quickly destroy the plasmodia.

Remarks.—Although modifications of the Bass and Johns' method have been recommended, excellent results can be obtained with this method if the technique be carefully followed. Enormous numbers of plasmodia may develop in such cultures and beautiful preparations may be made from them for instruction purposes, if desired.

Row's Method.—In 1917, Row described a method of cultivating the malaria plasmodia in which a single generation of parasites can be obtained by using only a few drops of blood from the patient obtained by puncture of the ear or finger. The technique is as follows:

The blood is drawn from a puncture in the ear lobe or finger into a small tube in which it is defibrinated. The defibrinated blood is then transferred by a pipette to a small, preferably flat bottomed tube, containing sterile blood serum to which the amount of dextrose recommended by Bass and Johns has been added. This small tube, contain-

ing the mixture of infected blood, serum and dextrose is then placed in a larger tube which contains a constriction, upon which the small tube rests. Below the constriction the large tube should contain a solution of pyrogallic acid and to this, just before the small tube is placed in the large tube, some sodium hydrate should have been added. The large tube is then tightly corked with a rubber cork and placed in the incubator at 37° C. (98.6° F.). The growth of the plasmodia in the cultures thus occurs in an oxygen-free atmosphere.

Remarks.—Row's method of cultivating the malaria plasmodia is a useful one if only one generation of the parasites is desired, as in diagnosis, but it is not as efficient a method as that of Bass and Johns.

Sinton's Method.—Sinton (1922) has devised a method for cultivating the plasmodia in which a specially prepared culture tube is employed. The reader is referred to his description of the technique which is rather complicated, especially the preparation of the culture tube. While, in his hands, the method has given satisfactory results, it is not as generally useful as the Bass and Johns or the Row methods of cultivation.

Tissue Culture Methods.—Two tissue culture methods have been devised for the cultivation of the malaria plasmodia that have proven successful and with these methods it is possible to cultivate both the exo-erythrocytic and erythrocytic forms of the plasmodia.

Hawking's Method.—This method was devised by Hawking (1944–1945) for the cultivation of the exo-erythrocytic forms of the avian plasmodium, *Plasmodium gallinaceum,* but it may be employed for the cultivation of other plasmodia and probably for the cultivation of the erythrocytic forms also.

The medium consists of Tyrode's solution and embryo extract and in this medium plasmodia could be demonstrated for as long as ninety days after inoculation.

Ball, Anfinsen, Geiman, McKee and Ormsbee's Method.—This method has been successful in the cultivation of the erythrocytic forms of *Plasmodium knowlesi* and will undoubtedly prove as successful in the cultivation of the plasmodia of man. With it the plasmodium could be cultivated for long periods of time but the method is a very complicated one and the reader is referred to the original paper by the observers mentioned (see page 366).

The method described by Ball and his colleagues gives promise of eventual discovery of a method of culturing the malaria plasmodia indefinitely and further research will probably result in the discovery of less complicated methods applicable to the ordinary clinical laboratory. At the present time only *Plasmodium knowlesi* has been thus cultivated but there would appear to be no reason why the various plasmodia infecting man should not be susceptible of cultivation with this method.

CHAPTER XXII

THE SEROLOGICAL DIAGNOSIS OF THE MALARIA PLASMODIA

COMPLEMENT FIXATION TESTS—PRECIPITIN TESTS—OTHER SEROLOGICAL TESTS—CRITIQUE OF DIAGNOSTIC METHODS

Introduction.—Several authorities have endeavored to devise serological tests that would be specific in the diagnosis of malarial infections and some of those devised have proven to be of value although none of them can replace the microscopic examination of the blood and the demonstration of the plasmodia therein, and none of them have been generally adopted as diagnostic measures by malariologists or the medical profession. A review of what has been accomplished in this direction and a description of the most important of these tests follows:

COMPLEMENT FIXATION TESTS

After the development of the Wassermann test for syphilis several authorities claimed that a positive reaction with this test occurred in certain malarial infections during the febrile paroxysm. This subject has been studied by many observers especially by Bates (1912), Fletcher (1914), Sutherland and Mitra (1915), Craig (1918–1921), Thomson and Mills (1919), St. John (1921), Asbelew (1925), and Lloyd and Mitra (1926). Some of these authorities have reported positive results with this test in malaria while others have obtained negative results, but with the more modern standard technique for the Wassermann test it is now the consensus of opinion that positive results do not occur in malarial patients unless there is a complicating syphilitic infection.

Many authorities have endeavored to devise a specific complement fixation test for the diagnosis of malarial infections and, until quite recently, with disappointing results. The chief difficulty in securing such a specific complement fixation test has been in obtaining a standardized antigen of specific nature. Many attempts have been made to evolve such an antigen from the initial one of De Blassi (1907), who, with an antigen prepared by extracting red blood cells in severe infections with the malaria plasmodia, claimed to have secured positive specific reactions in clinical infections. Mircoli, in 1908, using a similar antigen, also obtained positive results, while Ferrannini (1911), employing antigens made by extracting malarial spleens with both water and alcohol, obtained positive results in a large percentage of cases of malaria.

Gasbarrini (1913–1914) prepared an antigen by washing heavily infected red blood corpuscles three times in 0.9 per cent sodium chloride solution, after which they were laked in distilled water, dried in a desiccator and used in a 1 to 30 dilution in normal saline as an antigen. He found that by absorbing the natural anti-sheep hemolysin present in human blood serum by adding sheep red blood cells, before making the complement fixation test, he obtained 24 positive reactions in 28 malarial cases, and later determined that complement fixing bodies occur in the blood of malarial patients during the febrile stage and in the latent periods but disappeared during chronic infections.

After the successful cultivation of the malaria plasmodia by Bass and Johns, J. G. Thomson (1918–1919) prepared an antigen by extracting parasitized red blood corpuscles obtained from cultures and reported positive reactions with sera obtained from malarial patients but the reactions were weak and did not differentiate between the species of plasmodia. He also tried extraction of malarial spleens as a source of antigen but with unsatisfactory results.

The observations of Savtchenko and Baronoff (1926), who employed antigens made by extracting various organs from malarial patients resulted in apparently demonstrating that extracts of malarial spleens were very weak in antigenic properties while those of malarial livers gave much better results, and those of Kingsbury (1927), who employed saline extracts of spleen, liver, brain, heart and blood of malarial patients, showed that the extracts of washed and hemolyzed infected blood gave specific positive results in from 48 to as high as 67 per cent of known malarial patients and negative results with the blood serum of patients suffering from other diseases.

Recently Coggeshall and Eaton (1938–1939) have devised a complement fixation test for the diagnosis of malarial infections that has given most promising results, results which justify a detailed description of the technique, which follows:

Coggeshall and Eaton's Complement Fixation Test.—In 1938, Coggeshall and Eaton described a complement fixation test in monkey malaria in which they used an antigen made by extracting with saline solution, the spleen or blood of monkeys infected with *Plasmodium knowlesi*, a species of plasmodium that causes disease in monkeys but which may be experimentally transmitted to man. With this antigen they demonstrated that a specific complement fixation reaction for *Plasmodium knowlesi* occurred in *rhesus* monkeys infected with this parasite and that the specific complement fixing bodies appeared early in the infection and persisted during the chronic infection which followed. The successful results obtained by them with this antigen led them to use it in complement fixation tests upon the blood sera of human patients suffering from malarial infection and infected with *Plasmodium knowlesi, Plasmodium vivax* and *Plasmodium falciparum*, with the results which will be found detailed upon page 335 of this

work. The method of preparing malaria antigens according to Coggeshall and Eaton is as follows:

PREPARATION OF MALARIA ANTIGENS.—Coggeshall and Eaton prepared malaria antigens by extraction of parasitized red blood cells and spleens obtained from monkeys dying of infection with *Plasmodium knowlesi*. They found that antigens prepared from parasitized red blood cells were more sensitive and less anti-complementary than the ones prepared from malarial spleens, although all of the antigens they used gave specific reactions when properly titrated and none of them were hemolytic. The most efficient antigen that they used was prepared as follows:

Blood from the infected monkey containing from 20 to 50 per cent of parasitized cells is collected in a 2 per cent sodium citrate solution, centrifugalized to separate the serum, and the serum is then pipetted off and discarded. The red blood corpuscles are then washed twice with normal salt solution (0.85 per cent) by centrifugalization, the packed red cells being then frozen, dried and preserved in sealed glass tubes as a source of antigen. The antigenic solution is prepared by adding to 1 cc. of the packed red blood cells 10 cc. of normal saline and then freezing and thawing this suspension four times, after which it is centrifugalized and the supernatant fluid is used as the antigen for the complement fixation tests. An alternative method of preparing the antigen is to grind the red blood cells containing the plasmodia in a ball mill and extract with normal saline, the proportion of dried cells to saline being the same, but the antigen so prepared was found to be slightly anti-complementary.

TITRATION OF THE ANTIGENS.—The antigens are titrated against hyperimmune monkey serum in order to determine the relative amounts of specific and non-specific antigenic substances present. The necessary qualifications for an efficient malaria antigen are low anti-complementary activity, a relatively low content of non-specific material, and a relatively high content of specific malaria antigen, according to Coggeshall and Eaton. In making complement fixation tests upon human blood serum the antigenic extracts are diluted in the proportion of 1 part of antigenic extract to 4 parts of normal saline solution.

TECHNIQUE OF PERFORMING THE TEST.—The following is the technique used by Coggeshall and Eaton as described by them:

"The hemolytic system consisted of 5 per cent sheep cells and anti-sheep rabbit serum. The unit of amboceptor was taken as the smallest amount which produced complete hemolysis in the presence of an excess of complement (fresh or frozen and dried guinea-pig serum). The complement was titrated on each day before the tests were set up, using 2 units of the amboceptor. Complement having a unit greater than 0.15 cc. of a dilution of 1 to 10 was discarded. The complement fixation test was set up as follows:

0.1 cc. patient's serum undiluted.
2 units of complement.
0.25 cc. of antigen diluted 1 to 4 with normal saline.

Controls: 1. Normal blood serum with antigen and complement.
2. Antigen at dilution used in the test with complement and 0.25 cc. saline solution in place of serum. (Antigen control.)

"The tubes thus prepared are incubated for one hour in a water-bath at 37° C. (98.6° F.) and then add 0.5 cc. of a mixture of equal volumes of 5 per cent sheep red cells and amboceptor diluted so that 0.25 cc. contains 2 units.

"After adding the hemolytic system the tests are read as soon as the controls have cleared, usually between fifteen and thirty minutes, and the results . . . recorded as ++++, +++, ++, +, +- and —, according to amount of unhemolyzed cells remaining."

Results of the Complement Fixation Test.—Coggeshall and Eaton tested blood sera from normal human beings, patients having syphilis, patients with paresis who were receiving malaria treatment with *Plasmodium knowlesi*, *Plasmodium vivax* or *Plasmodium falciparum*, and patients suffering from normally acquired malaria. They found that antigens prepared from parasitized red blood cells of monkeys infected with *Plasmodium knowlesi* gave positive complement fixation reactions in patients infected with *Plasmodium vivax* and *Plasmodium falciparum*, thus proving that this is a group reaction and confirming the finding of Thomson (1918) and Kingsbury (1927) who found that antigens prepared from *Plasmodium vivax* gave positive results when tested with blood of patients suffering from infections with *Plasmodium falciparum* and *vice versa*. The fact that the *Plasmodium knowlesi* antigens gave positive results with the blood sera of human beings infected with the species of plasmodia causing disease in the human host is most important, as it renders possible the production of an efficient malaria antigen by the use of experimental animals, *i. e.*, monkeys.

With syphilitic and normal blood serum the malaria antigens gave negative results in from 70 to 80 per cent and weak or doubtful reactions in from 20 to 30 per cent. In known cases of malarial infection strong positive reactions were obtained in over 80 per cent of those tested, while in experimental infections with *Plasmodium knowlesi* in man, 100 per cent of the tests gave a positive result.

Complement fixing bodies begin to appear in the blood in about two weeks after infection and increase in titre until a maximum is reached in about one month in *Plasmodium knowlesi* infections, after which they gradually decrease and after four months diminish rapidly but may be present for twelve months or even longer. In man, the complement fixation reaction for *Plasmodium knowlesi* remains positive for some time after blood examinations and monkey inoculations are negative.

Most of the cases of infection with *Plasmodium vivax* and *Plasmodium falciparum* which were tested were treated with quinine and relapses did not occur, so that it was impossible to determine whether the titre of the complement fixing antibodies rose during a relapse but

it was determined that in human infections with *Plasmodium knowlesi*, a latent stage is soon reached and the titre of the complement-fixing antibodies diminished.

Stratman-Thomas and Dulaney (1940) have confirmed the work of Coggeshall and Eaton and found that a positive reaction coincided with the presence of plasmodia in the blood but that a negative reaction is not conclusive. In a later contribution, Dulaney, Stratman-Thomas and Warr (1942) reported their results with complement fixation in 675 persons, using an antigen similar to that of Coggeshall and Eaton. Of 125 patients with plasmodia in their blood, 102 or 81.6 per cent gave a three or four plus positive reaction, 23 gave a negative reaction, and 15 gave a positive one at times when plasmodia were absent from the blood. Sera from individuals with leprosy, amebic dysentery and Chagas' disease gave a high percentage of positive reactions but only 2 of 45 sera from patients with acute febrile diseases gave such a reaction. They conclude that the complement fixation tests with this type of antigen possesses a high specific value and may prove useful as a supplementary test to blood examinations.

Dulaney and Watson (1945) using the antigen of Coggeshall and Eaton, made 6507 tests of which 4007 gave a positive reaction, or 62 per cent, while only 854 blood examinations were positive for the plasmodia, 13 per cent. Those tested were all relapsing *vivax* cases and they conclude that the complement fixation test is more sensitive than blood examinations in detecting chronic malarial infections.

Kligler and Yoeli (1941) have confirmed the work of Coggeshall and Eaton. They examined 309 blood sera from human cases of malaria, testing them with an antigen supplied by Coggeshall, and found the antigen to be efficient in dilutions of 1 to 120 and 1 to 320. They have found that about 98 per cent of cases of malaria gave a positive reaction and that in hyperendemic areas 98 per cent of the children up to the age of twelve years gave a positive reaction, even though the blood was negative, that infected adults in the same area gave similar results, and that healthy adults in the same region gave only 10 per cent of positive reactions. They suggest, that in view of these findings, the test may prove useful in making malaria surveys.

Kligler found that, with an antigen prepared by extracting the dried blood of a chicken heavily infected with *Plasmodium gallinaceum*, he obtained practically the same results as when using the *Plasmodium knowlesi* antigen of Coggeshall and if further observations confirm these findings, he states that a readily available source of antigen for the complement fixation tests for human malaria will have been discovered. The method of preparing the *Plasmodium gallinaceum* antigen was similar to that employed by Coggeshall and Eaton in preparing their *Plasmodium knowlesi* antigen.

Remarks.—The results obtained with the Coggeshall-Eaton complement fixation tests for malaria appear most promising but as they say:

"The value of the complement fixation tests as a diagnostic aid in malaria can be determined only after an extensive study of the reactions of sera from human beings with known malaria in places where the disease is endemic."

PRECIPITIN TESTS IN MALARIAL DIAGNOSIS

Attempts have been made by several observers to devise a satisfactory precipitin test for the diagnosis of malarial infections. The most extensive work upon this subject is that of W. H. and L. G. Taliaferro and Fisher (1927) and W. H. and L. G. Taliaferro (1928) which was done in Honduras. The following review of this work is abstracted from the summary published by W. H. Taliaferro, in his book entitled: The Immunology of Parasitic Infections.

The best results were obtained with an antigen prepared in the following manner:

A placenta heavily infected with *Plasmodium falciparum* was minced by passing it through a meat grinder and extracting the ground material for several weeks in an equal volume of ether. The ether was then poured off and the wet ether residue extracted in Coca's solution, in the proportion of 10 gm. of placenta to 50 cc. of the solution, for about one week, after which it was filtered through a hard filter paper and the clear filtrate used as the antigen. The Coca solution employed had the following formula:

NaCl . .	0·50 gm.
$NaHCO_3$ . .	0·05 gm.
Carbolic acid .	0·40 gm.
Distilled water	100·00 cc.

Another antigen they used was prepared as above but the extraction of the ether-extracted placental material was made with equal parts of Coca's solution. This antigen had to be adjusted to a pH of about 7.8 before use, because of its acidity.

Results.—The tests were done by the ring-test method and 86 cases were tested with the first antigen described above and 64 cases with the second. Of the first series of cases 54 were infected with malaria and of these 45 gave positive results, 2 doubtful results and 7 gave negative reactions. Thirty-two individuals not suffering from malarial infections were tested, 6 gave a positive reaction, 1 doubtful and 25 gave negative reactions. In the second series, in which the second antigen described above was employed, 32 individuals infected with malaria were tested, of whom 30 gave a positive reaction, 1 doubtful, and 1 a negative reaction. With the same antigen, of 32 individuals negative for malaria, 1 gave a positive reaction, 4 doubtful reactions, and 27 gave negative reactions.

In ascertaining freedom from malarial infection the blood was examined by means of the thick film method and a negative result was

considered as indicating freedom from infection. As it is well known that the thick film method does not always demonstrate the presence of plasmodia it is possible that some of the positive results obtained in individuals apparently free from malaria could thus be explained.

As the antigens prepared as described above were found to be perishable, an antigen in powder form was prepared and with this antigen the blood sera of 34 individuals whose blood showed malarial plasmodia were tested of which 29 gave positive reactions, and 5 gave negative reactions, while the sera of 44 persons whose blood was negative for plasmodia gave 9 positive reactions, 3 doubtful and 32 negative. In another series, using the dried concentrate, extracted in Coca's solution, 23 sera from infected individuals gave 22 positive reactions and 1 doubtful, while 9 sera from uninfected individuals gave 5 positive and 4 negative reactions.

Summing up the results obtained with all of the antigens used we have the following data: Of 143 individuals tested whose blood showed malaria plasmodia, 126 gave a positive reaction, 4 gave a doubtful reaction and 13 gave a negative reaction, the percentage of positive reactions being 88 per cent. Of 117 individuals whose blood gave negative results by the thick film method of examination for the plasmodia, 21 gave a positive reaction, 8 a doubtful reaction and 88 a negative reaction. The number of apparent false positive reactions is high but it may be that some of these individuals had a latent malarial infection.

Remarks.—The results obtained with this precipitin test certainly warrant further research along these lines as such a test, if it could be perfected so as to give consistent results, would be most useful in diagnosis, as the technique is comparatively simple. At the present time the test is mostly of academic interest and has not been employed in the routine diagnosis of malaria to the writer's knowledge.

Stratman-Thomas and Dulaney (1940) prepared antigens from infected human and monkey blood and made 388 precipitin tests, 287 of which were made upon blood from malarial patients. In no case did they obtain a positive reaction.

OTHER SEROLOGICAL TESTS FOR MALARIAL INFECTION

The following serological tests have been recommended in the diagnosis of malarial infections in addition to the complement fixation and precipitin tests:

Akashi and So's Flocculation Test.—A simple flocculation test for the diagnosis of malaria has been devised by Akashi and So (1941). The technique follows:

To 2 cc. of the suspected individual's blood serum add 0.1 cc. of distilled water made slightly alkaline by phenolphthalein, and 0.1 cc. of a 0.5 per cent solution of formalin. Shake the mixture thoroughly

and allow the tube containing it to stand at room temperature for two hours. If the test is positive a flaky appearance of the mixture can be observed, using a lens for the examination.

The authors state that the test is always positive from three to four days after the occurrence of symptoms of malaria and is especially marked in chronic infections. It becomes negative within a month or two after the elimination of the malarial infection. The authors admit it also gives a positive reaction with cases that are Wassermann positive and is also strongly positive in some forms of jaundice, hepatic cirrhosis, the eruptive fevers, and kala-azar and slightly so in some cases of splenomegaly, pernicious anemia, leukemia, typhoid, rheumatic arthritis and malignancy.

Remarks.—Owing to the large number of disease conditions in which this test gives a positive reaction it would appear to have very little value, if any, as a positive reaction with it would always have to be regarded with suspicion and all of the conditions mentioned would have to be eliminated should a positive reaction be obtained.

Chorine's Flocculation Test.—Chorine, in 1933, and Chorine and Prudhomme (1934) showed that when the blood serum of malarial patients is mixed with distilled water flocculation results and Chorine has devised a simple test based upon this phenomenon, in which the melanin reagent employed by Henry is omitted. The technique of this test is as follows:

The patient is bled from a vein in the arm, the serum allowed to separate, and 0.2 cc. of the serum is diluted 1 to 10 with distilled water and the opacity of the mixture is read with a Vernes, Bricq and Yvonne photometer. The mixture is then placed in an incubator or water-bath and incubated at 37° C. (98.6° F.) for three hours, allowed to stand for twenty minutes at room temperature and the optical density again read with the photometer. The difference in the two readings, as expressed in photometric degrees, is the index for the serum and the following indices have been established by Chorine as guides to the results of the test: Indices up to 10 are regarded as negative; 10 to 20 are doubtful reactions; and above 20 and up to 100 are regarded as positive for malaria.

Results.—The results obtained with this test closely parallel those obtained with the Henry melano-reaction, the percentage of false positives, according to Chorine, being 6 to 8 per cent, as compared with 5 to 6 per cent with the Henry test.

Remarks.—The Chorine test has not been as extensively employed in malarial diagnosis as the Henry test but its simple technique and the apparent slight differences in the results would appear to favor its more extensive employment when the use of such tests is necessary. The need of employing a photometer in securing accurate readings of the test renders impossible its use outside of well-equipped diagnostic laboratories.

Henry's Flocculation Test.—In 1927, Henry described a diagnostic test for malarial infections based upon the assumption that the pigments occurring in such infections, *i. e.*, melanin and hemosiderin, were active antigenic substances producing antibodies in the patient's blood serum which could be demonstrated by adding to the serum a solution of choroidal melanin and certain organic iron compounds which resulted in a flocculation reaction specific for malarial infections. At first Henry ran both a melanin and iron test concurrently, but owing to the poor sensitivity of the iron test he abandoned it and at present only the melanin flocculation test is employed in diagnosis. Since the description of the test several observers, notably Adant (1934) and Chorine and Gillier (1934), have proven that neither melanin or iron compounds present in malaria are specific and that animals injected with choroidal melanin do not produce antibodies and their blood serum does not give a positive Henry reaction. In addition, Sinton and Ghosh (1934) have shown that the malaria pigment, or hemozoin, is not identical chemically with the melanin of the eye or skin but, in all probability, with hematin, so that it would not appear possible that choroidal melanin could act as a specific antigen in the Henry test.

While it is true that the antigen-antibody theory of the Henry reaction has been disproven, it is a fact that a very large proportion of blood sera from malarial individuals gave a positive flocculation reaction with the test and it has been very largely used in Europe in the diagnosis of these infections and an enormous mass of literature has accumulated regarding its value, the consensus of opinion being that it is a valuable diagnostic measure if other conditions in which it gives a positive reaction can be eliminated. The Henry test has not been used in this country to any extent, largely because of the difficulty of securing melanin and the expense connected with the laboratory equipment necessary for the proper reading of the reactions. The preparation of the melanin reagent and the reading of the different degrees of flocculation require the use of a photometer, that of Vernes, Bricq and Yvonne being recommended by Henry.

Technique of the Test.—The melanin used in the melanin reagent is obtained from the choroid of the eye of the ox and is prepared by removing the lens and scraping the choroidal membrane, the resulting material being mixed, along with the corpus vitreum, with distilled water to which 1 part of phenol has been added to 200 parts of water. This stock suspension is then tested for opacity and so adjusted that a 1 to 10 dilution will correspond to an optical density of 48 to 49 photometric degrees, using a Vernes, Bricq and Yvonne photometer.

In making the test, 1 part of the individual's blood serum is mixed with 4 parts of melanin suspension either in distilled water, or, if the serum being tested flocculates, in a 1 to 5 dilution of distilled water, with 0.3 per cent saline solution. A serum control and a control of the melanin reagent are also prepared. The various tubes should be stop-

pered and incubated at 37° C. (98.6° F.) for three hours and then allowed to stand for thirty minutes before the readings are made on the photometer. According to Henry, the following are the photometric indices for his test: One to 12 degrees is a negative reaction; 13 to 18 degrees, a doubtful reaction; and 19 to 100 degrees a positive reaction for malaria.

Results of the Test.—It is now the consensus of opinion that the Henry reaction is not a true antigen-antibody reaction but that it is a biochemical reaction caused by a disequilibrium of serum proteins in sera in which there is an increase in serum euglobulin. It follows that the test may be positive in other disease conditions in which the serum euglobulin is increased but clinically such conditions can usually be differentiated and seldom cause confusion. False positive reactions may occur but Chorine (1935) states that they do not exceed from 5 to 6 per cent of the cases tested and most observers who have used this test extensively agree that between 90 to 95 per cent of individuals infected with the malaria plasmodia give a positive reaction. The flocculation is greater during the apyretic periods and may be absent during the malarial paroxysm.

Remarks.—The Henry test would appear to have considerable value in the diagnosis of malarial infections in which the plasmodia cannot be demonstrated and as a control of the efficiency of treatment, as the positive reaction disappears if treatment has been effective in ridding the patient of plasmodia. As it gives a positive reaction in conditions in which the euglobulin is increased in amount, especially in syphilis, this should be borne in mind in interpreting positive findings, and such conditions should be ruled out if possible. While a photometer is not absolutely necessary in reading the results of the test, the great difficulty of reading the degree of flocculation without the use of this instrument, and the question of the personal equation which enters into every flocculation or precipitin test when it concerns the reading of the various types of reaction, renders the use of a photometer very important in evaluating the diagnostic efficiency of the Henry test.

Naidu, Rao and Rajagopal's Flocculation Test.—The authors state (1942) that this test has been used by them for many years as a diagnostic test for malarial infection. It is performed as follows:

The blood serum of the suspected individual is added to a solution composed of 8.5 cc. distilled water, which must be freshly distilled, and 1.5 cc. of absolute alcohol.

A positive reaction consists in a flocculation of the blood serum and the test is claimed to be of significance if a negative reaction is obtained and is useful in cases where the blood is negative for the plasmodia. The authors believe that in such cases either a positive or negative reaction is indicative of the presence or absence of malarial infection.

Remarks.—This simple test would appear to have little value in the diagnosis of malaria except from a negative standpoint. No confirma-

tion of the results claimed by the authors has come to the writer's attention.

Proske and Watson's Protein-Tyrosin Test.—In 1939, Proske and Watson described the technique of a biochemical serum test for the diagnosis of malarial infections based, as they say: "on the fact that proteins possess a chromogenic property which can be measured quantitatively against the color produced by pure tyrosin in the presence of a phenol reagent." The technique of this test is comparatively simple and a photometer is not needed to read the results accurately.

Technique.—The following solutions are required for the test:

1. Sodium sulphate solution, 14 per cent. This is prepared by dissolving 70 gm. of C.P. anhydrous sodium sulphate in 300 cc. of freshly distilled water which is then made up to 500 cc. at a temperature of 37° C. (98.6° F.). This solution will keep indefinitely but should be stored in an incubator at 37° C.

2. Five-normal sodium hydroxide solution, prepared by diluting saturated, carbonate free sodium hydroxide solution to 20 per cent.

3. Tyrosin standard solution. Prepared by dissolving 200 mg. of pure tyrosin (Phanstiehl Co.) in 1000 cc. of approximately 0.1 normal hydrochloric acid; 5 cc. contains 1 mg. of tyrosin.

4. Phenol reagent of Folin and Ciocalteu, prepared as follows: Into a 1500 cc. Florence flask place 100 gm. of sodium tungstate ($Na_2WO_42H_2O$), 25 gm. of sodium molybdate ($Na_2MoO_4H_2O$), 700 cc. water, 50 cc. 85 per cent phosphoric acid, and 100 cc. concentrated hydrochloric acid. Reflux gently for ten hours, add 150 gm. lithium sulphate, 50 cc. water, and a few drops of bromine, and boil the mixture for fifteen minutes without condenser to remove the excess of bromine. Cool the mixture and dilute to 1000 cc. and filter. The completed reagent should have no greenish color.

The following is the method of making the test in the words of Proske and Watson (1939):

"Measure 3 ml. of 14 per cent sodium sulfate solution into a small test tube, 75 by 10 mm.; from an accurately calibrated pipette add 0.1 ml. of unheated, clear, nonhemolysed, nonchylous serum; mix by inverting a dozen times, avoiding air bubbles; stopper the tube and place in the incubator at 37° C. for three hours. Centrifuge at 1,500 r. p. m. for ten minutes; completely pipette off the supernatant fluid; wash the precipitate twice with fresh sodium sulphate solution by centrifugation; dissolve the washed precipitate in 1.75 ml. of distilled water; and add 0.1 ml. of 5-normal sodium hydroxide.

"At this point prepare the stock standard by introducing into a test tube, graduated at 20 ml., 2 ml. of the tyrosin solution, 5 ml. of water, and 1.0 ml. of 5-normal sodium hydroxide. Heat the unknown and the standard in boiling water for ten minutes and allow to cool. Now add to the unknown 0.15 ml., and to the stock standard 1.5 ml. of the phenol reagent and make up the standard to the 20 ml. mark with distilled water. While the color is developing, set up a series of small test tubes, 75 by 10 mm., and mark the tubes with a wax pencil 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10. Prepare the sub-standards in these tubes according to the following scheme:

Sub-standards, per cent	100	90	80	70	60	50	40	30	20	10
Stock standard, ml.	2.0	1.8	1.6	1.4	1.2	1.0	0.8	0.6	0.4	0.2
Water, ml.	0	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8

"Compare the color intensity of the unknown with these standards; if the color of the unknown falls between two whole gradations, the color value is intermediate between the two. For example, if the unknown falls between 60 and 50, then the reading is 55.

"The writers have examined 2,941 consecutive serums with the above method, from the results of which they propose the following tyrosin indices (TI) for serum euglobulin:

TI	50 to 80	Normal serums.
TI	80 to 100	Doubtful for malaria. In this range fall new malaria cases which have experienced only one paroxysm, treated cases, and a few cases of syphilis.
TI	105 and over	Presumptively positive for malaria.

"The following precautions should be observed: All glassware must be chemically clean, but sterility is not necessary. The serum pipettes should have fine tips, because small droplets of serum adhering to blunt tips may cause considerable errors. Serums should be clear. Hemolysed serums give too high tyrosin values owing to their globin content; chylous serums give too low values owing to the fact that chyle interferes with the protein precipitation."

Results.—The authors examined with this test 116 cases of malaria comparing the results with the results of the examination of thick blood films in the same cases, taking a tyrosin index of 105 as the lowest reading indicating malaria. Three of the patients had suffered from but one malarial paroxysm and in these cases the tyrosin index was 80 and the blood films were positive. In the remaining 113 cases the tyrosin index ranged between 105 and 270 and in 18 of these patients the thick blood films were negative. In these 113 patients 97.4 per cent were positive for malaria by the tyrosin test and 81.9 per cent showed plasmodia in thick blood films.

The tyrosin index for over 2000 normal bloods was found to vary between 50 and 80, while that for blood serum from malarial patients varies between 80 to 280 or higher.

Remarks.—The protein-tyrosin test of Proske and Watson would appear to be of considerable value in the diagnosis of malarial infections although the later researches of Swartzwelder and Adams (1941) have shown that positive reactions occur in several other conditions, most of which, however, can be differentiated either clinically or by laboratory methods. They modified the test by using 0.5 cc. of blood serum, instead of 0.1 cc. and a dilution of 1 to 10 (0.5 cc. of serum in 5 cc. of 15.4 per cent sodium sulphate) instead of a 1 to 30 dilution (0.1 cc. of serum in 3 cc. of sodium sulphate) used by Proske and Watson. They found that while the tyrosin indices were above 65 (which they took as the upper limit of the normal range) in most cases of malaria, the index was also much above normal in 6 patients suffer-

ing from paresis; in 9 of 12 cases of tuberculosis; in 7 of 8 cases of leprosy and in 3 of 4 cases of typhoid fever. They concluded, as a result of their studies of this test that "the potential value of the protein tyrosine reaction is not great and that its value in the diagnosis of malaria is likely to be little more than supplemental."

It would appear that the value of this test as a guide in the treatment of malaria might be greater than in diagnosis, and that it could well be used as a control of the efficiency of treatment in malarial infections and as a guide to the efficiency of new drugs that may be recommended in treatment. The real value of the test remains to be proven and the results so far reported warrant further research.

Wolff's Test.—Wolff (1940) has devised a serological test based upon the precipitation of the euglobulin fraction of the blood serum by buffer solutions. He has ascertained that precipitation in normal serum disappears when the pH is about 7.4 but that serum from malarial patients shows precipitation much further toward the alkaline side, and uses this fact in the diagnosis of malaria. Two solutions are employed in this test:

A. Test Buffer Solution

Stock buffer solution pH 7.7	1 part
Glass-distilled water	4 parts
Formalin	0 2 per cent

B. Control Solution

Stock buffer solution pH 7.0	1 part
Glass distilled water	4 parts
Normal saline	5 parts
Formalin	0.2 per cent

The stock buffer solution pH 7.0 and 7.7 should be prepared by mixing acid potassium phosphate with sodium hydroxide according to Clark and Lub's formula for the preparation of phosphate buffers.

Technique of the Test.—About 2 cc. of blood is collected from the patient's arm and the serum allowed to separate. Test tubes 4 inches by ½ inch are placed in two rows in a rack, the anterior row to contain the serum to be tested and the posterior the normal serum control. The buffer solutions are first placed in the tubes, 1 cc. to each tube, the test buffer solution in the anterior tube and the control solution in the posterior tube. In each of the tubes there is now placed 2 drops of the serum to be tested, the tubes well shaken and allowed to stand at room temperature, and the results read in from half an hour to two hours. The average positive case may be read almost at once but weak reactions develop slowly and in such cases reading should be deferred.

The reading should be based upon the comparison of the test tube and the control tube and usually the results are clear cut. In very strong positive sera some precipitation may occur in the control tube but always much less than in the test tube.

Results.—Wolff regards this simple test as of value in the diagnosis of malarial infections and states that the only other infection in which positive results are regularly obtained is kala-azar. He regards a negative reaction, if repeated, to be very valuable but warns against collecting the patient's blood during the malarial paroxysms as fever markedly "diminishes the strength of the reaction."

Remarks.—As this test has been so recently recommended its real usefulness in the diagnosis of malarial infections is still undetermined but the results obtained by Wolff warrants further use of this very simple serological method. Bogen (1945), in more than 3000 tests found that the reaction is most marked in active malarial infections between the paroxysms and in recently recovered patients. It is apparently a test indicating past rather than present infection and it may be serviceable in making surveys, in prognosis, in the evaluation of cure and as a guide to therapeutic results.

It should be understood that none of the serological tests described are recommended by their authors as substitutes for the microscopic examination of the blood and the demonstration of the plasmodia, which still remains the most accurate method of diagnosis.

CRITIQUE OF DIAGNOSTIC METHODS

The diagnosis of malarial infections should, if possible, be based upon the demonstration of the malaria plasmodia in the blood of the suspected individual or in material obtained by sternal puncture and, at the present time, it is very doubtful if such a diagnosis should be accepted unless the plasmodia are demonstrated to be present unless malaria pigment, both free and ingested in leukocytes, can be demonstrated. Fortunately, in the vast majority of symptomatic malarial infections the plasmodia may be demonstrated in either thin or thick blood films, so that it is rarely necessary to employ other diagnostic methods. It is in latent malarial infections that it is sometimes impossible to demonstrate the plasmodia in the peripheral blood and dependence must be placed upon some other method of diagnosis. Not only should the diagnosis of malaria be based upon the demonstration of the plasmodia but the particular species of plasmodium present should be identified if possible, as such identification is very important from the standpoints of treatment, prophylaxis and epidemiology. Such a differentiation is only possible after the careful study of the differential morphology of *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariæ* and *Plasmodium falciparum* and such study should never be neglected by those having to do with the microscopic diagnosis of malaria.

The employment of provocative methods for forcing the plasmodia into the peripheral blood may sometimes be useful if the examination of thick blood films has proven negative and the concentration of the

plasmodia by the centrifugalization of the blood may prove successful in the demonstration of the parasites when examinations of thin blood films have proven negative and, more rarely, when thick blood films have failed to reveal the plasmodia.

The *puncture of the spleen or liver* and *sternal puncture* have proven useful in the hands of some authorities but the writer has never had to resort to these measures for the diagnosis of malaria in an experience of nearly fifty years and believes their use to be very rarely indicated. Of the three mentioned, sternal puncture is the safest and should be preferred.

Cultivation methods are of very little use in the diagnosis of malaria and are very rarely employed for this purpose. In rare instances such methods might conceivably prove useful, as in infections in which the plasmodia cannot be demonstrated in either thin or thick blood films or in blood concentrates, but practically it has never been demonstrated that cultivation methods are of much service in malarial diagnosis.

Serological reactions have yet to prove their practical value in the diagnosis of malaria. Of the specific antigen-antibody tests, the *complement fixation* test of Coggeshall and Eaton would appear to give the best promise of being a valuable diagnostic measure, while *precipitin tests* have given results which are encouraging. At the present time, however, neither complement fixation or precipitin tests are employed in the routine diagnosis of malarial infections.

Serological tests, non-specific in nature, as the *Henry melano-flocculation test*, the *Chorine test*, the *Proske-Watson protein-tyrosin test* and the *Wolff test* are still of uncertain value. The melano-flocculation test of Henry has been very largely used in Europe and is believed by many to be a valuable diagnostic measure but cannot replace the demonstration of the plasmodia in the patient's blood as an accurate diagnostic method. The fact that all of these tests are positive in conditions other than malaria renders a diagnosis of malaria based upon the results of these tests *alone* always open to question.

Procedure for the Diagnosis of Malarial Infections.—The following procedure is suggested for the laboratory diagnosis of malarial infections:

Prepare at least two thin blood films and one thick blood film from each patient and stain the thin films with the Wright or other modification of the Romanowsky stain. Examine the thin films carefully, covering every portion of the stained smear, by using a mechanical stage. If these films prove negative, stain the thick blood film with the Field stain method and examine. In practically all cases of symptomatic malaria, provided quinine or other antimalarial drugs have not been administered, either the thin or thick films will show plasmodia but if such drugs have been administered the result is often negative. If the malarial infection is latent, the thin blood films may show a few

plasmodia but in such cases the thick blood films are often positive when the thin films are negative.

If both thin and thick blood films are negative blood should be taken from a vein in the arm and centrifugalized, as recommended by Bass and Johns (see page 308) and films made from the material so obtained. If such films are negative, one of the provocative methods (see page 307) as the injection of adrenalin or ephedrin should be tried and blood films, both thin and thick, prepared and examined. If such blood films are also negative, cultures may be made or, if available, complement fixation tests or the other serological tests which have been described, may be tried, always remembering, however, that a diagnosis of malaria based upon the results of these tests alone is open to question.

In cases in which other measures have failed resort may be had to sternal or splenic puncture and the examination of material so obtained.

In the *control of the treatment of malaria*, if thick blood films are negative, the cultivation of the plasmodia has been found to give positive results in cases in which both thin and thick blood films are negative. If facilities are available cultures can be employed to determine whether a malarial infection has been eliminated. In this respect, the Henry melano-flocculation and the Proske-Watson protein-tyrosin tests may eventually prove to be valuable, as well as complement fixation tests.

In making *surveys* to ascertain the parasite index of malaria the thick film method of diagnosis should always be used, if possible, for while thin blood films will prove positive for plasmodia in many cases a much larger percentage of positive results will be obtained by the microscopic examination of thick blood films. Such blood films can be easily prepared in the field, placed in a slide box, and stained and examined after return to the laboratory. Thick blood films should always be used in studies of the *efficiency of drugs* for the *suppression* or *elimination* of malarial infections.

PART VI

Laboratory Diagnosis of Balantidiasis. Balantidium Coli

CHAPTER XXIII

LABORATORY DIAGNOSIS OF BALANTIDIASIS—MORPHOLOGY OF BALANTIDIUM COLI

Introduction.—Balantidiasis is the name usually used in referring to infections with a protozoan organism called *Balantidium coli.* In man this parasite may be apparently harmless but it may produce severe diarrhea and dysentery, practically indistinguishable clinically from diarrhea and dysentery produced by the pathogenic ameba, *Endamœba histolytica.* While essentially a parasite of hogs, infections sometimes occur in man and the recognition of the organism is, therefore, of importance in clinical pathology.

Balantidiasis is a rare infection in man but sufficiently common to be of interest to the physician and to render a knowledge of the morphology of the parasite essential to the laboratory diagnostician.

MORPHOLOGY OF BALANTIDIUM COLI

The morphology of *Balantidium coli* has been studied and described by many observers, notably Strong (1904), Brumpt (1909), Walker (1913), and McDonald (1922), and it was first described by Malmsten, in 1857. It belongs to the Class Ciliata, of the Protozoa, and is the only ciliate that has unquestionably been demonstrated to be a parasite of man. It lives in the large intestine of man and is most numerous in the cecum. It is really a parasite of the hog but, in addition to man, balantidia have been found in orang-outangs and monkeys (Brooks, 1902), and in rats (Awakian, 1937), which are identical morphologically with *Balantidium coli.* Infection of man usually occurs through swallowing food or drink contaminated by fecal material containing the parasite, although a common mode of infection in the instance of butchers is the direct transference of contaminated fecal material by the hands in handling the intestines of infected hogs. Multiplication in the intestine occurs by binary transverse division. When conditions are unfavorable for such multiplication the balantidium encysts but no reproduction occurs within the cyst. Rarely cysts may be observed containing two balantidia but these represent conjugating parasites that have become encysted, according to most authorities.

In describing the morphology of *Balantidium coli* it is necessary to describe the morphology of the trophozoites and cysts, in both unstained and stained preparations.

Morphology of the Trophozoites and Cysts of Balantidium Coli.—UNSTAINED PREPARATIONS.—The *trophozoites* of *Balantidium coli* are the largest protozoan parasites occurring in man and the larger specimens may just be seen by the naked eye although it is necessary to use the microscope in order to observe structural details essential for a diagnosis.

In unstained preparations of fecal material the trophozoites are seen as large, oval, slightly greenish bodies, having a marked motility which enables them to swim about very actively in the material which is being examined. This motility is made possible by the cilia, which are short, very delicate fibrils which cover the entire body of the parasite. One end of the organism is more pointed than the other and at this end there is a cleft in the body which is called a peristome and which leads into a mouth, or cytostome, which, in turn, is connected with a gullet, or esophagus. The size of the trophozoites varies considerably, as given by different observers, but is usually stated to vary from 50 to 100 microns in length by 40 to 60 microns in breadth.

The body of the trophozoite is covered by a delicate pellicle covering the clear, hyaline ectoplasm which encloses the more granular appearing endoplasm, the latter composing most of the body of the organism. The endoplasm contains food vacuoles and two contractile vacuoles, one, the larger, situated anteriorly, and the other, which is much smaller, situated posteriorly. These vacuoles are easily recognized, as they pulsate regularly, and the posterior one apparently empties into a minute tube connected with the outer surface of the organism through the anal opening, or cytopyge.

Lying within the endoplasm, usually near the middle of the body, is a large kidney-shaped nucleus, called the macronucleus, and a small, spherical body, lying within the concavity of the macronucleus and in contact with it, which is called the micronucleus. The endoplasm, in unstained preparations, appears somewhat granular and has a slightly greenish tinge.

The *cysts* of *Balantidium coli* are the largest protozoan cysts occurring in the intestine of man, measuring from 45 to 65 microns in longest diameter. They are spherical or oval in shape, having a double outlined cyst-wall and are of a slightly greenish or yellowish color. The younger cysts contain a single balantidium which may be clearly differentiated, but as the cysts become older, the outline of the organism is lost and the cyst appears to be filled with a mass of granular, colorless cytoplasm containing a macronucleus and a single contractile vacuole. The oldest cysts contain a mass of granular cytoplasm enclosing a large nucleus, the macronucleus. In the younger cysts the contained balantidium is freely movable within the cyst while in the

cysts containing simply a mass of cytoplasm motility is absent. It is probable that such cysts are not viable, degeneration of the contained balantidium having occurred, but this has not been proven.

STAINED PREPARATIONS.—In properly stained preparations of *Balantidium coli* the structural details observed in unstained preparations are more clearly distinguished and others are rendered visible. In preparations wet-fixed and stained with hematoxylin it will be noted that the entire body of the *trophozoite* is covered with a thin resistant "skin," or pellicle, turning in at the anterior end and forming a lining to the periostome and cytostome. The pellicle is striated obliquely or longitudinally, due to regular rows of cilia, each of which emerges through a minute opening in the pellicle and arises from a minute granule, stained

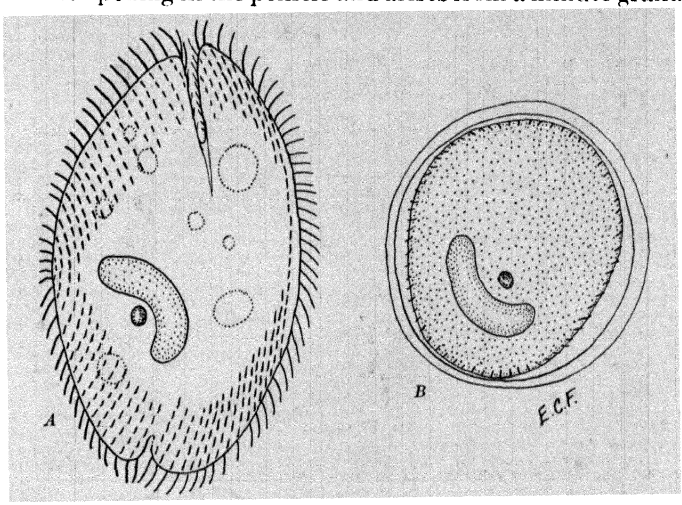


FIG. 55.—*Balantidum coli*. *A*, Stained trophozoite; *B*, cyst. × 750. (After Faust, in Jour. Lab. and Clin. Med.; courtesy of C. V. Mosby Company.)

black, and situated in the ectoplasm. After emerging the cilia become free and stain very poorly, usually appearing as obscure, cloudy, irregular masses of adherent threadlike cilia. The cilia also line the peristome and cytostome and these cilia are longer than those covering the body. These also arise from minute granules in the ectoplasm and emerge through apertures in the pellicle to become free cilia.

According to McDonald (1922) unstained preparations show that the ectoplasm, except in the region of the peristome, is divided into alternate light and dark spiral bands and in stained preparations the cilia, which usually stain very poorly, may be located by noting the arrangement of the basal granules from which they originate. These granules are situated just beneath the pellicle, in the hyaline, or bright, band of the ectoplasm and stain almost black with hematoxylin stains. According to this observer, a ciliary root arises from each granule and

extends in a diagonal direction into the dark band of the ectoplasm, terminating in a secondary basal granule.

In stained preparations the vacuoles remain unstained and the kidney-shaped macronucleus shows a blue or black nuclear membrane but this is usually indistinguishable in such preparations. The entire macronucleus is filled with a solid mass of dark staining chromatin which, in well-stained preparations, may be observed to be broken up into several irregular clumps. The micronucleus stains a bluish black color but is very often invisible except in very well differentiated specimens, as it lies in contact with the macronucleus and the dense staining of the latter usually obscures the small micronucleus.



FIG. 56.—*Balantidum coli.* Stained trophozoite in feces. (Photomicrograph, Army Medical Museum, from preparation from collection of Drs. James and Getz, Panama, R. deP.)

The *cysts* stain poorly, the cyst-wall remaining unstained, and the enclosed balantidium stains rather indistinctly, although the macronucleus may usually be seen as a deep bluish-black, kidney-shaped body lying within the cyst. In very well stained specimens the cilia covering the body of the enclosed balantidium may be distinguished but this is unusual.

Balantidium coli is best studied in the living, unstained condition as it is very difficult to obtain well-stained preparations and all of the essential morphological features necessary for a diagnosis may be seen in unstained specimens of this organism. The only organisms with which *Balantidium coli* might be confused in fecal material are certain free-living ciliates, as *Paramœcium,* but such a mistake could be made only by one having little experience in the study of the intestinal protozoa of man.

CHAPTER XXIV

PREPARATION OF MATERIAL FOR EXAMINATION FOR BALANTIDIUM COLI

UNSTAINED PREPARATIONS—STAINED PREPARATIONS—STAINING METHODS—CULTIVATION OF BALANTIDIUM COLI—CULTURE MEDIA AND METHODS—CRITIQUE OF DIAGNOSTIC METHODS

THE diagnosis of infections with *Balantidium coli* must be based upon the demonstration of the parasite in the stools of the suspected individual as a clinical diagnosis is impossible. The balantidium may be demonstrated in either unstained or stained preparations of the stools or in cultures but the most useful method is the microscopic examination of the unstained material from the stools.

Preparation of Material for Examination.—UNSTAINED PREPARATIONS.—If the stool is diarrheal in character a loopful or two is placed upon a microscopic slide, immediately covered with a cover-glass and examined with a low, dry objective, the 16 mm. ($\frac{2}{3}$ inch) objective being suitable. If the preparations are made from a freshly passed stool the very motile trophozoites are seen swimming about and, if cysts are present, they are easily distinguishable by their large size and the presence within some of them of a single balantidium which may revolve within the cyst-wall in some instances. The large size of this organism clearly differentiates it from the trophozoites or cysts of the intestinal amebæ and flagellates.

STAINED PREPARATIONS.—Staining is not necessary for the diagnosis of *Balantidium coli* but stained preparations are useful in the study of the minute morphology of the organism and for class use in fresh material is not available. The various modifications of the Romanowsky stain, *i. e.*, the Wright, Giemsa or Leishman stains, give poor results and the best methods depend upon wet-fixation, as for *Endamœba histolytica* (see page 52) and staining with one of the hematoxylin stains, of which Heidenhain's iron hematoxylin stain gives perhaps the best results (see page 57), although any of the hematoxylin stains may be used to advantage. It is always difficult to secure stained preparations showing all of the minute morphology of the parasite but such preparations can be secured if one is willing to devote the time that is necessary and does not become discouraged by frequent failures.

CULTIVATION OF BALANTIDIUM COLI

In routine diagnosis it is not necessary to cultivate *Balantidium coli* but for the study of the organism and for class demonstration cultures

are very useful and, owing to the rarity of infections, almost essential unless access can be had to a slaughter house where pigs are slaughtered. As this parasite is primarily a parasite of the hog and a considerable percentage of hogs are infected, material for study and class use can be easily obtained by collecting the contents of the cecum of these animals.

Culture Methods and Media.—Several authorities have devised methods and media for the cultivation of *Balantidium coli* and the most useful are described below, in alphabetical order:

Atchley's Method.—Atchley has employed the following medium in the cultivation of *Balantidium coli:*

Fecal material . .	. 1 part
Ringer's solution	. 4 parts

This mixture is allowed to stand for twenty-four hours, then filtered through gauze, centrifugalized, filtered through filter paper and infusorial earth, and finally sterilized by passing it through a Seitz filter.

The material to be examined is placed in tubes containing this medium and incubated at 37° C. (98.6° F.). A drop or two of the material should be used and the tubes examined daily.

Remarks.—This medium gives excellent results but the filtration through infusorial earth is apparently unnecessary as just as good results are obtained when this is omitted.

Barret and Yarbrough's Method.—The medium used in this very simple method for the cultivation of *Balantidium coli* has the following formula:

Human blood serum, inactivated	1 part
Sodium chloride solution, 0.5 per cent	16 parts

The medium is sterilized by filtration and distributed in 8 cc. portions in suitably sized test tubes. The tubes are inoculated by placing 0.1 cc. of the feces containing the organism at the bottom of the tubes by means of a pipette. The tubes are then incubated at 37° C. (98.6° F.) and examined at the end of twenty-four hours. Transfers are made every twenty-four or forty-eight hours and, in doing so, it should be remembered that the media near the bottom of the tubes contains the greatest numbers of the organism, and material for examination or for transfer should be obtained from this portion of the culture. Cysts may appear in the cultures.

Remarks.—With this medium, Barret and Yarbrough were able to maintain *Balantidium coli* during a period of thirty-two days, making 11 subcultures during that time.

The writer has found that the substitution of Locke or Ringer solution without dextrose for the salt solution gives better results and the cultures can be maintained for a longer time. The morphology of *Balantidium coli* in cultures is like that observed in the stools.

Jameson's Method.—Jameson found that *Balantidium coli* obtained from the hog grew without difficulty in Dobell and Laidlaw's medium used in the cultivation of *Endamœba histolytica* (see page 82). In this medium the organism grew without difficulty and could be maintained for many weeks. In starting the cultures he recommended that transfers be made every forty-eight hours but afterwards every three to five days. The pH of the medium should be between 5.4 and 8.

Remarks.—In this medium no cysts were observed but conjugation of the trophozoites occurred periodically and encystment did not follow conjugation. The conjugating parasites were smaller than the other trophozoites and frequently unequal in size. Otherwise the morphology of the organisms was like that observed in the stools.

This medium apparently gives excellent results in the cultivation of *Balantidium coli* and may be recommended for that purpose.

Nelson's Method.—This method is based upon the use of the intestinal contents of the hog as a base for the culture medium. The method of preparing the medium is as follows:

The cecal contents of an infected pig is mixed with Ringer's solution (see page 81) in the proportion of 1 part of cecal contents to 9 parts of Ringer's solution. The mixture is strained through a copper sieve to remove the coarse particles and then filtered through cotton in a glass funnel. The resulting filtrate constitutes the culture medium and is immediately tubed or stored in a flask and tubed as required. It is best to keep the medium in the ice-box but it keeps well even at room temperature and Nelson states that flasks of this medium have been kept at room temperature for over two months without deterioration.

The pH of the medium is important and Nelson found that a cecal medium having a pH of 6.2 resulted in the balantidia living within it for an average of two days whereas a medium having a pH of 6.8 showed viable balantidia after an average period of nine days, and when the pH of the medium reached 8.0 the average period of survival of the organisms was twenty-eight days and some cultures lived for thirty-four days in this medium. A pH of about 5.0 is fatal to the balantidia.

In routine work 10 cc. of the medium is placed in each culture tube. It was found that autoclaving the medium rendered it almost useless as bacteria appear to be necessary for the continuance of life of the balantidia in cultures. Nelson showed by a series of experiments that some substance produced by bacteria is essential for the growth of the balantidia and when bacteria are removed by sterilization the balantidia perish within a few hours and cultures cannot be maintained for any length of time.

Balantidium coli may be cultivated in flasks, using this method, and Nelson recommends placing 200 cc. of the medium in Erlenmeyer flasks and adding a small amount of rice starch every two or three days after inoculation. Such flask cultures showed numerous balan-

tidia after periods varying from fourteen to as long as thirty-four days after inoculation.

Remarks.—Nelson states that with this medium it is never necessary to make transfers because of the growth of bacteria, as in media containing blood serum, and he made transfers only when the pH of the medium approached the lethal point, *i. e.*, about 5.0. Using this medium he was successful in maintaining 30 strains of *Balantidium coli* in cultures for periods up to one hundred and thirteen days, when the work was discontinued. During this time 7 subcultures were made. The largest number of balantidia observed in routine counts of the culture was 7800 and in routine cultures the organisms usually vary in number between 4000 to 5000 per cubic centimeter. Rice starch should be added to the culture tubes every two or three days or the balantidia will perish, especially if the organisms are very numerous in the cultures.

This method of cultivating *Balantidium coli* would appear to be an excellent one if the cecal contents of hogs are available. The technique is very simple and the organism can be maintained in cultures for long periods of time, as shown by Nelson.

Rees' Medium.—Rees, in 1927, described the following medium and he was the first to successfully cultivate *Balantidium coli*, employing this medium. The formula follows:

A modified Ringer solution is prepared as follows:

NaCl	6.50 gm.
KCl	0.14 gm.
$CaCl_2$	0.12 gm.
$NaHCO_3$	0 20 gm.
Na_2HPO_4	0 01 gm.
Distilled water	1.00 liter

Eighteen cc. of this solution is placed in test tubes and autoclaved and to this is added 2 cc. of human or horse serum and a sprinkle of rice starch, which must be sterilized.

After inoculation the tubes are kept in an incubator at 36° C. (96.8° F.) and transfers are made at three-day intervals.

Remarks.—Upon this medium Rees was successful in cultivating *Balantidium coli* for several months and it would appear to be well suited for the purpose.

Shumaker's Method.—Shumaker employed a medium composed of 1 part of sterile horse serum and 9 parts of Ringer's solution, to which rice starch has been added in small amounts. This medium is tubed and a minute amount of rice starch is added to each tube, after which the tubes are inoculated with a small amount of the stool containing the balantidium. Subcultures should be made every two or three days if this medium is employed.

Remarks.—*Balantidium coli* will multiply in this medium but the serum present greatly facilitates bacterial growth and this becomes so

abundant within a few hours that transfers must be made frequently if it is desired to maintain the organism in the cultures.

Tanabe's Method.—This method is described on page 86.

Remarks.—This culture method is an excellent one for *Balantidium coli.*

CRITIQUE OF DIAGNOSTIC METHODS

The diagnosis of infections with *Balantidium coli* must be based upon the demonstration of this parasite in the stools of the suspected individual. This demonstration is easily accomplished by the microscopic examination of fresh, unstained fecal material from the suspected individual and stained preparations are not necessary for a diagnosis. All that is necessary is to place a small drop of the fecal material upon a microscopic slide, cover it with a cover-glass and examine the preparation with a 16 mm. ($\frac{2}{3}$ inch) microscopic objective. If the examination of several such preparations results negatively, cultures may be made but the writer has never observed a case in which the fecal preparations were negative and cultivation resulted in the demonstration of the organism. If cultures are used the simple media recommended by Rees or Nelson would appear to be preferable.

For teaching purposes stained preparations are best prepared by wet-fixation and staining with one of the hematoxylin stains and for teaching purposes and the study of the biology of the parasite cultures are very useful and should be employed. If it be desired to study the balantidia in the tissues of the bowel sections should be prepared and stained as recommended for the demonstration of *Endamœba histolytica* in the tissues (see page 65).

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