Basic Histology

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3rd Edition

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Preface

The third edition of *Basic Histology* represents the authors' continuing effort to present in compact but amply illustrated format the foundations of histology for medical students and others in the biologic sciences. Again we emphasize cellular biology as the most fundamental approach to the study of tissue physiology.

We have been most gratified by the success this book has achieved since the first appearance of the English language edition in 1975. We gratefully acknowledge our dependence on our readers' suggestions for changes and additions in the ongoing effort to keep up to date with advances in the field.

We are very glad to be able to say that in preparing this new edition we have had the assistance of Bruce Lipton, PhD, Associate Professor of Anatomy at the University of Wisconsin–Madison. Dr Lipton has reviewed every word of our revisions, making further revisions as required, and has worked closely with our publishers in California to make certain that *Basic Histology* is up to date and accurate in all important respects. We look forward to a continued close relationship with this young scientist and scholar in future editions of this work.

As the third edition goes to press we are pleased to be able to announce that an Italian edition has been published and that translations are going forward in French, German, Japanese, Serbo-Croatian, Dutch, and Indonesian.

—LCJ
—JC

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Methods of Study | 1

Familiarity with the tools and methods of any branch of science is essential for proper understanding of the subject. Some of the more common methods used to study cells and tissues and the principles involved in these methods will be reviewed here: units of measurement, preparation of tissues for examination, optical microscopy, phase contrast microscopy, polarizing microscopy, electron microscopy, radioautography, examination of living cells and tissues, differential centrifugation, and problems in interpretation of tissue sections.

The most important units of measurement used in histology are given in Table 1–1. At a recent international conference, it was recommended that the **Ångström unit** (Å; 10^{-10} meter) be abandoned in favor of the **nanometer** (nm, 10^{-9} meter) and that the nanometer be used in place of the **millimicron** ($m\mu$, 10^{-9} meter). In this book, the nanometer will be used in place of the Ångström unit (1 nm = 10 Å). The **micron** (μ) is now called a **micrometer** (μm), with value (10^{-6} meter) unchanged.

Table 1–1. Units of measurement used in light and electron microscopy.*

SI Unit*	Symbol and Value
Micron (micrometer)	μ (μm) = 0.001 mm, 10^{-6} m
Millimicron (nanometer)	$m\mu$ (nm) = 0.001 μm, 10^{-9} m
Ångström	Å = 0.1 nm, 10^{-10} m

*The preferred SI *(Système International)* units (in parentheses) will be used throughout this book.

PREPARATION OF TISSUES FOR MICROSCOPIC EXAMINATION

The most common procedure used in the study of tissues is the preparation of permanent histologic slides that can be studied with the aid of the optical microscope. Under the optical microscope, tissues are examined by transillumination. Since tissues and organs are usually too thick for transillumination, technics have been developed for obtaining thin, translucent sections. In some cases, very thin layers of tissues or transparent membranes of living animals (eg, the mesentery, the tail of a tadpole, the wall of a hamster's cheek pouch) can be observed in the microscope. In such instances, it is possible to study these structures for long periods and under varying physiologic or experimental conditions. If a permanent slide preparation is desired, small fragments of these thin structures can be fixed, spread on a glass slide, stained and mounted with resin, and examined under the microscope. In most cases, however, tissues must be sliced into thin sections before they can be examined. These sections are cut by precision fine cutting instruments called **microtomes**, and the organ or tissue must be fixed and prepared for sectioning. (See Table 1–2.)

The ideal microscope tissue preparation would of course be preserved with suitable chemicals so that the tissue on the slide would have the same structure and chemical composition as it has in the body. This is sometimes possible but, as a practical matter, seldom feasible, and artifacts resulting from the preparation process are almost always present.

Table 1–2. Stages through which the tissues must pass before paraffin impregnation. (The next steps are microtome sectioning, staining, and mounting.)

Stage	Purpose	Duration
1. Fixation in simple or compound fixatives (Bouin's, Zenker's formalin)	To preserve tissue morphology and chemical composition	About 12 h, according to the fixative and the size of the piece of tissue
2. Dehydration in graded concentrated ethyl alcohol (70% up to 100% alcohol)	To remove cell water	6–24 h
3. Clearing in benzene, xylene, or toluene	To impregnate the tissues with a paraffin solvent	1–6 h
4. Embedding in melted paraffin at 58–60 C	Paraffin penetrates all intercellular spaces and even into the cells, making the tissues more resistant to sectioning	½–6 h

Fixation

In order to avoid tissue digestion by enzymes (autolysis) or bacteria and to preserve physical structure, pieces of organs should be promptly and adequately treated prior to or as soon as possible following removal from the animal's body. This treatment—**fixation**—usually consists of submerging the tissues in chemical substances or perfusing them with those substances in order to preserve as much as possible of their morphologic and chemical characteristics.

The chemical substances used to fix tissues are called **fixatives.** Some fixatives (eg, mercuric chloride, picric acid) promote the precipitation or clumping of proteins. Others (eg, formalin, glutaraldehyde) promote coagulation but not coarse precipitation of proteins. All fixatives have both desirable and undesirable effects. The goal of combining the desirable effects and minimizing the undesirable ones has led to the development of several mixtures. The most commonly used mixtures are **Bouin's fluid,** composed of picric acid, formalin (a saturated solution—37% by weight of formaldehyde gas in water), acetic acid, and water; and **Zenker's formalin (Helly's fluid),** containing formaldehyde, potassium dichromate, mercuric chloride, and water. The simple fixatives most commonly used are a 10% solution of formalin in saline and a 2–6% solution of buffered glutaraldehyde.

The chemistry of the process involved in fixation is complex and not well understood. However, formaldehyde and glutaraldehyde are known to react with the amine groups (NH_2) of tissue proteins. In the case of glutaraldehyde, the fixing action is reinforced by the fact that it is a dialdehyde and can cross-link.

In view of the high resolution afforded by the electron microscope, greater care is necessitated in fixation in order to preserve ultrastructural detail. Toward that end, a double fixation procedure, using a buffered glutaraldehyde solution first, followed by a second fixation in buffered osmium tetroxide, has become a standard procedure in preparations for fine structural studies.

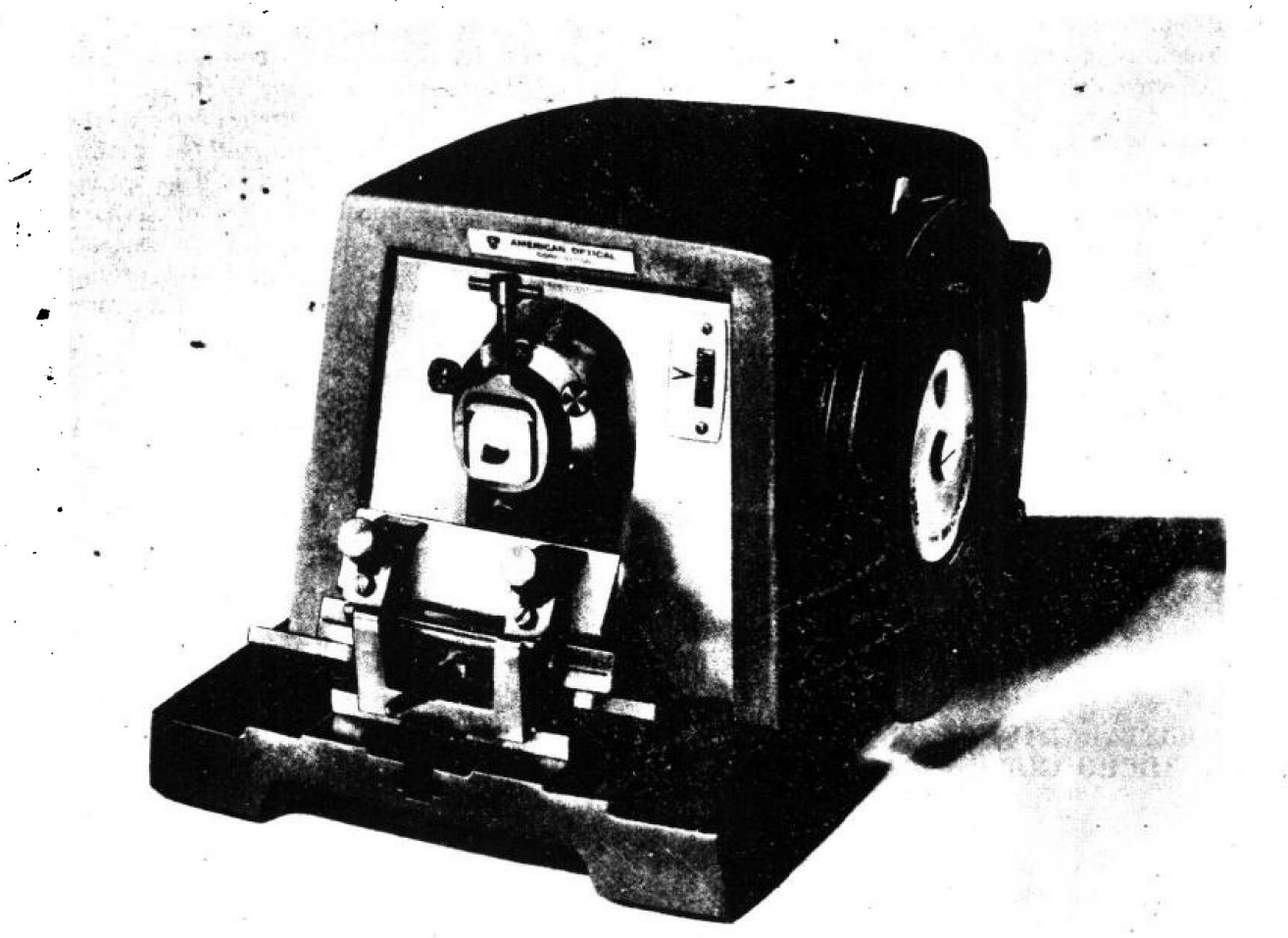

Figure 1–1. Microtome for paraffin-embedded tissues. Rotation of the drive wheel—seen with a handle on the right side of the instrument—moves the tissue block holder up and down. Each turn of the drive wheel advances the specimen holder a controlled distance, generally 3–8 μm, and the block strikes the knife edge, cutting the sections. The sticky paraffin sections adhere to each other, producing a ribbon which is collected and fixed on a slide. (Courtesy of American Optical Corp.)

Embedding

In order to be able to obtain thin sections with the microtome, tissues must be infiltrated after fixation with a substance that will impart a firm consistency necessary for cutting. This can be gelatin, celloidin, paraffin, resins, or other plastic materials.

Paraffin is used routinely for light microscopy; resins of the epoxy type (Epon or Araldite) are more commonly employed for electron microscopy.

The process of embedding or tissue impregnation is usually preceded by 2 main steps: **dehydration** and **clearing.** The water of the fragments to be embedded is first extracted by bathing successively in a graded series of mixtures of ethanol with water (usually from 70% to 100% ethanol). The ethanol is then replaced by a lipid solvent. (In paraffin embedding, the solvent used is xylene or benzene.) As the tissues become impregnated with the solvent, they usually become transparent in a step called **clearing.** Once the tissue is impregnated with the solvent, it is placed in melted paraffin in the oven, usually at 58–60 C. The heat causes the solvent to evaporate, and the space becomes filled with paraffin. Tissues to be embedded for electron microscopy are also dehydrated in ethanolic solutions. However, instead of using the lipid solvents as in paraffin embedding, the tissues are subsequently infiltrated with plastic solvents such as propylene oxide. These solvents are miscible with and later replaced by plastic solutions (eg, Epon, Araldite) hardened by means of cross-linking polymerizers or heat. This is the infiltration or embedding procedure.

The small blocks of paraffin containing the tissues are then sectioned by the steel blade of the microtome to a thickness of 3–8 μm* (Fig 1–1). The sections are laid out on warm water and transferred to glass slides. For electron microscopy, much thinner sections are necessary (0.02–0.1 μm); embedding is therefore performed in a hard epoxy plastic. The blocks thus obtained are so hard that glass or diamond knives are usually necessary to section them. Since the electron beam in the microscope cannot penetrate glass, the extremely thin plastic sections are collected on small metal (usually etched copper) screens. Those portions of the sections spanning the holes in the mesh of the screen can be examined in the microscope.

Immersion of tissues in lipid solvents such as benzene or xylene dissolves the tissue lipids, which is an undesirable effect when these compounds are studied. To prevent this, a **freezing microtome** has been devised in which the tissues are hardened at low temperatures in order to provide the rigidity necessary to permit sectioning. The freezing microtome—and its more elaborate and efficient successor, the **cryostat**—permit sections to be obtained quickly without going through the embedding procedure described above. They are often used in hospitals, for they allow rapid study of pathologic specimens during surgical procedures. They are also effective in the histochemical study of very sensitive enzymes or small molecules, since freezing does not inactivate enzymes and hinders the diffusion of small molecules.

*For investigative work this may vary from 1–20 μm.

Staining

With few exceptions, most tissues are colorless, so that observing them unstained in the light microscope is difficult. Methods of staining tissues have therefore been devised that not only make various tissue components conspicuous but also permit distinctions to be made among them. This is done by using mixtures of dyes which stain tissue components more or less selectively. Most dyes used in histologic studies behave like acidic or basic compounds and have a tendency to form electrostatic (salt) linkages with ionizable radicals of the tissues. Tissue components that stain more readily with basic dyes are termed **basophilic;** those with an affinity for acid dyes are termed **acidophilic.**

Examples of basic dyes are toluidine blue and methylene blue. Hematoxylin behaves in the manner of a basic dye, ie, it stains the tissues basophilically. The main tissue components that ionize and react with basic dyes do so because of acids in their composition (nucleoproteins and acid mucopolysaccharides). Acid dyes (eg, orange G, eosin, acid fuchsin) stain mostly the basic components present in cytoplasmic proteins. The basic or acid character of a dye usually explains the staining reaction on a chemical basis, but a physical basis is sometimes also present.

Of all dyes, the combination of hematoxylin and eosin (H&E) is most commonly used. Many other dyes are used in different histologic procedures. Although they are useful in visualizing the different tissue components, they usually provide no insight into the chemical nature of the tissue being studied.

Besides tissue staining with dyes, impregnation with such metals as silver and gold is a much used technic, especially in the study of the nervous system. Table 1–3 summarizes some staining technics used in preparing microscope slides.

Since the electrons in the beam of an electron microscope are not of wavelengths in the visible spectrum, colored dyes are not applicable in fine structural studies. In view of their ability to scatter or absorb electrons, heavy metal salts such as lead citrate and uranium acetate are the primary stains used in electron microscopy.

THE LIGHT MICROSCOPE

With the light microscope, stained preparations are usually examined by transillumination.

Table 1–3. Examples of staining technics commonly used in histology.

Technics	Components	Nucleus	Cytoplasm	Collagen	Elastic Fibers	Reticular Fibers
H&E	Hematoxylin and eosin	Blue	Pink	Pink	Irregular	. . .
Masson's trichrome	Iron hematoxylin, acid fuchsin, Ponceau 2R, light green	Black	Red	Green	. . .	Green
Weigert's elastic stain	Resorcin and fuchsin, HCl, hematoxylin, Ponceau's picric acid, glacial acetic acid	Gray	Yellow	Red	Black	. . .
Silver impregnation for reticular fibers	Silver salt solution	. . .	. . .	Dark brown	. . .	Black

The microscope is composed of both mechanical and optical parts. The mechanical components are illustrated in Fig 1–2. The optical components consist of 3 systems of lenses: condenser, objective, and ocular. The **condenser** projects a cone of light to illuminate the object to be observed. (The role of the condenser is usually underestimated because it does not contribute to the magnification; however, its proper use influences the quality of the image observed.) The **objective** lens enlarges the object and projects its image in the direction of the ocular lens. The **ocular** lens further amplifies this image and projects it onto the viewer's retina or onto a screen or photographic plate. The degree of total magnification is obtained by multiplying the magnifying power of the objective and ocular lenses.

Resolution

The critical factor in obtaining a good image with the microscope is the resolution, which is the

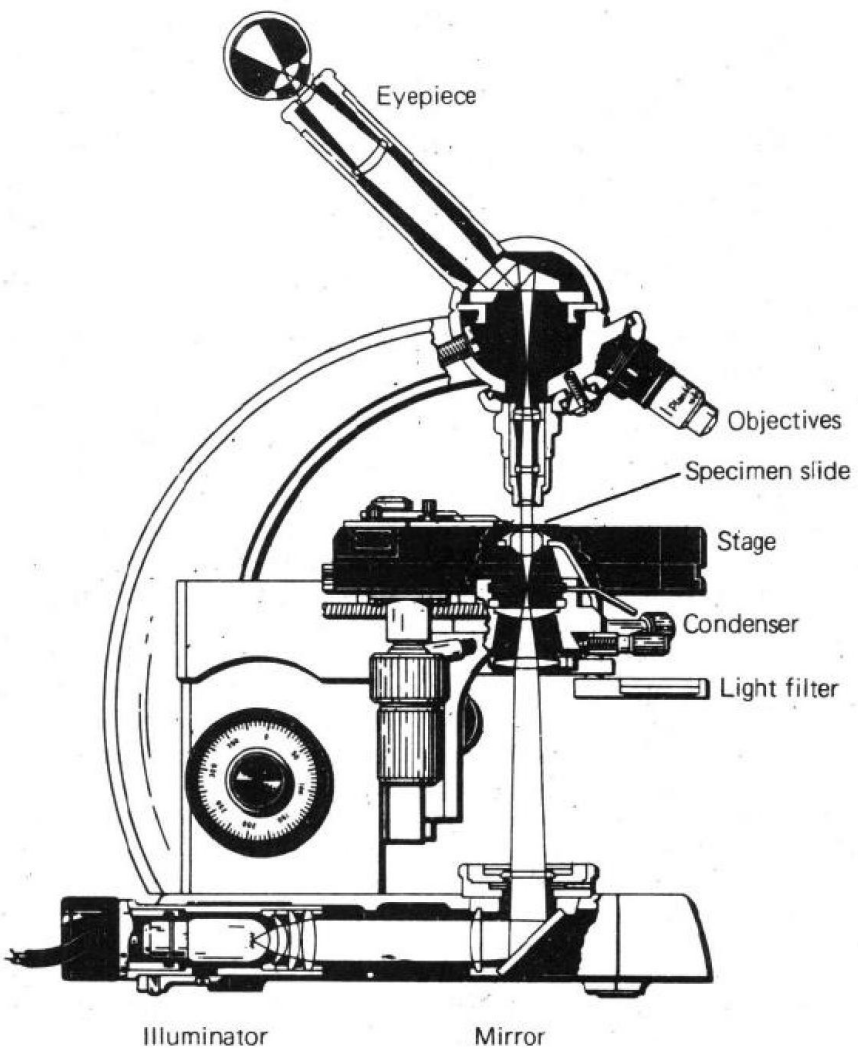

Figure 1–2. Schematic drawing of a student's light microscope showing its main components and the pathway of light from the source (substage lamp) to the eye of the observer. (Courtesy of Carl Zeiss Co.)

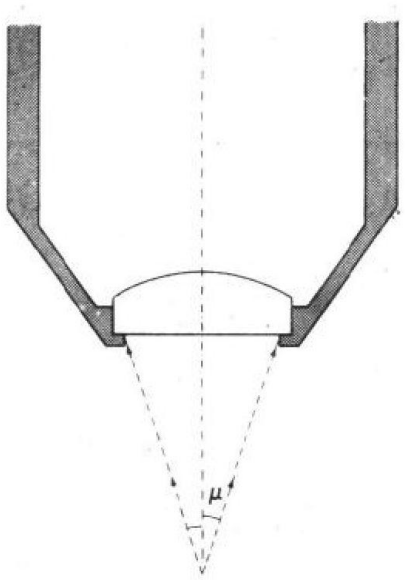

Figure 1–3. Drawing of the light beam which enters the objective lens to show the semiangle of aperture (μ) from which the numerical aperture can be calculated.

smallest distance between 2 particles that can be distinguished from each other. For example, 2 particles will appear distinct if they are separated by a distance of 0.3 μm and the microscope has a resolution factor of 0.2 μm. However, if the same particles are examined with a microscope that has a resolution factor of only 0.5 μm, they will appear as a single point. The resolving power of the best light microscopes is approximately 0.2 μm.

The quality of an image—its clarity and richness of detail—depends on the microscope's resolving power. The **magnification** is independent of its resolving power and is only of value when accompanied by a high resolution capacity. The resolving power of a microscope depends mainly on its objective lens. The ocular lens only enlarges the image obtained by the objective; it does not improve resolution. Thus, high magnification with low resolution gives blurred images of little value.

Numerical Aperture

One of the main characteristics of an objective lens is its numerical aperture (NA), for resolution is a function of NA and of the light wavelength employed (Fig 1–3). NA can be defined as the smallest refractive index (n)* observed between the microscopic preparation and the objective multiplied by the sine of the semiangle of aperture of the lens (μ): NA = n × sine μ (Fig 1–3).

The resolution of an objective can be defined by the equation:

$$R = \frac{K \times \lambda}{NA}$$

where K is a constant of 0.61 and λ is the wavelength. Resolution is directly proportionate to the wavelength used and inversely proportionate to the NA. To calculate the resolution when working with white light, a wavelength of 0.55 μm is most often used. This corresponds to yellowish-green, a color to which the human eye is very sensitive. Fig 1–4 is an example of the importance of resolution in microscopy.

*The refractive index is a measure of the optical density of an object. A light wave traverses an object readily or otherwise depending on the object's optical density.

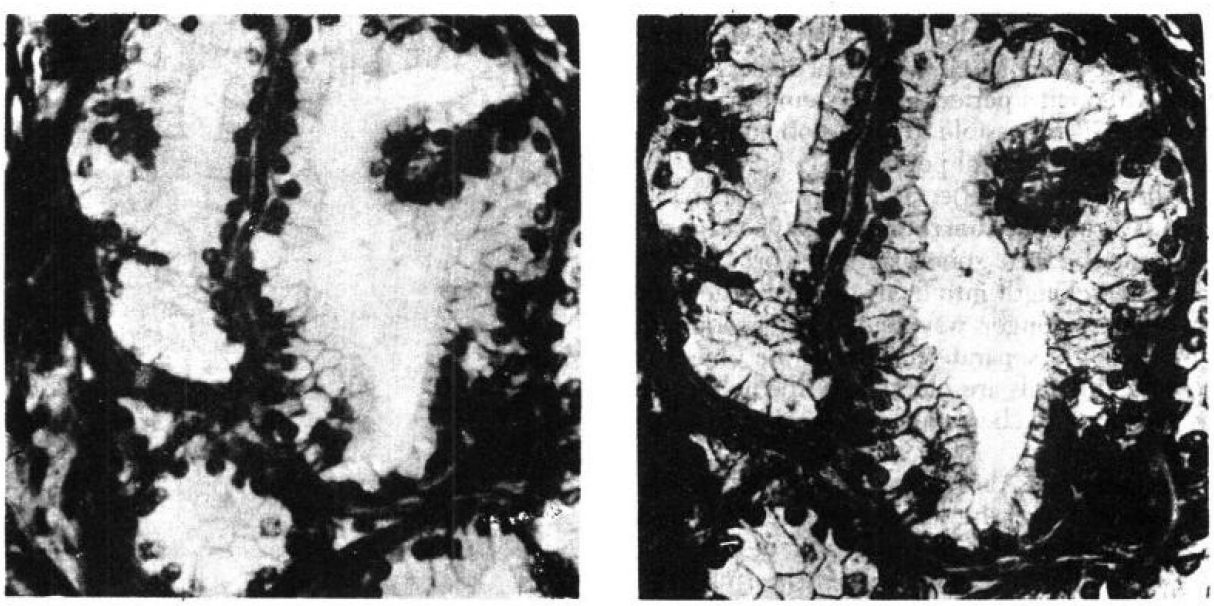

Figure 1–4. Photomicrographs of the same microscopic field at the same magnification (× 350) but with objectives of different numerical apertures (NA). The photomicrograph on the left was made with an objective of NA = 0.22; the one on the right was made with an objective of NA = 1.0. Dog prostate gland stained by Masson's trichrome stain. Observe that the picture at right (NA = 1.0) shows more detail and is sharper than the one on the left.

Figure 1–5. Drawing of an objective with the following characteristics: magnification × 25, NA = 0.45, planachromatic, corrected for 160 mm tube and for 0.17 mm coverslips.

An objective lens system often has several numbers engraved on it (Fig 1–5). The first number (upper left) refers to the enlargement; to its right is the NA. The number on the left in the second line is the tube length in millimeters; the number on the right indicates the thickness (in millimeters) of the coverslip for which the objective is corrected. The thickness of the coverslip is important in dry field examination, but when oil immersion is used the oil equalizes the refractive index of the light path between the coverslip and the objective, and the thickness between the usual limits of the coverslip becomes irrelevant.

Objective & Ocular Lenses

Objective and ocular lenses are formed by systems of lenses put together in order to achieve partial correction of their individual defects (aberrations). Although a perfect lens system has not been developed, it is possible to devise objective lenses with increasing optical perfection.

Three common aberrations are as follows:

A. Chromatic Aberration: This type of aberration occurs because spherical lenses bring light of shorter wavelength into focus closer to the retina than light of longer wavelength. Consequently, several slightly separate images of the object are formed and details are blurred. In the **achromatic** lens system, this aberration is corrected to a large extent.

B. Spherical Aberration: In spherical aberration, the quality of the image is hindered because the optical properties of the center of a lens are somewhat different from those of its periphery. In **apochromatic** objective lens systems, complete correction of chromatic and spherical aberrations has been achieved.

C. Curvature of Field: Lenses with this aberration produce an image in which the central field is in focus while the peripheral field is out of focus or vice versa. **Planar** lenses are corrected to provide "flat field" focus, in which the entire field is in focus.

PHASE CONTRAST MICROSCOPY

Unstained biologic specimens are usually transparent and difficult to view in detail since all parts of the specimen have almost the same optical density. Consequently, another form of microscopy—**phase contrast microscopy**—has been developed that produces in vivo visible images from transparent objects (Fig 1–6).

Phase contrast microscopy is based on the fact that light passing through media with different re-

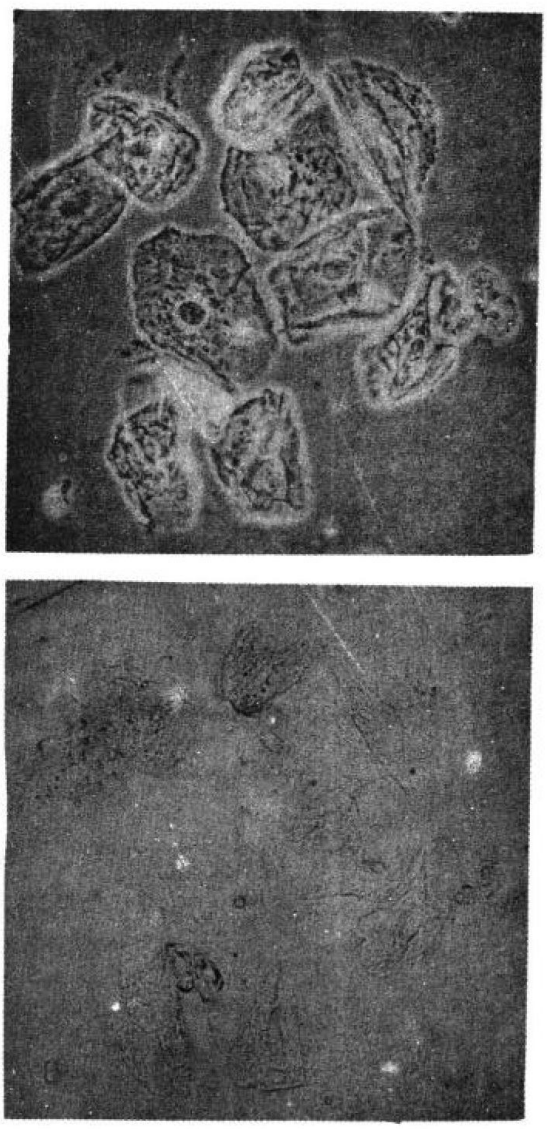

Figure 1–6. Desquamated cells from the oral mucosa. (Unstained fresh preparation.) The top photomicrograph was taken with the phase contrast microscope; the bottom photomicrograph with the standard light microscope. × 300.

fractive indexes slows down and changes direction. Within the cell, different organelles—such as the nuclei, mitochondria, and secretion granules—exhibit different refractive indexes and consequently alter the light passing through them. This forms phase differences between 2 adjoining regions. These phase differences are—by means of a special optical system—transformed into differences of light intensity so that the image becomes visible (Fig 1–6). The examination of fresh tissue or living cells has been facilitated by the development of phase contrast microscopy.

THE POLARIZING MICROSCOPE

When light passes through certain substances or body tissues, it divides in a way that produces 2 light rays from one. This is called **polarization.** It occurs with substances whose atoms have a periodic arrangement. Whether or not this arrangement is apparent, these substances are **crystalline (birefringent).** Substances that do not belong to the crystalline group are **amorphous (monorefringent).**

The velocity with which light travels through amorphous substances is always the same regardless of the direction. Therefore, the substance has only one refractive index. In crystalline substances, light velocity changes according to the direction of propagation; from one light ray, 2 refracted rays result. They are polarized rectilinearly, ie, the direction of light vibration follows a determinate direction.

Crystalline calcium carbonate (calcite) is highly birefringent. The **ordinary** ray follows the law of isotropic substances (Descartes' law); the **extraordinary** ray follows slightly different laws.

In the polarizing microscope, the properties of the extraordinary ray are utilized whereas those of the ordinary ray are not. This is achieved with the use of the Nicol prism, made from calcite and balsam. The Nicol prism permits only the passage of rectilinearly polarized light; the ordinary ray is eliminated by total reflection.

Sheets of **Polaroid film** are most often used at present. They contain special organic compounds so disposed that ordinary vibration is totally absorbed, resulting in a uniform field superior to that provided by the Nicol prism.

If this polarized light is transmitted to a seeond Nicol prism or Polaroid plate similar to the first, it does not pass through when the main axes of the 2 prisms or plates are crossed. In any other position, light is transmitted with greater or lesser intensity.

Principle of the Polarizing Microscope

The polarizing microscope contains a rotating stage with 2 polarizing elements: one located under the stage—the **polarizer**—and the other located above it, adjacent to the eyepiece on the analyzer.

The polarizer and the analyzer are placed so that their main axes are perpendicular, thus preventing the appearance of light in the eyepiece. When the stage contains an amorphous object, there is no light because the light rays are not modified. However, when a crystalline or birefringent object is placed on the stage, light appears with greater or lesser intensity in the microscope field, depending on the orientation of the analyzer. The usual test is to rotate the specimen to find the points of maximum and minimum brightness.

With the polarizing microscope it is therefore possible to distinguish between monorefringent and birefringent substances. With birefringent substances, it is now possible to discern their internal arrangement and their orientation at the submicroscopic level.

Although the birefringence observed in biologic specimens is generally weak, such crystalline or semicrystalline substances as bone tissue, cellulose walls, structures with linear symmetry (collagen, muscle fibers, nerve fibers, cilia, flagella), and structures with radial symmetry (starch granules, lipid droplets) can be easily studied by making use of this principle.

ELECTRON MICROSCOPY

The principle upon which electron microscopy is based can be understood by referring to the following equation (used above to calculate the resolution in the light microscope):

$$R = \frac{K \times \lambda}{NA}$$

where K is a constant of 0.61. The wavelength (λ) of an electron beam accelerated by 60 kV is approximately 0.005 nm, which gives a very high theoretic resolution. In practice, however, a resolution of 1 nm in tissue sections is considered to be quite satisfactory. This by itself permits enlargements to be obtained up to 200 times greater than those achieved with the light microscope.

The electron microscope functions on the principle that a beam of electrons can be deflected by electromagnetic fields in a manner similar to light deflection in glass lenses. Electrons are produced by high-temperature heating of a metallic filament (cathode) in a vacuum. The electrons emitted are then submitted to a difference of potential of approximately 60–100 kV or more between the cathode and the anode (Fig 1–7). The anode has the shape of a metallic plate with a small hole in its center. Electrons are accelerated from the cathode to the anode. Some of these particles pass through the central orifice of the anode, forming a constant

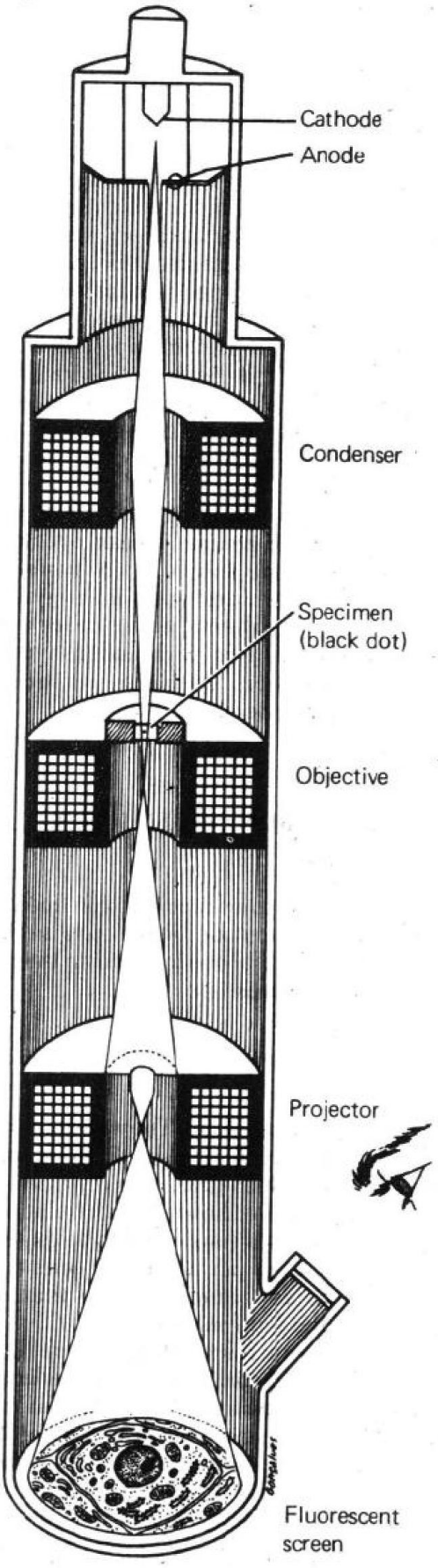

Figure 1–7. Pathway of the electron beam in the electron microscope: The ultrathin section is placed just over the objective electromagnetic lens. The image is projected on a fluorescent screen and observed directly or through a × 10 magnifying optical system.

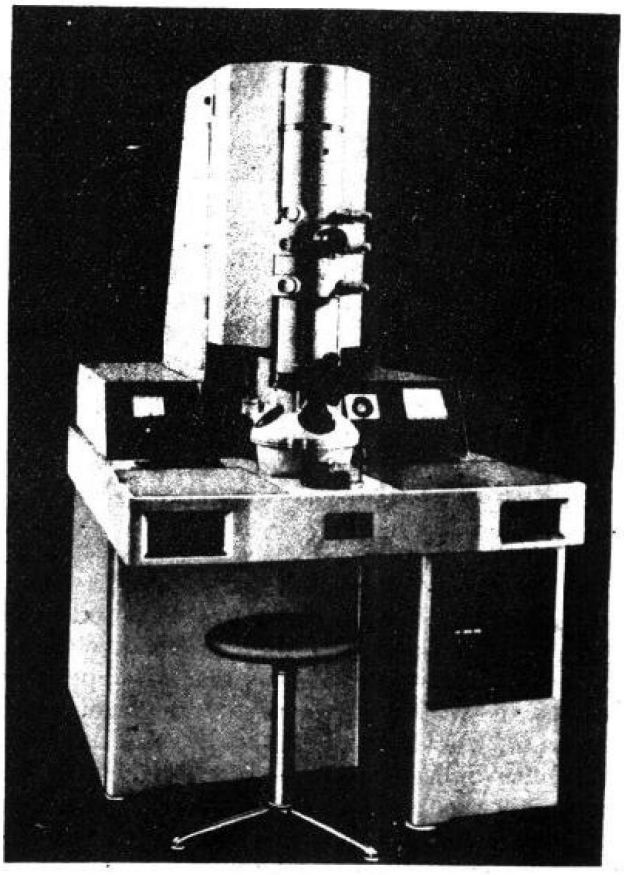

Figure 1–8. Photograph of the Zeiss model EM 9A electron microscope. (Courtesy of Carl Zeiss Co.)

stream (or beam) of electrons. This beam is deflected by electromagnetic lenses in a way roughly analogous to that which occurs in the optical microscope. Thus, the condenser focuses the beam at the object plane and the objective forms an image of the object. The image obtained is further enlarged by 1–2 projecting lenses and is finally projected on a fluorescent screen or photographic plate (Figs 1–7 and 1–8).

Differences Between Electron & Light Microscopes

In contrast to what happens in the light microscope, the enlargement produced by the objective in the electron microscope is fixed (or unvariable). The enlargements are produced by changes in the magnetic field of the projecting "lenses," which are analogous to the "zoom" ocular lens in the light microscope.

Because electrons are easily scattered or absorbed by the object, one must use very thin sections of tissue—usually 0.02–0.1 μm. Another characteristic of the electron microscope is that the electrons are scattered or absorbed by portions of the object with high molecular weight, whereas in the light microscope light is absorbed by stained structures. The scattered electrons are absorbed by the aperture of the objective lens (usually a diameter of 25–100 μm). The aperture filters out the

scattered electrons that thus do not contribute to image formation. The structures that scatter electrons thus appear in the fluorescent screen as dark bodies (electron-dense regions). The capacity to scatter electrons depends on the molecular weight (and therefore the density) of a given particle. Heavy metals (eg, uranium, lead) are therefore used to impregnate tissue sections; they increase contrast and permit better images.

Limitations in the Use of the Electron Microscope

The nature of the electron beam requires that work with the electron microscope be done in high vacuum with very thin sections. These conditions preclude the use of living material. Additionally, the action of an electron beam on an object can damage it and can produce unwanted changes in tissue structures. Electron microscopy is a rapidly developing field, however. Recent advances include the use of high-voltage (500,000–1,000,000 V) electron microscopes in which the high speed of acceleration of the electrons in the beam allows the penetration and consequently the visualization of relatively thick plastic sections (1–5 μm). The development of a phase (Crewe) electron microscope has permitted direct visualization of atoms. Other recent advances in electron microscopy include instruments that provide higher resolution and the use of live specimens.

PROBLEMS IN THE INTERPRETATION OF TISSUE SECTIONS

During the study and interpretation of stained tissue sections in microscope preparations, it should be remembered that the observed product is the end result of a series of processes which considerably distort the image observable in the living tissue, mainly through shrinking and retraction. As a consequence of these processes, the spaces frequently seen between the cells and other tissue components are artifacts. Furthermore, there is a tendency to think in terms of only 2 dimensions when examining thin sections, whereas in actuality the structures from which the sections are made have 3 dimensions. In order to understand the architecture of an organ, it is therefore necessary to study sections made in different planes and to reason accordingly (Fig 1–9).

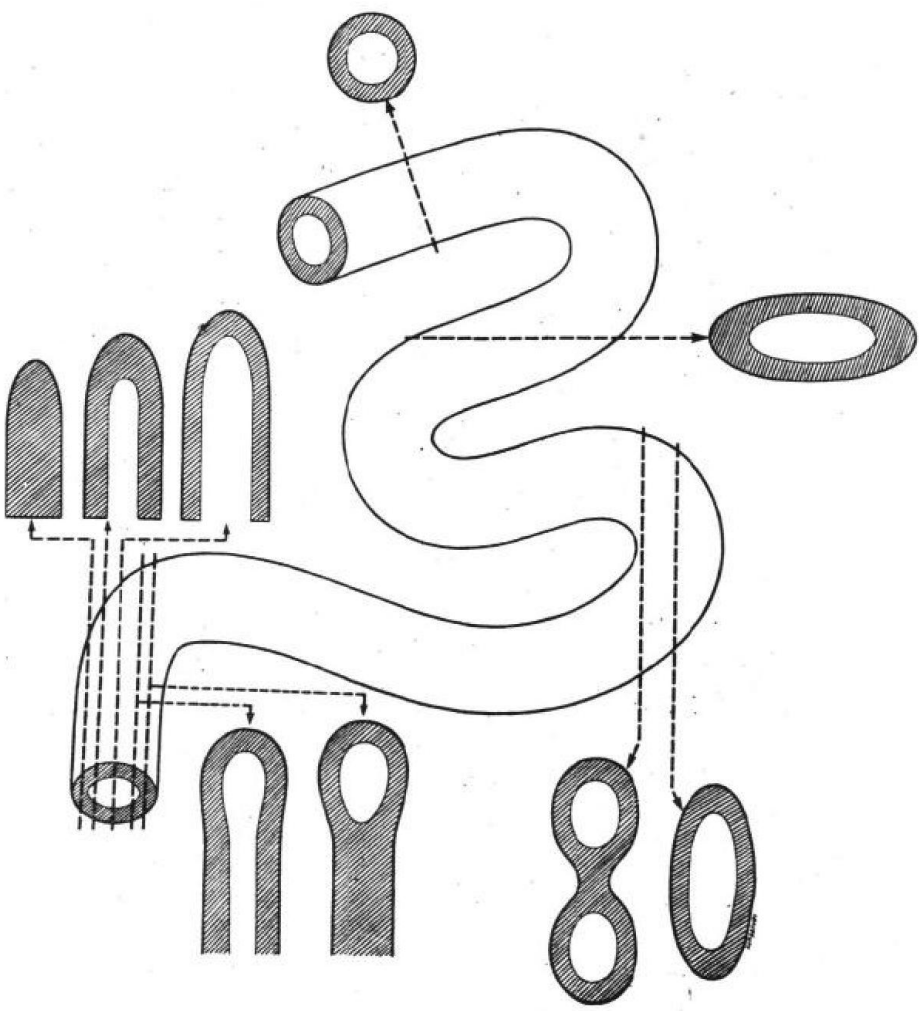

Figure 1–9. Some of the aspects a tube-shaped organ might exhibit when sectioned. The arrows indicate what is seen under the microscope in each particular section plane.

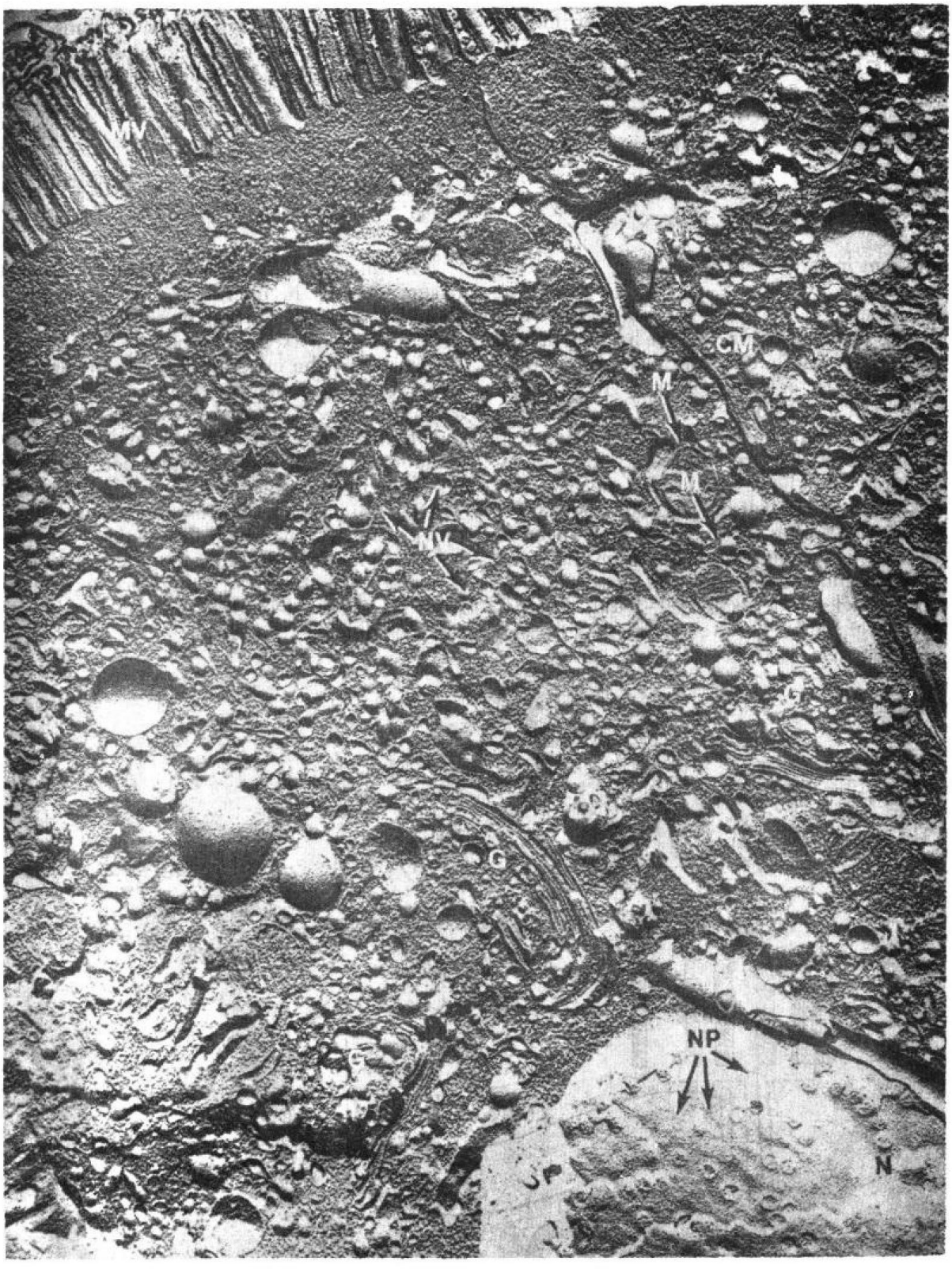

Figure 1–10. Electron micrograph of a mouse intestinal epithelial cell. This picture was obtained by a process called freeze etching. It consists of freezing a fragment of tissue to very low temperatures and fracturing it with a sharpened metal blade. The fractured surface is kept at low temperature in a vacuum environment. A portion of the water in the surface thus sublimates, giving a bas-relief effect (etching). A replica of this surface is then obtained by covering it with a layer of platinum and carbon. In this picture, one can observe in material that has not been submitted to the processes of embedding and sectioning the presence of the various cell components described by classic transmission electron microscopy, eg, microvilli (MV), cell membrane (CM), mitochondria (M), Golgi apparatus (G), nucleus (N), nuclear pores (NP), and nuclear vesicles (NV). × 24,000. (Courtesy of LS Staehelin.)