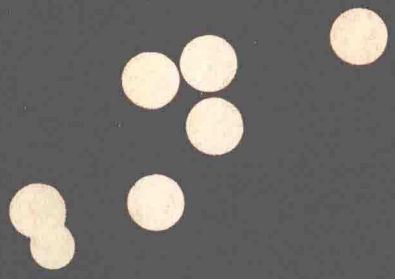

MICROBES IN ACTION

A LABORATORY MANUAL OF MICROBIOLOGY

THIRD EDITION

Harry W. Seeley, Jr.
Paul J. VanDemark

Microbes in Action

A Laboratory Manual of Microbiology

THIRD EDITION

Harry W. Seeley, Jr.
Paul J. VanDemark
Cornell University

W. H. FREEMAN AND COMPANY
San Francisco

Project Editor: Pearl C. Vapnek
Copyeditor: Stephen McElroy
Interior Designer: Marie Carluccio
Cover Designer: Sharon Helen Smith
Production Coordinator: Linda Jupiter
Illustration Coordinator: Audre W. Loverde
Compositor: Interactive Composition Corporation
Printer and Binder: Webcrafters, Incorporated

Printed in the United States of America

ISBN 0-7167-1159-8

9 8 7 6 5 4 3 2 1

CONTENTS

Preface ix
Suggestions and Regulations xi

I The Microscope 1
Magnification 1
Resolving Power 3
Illumination 4
Use of the Microscope 4
Special Precautions 5
1 Examination of Natural Infusions 6
2 Examination of Living Bacteria 7
Phase Microscopy and Dark-Field Microscopy 8
The Electron Microscope 10
3 Examination of Stained Cells 15
4 Microscopic Measurements 16

II The Culture of Microorganisms 17
5 Broth Culture 19
6 Agar Slope 20
7 Pure Culture Techniques 21
Streak Plate 22
Pour Plate 23
Isolation of a Bacterial Culture 25
Preservation of Laboratory Cultures 27

III The Staining of Microorganisms 29
Simple Staining Techniques 29
8 Direct Staining with Basic Dyes 30
9 Negative or Indirect Staining 32
Differential Staining Techniques 34
10 Gram Stain 34
11 Acid-Fast Stain 36
12 Structural Stains 37

IV The Preparation of Media and Methods of Sterilization 42
Media 42
The Meaning of pH 42
13 Preparation of Culture Media 43
14 Sterilization Methods 44

V The Determination of Microbial Numbers 49
15 Quantitative Plating Methods 50
Spread Plate 50
Pour Plate 53
16 Turbidimetric Estimation of Bacterial Growth 55
17 Growth Curve 57

VI Environmental Influences 59
18 Effect of Temperature on Growth 59
19 Heat Resistance of Vegetative Cells and Spores of Bacteria, Yeasts, and Molds 61
20 Effect of Osmotic Pressure on Growth 61
21 Effect of pH of Medium on Growth 63
22 Effect of Energy Source and Buffer on Growth 63
Microbial Nutrition 65
23 Microbiological Assay 66
24 Bacterial Photosynthesis 67
25 Relation of Free Oxygen to Microbial Growth 68
26 Anaerobic Culture of Bacteria 70
27 Antiseptic and Disinfectant Action 73
28 Lethal Action of Ultraviolet Light; Photoreactivation 74
29 Antimetabolites: Inhibition by Sulfanilamide 77

VII Microbial Interrelationships 79
30 Antibiosis 79
Antibiotic Susceptibility 79
Isolation of an Antibiotic Producer 80
31 Symbiosis 81
32 Winogradsky Column 82

VIII Enzymatic Reactions 85
Carbohydrates 85
33 Carbohydrate Fermentation 86
34 Starch Hydrolysis 88
Proteins and Amino Acids 89
35 Casein Hydrolysis 89
36 Gelatin Hydrolysis 89
37 Utilization of Amino Acids 90
Decarboxylation and Amine Production 91
Deamination 92
Indole Production 92
Hydrogen Sulfide Production 93
Lipids 93
38 Lipid Hydrolysis 94
Respiratory Enzymes 94
39 Catalase Activity 95
40 Oxidase Test 96
41 Action of Bacteria on Milk 97

IX The Isolation and Identification of Bacterial Cultures 99
Use of Selective and Enrichment Culture 100
42 Differential Plating 101
43 Selective Plating 102
Identification of Important Groups of Bacteria 103
44 Identification of Unknown Bacterial Cultures 105
Appendix to Exercise 44 107
45 Miniaturized Multitest Methods for the Identification of Enterics 109
46 Morphological Identification of Some Unique Bacteria 112
Isolation of Bacteria 113
47 Bacillus Isolation 113
48 Pseudomonad Isolation 115
49 Staphylococcus Isolation 117
50 Lactic Acid Bacteria Isolation 118

X Bacterial Variation, Mutation, and Recombination 121
51 Bacterial Variation 122
52 Enzyme Induction 122
53 Genotypic Change 124
Bacterial Mutation: Isolation of a Streptomycin-Resistant Mutant 124
Plasmid-Mediated Lactose Fermentation by *Streptococcus lactis* 125
54 Bacterial Conjugation 125
55 Bacterial Transformation 127

XI The Viruses 129
56 Bacteriophage Isolation and Characteristics 129
57 Bacteriophage Production: Single-Step Growth 131
58 Tobacco Mosaic Virus: Isolation and Infection of Plants 133
59 Virus Culture in Embryonating Chicken Eggs 135
60 Cultivation of Viruses in Tissue Culture 137
61 Enumeration of Viruses: Hemagglutination 139

XII The Eucaryotes 141
Protozoa 141
62 Observation of Some Protozoa 142
Algae 143
63 Observation of Phytoplankton 143
Fungi: Molds and Yeasts 145
64 Morphology and Reproduction of Molds 147
65 Morphology and Reproduction of Yeasts 149
66 Identification of Fungi 151
67 Cellular Slime Molds 152

XIII Water Microbiology, Sanitation, and Pollution 155
68 Standard Analysis of Water 156
69 Membrane Filter Technique in Water and Air Analysis 158
Water Analysis 158
Air Analysis 161

XIV Food Microbiology 163
70 Quantitative Examination of Bacteria in Raw and Pasteurized Milk 163
71 Direct Microscopic Determination of Bacteria in Milk 164
72 Fermented Foods 166
Sauerkraut 166
Beverages 167
Cheese 168

XV Soil Microbiology 169
73 Microbial Population of Soils 170
74 Nitrogen Cycle 170
Ammonification 170
Nitrification 171
Denitrification 172
Nitrogen Fixation 172

XVI Medical Microbiology and Immunology **175**
75 Normal Throat Flora 175
76 Identification of Pathogenic Staphylococci 177
Coagulase Test 177
Heat-Stable DNase Test 178
77 Sensitivity Discs in the Therapeutic Use of Antibiotics: Kirby–Bauer Technique 178
78 Demonstration of Koch's Postulates 180
79 Phagocytosis 182
80 Slide Agglutination Test 183
81 Precipitin Test 184
82 Immunodiffusion: Ouchterlony Plate 185
83 Complement Fixation 186
84 Fluorescent-Antibody Technique 188
85 Serological Test for Infectious Mononucleosis 190
86 Detection of Chemical Carcinogens: Ames Test 191

Appendix 193
Stains and Reagents 193
Indicators 195
Media 195
Reports 201
Index 381

PREFACE

In recent years few sciences have grown and changed as rapidly as microbiology. In this edition of *Microbes in Action* we have included laboratory exercises to bring the student abreast of these recent developments.

Advances over a broad front of microbiology and related fields depend on a basic knowledge of microorganisms. Therefore, in this revision we have continued to emphasize classical methods such as aseptic techniques and enrichment and selective culture. Our primary objective is to provide a basic training in handling and understanding microorganisms.

The introduction of new exercises in areas such as microbial genetics, virology, and immunology is intended to bring the manual up to date and to prepare the student for further work in these disciplines. As in the previous editions, our basic approach is ecological; that is, we emphasize the natural relationship between organisms and their environments. To strengthen this approach, new exercises have been added, for example, the isolation and identification of representative species of various bacterial groups, the Winogradsky column, and the microbial analysis of air.

In introducing these new and more complex exercises, we have tried to make certain that they can be presented practically and are repeatable in the average introductory laboratory. Their success in our introductory laboratory course over a period of several semesters indicates that the projects are feasible.

In designing the exercises, we have carefully chosen cultures and conditions that minimize the exposure of students to potentially pathogenic microorganisms and hazardous chemicals. The growing awareness and concern about the effects of many chemicals on human health has prompted us to eliminate certain procedures or reagents from exercises, for example, the use of pyrogallol for anaerobic growth. Where such cultures or chemicals are employed, cautions concerning possible dangers to the student and instructor are emphasized.

We are deeply grateful to the instructors and students in our classes over many years whose performance has been essential to the development of this manual. We are particularly indebted to Carole Rehkugler, Linda Flinton, and Rosalie McDermid for modification and design of many exercises that add immeasurably to providing a sound and interesting laboratory training. Additional thanks go to Mary Carlisle for her excellent typing of the manuscript.

Harry W. Seeley, Jr.
Paul J. VanDemark

November 1980

SUGGESTIONS AND REGULATIONS

General Suggestions and Information

1. Wear a coat, smock or apron to protect your clothing.

2. Before each laboratory period, read over the exercises to be done and plan your work carefully. Know how each exercise is to be done and what basic principles it is intended to convey.

3. Each laboratory meeting will begin with a short discussion and instruction period. Do not begin work until you have received your instructions. Ask questions when you do not understand the method and purpose of any experiment. Good laboratory technique depends primarily on knowing what you are to do.
4. Properly record all observations at the time they are made. Laboratory examinations will cover both the information given by your laboratory instructor and that contained in the manual as well as your own observations and deductions. Answer the questions following each exercise on the report sheet.

Laboratory Regulations

1. Sponge off the top of your laboratory desk with germicide solution at both the beginning and the close of each laboratory period.

2. Keep your desk free of nonessential materials at all times, and at the end of the period leave it free of all materials and equipment.
3. Place all solid waste material in the waste cans and all dirty glassware in the trays. Your laboratory grade will depend to some extent on your technique, orderliness, and cleanliness.
4. Because many of the microorganisms with which you will be working are potentially pathogenic, it is imperative to develop aseptic techniques in handling and transferring them. Avoid any hand-to-mouth operations such as smoking, eating, or moistening labels with the tongue.

5. Report immediately all accidents such as cuts, burns, or spilled cultures to your instructor. Take all precautions to avoid such accidents.

6. Some of the chemicals employed in the laboratory can be hazardous if not handled properly. We have selected the experiments to minimize the use of such substances; however, where they are necessary, make certain to observe the precautions pointed out by your instructor.

MICROBES IN ACTION

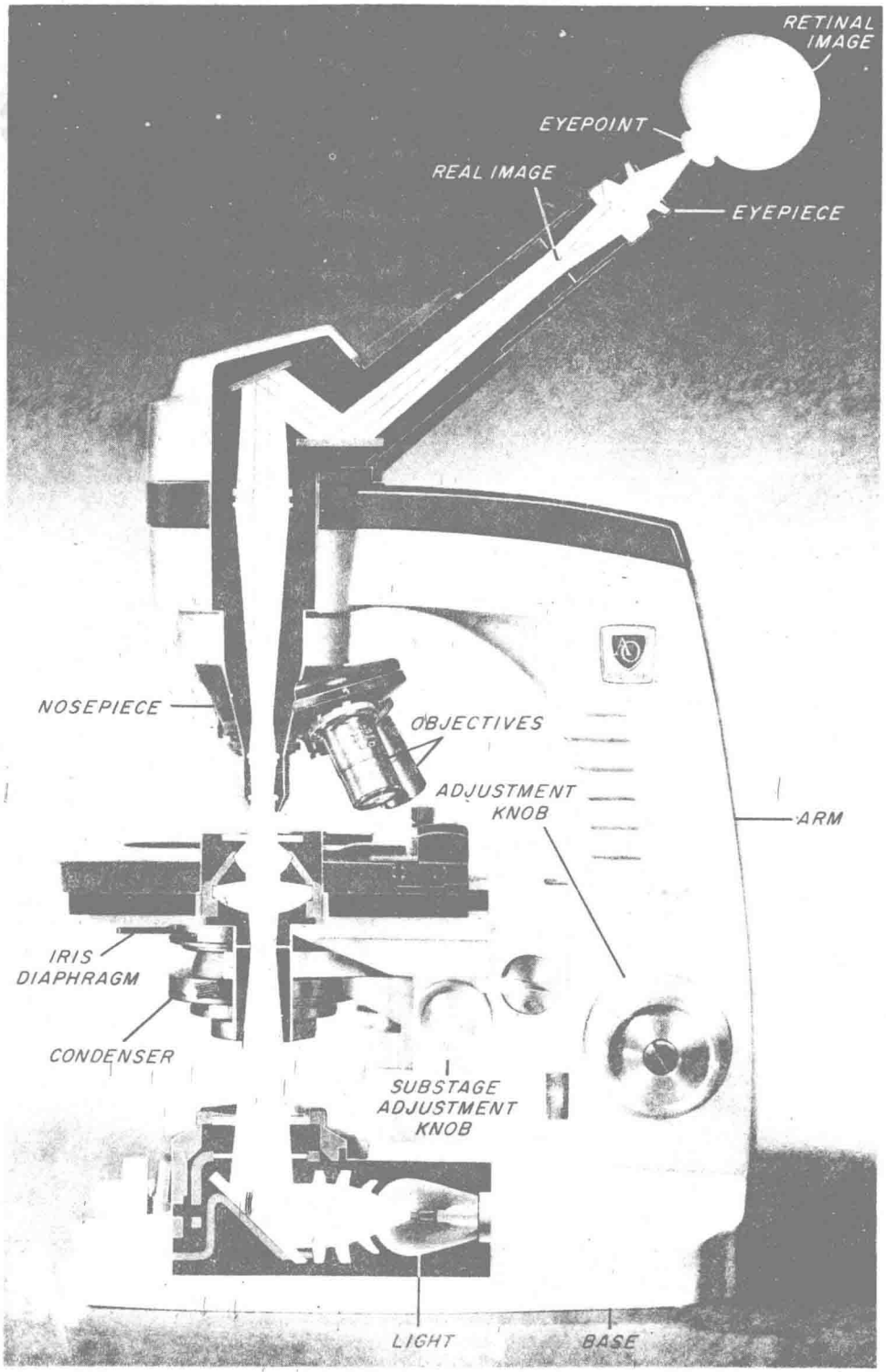

Figure I-1 The compound microscope. (Courtesy of the American Optical Corporation.)

I

THE MICROSCOPE

Microbiology is the science concerned with living organisms too small to be seen with the naked eye; thus the advent of microbiology dates from the invention of the microscope. A **simple microscope** is little more than a biconvex lens, but a **compound microscope** employs two separate lens systems to magnify the object, thereby achieving greater magnification. Since the compound microscope is the primary tool in microbiology, a thorough understanding of the basic principles of microscopy and a skill in the use and manipulation of this instrument are prerequisites to any study in this science.

From the standpoint of construction and operational details, there are many different types of compound microscopes, but the principles underlying all these instruments are the same. The microscope is basically an optical system (for magnification) and an illumination system (for rendering the specimen properly visible). To comprehend the operation of the optical and illumination systems, you must thoroughly understand the principles of and the relationship between magnification, resolving power, and illumination. Based upon different physical principles than light microscopy, electron microscopy is capable of much greater magnification and resolving power.

MAGNIFICATION

Magnification in the compound microscope is obtained by a series of two lens systems (see Figure I-1). The lens system nearest the specimen, called the **objective,** magnifies the specimen and produces a **real image.** The **ocular** or eyepiece lens system magnifies the real image, yielding a **virtual image** that is seen by the eye. The total magnification is equal to the product of the ocular magnification and the objective magnification.

The objective lens system is a combination of convex and concave lenses of different types of glass that correct for various chromatic and spherical aberrations inherent in a simple convex lens. Microscopes in most bacteriological laboratories are equipped with three objectives of different focal lengths: the **low-power** objective (16 mm), the **high-dry** objective (4 mm), and the **oil-immersion** objective (1.8 mm). (The desired objective is rotated into place by means of the **revolving nosepiece.**) The 16 mm, 4 mm, and 1.8 mm designate the **focal length** of each objective. As shown in Figure I-2, the shorter the focal length of the objective, the shorter is the **working distance** of the lens, that is, the distance between the specimen and the objective.

The objective lens focuses the light rays from the specimen to form a real image within the body tube. The real image is further magnified by the ocular lens system, which is situated at the top of the draw tube and which comprises two lenses. The lower or **field lens** places the real image in the focal plane of the upper or **eye lens,** which serves as a simple magnifying lens, enabling the eye to focus on the virtual image of the specimen.

The total magnification obtained with a compound microscope is found by multiplying the initial magnification of the objective being employed by the magnification of the ocular. The initial magnification of an objective is engraved on the objective mount, and the magnification of the ocular is usually marked on the top of the eye-lens mount or on the side of the eyepiece. The total magnification obtained with the objectives listed above is as follows: the 16-mm objective with a 10× eyepiece gives a total magnification of 100 diameters; the 4-mm

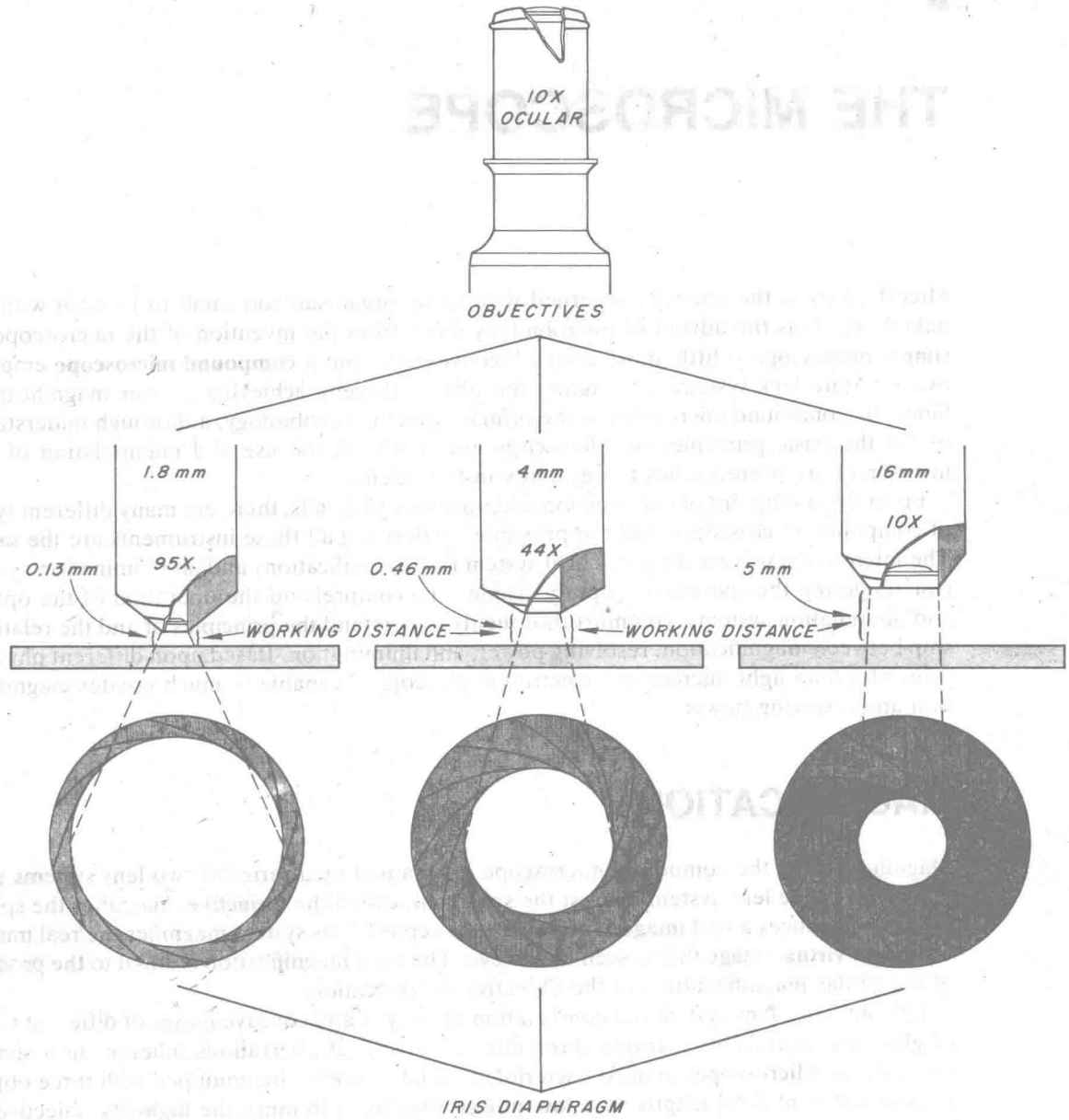

Figure I-2 Relationship between working distance of objective lens and adjustment of iris diaphragm. The shorter the working distance, the more open the diaphragm.

objective with a 10× eyepiece gives a total magnification of 440 diameters; the 1.8-mm objective with a 10× eyepiece gives a total magnification of 950 diameters.

Two adjustment wheels focus the lens systems on the specimen. The **coarse adjustment** moves the body tube (or the stage on some models) over a greater vertical distance and brings the specimen into approximate focus, and the **fine adjustment** moves the body tube more slowly for precise final focusing.

RESOLVING POWER

Since the total magnification of the compound microscope is the product of the magnification of two lens systems, we might expect that the total magnification would be indefinitely increased by the use of additional lenses. However, this is not so, owing to a property of lenses called **resolving power** (see Figure I-2). The resolving power of a lens is its ability to show two closely adjacent points as distinct and separate. This characteristic of a microscope is a function of the wavelength of the light used and a characteristic of the lens system known as its **numerical aperture:**

$$\text{resolving power} = \text{diameter of smallest structure visible} = \frac{\text{wavelength}}{\text{numerical aperture}}$$

Thus, the shorter the wavelength of light used, the smaller the structure visible; for example, blue light will give a greater resolution than will red light. However, since the spectrum of visible light is relatively narrow, increasing the resolution by decreasing the wavelength of the light used is of limited value. The greatest increase in resolution of a light microscope is brought about by increasing the numerical aperture. The numerical aperture is a function of the effective diameter of the objective in relation to its focal length and the light-bending power of **refractive index** of the medium between the specimen and the objective. Optical factors limit the degree to which the objective may be altered to increase its numerical aperture.

Because the refractive index of air is less than that of glass, light rays are refracted or bent as they pass from the microscope slide into the air. Thus, many of the light rays reflected from the specimen are refracted at so great an angle that they completely miss the objective. By interposing immersion oil, which has essentially the same refractive index as glass, between the slide and the objective lens, we greatly decrease refraction, and a far greater percentage of the light rays from the specimen pass directly into the objective, resulting in greater resolution and a clear image (see Figure I-3).

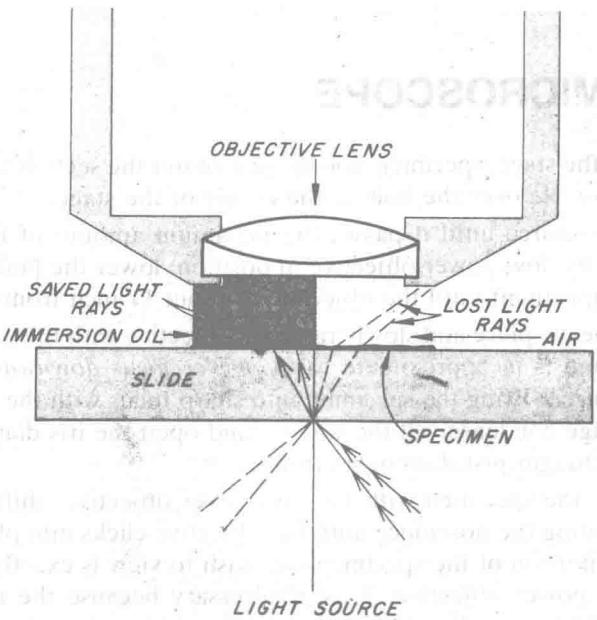

Figure I-3 How the oil-immersion objective increases the amount of light passing from the specimen into the objective lens.

The relationship between the wavelength of light used and the numerical aperture in determining resolving power holds only for parallel light rays. When the specimen is illuminated with oblique light rays in addition to direct light rays, the relationship becomes

$$\text{resolving power} = \frac{\text{wavelength}}{2\times \text{ numerical aperture}}$$

The substage condenser lens provides both oblique and direct illumination and further increases the resolution of a light microscope.

ILLUMINATION

Just as darkness or the glare of direct sunlight can blind vision, poor illumination can obscure the field of view of a microscope. Proper illumination is essential for the efficient utilization of the magnification and resolution of a microscope.

The readiest available source of illumination is ordinary daylight, but since the intensity of daylight varies greatly, artificial light sources (generally a tungsten lamp) are most often used. The most precise of such light sources control the intensity, color, and size of the light beam.

As shown in Figure I-1, the light from the illuminating source passes into the substage condenser. The size of the cone of light passing into a microscope differs with each objective. As the magnification of the objective lens increases, the working distance decreases (Figure I-2) and the angle of aperture of the objective increases. Therefore, with increasing magnification a larger cone of light must enter the objective. The size of the light cone is controlled by the iris diaphragm, located just above the substage condenser. When the low-power and high-dry objectives are used, which magnify 10 and 44 times respectively, the iris diaphragm is not opened fully since at these magnifications definition and detail are most clear when the light is not too intense. When the oil-immersion objective, which magnifies 95 times, is used, the working distance is the least and the iris diaphragm is opened more (Figure I-2).

In phase-contrast and dark-field microscopy, the systems of illumination differ from that just described. These topics will be discussed between Exercises 2 and 3.

USE OF THE MICROSCOPE

1. Place a slide on the stage, specimen side up, and center the section to be examined as accurately as possible over the hole in the center of the stage.
2. Adjust the light source until it passes the maximum amount of light through the specimen. With the low-power objective in position, lower the body tube by means of the coarse adjustment until the objective is about ¼ inch from the slide.
3. Look through the eyepiece and slowly raise the objective with the coarse adjustment until the specimen is in approximate focus. *Never focus downward while looking through the eyepiece.* Bring the specimen into sharp focus with the fine adjustment. Raise the substage condenser all the way up and open the iris diaphragm until the edge of the diaphragm just disappears from view.
4. After examining the specimen with the low-power objective, shift to the high-dry objective by rotating the nosepiece until the objective clicks into place, first making certain that the portion of the specimen you wish to view is exactly centered in the field of the low-power objective. This is necessary because the microscopic field diameter under high magnification is proportionately less than that under low magnification. *Caution:* Do not touch the lenses of the objectives.
5. The object should be nearly in focus. Look through the eyepiece and slowly adjust the focus with the coarse adjustment until the specimen comes into approximate

focus. Then bring the image into final accurate focus by using the fine adjustment. *Remember:* Never attempt to bring the specimen into focus by lowering the body tube while looking through the ocular. Always observe the objective lens from the side while bringing it toward the slide, and then focus by moving the objective lens away from the object on the slide.

6. Focusing of the oil-immersion objective requires more care than that of the other objectives, but the procedure is essentially the same. First use the low-power objective to locate the portion of the specimen to be examined. Be extremely careful to locate the portion in the exact center of the low-power field since the field diameter is much less with the oil-immersion objective than with either of the other objectives. Raise the body tube, and then rotate the nosepiece until the oil-immersion objective clicks into position. Now place a drop of immersion oil on the portion of the slide directly under the objective. Watching the objective from the side, carefully lower it into the oil. *Caution:* Do not allow the objective to touch the slide. Look through the ocular and slowly focus upward with the fine adjustment until the image appears. The image will come into view quickly since the working distance of the oil-immersion objective is relatively short. Once it appears, obtain the clearest possible image by critically focusing with the fine adjustment and by adjusting the light source and the iris diaphragm to obtain optimum illumination. If you have difficulty in bringing the image into view, move the stage adjustment while focusing. The motion will make it easier to perceive the image as it comes into focus.

   After each use of the oil-immersion objective, clean the oil from the objective lens with lens paper.

SPECIAL PRECAUTIONS

To keep the microscope and lens systems clean:

1. *Never touch the lenses.* If the lenses become dirty, wipe them gently with lens paper.
2. Never leave a slide on the microscope when it is not in use.
3. Always remove oil from the oil-immersion objective after its use. If by accident oil should get on either of the lower power objective, wipe it off immediately with lens paper. If oil becomes dried or hardened on a lens, you may remove it with lens paper lightly moistened with xylol. *Caution:* Too much xylol will dissolve the cement holding the lens.
4. Keep the stage of the microscope clean and dry. If any liquids are spilled, dry the stage with a piece of cheesecloth. If oil should get on the stage of the microscope, moisten a piece of cheesecloth with xylol, clean the stage, and wipe it dry.
5. Do not tilt the microscope when working with the oil-immersion system. The oil may flow under the mechanical stage system where it will be difficult to remove, or it may drip onto the substage condenser and harden there.
6. When the microscope is not in use, keep it covered and in the microscope compartment.

To avoid breaking the microscope:

1. Never force the microscope. All adjustments should work freely and easily. If anything does not work correctly, do not attempt to fix it yourself, but immediately call your instructor.
2. Never allow an objective lens to touch the cover glass or the slide.
3. Never lower the body tube with the coarse adjustment while you are looking through the microscope.

4. Never exchange the objectives or oculars of different microscopes, and never under any circumstances remove the front lenses from objectives.
5. Store your microscope when not in use in its cabinet. Before storing it, rotate the nosepiece with the low-power objective into position, and be sure the mechanical stage does not extend beyond the edge of the microscope stage.

1 Examination of Natural Infusions

If the human eye could magnify to the same degree as does the compound microscope, microorganisms would be readily visible practically anywhere. To prove this point let us examine some natural materials known to contain large numbers of microbes—a hay infusion, rumen fluid, or water from a stagnant pond.

In this exercise you will use only the low-power and high-dry objectives, and so the numbers and types of microorganisms you will see are limited by the magnification of these objectives.

PROCEDURE

Make a wet-mount preparation by placing a loopful of the sample provided on a slide and covering it with a coverslip. Apply the coverslip at one edge; then let it drop onto the culture so air bubbles are not incorporated. Examine the preparation under the 16-mm and 4-mm objectives.

Most of the microorganisms you will see will be protozoa. Can you identify representatives of the three main groups of protozoa—amoebae, ciliates, and flagellates? By observing closely, you may be able to detect some smaller moving bodies. They will be forms of larger bacteria. Make drawings on the report sheet of what you see.

Like all small particles, nonmotile microorganisms will exhibit **Brownian movement,** a motion resulting from collision with the molecules of the surrounding fluid. Thus, the aimless bouncing of cells for short distances is Brownian movement, not true motility. Though you will sometimes see spinning or rolling, motile organisms usually move for considerable distances in given directions, independently of other organisms.

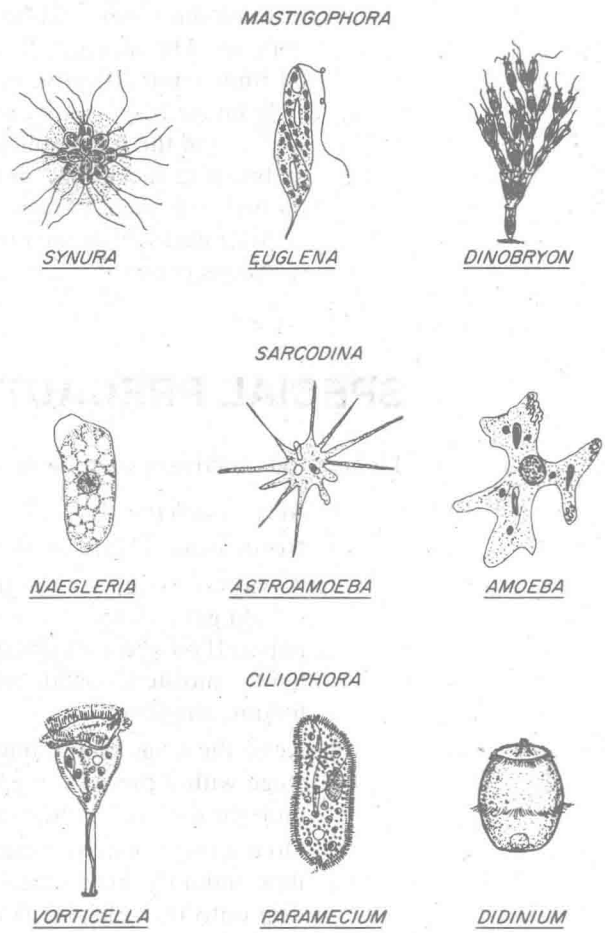

Figure 1-1 Some types of protozoa.

QUESTIONS

1. What classic experiments in microbiology does this exercise resemble?
2. In these classic experiments what natural infusions were studied and what microbes were observed?
3. What is the size range of the protozoa?
4. Why were the majority of microorganisms that you saw protozoa?