Experimental

PATHOGENIC MICROBIOLOGY

SLACK - GERENCSER - DEAL

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by

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PREFACE

This manual is dedicated to the student and it is our sincere desire that it will serve him well in the performance of his laboratory experiments making them both interesting and knowledgeable.

Students of Dentistry, Pharmacy, Medicine, Medical Technology, Nursing and Dental Hygiene must of necessity receive much of the same basic material in Microbiology. However, each profession has certain areas which require special types of experiments; thus, this manual has been designed in such a manner that it contains the common basic material as well as experiments designed to meet the needs of the specialty groups. In addition, there are adequate provisions for the Instructor to add supplemental experiments of his own choosing.

Some of this material was first published in 1941 with Dr. M. F. Gunderson and in thumbing through that early edition it is interesting to note that although some of the experiments were essentially the same, there was no mention made of the electron microscope or phase microscopy; fluorescent antibody techniques were yet to come; antibiotics were not included; decarboxylases were not appreciated; and no virus experiments were performed. Such comparison makes one appreciate the progress that has been made in this relatively short time and serves as a reminder of the dynamics of Microbiology.

The present edition represents a complete review and editing of all the experiments with almost every one being changed or rewritten. New experiments were added in genetics, virology, oral flora and mycology. Recently established techniques as the Elek plate, decarboxylase determinations and optochin discs have also been added. All of these changes were made so as to provide the student with experiments that incorporate present day methodology.

The authors wish to take this opportunity to express our appreciation to Dr. C. C. Mascoli, Dr. R. G. Burrell, Mr. Dane Moore, Jr., and Miss Helen Pavlech for their assistance in the preparation of this manual.

John M. Slack
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THE MICROSCOPE

THE LIGHT MICROSCOPE

The microscope (Greek: micros - small and skopein - to see) is a complex optical instrument which has been developed to enable one to examine small objects or the fine detail or structure of objects. It consists of a series of lenses so arranged that in the eye a clearly focused image is formed which is larger than the unaided eye could produce.

For the normal and unaided eye the distance of clearest vision is 10 in. or 25 cm. However, with the aid of a simple microscope (magnifying glass) it is possible to bring an object much nearer and to see it clearly. Finally, if the magnifying glass is brought very close to the eye and if the distance between the object and the glass is less than the focal length of the lens, the rays from the object may be made to enter the eye as though they come from an enlarged object situated at the distance of clearest vision (Fig. 1). We speak of such an image as being virtual because the image is imaginary and no light rays actually arise from this point.

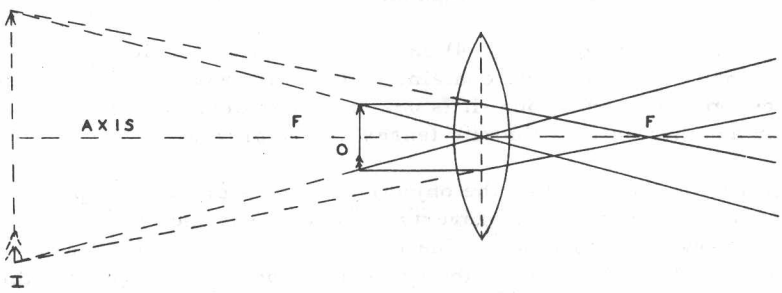

FIGURE 1

O – OBJECT; I – VIRTUAL IMAGE; F,F – PRINCIPAL FOCI OF THE LENS

It is impossible to use a single lens for magnification above about 8 times because of certain defects. The two main defects involved are spherical and chromatic aberration. By spherical aberration (Fig. 2) we mean that there is no definite point focus for all the rays. Chromatic aberration (Fig. 3) is due to the fact that the index of refraction varies with the wave length. Thus, violet light would come to a focus closest to the lens and red light farthest. This would result in a series of colored foci extending along the axis.

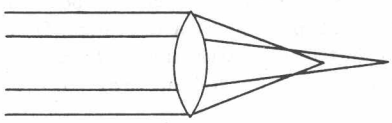

FIGURE 2

SPHERICAL ABERRATION

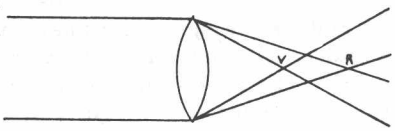

FIGURE 3

CHROMATIC ABERRATION

Spherical aberration is corrected by selecting convex and concave lenses of different materials (e. g. , crown and flint glass) and hence of different refractive power. It can also be corrected by combining lenses of the same type of glass or by using a plano-convex lens.

Chromatic aberration is corrected by using a converging lens of crown glass and cementing to it a diverging lens of flint glass. The lenses must have equal dispersion but the diverging lens has a smaller power thus, the dispersion is neutralized without entirely eliminating the converging powers. Such a combination is called an "achromatic" doublet. However, this combination corrects only for two colors and with high magnifications there may be some color present.

Recently fluorite has been substituted for some of the glass elements and this permits a partial correction of the secondary spectrum. If several fluorite lenses are combined the entire secondary spectrum can be eliminated. In the case of objectives these are called "apochromatic".

The smallest interval that the unaided eye can distinguish is from 70 to 140 microns. Thus, to observe smaller objects or the details of large objects it is necessary to employ some means of magnification. As stated before, simple lenses can be used for magnifying up to eight times but beyond this compound lenses are necessary.

The compound microscope (Fig. 4) consists of two sets of lenses, the objective set and the eyepiece set. The objective contains a series of lenses and is corrected for both spherical and chromatic aberration. It is designated as achromatic if crown and flint glass is used or apochromatic if fluorite lenses are employed.

The magnification obtained by the objective depends primarily upon the resolving power. This may be defined as the property of showing distinctly and separately two points. which are very close together. The resolving power can be measured by the numerical aperture (N. A.). The higher the numerical aperture the greater the resolving power and the finer the detail it can reveal. The numerical aperture in turn depends upon the wave length of the light, the focal length of the lens and upon the refractive index of the substance which lies between the lens and the object. Thus, the oil immersion lens has a greater magnifying power because the index of refraction is increased by the use of immersion oil which has the same refractive index as glass. To summarize, the magnification obtained by the objective depends primarily upon the wave length of light, the focal length of the lens and upon the refractive index of the substance between the lens and the object.

Microscopes used in microbiology usually have three objectives which have a focal length of 16 mm. , 4 mm. and 1. 8 mm. Their respective magnification is approximately 10, 43 and 97 times

The Huyghens eyepiece bends the cone of rays coming from the objective so that they focus closer to the axis where more of them may send light to the eye and thus increase the field of view. Such an eyepiece usually has only two plano-convex lenses which are so shaped and spaced that the light passage through them is as symmetrical as possible in order to reduce the spherical aberration. The lenses are placed at a distance from each other equal to half the sum of their focal lengths, so that chromatic aberration is nearly eliminated. The so-called "compensating" eyepieces are corrected further for color. The usual eyepieces will magnify 5, 10, or 15 times.

Turning to Fig. 4 let us trace the light rays through the microscope and see how the final virtual image is formed. The mirror (use the plane side) focuses the light rays through the condenser diaphragm into the lenses of the condenser. The rays are then bent so that

they focus in the plane of the object. The rays from the object are collected by the objective lenses and bent so that they cross in the barrel of the microscope. These rays are in turn collected by the field lens of the eyepiece and focused in the plane of the eyepiece diaphragm where a reversed image of the object is formed within the focal length of the eye lens. The rays then pass through the eye lens which acts as a magnifying glass and the eye sees an enlarged virtual image of the object which appears to come from a point about 25 cm. away. The final magnification is the product of the magnification due to the objective and the magnification due to the eyepiece (e. g., the total magnification of a 97x objective and a 10x eyepiece would be 970x).

THE PHASE MICROSCOPE

When light rays go through a transparent object some rays are retarded because of different densities in the object. This retardation is often about 1/4 wave length although it may be more or less. This retardation causes these rays to be out of phase with the remainder of the light. We are unable, however, to see these phase differences with the ordinary microscope.

The phase microscope converts these phase differences into intensity differences which can be seen with the eye.

This is accomplished by placing an annular diaphragm in the condenser and a diffraction plate in the objective of the optical system. The annular diaphragm allows a circle of light to enter the condenser and the objective.

This light which passes through the transparent object is then retarded or thrown out of phase in varying degrees. This out of phase light and the unretarded light then passes through the diffraction plate.

The diffraction plate is made of glass upon which is deposited phase retarding material (dielectric film) and absorbing material (metal film). These films are deposited only on certain areas of the plate. Light passing through these coated areas is retarded 1/4 wave length, light passing through the uncoated areas is not changed. For bright contrast the undeviated light (S) passes through the diffraction plate in such a manner that it is retarded by 1/4 wave length while the deviated light (D) passes through the entire objective. This brings the deviated and undeviated light into phase with respect to each other. All of these rays are then brought to focus at the focal plane of the eyepiece at which point the amplitude of the diffracted waves is reinforced by the addition of the non-diffracted waves and the areas within the object from which these out of phase waves originated appear as areas of increased brightness.

MAGNIFICATION TABLE

LENS			EYEPIECE	
Focal Length	Numerical Aperture	Magnification	Magnification	Final Magnification
16 mm.	0.25	10x	10x	100x
4 mm.	0.65	43x	10x	430x
1.8 mm.	1.25 oil	97x	10x	970x
1.8 mm.	1.30 oil	98x	10x	980x

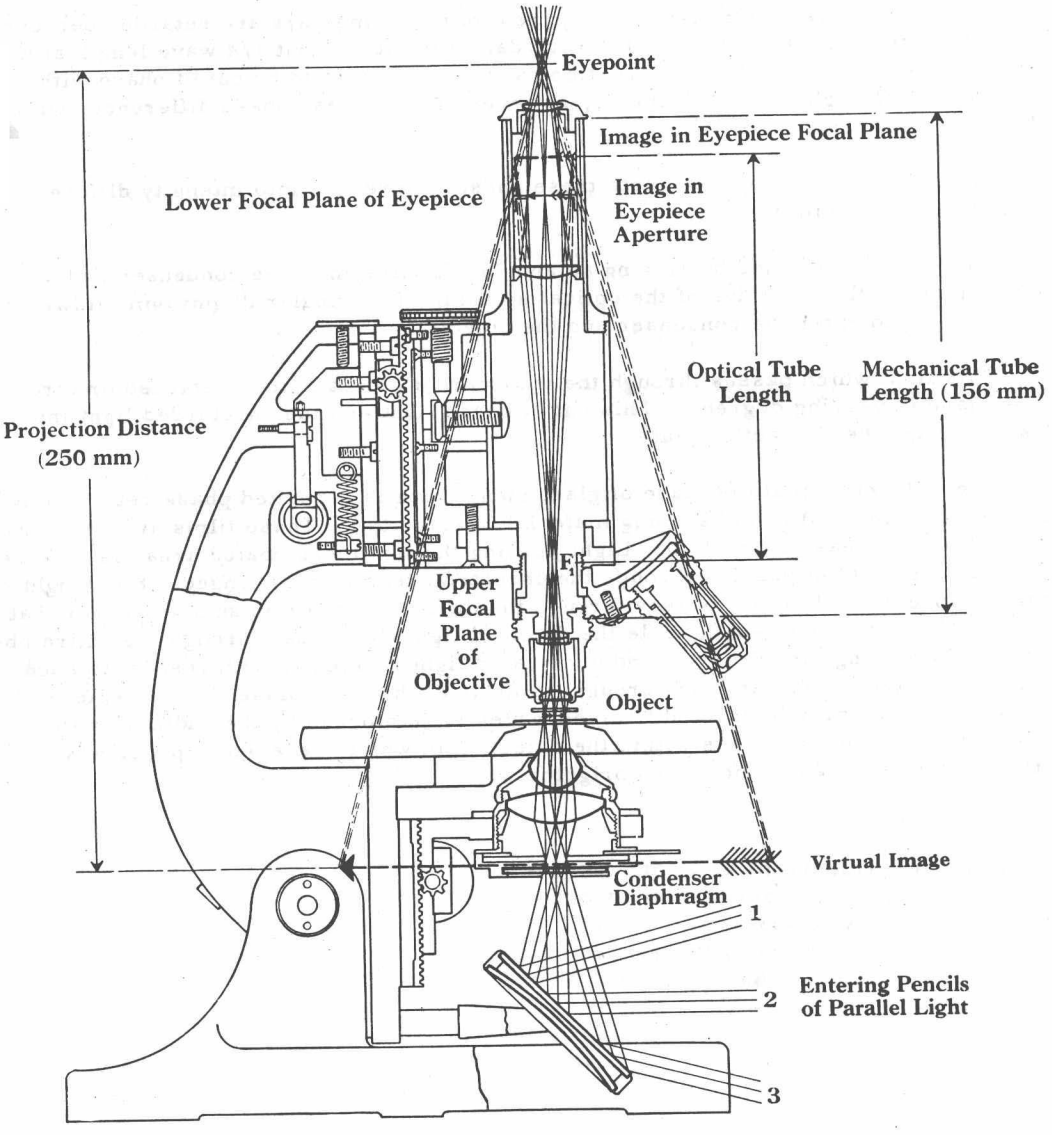

FIGURE 4

PATH OF LIGHT THROUGH THE MICROSCOPE

COURTESY OF BAUSCH AND LOMB OPTICAL CO.

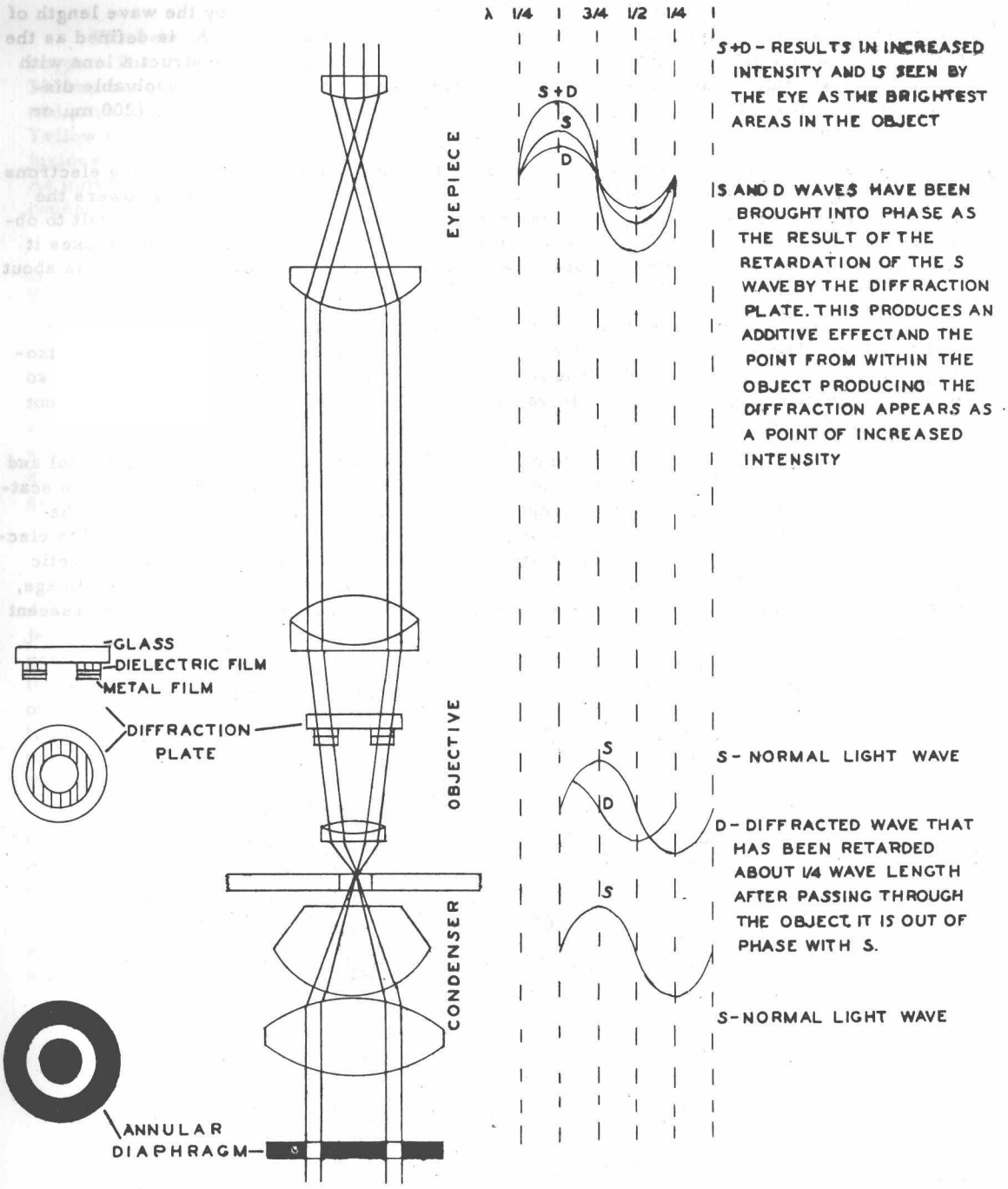

FIGURE 5

DIAGRAMMATIC REPRESENTATION OF THE BRIGHT PHASE MICROSCOPE

MODIFIED BY SPENCER

THE ELECTRON MICROSCOPE

The light microscope is limited in the powers of magnification by the wave length of light and the numerical aperture of the lens (numerical aperture or N. A. is defined as the ratio of the radius of a lens to its focal length). It is impossible to construct a lens with a numerical aperture much greater than 1. 5. Therefore, the minimum resolvable distance between two points is about 5/12 the wave length of light or about 0. 2μ (200 mμ or 2000 angstroms).

The electron microscope overcomes these two limitations, first, by using electrons instead of light rays, and second, by using magnets instead of lenses. This lowers the limit of resolution to a fraction of an angstrom; in practice, however, it is difficult to obtain resolution better than .001 - 0. 1μ (1-10 mμ or 10 - 100 angstroms). This makes it possible to observe even the smallest of the viruses such as the polio virus which is about 12 mμ in diameter.

The source of electrons is an electron gun which operates at a potential of about 60, 000 volts. These high velocity electrons are kept concentrated as a beam by electromagnets spaced along the barrel of the microscope. Electrons cannot penetrate air, so the entire electron pathway must be in vacuum. The electrons have a wave length about 100, 000 times shorter than visible light.

The object to be viewed is placed on a thin film of collodion or similar material and inserted into the vacuum tube so as to be in the path of the electrons. Electrons are scattered by matter, the degree of scattering depends upon the density of the object. The amount of contrast in the final image depends on the degree of electron scatter. The electrons which are scattered by the object are brought to a focus by the objective magnetic lens to form a primary image. The projector lens further magnifies the primary image, bringing the electrons to a focus as a secondary image which is observed on a fluorescent viewing screen or may be photographed.

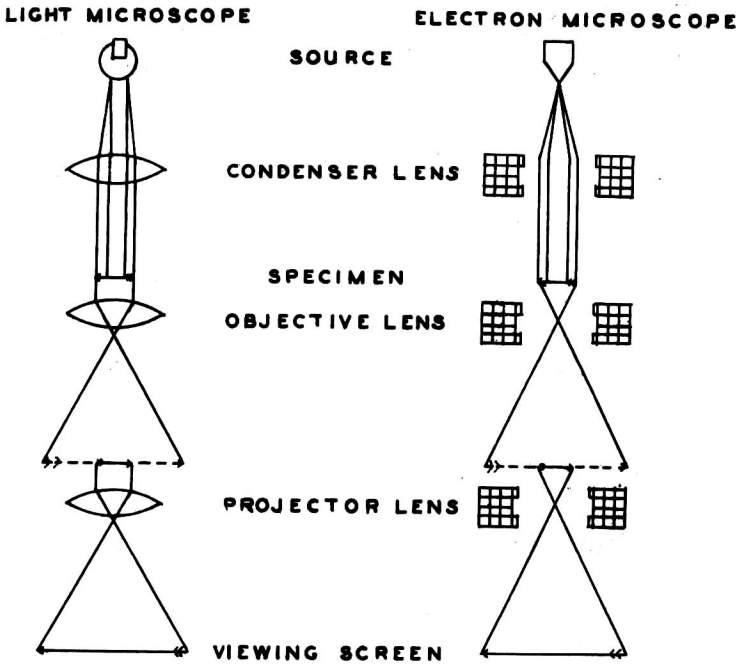

FIGURE 6
DIAGRAMMATIC REPRESENTATION OF IMAGE FORMATION IN LIGHT AND ELECTRON MICROSCOPES

THE FLUORESCENCE MICROSCOPE

Fluorescence microscopy employs the conventional monocular or binocular microscope. The light source is a mercury vapor lamp which emits ultraviolet rays in the 365 mμ range. Ultraviolet or exciter filters which allow only U-V rays and some infrared rays to pass through are placed between the lamp and the microscope objectives. Yellow barrier filters, placed in the eyepieces to remove the U-V rays which may be injurious to the eyes, allow the yellow-green rays of the fluorescent image to pass through. As most optical glass is transparent to light of wave length greater than 350 mμ, the usual condenser and objective lenses can be used for the work.

Many years ago this technique was used as an aid in the visualization of Mycobacterium tuberculosis. The fluorescent dye, auramine, was applied to the smear and when this smear was examined with U-V light the tubercle bacilli would fluoresce.

Recently the fluorescent-antibody technique has been developed in which case the antibody globulin is conjugated with fluorescein isothiocyanate or an other fluorochrome. This conjugated antiserum is applied to the homologous antigen and there results an antigen-antibody reaction with the retention of the fluorescent dye on the antigen. When a preparation of this antigen is examined under U-V light, the molecules of the dye reach a higher energy level which is released as visible fluorescent light. Such specimens are examined using the darkfield condenser; thus the antigen appears as a brilliant yellow-green object against a black background.

THE DARKFIELD MICROSCOPE

In darkfield microscopy light is concentrated at the specimen by the darkfield condenser in the form of a hollow cone of light at such an angle that none of it enters the objective unless a specimen is present to change its direction. As can be seen in the diagram, the condenser has two reflecting surfaces. These are situated in such a way that the entering light strikes the first surface in the center of the condenser and is reflected onto the second one. The latter reflecting surface then directs the light in such a way that it leaves the condenser at an angle to the specimen and the objective. Any organism present in the microscopic field will reflect some of the light rays into the objective and thus be outlined as a bright object against a dark background.

In using the darkfield microscope it is necessary to have an immersion fluid contact between the top level of the condenser and the lower surface of the microscopic slide. Also a funnel stop must be placed within the oil immersion objective to decrease its aperture to match that of the condenser.

The darkfield microscope is particularly useful for observing the spirochete of syphilis in direct smears and in the study of small cellular inclusions. A dark field and a bright field condenser are represented in the following drawings.

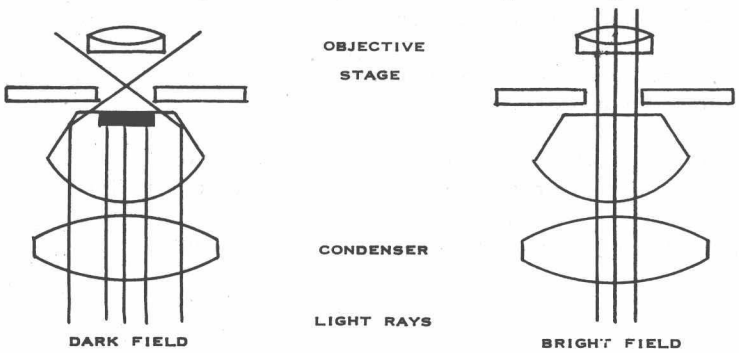

FIGURE 7

DIAGRAMMATIC REPRESENTATION OF DARKFIELD AND BRIGHT FIELD CONDENSERS

STUDENT SUPPLIES

1. The student will purchase the following supplies. Additions or subtractions from this list will be posted.

1 laboratory manual	1 orange glass marking pencil
1 box of precleaned micro slides	1 red drawing pencil
1 box of square cover glasses	1 blue drawing pencil
1 slide box of 100 slide capacity	1 box slide labels
1 book of combined lens and blotter paper	1 white laboratory coat

2. Each student will purchase a copy of the designated textbook. Your textbook is to be brought into the laboratory in order that you may read about the organisms being studied.

3. During the first day of lab, desks, microscopes and keys will be assigned. A check list of equipment will be provided for the student to sign. Lost lab keys will have to be replaced.

GENERAL DIRECTIONS FOR LABORATORY WORK AND CONDUCT

1. Your microscope is a valuable instrument and must be handled and used carefully. Pick it up and set it down gently. At the end of each period clean the eyepiece lens, oil immersion objective, other objectives if used and the stage. Then for storage turn the low power into place so that the oil immersion lens will not be cracked by being racked down into the condenser.

2. All slides prepared in this course are to be properly labeled with the name of the organism, type of stain, date and your initials.

3. All properly labeled slides are to be placed in your slide box and recorded in the index.

4. Each student is responsible for all drawings. All Morphology and Physiology data must be complete. Whenever writing the name of a bacterium write out both the generic and species name - do not abbreviate.

5. Directions will be given for the discarding of used materials. These instructions are to be followed explicitly.

6. Demonstration preparations will usually supplement each exercise. It is the responsibility of each student to review and study these demonstrations.

7. The use of food or tobacco is prohibited in the laboratory.

A WORD OF CAUTION

Throughout this course you will be working with pathogenic microorganisms which are capable of causing infections in yourself as well as in your neighbor. There is no danger if you learn to carry out the laboratory techniques carefully, however careless procedures on your part will endanger yourself and others.

The following procedures are to be strictly observed:

1. Burn your inoculating wire before and after each use.

2. Flame the lips of glassware before and after use. Flame pipettes before using.

3. Never completely remove a Petri dish lid.

4. Immediately report any accidents to the Instructor. A disinfectant will be applied to the contaminated area. You will wash your hands thoroughly with soap and water and if necessary apply a disinfectant.

5. Before and after each laboratory period wipe off your laboratory desk top with a disinfectant such as Lysol.

SUPPLEMENTARY WORK

During the course students will be assigned certain supplementary exercises. The number and extent of these exercises will vary depending upon the available material. Record of this work will be kept on the Supplementary Work Sheets at the back of this manual.

This work will be mainly of two types:

1. Diagnostic, autopsy or dental clinic material will be issued to groups of students. The required laboratory procedures will be done under the guidance of the teaching staff. Special media or the necessary animals will be supplied. Keep adequate records of these procedures which should indicate the type of material received, the procedures used and the results.

2. Groups of students will be assigned to animal work such as immunization, pathogenicity tests and protection tests. In such experiments be certain to record the type animal used, its weight and sex. Record the kind and amount of material injected and the date. Finally, record the results of your injections and the fate of the animal.

3. From time to time unknowns consisting of pure or mixed cultures will be distributed. Each student will receive an unknown and directions for handling will be given at the time of distribution.

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Experiment 1
BLOOD STREAM COMPONENTS AND BACTERIAL MORPHOLOGY

I. DEMONSTRATION

A. There will be an introductory discussion concerning the blood and the part which the various blood elements play in defending the body against infection.

B. The microscope, its use and care, will be demonstrated.

C. Slides of stained blood cells and bacteria will be demonstrated.

II. LABORATORY WORK

A. Preparation and staining of blood films.

1. Wash the middle finger with 70% alcohol and allow it to dry. Then pierce the finger with a lancet -- be certain to cut across the lines of the skin well into the corium. Wipe off the first drop of blood with a pledget of cotton and gently squeeze the finger until a second drop forms.

2. Touch this drop near the end of a pre-cleaned slide. Place this slide on the table and touch the end of the second clean slide, to be used as a spreader, to the drop. Holding this second slide at an angle of 45°, advance it with a clean sweep to the edge of the horizontal slide. The blood is pulled or drawn behind the end of the advancing slide. Allow the film to dry in air and stain as follows:

   a. Cover the slide with 7-10 drops of Wright's stain and allow this to stand 1 minute.
   b. Add carefully, drop by drop, an equal amount of distilled water or buffered solution. A metallic film should appear on the surface. Allow this to stand for 7-10 minutes.
   c. Wash in tap water, blot dry, and examine under oil immersion.

   Draw the various types of cells seen, as red blood cells, lymphocytes, polymorphonuclear leucocytes, eosinophiles, etc. A red blood cell is 7.4 μ in diameter.

B. Prepared slides

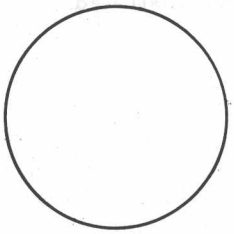
1. Slides showing stained blood cells and bacteria will be distributed. The bacteria are staphylococci and bacilli. Observe these under oil immersion. Draw showing the comparative sizes between bacteria and blood cells.

2. Slides showing various kinds of bacteria will be distributed. Examine carefully under oil immersion and draw the various bacteria. Leave these slides on your desk at the end of the laboratory period.

C. Optional Work

1. If the Instructor desires other environmental sources of microorganisms may be examined as: hay infusion, pond water, rumen fluid, fish tank water, etc.

DRAWINGS



WRIGHT'S STAIN
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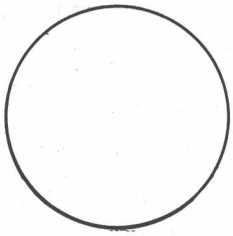

STAINED BACTERIA
STAINED BLOOD CELLS
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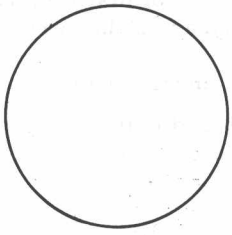

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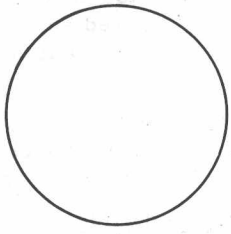

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