Chapter 4 The Microscopic Examination of Microorganisms

OUTLINE **Microscopes and Microscopy**
Bright-Field Microscopy • Dark-Field Microscopy • Fluorescence Microscopy • Phase-Contrast Microscopy • Transmission Electron Microscopy • Scanning Electron Microscopy

Limitations of Electron Microscopy

Preparations for Light-Microscope Examinations
The Wet-Mount and Hanging-Drop Techniques • Fixed, Stained Smears

The **microscope** is the instrument most characteristic of the microbiology laboratory. The **magnification** it provides enables us to see microorganisms and their structures otherwise invisible to the naked eye. The magnifications attainable by microscopes range from X100 to X400,000. In addition, several different kinds of microscopy are available, and many techniques have been developed by which specimens of microorganisms can be prepared for examination. Each type of microscopy and each method of preparing specimens for examination offers advantages for demonstration of specific morphological features. In this chapter we shall describe some of the microbiologists' methods for observing the morphological characteristics of microorganisms. The techniques used to make these examinations are provided in the laboratory manual.

MICROSCOPES AND MICROSCOPY

Microscopes are of two categories, **light** (or **optical**) and **electron**, depending upon the principle on which magnification is based. **Light microscopy**, in which magnification is obtained by a system of optical lenses using light waves, includes: (1) **bright-field**, (2) **dark-field**, (3) **fluorescence**, and (4) **phase-contrast** microscopy. The **electron microscope**, as the name suggests, uses a beam of electrons in place of light waves to produce the image. Specimens can be examined by either **transmission** or **scanning** electron microscopy.

In a first microbiology course, students perform most of their examinations, if not all, with the bright-field microscope. This is the most widely used instrument for routine microscopic work. The other types of microscopy are used for special purposes or research investigations. However, students should be acquainted with their applications, since each has some unique feature that is useful for demonstrating particular structures of the cell.

Figure 4-1. A stained preparation of bacteria as seen by bright-field microscopy.

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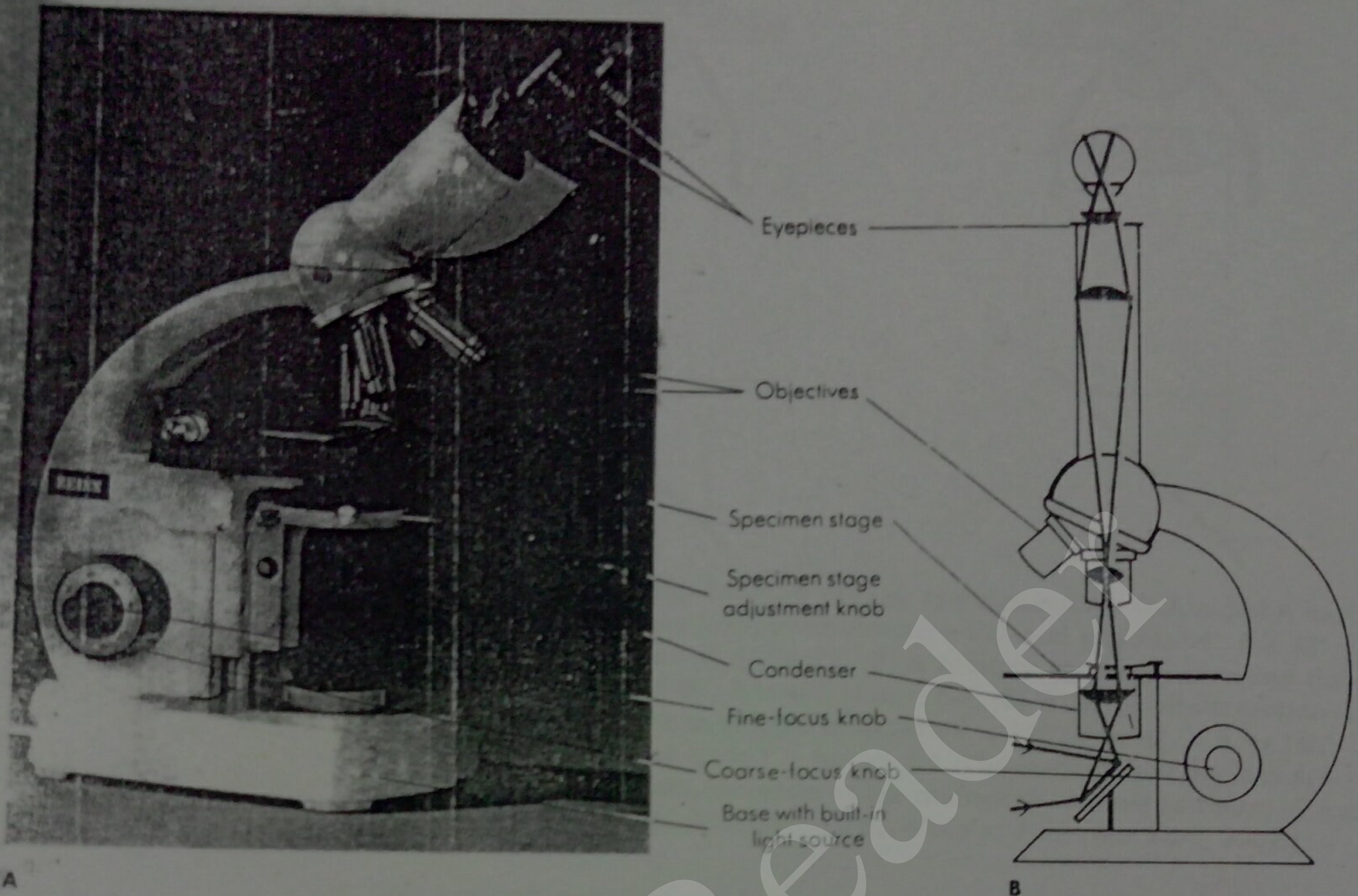

Figure 4-2. The student microscope. (A) Identification of parts. (B) Cutaway sketch of student microscope showing optimal parts and path of light. *(Courtesy of Carl Zeiss, New York.)*

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Bright-Field Microscopy

In bright-field microscopy, the microscopic field (the area observed) is brightly lighted and the microorganisms appear dark because they absorb some of the light. Ordinarily, microorganisms do not absorb much light, but staining them with a dye greatly increases their light-absorbing ability (Fig. 4-1), resulting in greater contrast and color differentiation. The optical parts of a typical bright-field microscope and the path the light rays follow to produce enlargement, or magnification, of the object are shown in Fig. 4-2. Generally microscopes of this type produce a useful magnification of about X1,000 to X2,000. At magnifications greater than X2,000 the image becomes fuzzy for reasons we will explain now.

Resolving Power

The basic limitation of the bright-field microscope is one not of magnification but of **resolving power,** the ability to distinguish two adjacent points as distinct and separate. Mere increase in size (greater magnification) without the ability to distinguish structural details (greater resolution) is not beneficial. To state it differently, the largest magnification produced by a microscope may not be the most useful because the image obtained may be unclear or fuzzy. The more lines

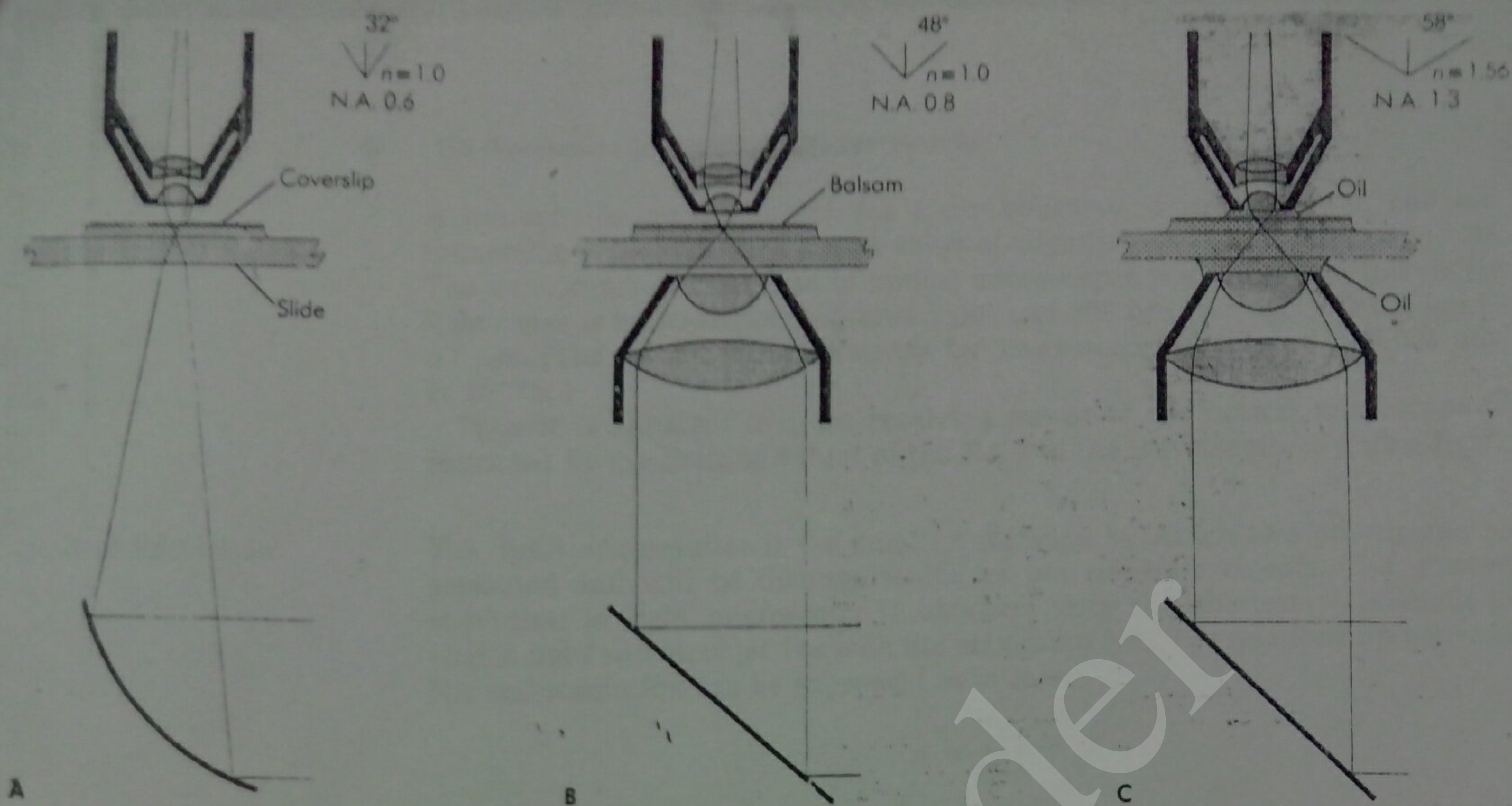

Figure 4-3. The relationship between angular aperture and resolution. (A) A narrow cone of light enters the low-power objective; the total angle is 64°, θ is 32°, and the numerical aperture (N.A.) is 0.6. (B) A substage condenser increases the size of the cone of light to 96°; θ is 48°, N.A. is 0.8. (C) With the oil-immersion objective, the size of the cone of light is increased to 116°; θ is 58°, N.A. is 1.3. The refractive index (*n*) for air is 1.0; for oil, 1.3. The resolution of the lens system, as described in the text, increases as the numerical aperture increases. *(From P. Gray, Handbook of Basic Microtechnique, McGraw-Hill, New York, 1964.)*

or dots per unit area that can be seen distinctly as separate lines or dots, the greater is the resolving power of the microscope system. The resolving power of a microscope is a function of the wavelength of light used and the numerical aperture (NA) of the lens system.

Numerical Aperture

The angle θ subtended by the optical axis and the outermost rays still covered by the objective is the measure of the **aperture** of the objective; it is the **half-aperture angle** (Fig. 4-3). The magnitude of this angle is expressed as a sine value. The sine value of the half-aperture angle multiplied by the refractive index *n* of the medium filling the space between the front lens and the cover slip gives the numerical aperture (NA): $NA = n \sin \theta$

With dry objectives the value of *n* is 1, since 1 is the refractive index of air. When immersion oil is used (Fig. 4-3) as the medium, *n* is 1.56, and if θ is 58°, then

$$NA = n \sin \theta = 1.56 \times \sin 58° = 1.56 \times 0.85 = 1.33$$

The degree to which microscope objectives can be altered to increase the NA

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is limited: the maximum NA for a dry objective is less than 1.0, and oil-immersion objectives have an NA value of slightly greater than 1.0 (1.2 to 1.4). The wavelength of light used in optical microscopes is also limited; the visible light range is between 400 nm (blue light) and 700 nm (red light), or 0.4 μm to 0.7 μm. (The abbreviation *nm* stands for *nanometer* and is equal to 0.001 μm, or 10^{-9}m.)

Thus it is apparent that the resolving power of the optical microscope is restricted by the limiting values of the NA and the wavelength of visible light.

Limit of Resolution

The *limit of resolution* is the smallest distance by which two objects can be separated and still be distinguishable as two separate objects. The greatest resolution in light microscopy is obtained with the shortest wavelength of visible light and an objective with the maximum NA. The relationship between NA and resolution can be expressed as follows:

$$d = \frac{\lambda}{2NA}$$

where d = resolution and λ = wavelength of light. Using the values 1.3 for NA and 0.55 μm, the wavelength of green light, for λ, resolution can be calculated as

$$d = \frac{0.55}{2 \times 1.30} = 0.21\ \mu m$$

From these calculations we may conclude that the smallest details that can be seen by the typical light microscope are those having dimensions of approximately 0.2 μm.

Magnification

Magnification beyond the resolving power is of no value since the larger image will be less distinct in detail and fuzzy in appearance. The situation is analogous to that of a movie screen: if we move closer to the screen the image is larger but is also less sharp than when viewed at a distance.

Most laboratory microscopes are equipped with three objectives, each capable of a different degree of magnification. These are referred to as the *oil-immersion*, *high-dry*, and *low-power objectives*. The primary magnification provided by each objective is engraved on its barrel. The total magnification of the system is determined by multiplying the magnifying power of the objective by that of the eyepiece. Generally, an eyepiece having a magnification of X10 is used, although eyepieces of higher or lower magnifications are available.

Dark-Field Microscopy

The effect produced by the dark-field technique is that of a dark background against which objects are brilliantly illuminated. This is accomplished by equipping the light microscope with a special kind of condenser that transmits a hollow cone of light from the source of illumination, as shown in Fig. 4-4. Most of the light directed through the condenser does not enter the objective; the field is essentially dark. However, some of the light rays will be scattered (diffracted) if the transparent medium contains objects such as microbial cells. This diffracted light will enter the objective and reach the eye; thus the object or microbial cell, in this case, will appear bright in an otherwise dark micro-

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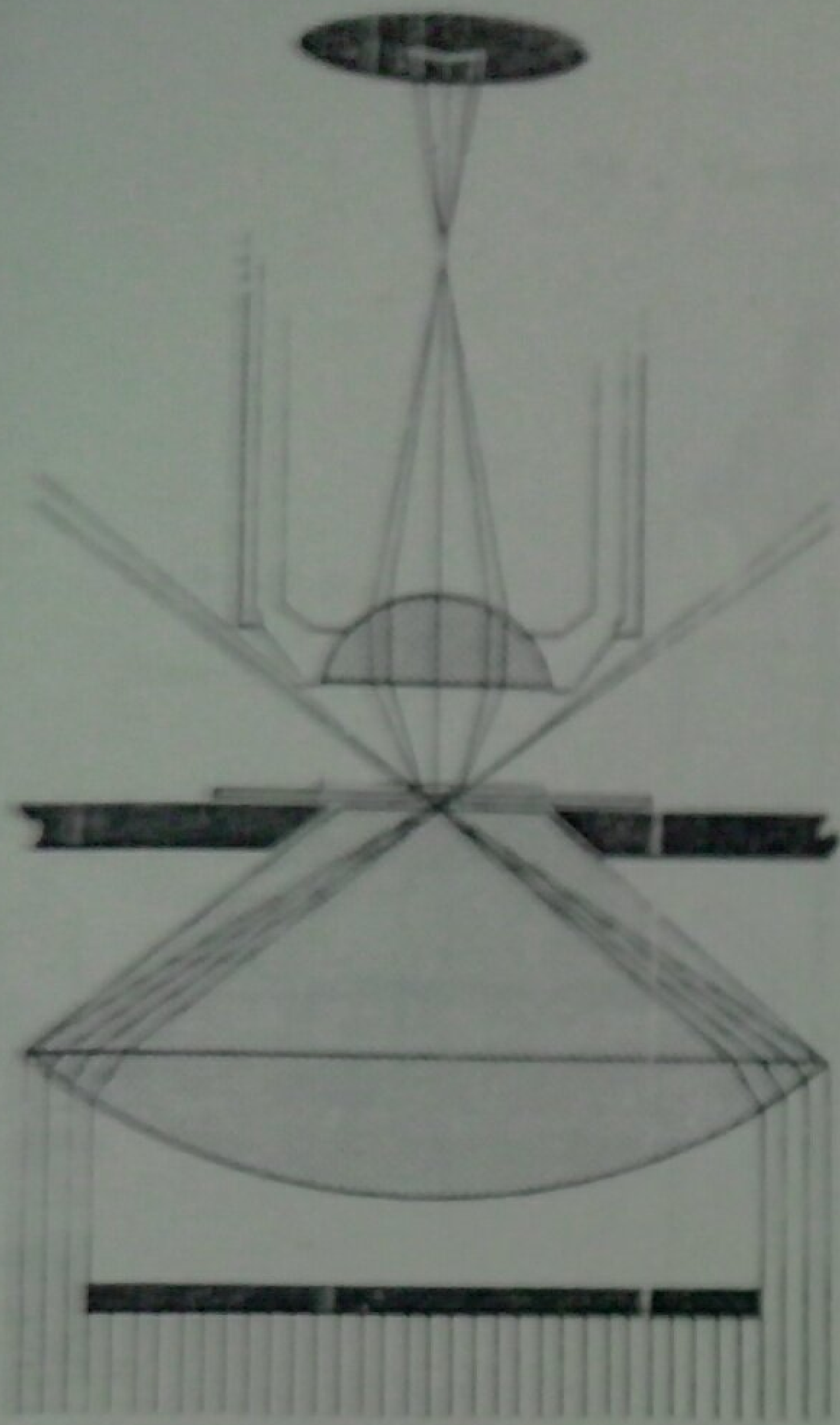
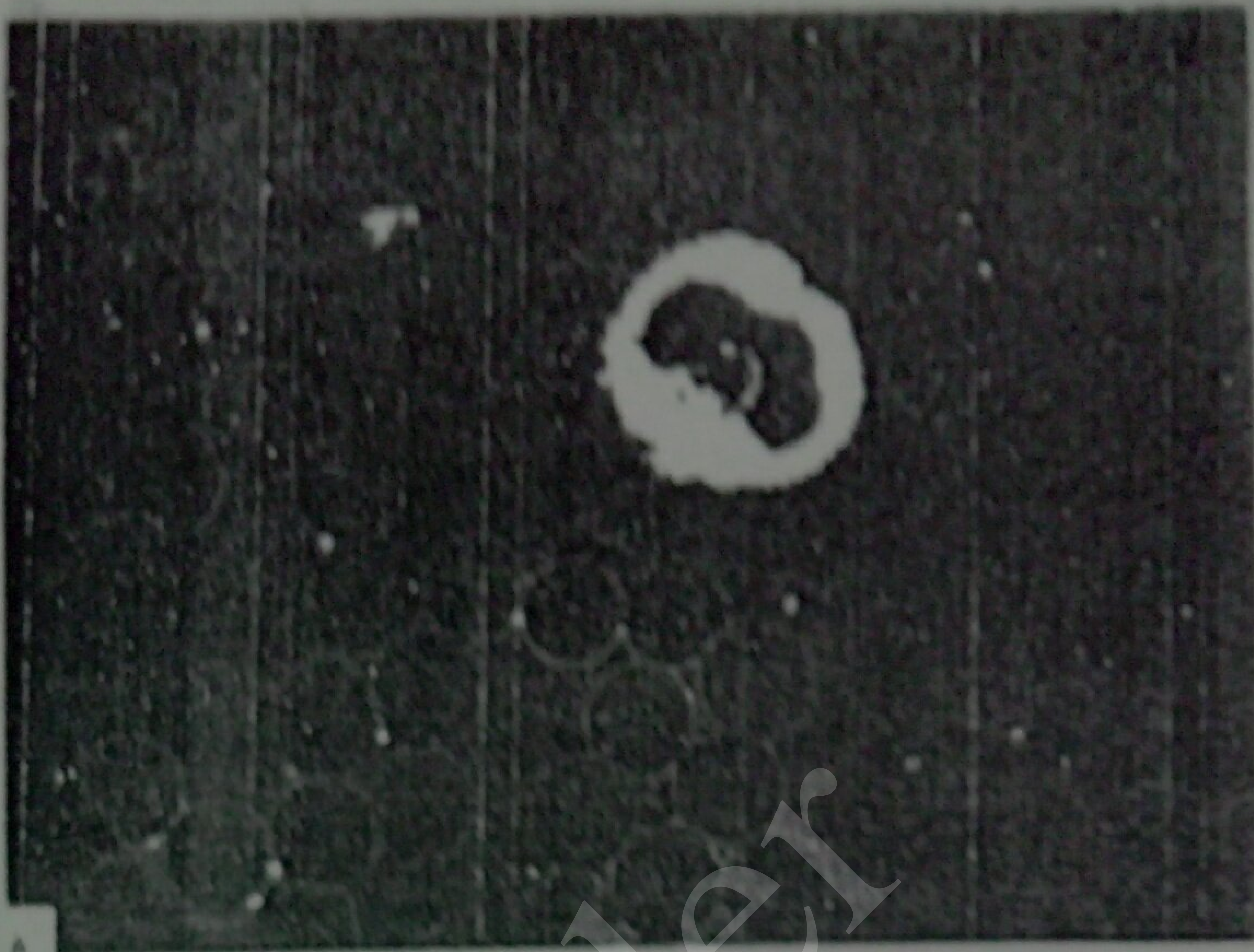


Figure 4-4. Path of light through a dark-field microscope system. Note that only those light waves which strike an object in the microscopic field are "bent" toward the observer's eye. *(Erwin F. Lessel, illustrator.)*



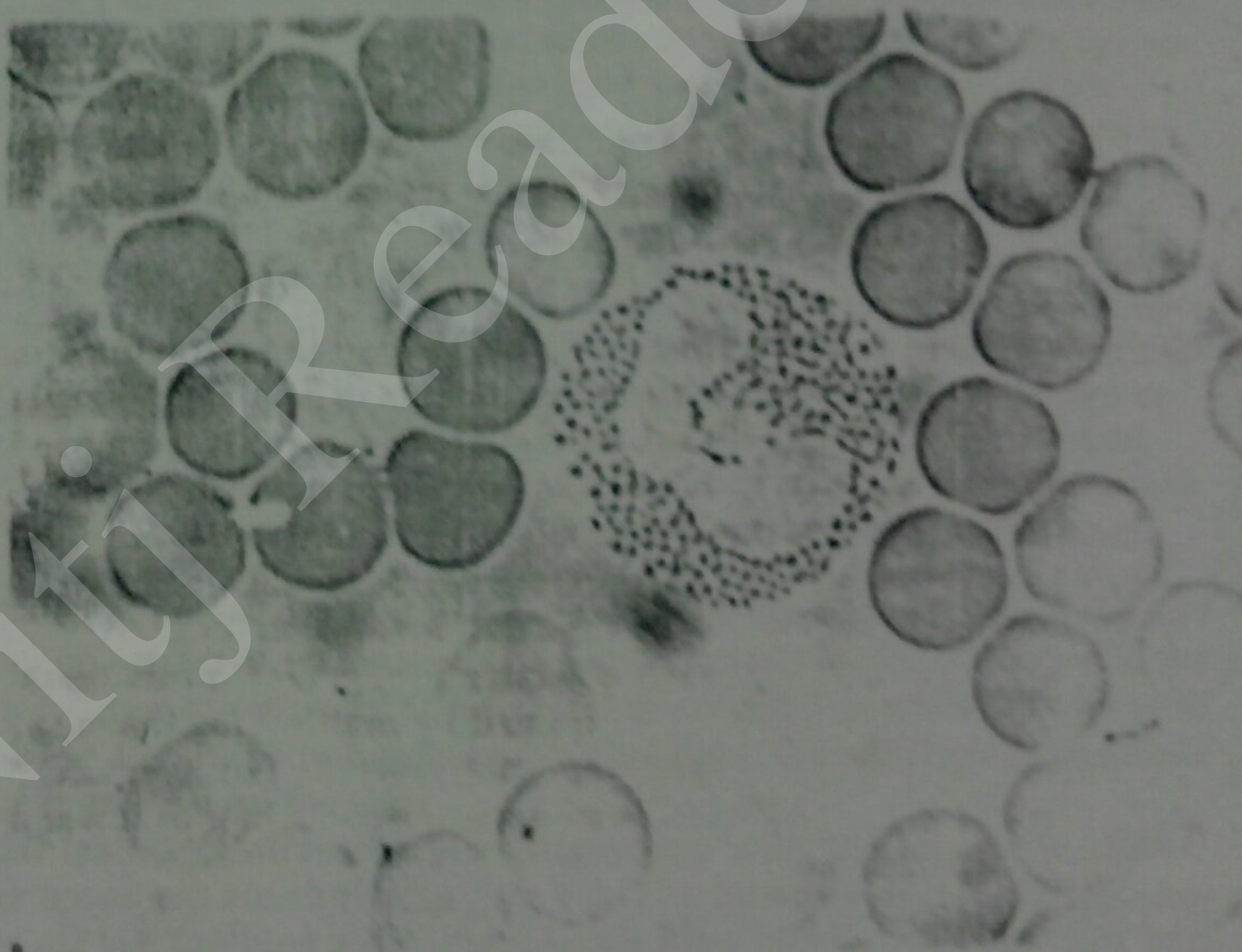


Figure 4-5. Dark-field and bright-field microscopy. The appearance of a white blood cell (eosinophil) surrounded by red blood cells, as viewed by (A) dark-field and (B) bright-field microscopy. *(From Scope, courtesy of The Upjohn Company.)*

scopic field (Fig. 4-5). Dark-field microscopy is particularly valuable for the examination of unstained microorganisms suspended in fluid—wet-mount and hanging-drop preparations.

Fluorescence Microscopy

Many chemical substances absorb light. After absorbing light of a particular wavelength and energy, some substances will then emit light of a longer wave-

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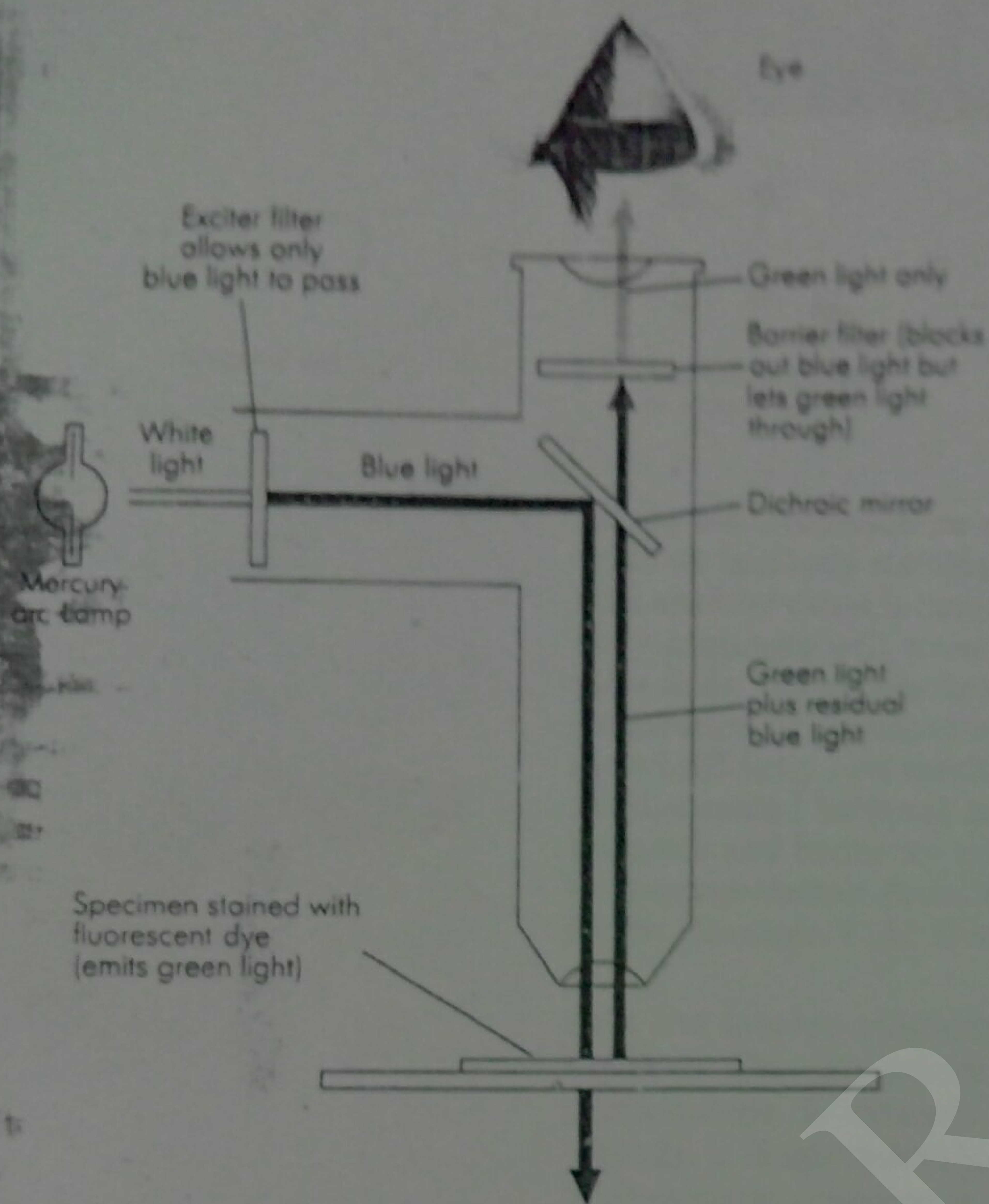

Figure 4-6. The special features of fluorescence microscopy. A high-intensity mercury lamp is used as the light source and emits white light. The exciter filter transmits only blue light to the specimen and blocks out all other colors. The blue light is reflected downward to the specimen by a dichroic mirror (which reflects light of certain colors but transmits light of other colors). The specimen is stained with a fluorescent dye: certain portions of the specimen retain the dye, others do not. The stained portions absorb blue light and emit green light, which passes upward, penetrates the dichroic mirror, and reaches the barrier filter. This filter allows the green light to pass to the eye; however, it blocks out any residual blue light from the specimen which may not have been completely deflected by the dichroic mirror. Thus the eye perceives the stained portions of the specimen as glowing green against a jet black background, whereas the unstained portions of the specimen are invisible. (Erwin F. Lessel, illustrator.)

length and a lesser energy content. Such substances are called **fluorescent** and the phenomenon is termed **fluorescence.** Application of this phenomemon is the basis of fluorescence microscopy. In practice, microorganisms are stained with a fluorescent dye and then illuminated with blue light; the blue light is absorbed and green light emitted by the dye.

The special features of fluorescence microscopy with the respect to illumination of the specimen are shown in Fig. 4-6. The function of the **exciter filter** is to remove all but the blue light; the **barrier filter** blocks out blue light and allows green light (or other light emitted by the fluorescing specimen) to pass through and reach the eye. Barrier filters are selected on the basis of the dye used.

An example of direct staining of bacteria with a fluorescent dye is shown in Fig. 4-7.

The Fluorescent Antibody Technique—Immunofluorescence

It is possible to chemically combine fluorescent dyes with antibodies, i.e., substances that combine with specific microorganisms. Antibodies to which a fluorescent dye is attached are referred to as **labeled antibodies.** Thus labeled antibodies can be mixed with a suspension of bacteria and then the preparation examined by fluorescent microscopy. The bacterial cells that have combined

Figure 4-7. *Mycobacterium tuberculosis* in a sputum specimen stained with fluorescent dye shows up a bright bacillus. (Courtesy Center for Disease Control, Atlanta, Ga.)

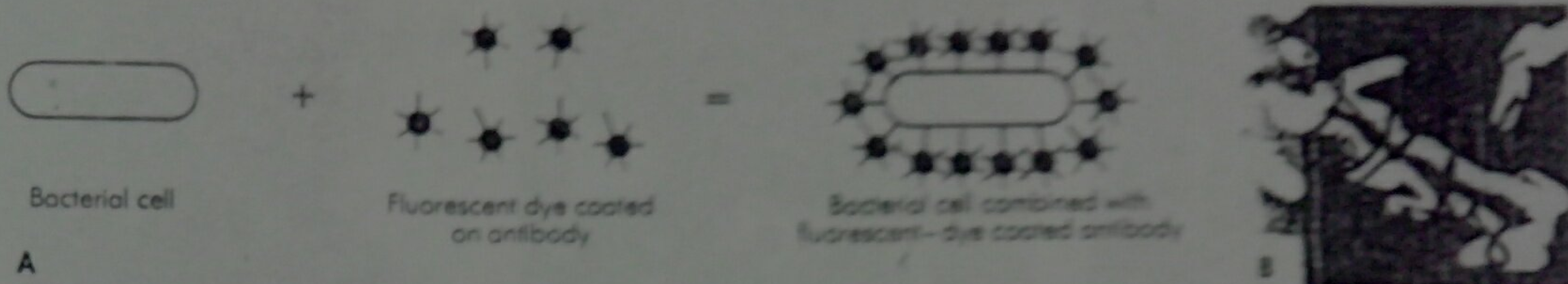

Figure 4-8. Fluorescence staining technique and microscopy. (A) The direct fluorescent antibody staining technique. When a bacterial cell is incubated with spe cific antibody that is conjugated (combined) with a fluorescent dye, the dye–ant body conjugate will cover the surface of the cell. The technique is performed on a glass slide, the excess fluorescent dye–antibody conjugate is washed off, and the preparation is examined by ultraviolet light microscopy. The bacterial cell will glow brilliantly as a result of fluorescence caused by the ultraviolet illumination o the dye-coated bacterial cell. Any bacterial cells not covered by the dye do not fluoresce and hence are not visible by this technique. (B) Photomicrograph of a fluorescent-stained *Proteus mirabilis* preparation as described above. (Courtesy of Judith Hoeniger, F. M. Clinits, and E. A. Clinits, *J. Bacteriol*, 98:226, 1969.)

with the labeled antibody will be visible in the microscopic preparation (se Fig. 4-8). This procedure is known as the **fluorescent antibody technique**; th phenomenon is termed **immunofluorescence**. Theoretically, it is possible t identify a **single microbial cell** by this procedure. The application of this tes in diagnostic procedures is discussed in Chap. 34.

Phase-Contrast Microscopy

Phase-contrast microscopy is extremely valuable for studying living unstainec cells and is widely used in applied and theoretical biological studies. It uses a conventional light microscope fitted with a phase-contrast objective and a phase-contrast condenser. This special optical system makes it possible to distinguish unstained structures within a cell which differ only slightly in their refractive indices or thicknesses.

In principle, this technique is based on the fact that light passing through one material and into another material of a slightly different refractive index and/or thickness will undergo a change in **phase**. These differences in phase, or wave-front irregularities, are translated into variations in brightness of the structures and hence are detectable by the eye.

With phase-contrast microscopy it is possible to reveal differences in cells and their structures not discernible by other microscopic methods. A compari-

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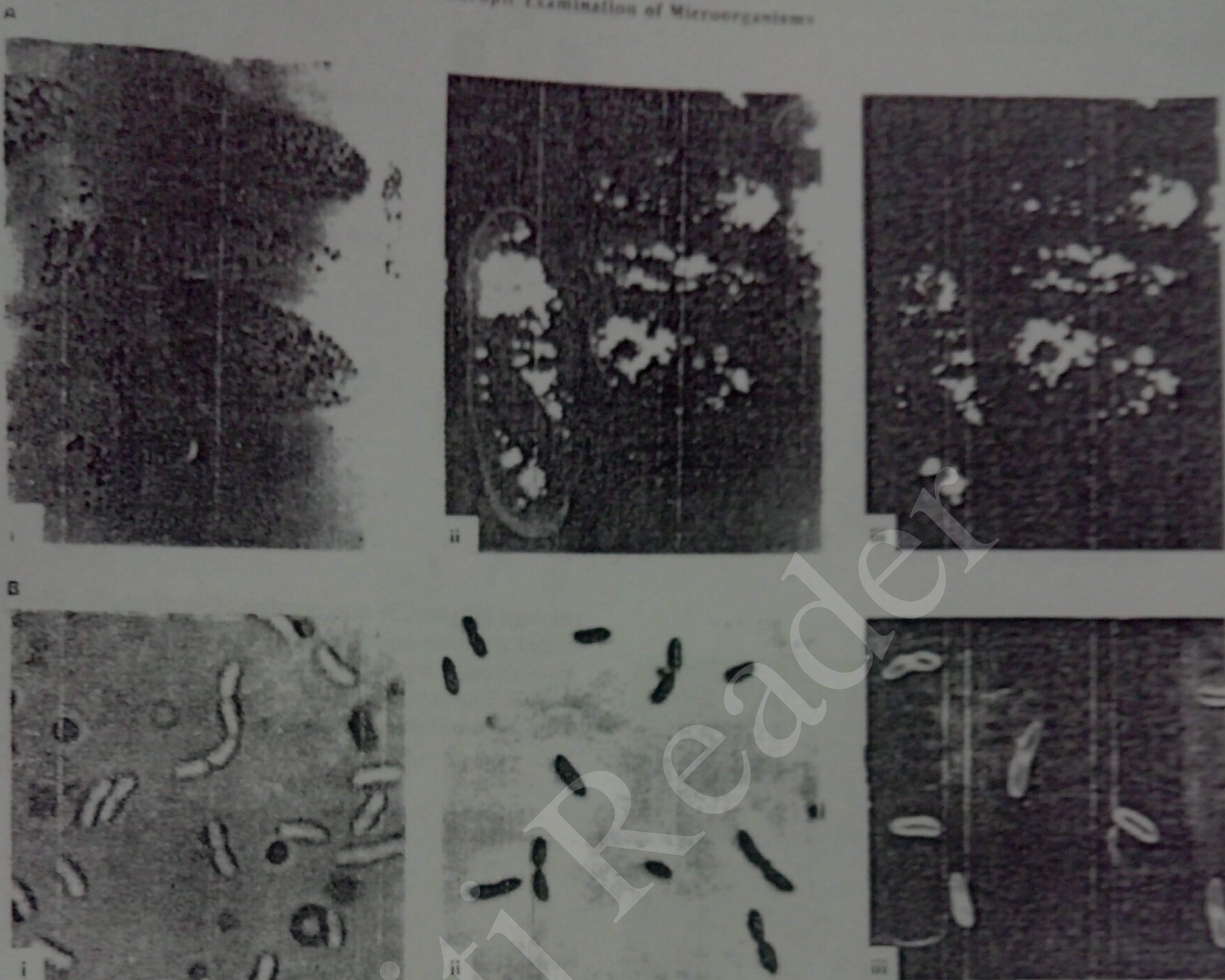

Figure 4-9. (A) Phase-contrast microscopy compared with bright-field and dark-field microscopy. The same specimen of a protozoan as seen by each method: (i) bright-field; (ii) phase-contrast; (iii) dark-field. *(Courtesy of O. W. Richards, Research Department, American Optical Company.)* (B) Photomicrographs of living unstained, rod-shaped cells of *Pseudomonas fluorescens*. The bacilli are 0.7 to 0. μm in width. They can be seen only indistinctly by ordinary bright-field microscopy (i) but are readily visible by phase-contrast (ii) or dark-field microscopy (iii *(Courtesy N. R. Krieg.)*

son of a specimen viewed by bright-field, dark-field, and phase-contrast micro copy is shown in Fig. 4-9.

Transmission Electron Microscopy

Electron microscopy differs markedly and in many respects from the optic microscopic techniques. The electron microscope provides tremendous usef magnification, because of the much higher resolution obtainable with the

Light microscope (upside down)
Light source
Condenser lens
Specimen
Objective lens
Projection lens or eye piece
Final image
A

Transmission electron microscope
Electron gun
Condenser lens
First aperture
Specimen
Objective lens
Second aperture
Intermediate lens
Projection lens
Final aperture
Final image
B

Scanning electron microscope
Electron gun
First aperture
First condenser lens
Second aperture
Second condenser lens
Third aperture
Scanning coils
Final condenser lens
Final aperture
Electron collector
Signal for electronic image formation
Specimen
C

Figure 4-10. Diagrammatic comparison of imaging systems in (A) optical microscope, (B) transmission electron microscope, and (C) scanning electron microscope. *(Courtesy of L. A. Bulla, Jr., G. St. Julian, C. W. Hesseltine, and F. L. Baker, Scanning Electron Microscopy, in Methods in Microbiology, vol. 8, Academic, New York, 1973.)*

Figure 4-11. A high-resolution electron microscope. *(Courtesy of George Hatjygeorge, Fordham University.)*

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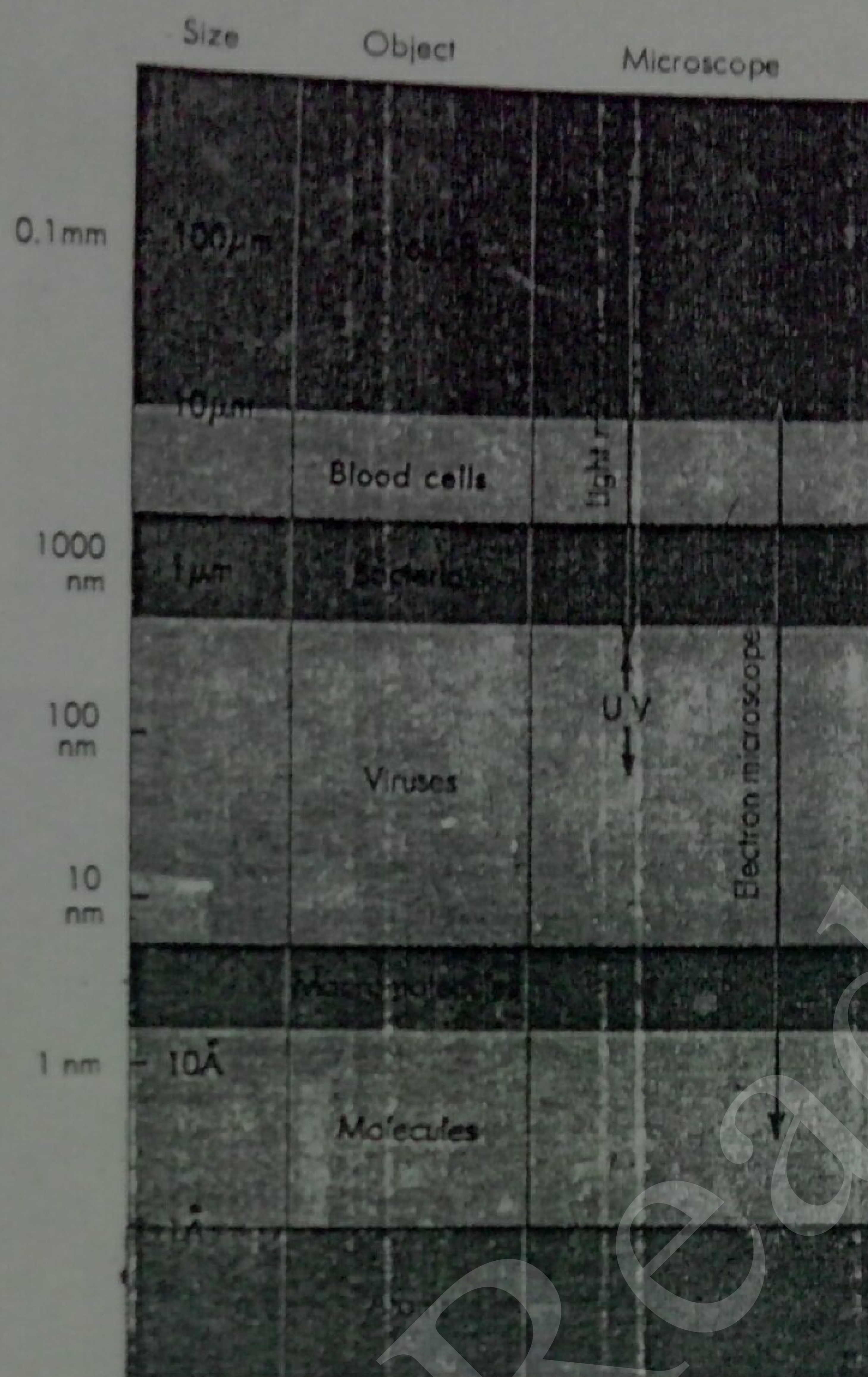

Figure 4-12. Relative size of microbes, molecules, and atoms is depicted here, together with an indication of the useful range of different types of microscopes. *(Courtesy of A. J. Rhodes and C. E. van Rooyen, Textbook of Virology, Williams & Wilkins, Baltimore, 1968.)*

tremely short wavelength of the electron beam used to magnify the specimen As shown in Fig. 4-10, the electron microscope uses electron beams and magnetic fields to produce the image, whereas the light microscope uses light waves and glass lenses.

With an electron microscope (Fig. 4-11) employing 60- to 80-kV electrons the wavelength is only 0.05 Å. Å is the abbreviation for **angstrom;** 1 Å equals 1/100,000,000 (10^{-8}) cm or 1/10,000 (10^{-4}) μm. (Compare this electron wavelength with the light wavelengths used for optical microscopes.) It is possible to resolve objects as small as 10 Å (Fig. 4-12). The resolving power of the electron microscope is more than *100 times* that of the light microscope, and it produces useful magnification up to ×400,000.

For electron microscopy, the specimen to be examined is prepared as an extremely thin dry film on small screens and is introduced into the instrument at a point between the magnetic condenser and the magnetic objective; this point is comparable to the stage of the light microscope. The magnified image may be viewed on a fluorescent screen through an airtight "window" or recorded on a photographic plate by a camera built into the instrument.

Numerous techniques are available for use with electron microscopy which extend its usefulness in characterizing cellular structure. Some of these are described below.

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Shadow-casting

This technique involves depositing an extremely thin layer of metal (e.g., platinum) at an oblique angle on the organism so that the organism produces a shadow on the uncoated side. The shadowing technique produces a topographical representation of the surface of the specimen (see Fig. 4-13).

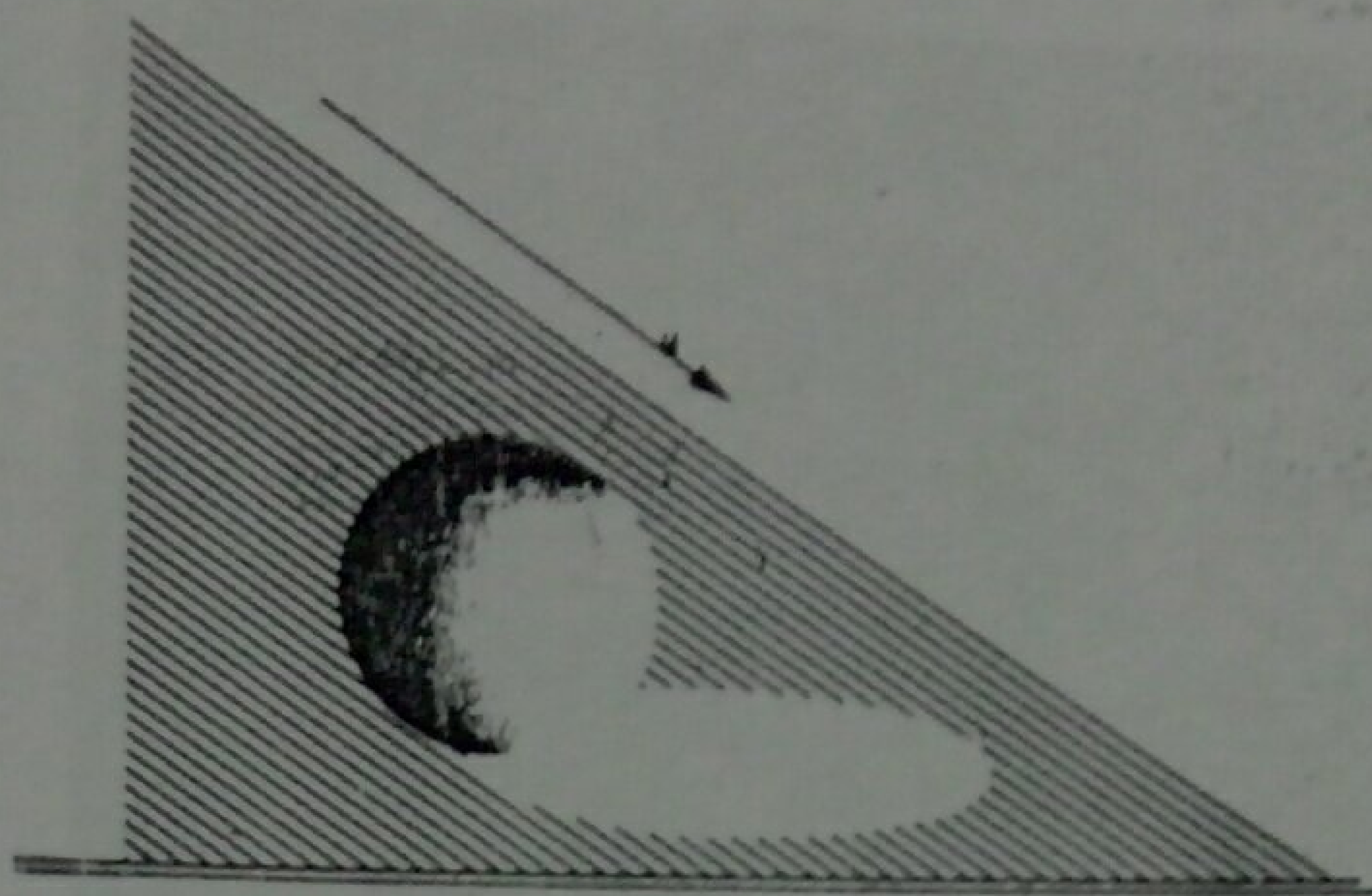

Figure 4-13. Shadow-casting technique. The specimen is dried on a special grid which is placed in a vacuum jar. Atoms of a heavy metal such as platinum are projected (from a highly heated filament) at an angle that produces a "shadow" behind the particles being examined. Examination of the shadowed image provides information as to the shape of the specimen particles. *(Erwin F. Lessel, illustrator.)*

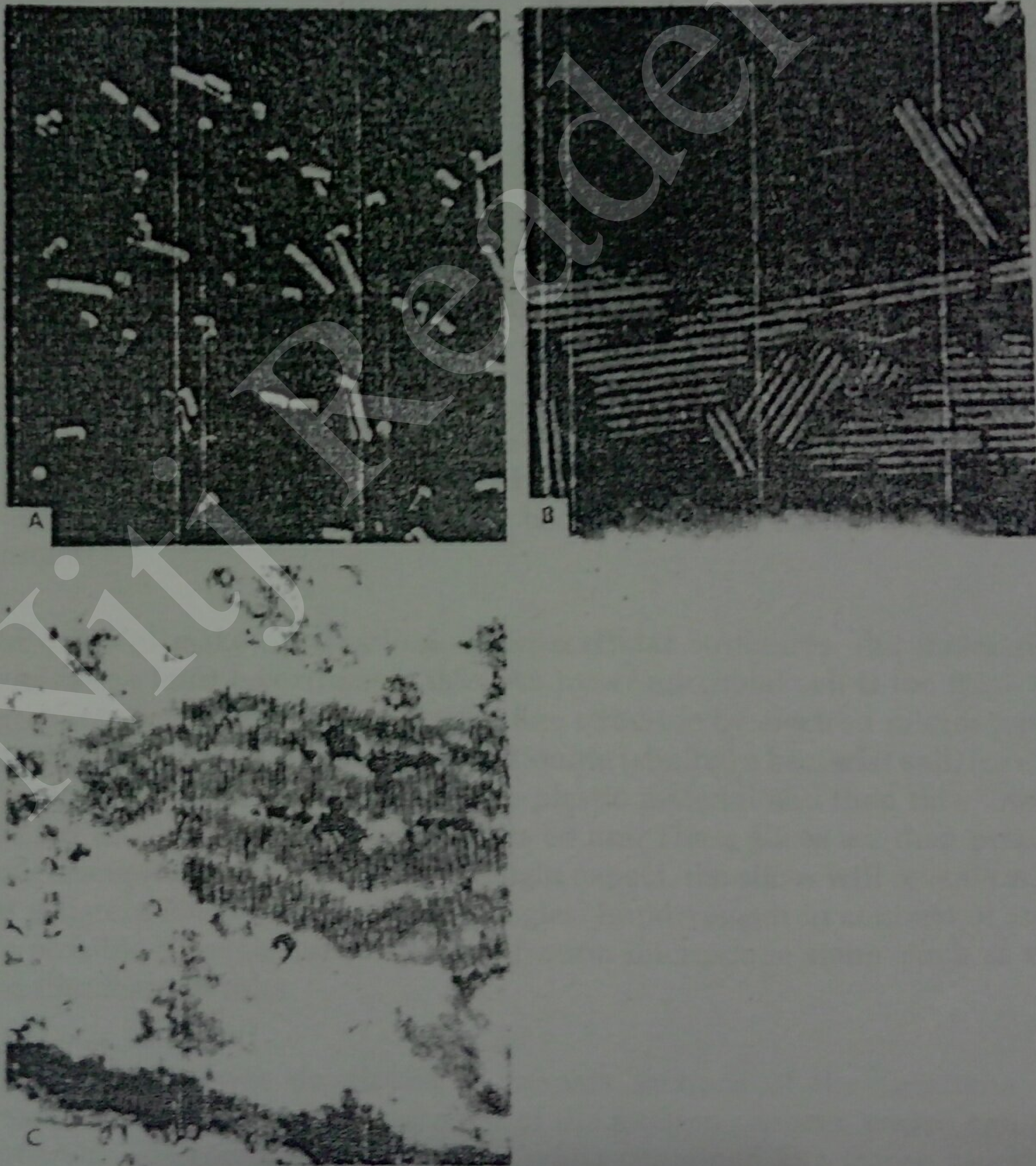

Figure 4-14. Electron micrographs of tobacco rattle virus as seen in three different preparations (A, B, and C). This virus characteristically appears as particles of two different sizes; the larger particle measures 184 by 25 nm and the smaller particle measures 74 by 25 nm. (A) Shadow-cast preparation using chromium. (B) Negative-stain preparation using potassium phosphotungstate. (C) Ultrathin section of infected leaf showing intracellular virus crystals, stained with uranyl acetate and lead citrate. *(Courtesy of M. Kenneth Corbett.)*

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Figure 4-15. Electron micrograph of freeze-etched preparation of *Neisseria gonorrheae*. This bacterium measures approximately 0.6 to 1.0 μm in diameter. (*Courtesy of Ivan L. Roth.*)

Negative Staining

An electron-dense material such as phosphotungstic acid can be used as a "stain" to outline the object. The electron-opaque phosphotungstate does not penetrate structures but forms thick deposits in crevices (see Fig. 4-14). Fine detail of objects such as viruses or bacterial flagella can be seen by this technique.

Ultrathin Sectioning

In order to make observations of intracellular structures, the material for examination must be extremely thin. An intact microbial cell is too thick to allow distinct visualization of its internal fine structure by electron microscopy. However, techniques are available for sectioning (slicing) a bacterial cell; for example, bacterial cells can be embedded in a plastic material and then this "block" can be cut into ultrathin slices, as thin as 60 nm. These slices are then prepared for microscopic examination. As you might expect, the slices will reveal cells sliced at different levels and at different angles. Improvement in contrast of structures is possible through use of special electron-microscope stains such as uranium and lanthanum salts.

Freeze-Etching

Freeze-etching was developed to prepare sections of the specimen without resorting to the chemical treatment of the fixation process, which can produce artifacts. The specimen is sectioned while contained in a frozen block. Carbon replicas of these exposed surfaces are then prepared which reveal internal structures of the cell (see Fig. 4-15).

Localization of Cell Constituents

Special techniques have been developed making it possible to locate chemical constituents of the cell. For example, thin sections of a cell can be treated with

ferritin-labeled antibody. Ferritin is an iron-containing substance of high density that markedly affects passage of the electron beam. The combination of this ferritin-labeled antibody with antigen in the cell produces a complex which manifests a higher contrast in the electron-microscope image.

Localization of Enzymes in Thin Sections

Electron-microscope techniques have been developed to locate the position of enzymes within cells. The intracellular localization of the enzyme isocitrate dehydrogenase of *Escherichia coli* is shown in Fig. 4-16. This was accomplished by first preparing ultrathin sections of the bacterial cells, followed by an immunochemical technique by which colloidal gold is affixed specifically to the isocitrate dehydrogenase enzyme.

Autoradiography

Autoradiography is a cytochemical method in which the location of a particular chemical constituent in a specimen is determined by observing the site at which radioactive material becomes positioned. The cells are first exposed to the radioactive substance to permit its uptake. In practice, the specimen prepared for microscopic examination is covered with a layer of photographic emulsion and stored in the dark for a period of time. The ionizing radiation emitted during the decay of the radioactive substance produces latent images in the emulsion, and, after photographic processing, the developed image is seen as grains of silver in the preparation.

Scanning Electron Microscopy

In scanning electron microscopy the specimen is subjected to a narrow electron beam which rapidly moves over (scans) the surface of the specimen. This causes the release of a shower of secondary electrons and other types of radiation from

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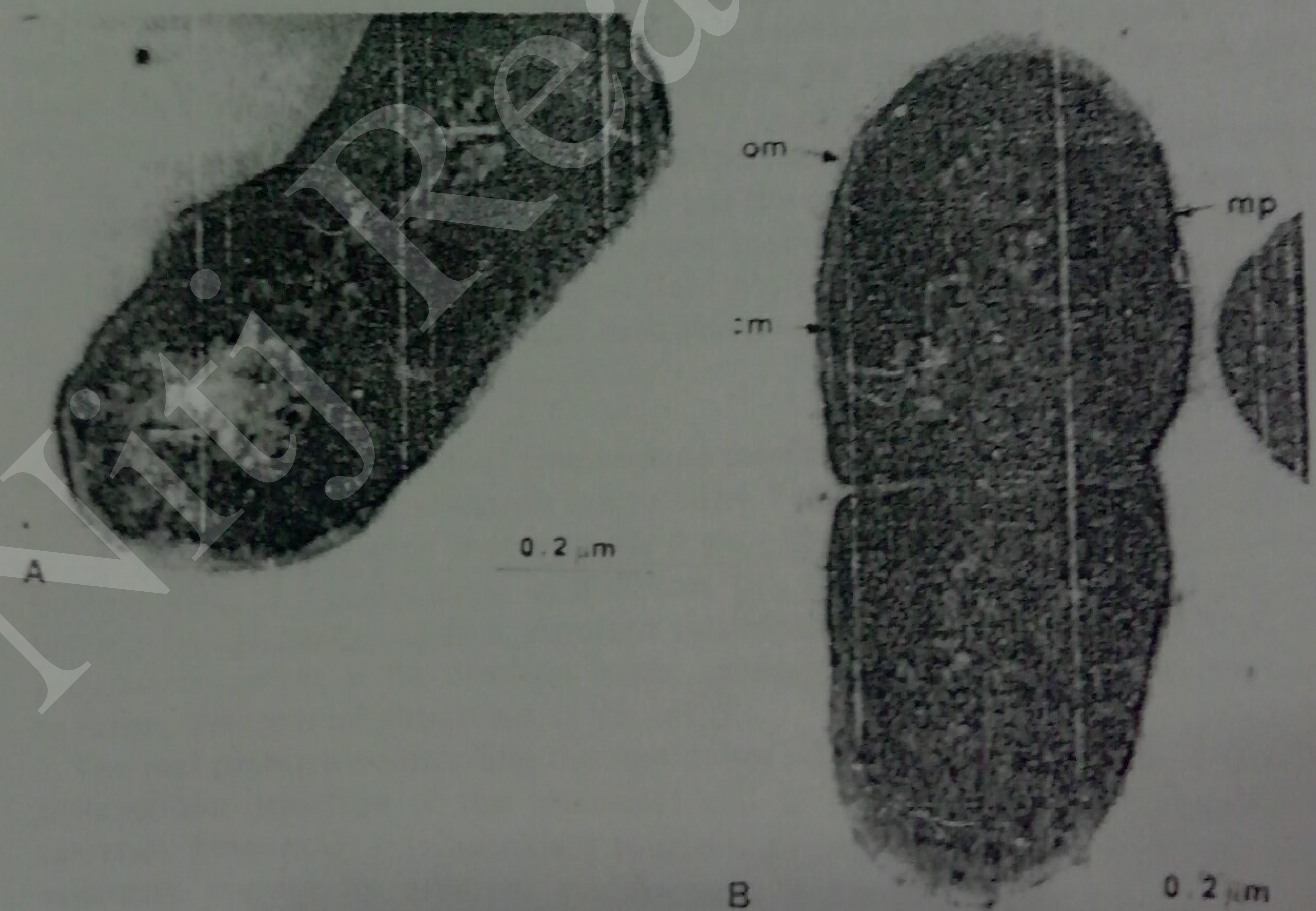

Figure 4-16. Intracellular localization of isocitrate dehydrogenase in *Escherichia coli* as seen by electron microscopy. (A) Ultrathin section of *E. coli* treated with a specific fraction of antiserum to the enzyme and then with a protein–gold particle complex which reacts with the antiserum. Location of gold particles and hence the enzyme is shown by arrows. (B) Control section treated with preimmune serum and protein–gold, showing outer membrane (om), peptidoglycan (mp), and cytoplasmic membrane (cm). (*Courtesy of J. R. Swafford et al., Science* **121**:295, 1983.)

Figure 4-17. Scanning electron micrograph of cells of *Pseudomonas aeruginosa*. The average size of this bacterium is 0.5 to 1.0 μm by 1.5 to 4 μm. (Courtesy of David Greenwood.)

the specimen surface. The intensity of these secondary electrons depends on the shape and the chemical composition of the irradiated object. The secondary electrons are collected by a detector which generates an electronic signal. These signals are then scanned in the manner of a television system to produce an image on a cathode ray tube. The image system for the scanning electron microscope is shown in Fig. 4-10.

The scanning electron microscope lacks the resolving power obtainable with the transmission electron microscope but has the advantage of revealing a striking three-dimensional picture. The surface topography of a specimen can be revealed with a clarity and a depth of field not possible by any other method. An example of a scanning electron microscope picture (micrograph) is shown in Fig. 4-17.

LIMITATIONS OF ELECTRON MICROSCOPY

Despite the great advantage of tremendous resolution and magnification, there are several limitations to electron microscopy. For example, the specimen being examined is in a chamber that is under a very high vacuum. Thus cells cannot be examined in a living state. In addition, the drying process may alter some morphological characteristics. Another limitation of the technique is the low penetration power of the electron beam, necessitating the use of thin sections to reveal the internal structures of the cell.

The real problem confronting the researcher who attempts to unravel the fine intracellular structure of the microbial cell is identification of intracellular material. Frequently it is necessary to correlate results obtained with the same organism viewed by different microscopic techniques, e.g., phase-contrast, bright-field (stained preparations), and electron microscopy. Each method contributes different kinds of information. Interpretation of this information, particularly comparison of what is revealed by each technique, makes it possible to identify cellular structures. But considerable experience in microscopy is required before a researcher can correctly interpret the results.

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Table 4-1. A Comparison of different Types of Microscopy

Type of Microscopy	Maximum Useful Magnification	Appearance of Specimen	Useful Applications
Bright-field	1,000–2,000	Specimens stained or unstained; bacteria generally stained and appear color of stain	For gross morphological features of bacteria, yeasts, molds, algae, and protozoa
Dark-field	1,000–2,000	Generally unstained; appears bright or "lighted" in an otherwise dark field	For microorganisms that exhibit some characteristic morphological feature in the living state and in fluid suspension, e.g., spirochetes
Fluorescence	1,000–2,000	Bright and colored; color of the fluorescent dye	Diagnostic techniques where fluorescent dye fixed to organism reveals the organism's identity
Phase-contrast	1,000–2,000	Varying degrees of "darkness"	For examination of cellular structures in living cells of the larger microorganisms, e.g., yeasts, algae, protozoa, and some bacteria
Electron	200,000–400,000	Viewed on fluorescent screen	Examination of viruses and the ultrastructure of microbial cells

Some of the major features of the several kinds of microscopy are summarized in Table 4-1.

PREPARATIONS FOR LIGHT-MICROSCOPE EXAMINATIONS

Two general techniques are used to prepare specimens for light-microscope examination. One is to suspend organisms in a liquid (the wet-mount or the hanging-drop techniques), and the other is to dry, fix, and stain films or smears of the specimen.

The Wet-Mount and Hanging-Drop Techniques

Wet preparations permit examination of organisms in a normal living condition. A wet mount is made by placing a drop of fluid containing the organisms onto a glass slide and covering the drop with a cover slip. To reduce the rate of evaporation and exclude the effect of air currents, the drop may be ringed with petroleum jelly or a similar material to provide a seal between the slide and cover slip. A special slide with a circular concave depression is sometimes used for examination of wet preparations. A suspension of microbial specimen is placed on a cover slip, then inverted over the concave depression to produce a "hanging drop" of the specimen.

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Examination of microorganisms in wet preparation is desirable in the following instances:

1 The morphology of spiral bacteria is greatly distorted when these bacteria are dried and stained; they should be examined in living condition. For example, in the examination of serous exudates suspected of containing the spirochete that causes syphilis, the wet preparations are examined by dark-field microscopy. This provides a sharp contrast between the organisms and the dark background. The normal arrangement of cells can also be better determined in a wet preparation.
2 The observation of bacteria to determine whether or not they are motile obviously requires that they be suspended in a liquid medium, free to move about.
3 To observe cytological changes occurring during cell division and to determine the rate at which the division occurs, the organisms must be examined in the living state (i.e., wet mount). Spore formation and germination must also be observed in living cells.
4 Some cell inclusion bodies, e.g., vacuoles and lipid material, can be observed readily by this method.

When wet preparations are examined by bright-field microscopy, it is extremely important to control the light source. The reason is that the lack of a stain makes the cells less distinctly visible; adjustment of the intensity of the light source can enhance their visibility. Partially closing the substage condenser diaphragm helps to increase contrast; however, some resolving power is lost. Dark-field and phase-contrast microscopy offer the distinct advantage of providing both high contrast and high resolving power for examination of unstained preparations.

Fixed, Stained Smears

Fixed, stained preparations are most frequently used for the observation of the morphological characteristics of bacteria. The advantages of this procedure are that (1) the cells are made more clearly visible after they are colored, and (2) differences between cells of different species and within the same species can be demonstrated by use of appropriate staining solutions (differential or selective staining).

The essential steps in the preparation of a fixed, stained smear are (1) preparation of the film or smear, (2) fixation, and (3) application of one or more staining solutions.

Microbiological Stains

A large number of colored organic compounds (dyes) are available for staining microorganisms. These compounds are generally rather complex in terms of molecular structure. On this basis they may be classified into groups such as **triphenylmethane dyes, oxazine dyes,** and **thiazine dyes.**

A more practical classification for the cytologist is one based on the chemical behavior of the dye; namely, **acid, basic,** or **neutral.** An acid (or anionic) dye is one in which the charge on the dye ion is negative; a basic (or cationic) dye is one in which the charge carried by the dye ion is positive. A neutral dye is a complex salt of a dye acid with a dye base, e.g., eosinate of methylene blue. Acid dyes generally stain basic cell components, and basic dyes generally stain acidic cell components.

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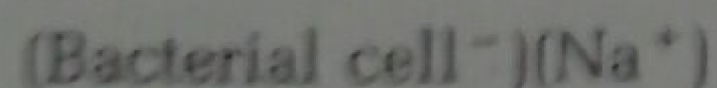
The process of staining may involve ion-exchange reactions between the stain and active sites at the surface of or within the cell. For example, the colored ions of the dye may replace other ions on cellular components. Certain chemical groupings of cell proteins or nucleic acids may be involved in salt formation with positively charged ions such as Na^+ or K^+. Thus we might view these peripheral areas of the cell as carrying a negative charge in combination with positively charged ions; for example,



$$(\text{Bacterial cell}^-)(Na^+)$$

In a basic dye like methylene blue, the colored ion is positively charged (a cation), and if we represent this ion by the symbol MB, the dye, which is actually methylene blue chloride, may be represented as

$$MB^+Cl^-$$

The ionic exchange which takes place during staining can be represented by the following equation, in which the MB^+ cation replaces the Na^+ cation in the cell:

$$(\text{Bacterial cell}^-)(Na^+) + (MB^+)(Cl^-) \rightarrow (\text{bacterial cell}^-)(MB^+) + (Na^+Cl^-)$$

Simple Staining. The coloration of bacteria by applying a single solution of stain to a fixed smear is termed **simple staining.** The fixed smear is flooded with a dye solution for a specified period of time, after which this solution is washed off with water and the slide blotted dry. The cells usually stain uniformly. However, with some organisms, particularly when methylene blue is used, some granules in the interior of the cell may appear more deeply stained than the rest of the cell, indicating a different type of chemical substance.

Differential Staining. Staining procedures that make visible the differences between bacterial cells or parts of a bacterial cell are termed **differential staining** techniques. They are slightly more elaborate than the simple staining technique in that the cells may be exposed to more than one dye solution or staining reagent.

Gram Staining. One of the most important and widely used differential staining techniques in microbiology is **Gram staining.** This technique was introduced by Christian Gram in 1884. In this process the fixed bacterial smear is subjected to the following staining reagents in the order listed: crystal violet, iodine solution, alcohol (decolorizing agent), and safranin or some other suitable counterstain. Bacteria stained by the Gram method fall into two groups: **Gram-positive** bacteria, which retain the crystal violet and hence appear deep violet in color; and **Gram-negative** bacteria, which lose the crystal violet, are counterstained by the safranin, and hence appear red in color. Why does this procedure stain some bacteria purple-violet and others red?

The most plausible explanations for this phenomenon are associated with the structure and composition of the cell wall. (See Chap. 5 for a discussion of the relative differences between the cell walls of Gram-negative and Gram-positive bacteria.) Differences in the thickness of cell walls between these two groups

MitjReader

may be important; the cell walls of Gram-negative bacteria are generally thinner than those of Gram-positive bacteria. Gram-negative bacteria contain a higher percentage of lipid than do Gram-positive bacteria. Experimental evidence suggests that during staining of Gram-negative bacteria the alcohol treatment extracts the lipid, which results in increased porosity or permeability of the cell wall. Thus the crystal violet–iodine (CV-I) complex can be extracted and the Gram-negative organism is decolorized. These cells subsequently take on the color of the safranin counterstain. The cell walls of Gram-positive bacteria, because of their different composition (lower lipid content), become dehydrated during treatment with alcohol. The pore size decreases, permeability is reduced, and the CV-I complex cannot be extracted. Therefore these cells remain purple-violet.

Another explanation, somewhat similar, is also based on permeability differences between the two groups of bacteria. In Gram-positive bacteria, the CV-I complex is trapped in the wall following ethanol treatment, which presumably causes a diminution in the diameter of the pores in the cell-wall peptidoglycan. Walls of Gram-negative bacteria have a very much smaller amount of peptidoglycan, which is less extensively cross-linked than that in the walls of Gram-positive bacteria. The pores in the peptidoglycan of Gram-negative bacteria remain sufficiently large even after ethanol treatment to allow the CV-I complex to be extracted. These two explanations are not mutually exclusive, and it is likely that both may contribute to the explanation of the mechanism of the Gram stain. Furthermore, if Gram-positive cells are treated with lysozyme (an enzyme) to remove the cell wall, the resulting structures, called **protoplasts**(cells lacking walls), will be stained by the CV-I complex. However, they are easily decolorized by alcohol. All this evidence points to the cell-wall structure of Gram-positive bacteria as the site of retention of the primary stain.

Although Gram-negative organisms consistently fail to retain the primary crystal violet stain, Gram-positive organisms may sometimes show variations in this respect, i.e., a **Gram-variable reaction**.For example, old cultures of Gram-positive bacteria lose the ability to retain the crystal violet and hence will be stained by the safranin. A similar effect may sometimes be due to changes in the environment of the organism or a slight modification in staining technique.

Within some groups of bacteria, such as the archaeobacteria (see Chap. 5), some are Gram-positive and others Gram-negative; yet the cell wall structure and chemical composition of these bacteria is very different from that of other groups of Gram-positive and Gram-negative bacteria.

Gram-positive bacteria differ from Gram-negative bacteria in other characteristics besides staining reaction. Gram-positive bacteria are usually more susceptible to penicillin and less susceptible to disintegration by mechanical treatment or exposure to some enzymes than Gram-negative bacteria. Gram-negative bacteria as a group are more susceptible to other antibiotics such as streptomycin. There are other differences between these two groups of bacteria.

The Gram stain has its greatest use in characterizing bacteria. This staining technique is not generally applicable for other groups of microorganisms such as protozoa and fungi; however, yeasts consistently stain Gram-positive.

Other Differential Stains. There are numerous other staining techniques de-

signed to identify some particular feature of cell structure or composition. These techniques are summarized here. Detailed descriptions of these procedures appear in the laboratory manual.

NAME OF STAINING TECHNIQUE	APPLICATION
Acid-fast stain	Distinguishes acid-fast bacteria such as *Mycobacterium* spp. from non-acid-fast bacteria
Endospore stain	Demonstrates spore structure in bacteria as well as free spores
Capsule stain	Demonstrates presence of capsules surrounding cells
Flagella stain	Demonstrates presence and arrangement of flagella
Cytoplasmic inclusion stains	Identifies intracellular deposits of starch, glycogen, polyphosphates, hydroxybutyrate and other substances
Giemsa stain	Particularly applicable for staining rickettsia and some protozoa

QUESTIONS

1. Define the following terms:

Resolving power	Fluorescence
Limit of resolution, *d*	Autoradiography
NA (numerical aperture)	Anionic dye
Angle θ	Cationic dye

2. What are the usual magnifications obtainable with light microscopy? What determines its useful limit?
3. Assume that a yeast cell is examined by (*a*) bright-field, (*b*) phase-contrast and (*c*) dark-field microscopy. Describe the likely differences in the appearance of the cell when viewed by these methods.
4. Why are microorganisms stained?
5. What is the function of oil when used with the oil-immersion objective?
6. Name several different staining techniques and describe their particular applications.
7. Compare the kind of image obtained with scanning electron microscopy with that obtained using transmission electron microscopy.
8. Compare the resolving power of the electron microscope with that of the light microscope.
9. Name two limitations of electron microscopy.
10. What are some major differences between Gram-positive and Gram-negative bacteria?
11. Why is the Gram stain one of the most important and widely used stains in bacteriology?
12. Compare the appearance of microorganisms as seen by dark-field and by phase-contrast microscopy.
13. Describe two special applications of fluorescence microscopy.

Nitj Reader

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