

FROZEN THIN SECTIONS OF FRESH TISSUE FOR ELECTRON MICROSCOPY, WITH A DESCRIPTION OF PANCREAS AND LIVER

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ABSTRACT

A simple method has been developed that allows frozen thin sections of fresh-frozen tissue to be cut on a virtually unmodified ultramicrotome kept at room temperature. A bowl-shaped Dewar flask with a knifeholder in its depths replaces the stage of the microtome; a bar extends down into the bowl from the microtome's cutting arm and bears the frozen tissue near its lower end. When the microtome is operated, the tissue passes a glass or diamond knife in the depths of the bowl as in normal cutting. The cutting temperature is maintained by flushing the bowl with cold nitrogen gas, and can be set anywhere from about -160°C up to about -30°C . The microtome is set for a cutting thickness of 540–1000 Å. Sections are picked up from the dry knife edge, and are placed on membrane-coated grids, flattened with the polished end of a copper rod, and either dried in nitrogen gas or freeze-dried. Throughout the entire process the tissue is kept cold and does not come in contact with any solvent. The morphology seen in frozen thin sections of rat pancreas and liver generally resembles that in conventional preparations, although freezing damage and low contrast limit the detail that can be discerned. Among unusual findings is a frequent abundance of mitochondrial granules in material prepared by this method.

Many methods have been developed over the last few years for localizing enzymes and other materials within cells at the electron microscope level. Some of the impetus for this rise in cytochemistry came from two technical advances: a method for performing autoradiography on electron microscope sections (9, 54), and the introduction of glutaraldehyde as a fixative (56). Both of these have yielded rich returns, but technical problems now stand in the way of further developments. Autoradiography has been valuable in localizing large, insoluble molecules, but smaller, more mobile molecules and ions usually are not maintained in place by current means of preservation. And although some enzymes survive glutaraldehyde fixation, a majority probably do not retain

enough activity for subsequent cytochemistry. It therefore seems important to find ways of preparing biological tissues for electron microscopy with minimal damage or displacement of enzymes and other substances.

One approach to this problem that has been attractive to light and electron microscopists over the years is that of cutting sections of frozen tissue. Although effective frozen sectioning techniques are available for light microscopy, no routine method has emerged for electron microscopy. Several workers have developed means of cutting frozen thin sections (the work of Fernández-Morán, Bernhard, and others will be described in the Discussion) but so far none of these methods has come into general use, probably because of the

expense or complexity of the technique, or of the difficulty in dealing with unfixed and unembedded tissue.

Any method for localizing soluble materials within cells by EM autoradiography must satisfy stringent requirements. Even at the light microscope level, Stumpf and Roth (66, 67) found that the localization of diffusible substances could be trusted only if performed on frozen sections of fresh tissue cut and freeze-dried below about -60°C and in the absence of any solvent. No technique has been available that would provide sections for electron microscopy satisfying these criteria.

This report describes a relatively simple means of preparing frozen thin sections of fresh tissue for electron microscopy. By this method, it is possible to cut, flatten, and freeze-dry the sections at low temperature without exposing them to any solvent. The morphology of rat pancreas and liver as seen in these frozen thin sections is described

MATERIALS AND METHODS

*The Cutting Bowl*¹

An unmodified Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.) was used for this work, but other current microtomes presumably could be adapted. To set up the sectioning device, the chuck and stage are removed from the microtome.

¹ The description in this paper pertains to the simple device I developed at Stanford, which lacked certain conveniences that were difficult to machine. However, since early 1969 I have been using a prototype produced by Ivan Sorvall, Inc., Norwalk, Conn., which is based on my model and was developed with my help. This instrument has been illustrated in two recent publications (11, 12). Some of the improvements found in this prototype are: (a) the ebony bar is replaced by an inverted "U" (composed of metal and of linen-based phenolic plastic) which allows sectioning in the axis of the cutting arm and is probably more stable; (b) the chucks are clamped rather than screwed into position; (c) there is a single knife holder that accepts either glass or diamond knives; (d) the knife angle is adjustable; (e) the bowl has micrometer advance and lateral traverse controls that allow more accurate positioning of the knife; (f) there is more room in the bowl. Figs. 1, 2, 4, 6, 7, 15, 16, and 17 in this paper pertain to my original model, while Figs. 8-14, and the inset to Fig. 17, are derived from sections cut with the Sorvall prototype.

The chuck is replaced with a bar of ebony (Fig. 1) attached to the cutting arm of the microtome by a 10-32 screw. The ebony bar extends down from the cutting arm about 6 cm and has a copper plate attached at its lower end. A small copper chuck, which will bear the tissue, is screwed into a hole in the plate.

The stage of the microtome is replaced with a bowl-shaped Dewar flask about 10 cm in outer diameter (Fig. 1). The wall of the bowl consists of three layers: an outer stainless-steel layer adapted from a 1200 ml stainless-steel beaker, a middle layer of foam rubber insulation, and an inner polypropylene layer made from a 400 ml Nalgene beaker (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N. Y.). Between the stainless steel and polypropylene in the bottom of the bowl is a plastic spacer about 1 cm thick, bearing several foam rubber-filled holes. The bowl is supported on a stainless steel baseplate that is milled so it can be clamped by the stage locking device of the microtome, but before being clamped it can be moved forward and backward and to some extent sideways. Part of the wall of the bowl must be cut away to accommodate the cutting arm and allow its movement. A hole is drilled through the wall obliquely in the lower left part of the bowl to accommodate an entry tube through which cold gas can be introduced into the bowl.

A knife holder for either a glass or diamond knife is mounted in the bottom of the bowl. The glass knife holder (Fig. 1) is made of brass and is designed to hold the uniform glass knife made by an LKB Knifemaker. The holder for a diamond knife (Fig. 4) is a brass plate with a screw passing up into the base of one of the commercial knife assemblies. Either knife holder is firmly attached to the bottom of the bowl by means of four stainless-steel screws that pass up through the baseplate and the bottom of the bowl, and screw into the knife holder.

When the microtome is operated, the chuck passes the edge of the knife in the depths of the bowl just as it does in normal cutting, and the thickness of sections or speed of cutting can be adjusted in the usual way. The glass and diamond knife holders are made so the knives tilt back 8° , which provides a small clearance angle in the particular geometry of this system. Lateral positioning of the knife with respect to the tissue is permitted by leeway in the clamping guide of the baseplate.

The method used in this study for freezing the tissue and for cutting, flattening, and drying the sections is summarized in Fig. 3. The steps will now be described in detail.

Freezing the Tissue

Tissue is frozen by being brought against a block of copper that is at liquid nitrogen temperature,

-196°C (Fig. 3, step 2, and Fig. 5). The copper block is 99.999+ % pure copper (courtesy of the Central Research Laboratories of the American Smelting and Refining Company, South Plainfield, N. J.), is about 1.5 cm in diameter, and is highly polished on its upper surface. A chuck bearing the tissue fits into the end of a wooden sliding rod, whose descent toward the copper block is controlled by a guide.

A small piece of fresh tissue is removed from an anesthetized animal (rat pancreas and liver were used in this study), and cut into bits 1-2 mm² with a sharp razor blade on a wax plate. A chuck may previously have been coated with a thin layer of vacuum grease (Dow Corning High Vacuum, Dow Corning Corp., Midland, Mich.) and then with fine cigarette ashes, in order to increase the rate at which the chuck will reach thermal equilibrium in liquid nitrogen (see Discussion). Ashes and grease are wiped off the tip of the chuck, and it is then fitted into the end of the sliding rod. A bit of tissue is placed on the end of the chuck (Fig. 3, step 1), and the sliding rod is started down the guide. The copper block, previously brought to liquid nitrogen temperature in a small Dewar flask, is now raised above the surface of the liquid nitrogen. The tissue is brought quickly but gently against the copper block for a few seconds (Fig. 3, step 2), and then both block and chuck are lowered back into the liquid nitrogen. In a minute or so the rod is removed, and the chuck is gripped with long-nosed pliers and quickly placed in liquid nitrogen until needed. Chucks with frozen tissue on them can be stored in a liquid nitrogen refrigerator.²

² A very convenient and inexpensive liquid-nitrogen refrigerator can be improvised using Kodak 35 mm film cans with holes punched in their sides as containers. The cans are kept in a nitrogen Dewar and

A similar approach has been developed in this laboratory for freezing tissues at liquid helium temperature (-268°C).³ The lower ends of the guide (with copper plug) and the sliding rod are shown at right in Fig. 5; they are modified to fit the long, narrow entry channel of helium Dewars. The method will not be described in detail, since preliminary results so far have failed to demonstrate sufficient advantage to warrant the additional difficulty and expense of working with liquid helium.

Cooling the Bowl

The bowl is cooled by being flushed continuously with a jet of cold nitrogen gas, evolved in a nearby 31 liter Dewar (Model LD-31, Linde Division, Union Carbide Corp., New York) by the boiling of liquid nitrogen (Fig. 2).⁴ The gas is conducted to the bowl through rubber tubing (3/8 inch inner diameter) that enters by means of a hole in the bowl's lower-left rear wall (Fig. 1). The temperature of the gas entering the bowl depends on the rate of boiling in the Dewar, which in turn is governed by the current through a heater that hangs down into the liquid nitrogen. The heater is a 150 w soldering iron with current controlled by a Variac. A plexiglass shelf with holes in it (Fig. 1) causes the cold nitrogen gas to circulate in the bottom of the bowl before rising, which gives more even cooling. Recently, the last 6 inches of the nitro-

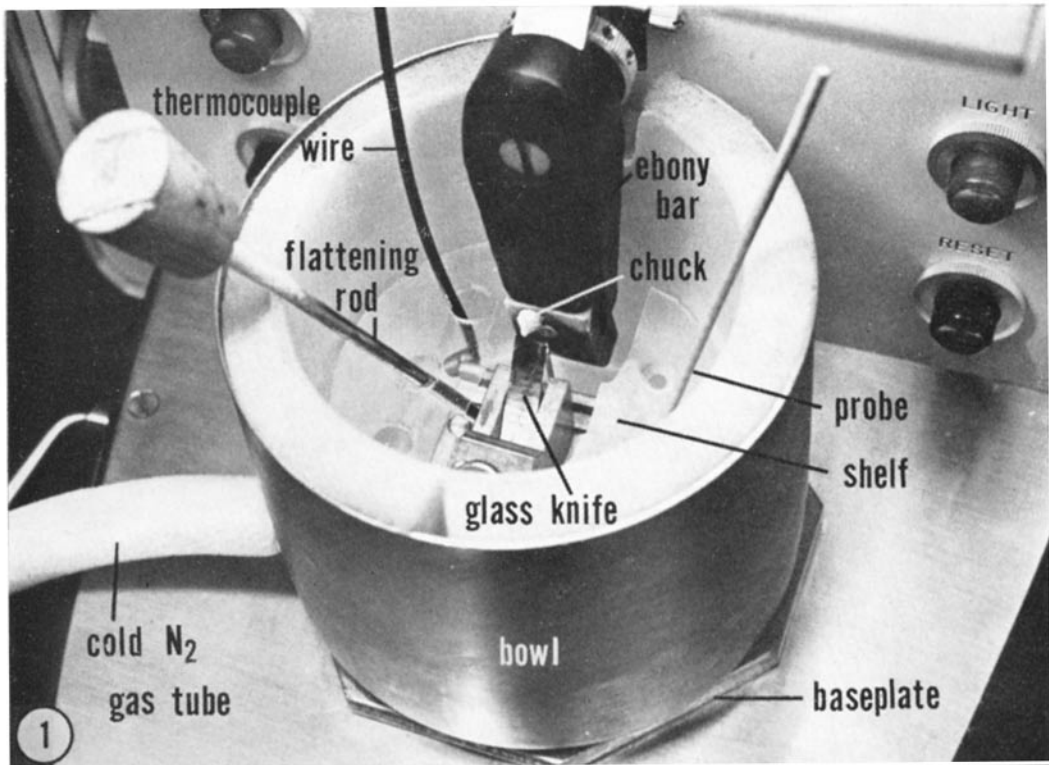
are removed by means of a labeled string that attaches to their lid and extends out of the Dewar. I learned of this from Dr. Daniel Branton.

³ I want to thank Mr. Robert J. Gillis, of the Cryogenic Laboratory of Stanford's Department of Physics, for making liquid helium available to me, and for his aid and advice in its use.

⁴ This means of cooling the bowl was suggested to me by Dr. Harold T. Meryman.

FIGURE 1 Device for cutting frozen thin sections. The stage of the ultramicrotome has been replaced by a bowl-shaped Dewar whose wall is insulated with foam rubber. The usual chuck of the microtome is replaced by an ebony bar with a copper plate at its lower end. Into the plate is screwed a small copper chuck that bears the frozen tissue. A knife holder, here with a glass knife, is mounted in the bottom of the bowl. The interior of the bowl is cooled with cold nitrogen gas which enters the bowl through the tube at left, circulates under a plexiglass shelf, and then rises to leave the bowl, excluding atmospheric moisture that could cause frost. The temperature is read with an iron-constantan thermocouple. A copper rod is used in flattening the sections after they have been carried from the knife edge to grids lying on the shelf. A probe is used for transporting and manipulating the sections and consists of an eyelash mounted at the end of a wooden stick.

FIGURE 2 The equipment. The bowl, situated on the microtome stage, receives cold nitrogen gas by way of a tube from a Dewar of liquid nitrogen. The gas arises as the liquid nitrogen is caused to boil by a heater, whose current is controlled with a Variac. The thermocouple is read with a potentiometer against an ice water reference. Extra-dry nitrogen gas from the tank at right is used for drying grids and for other tasks.



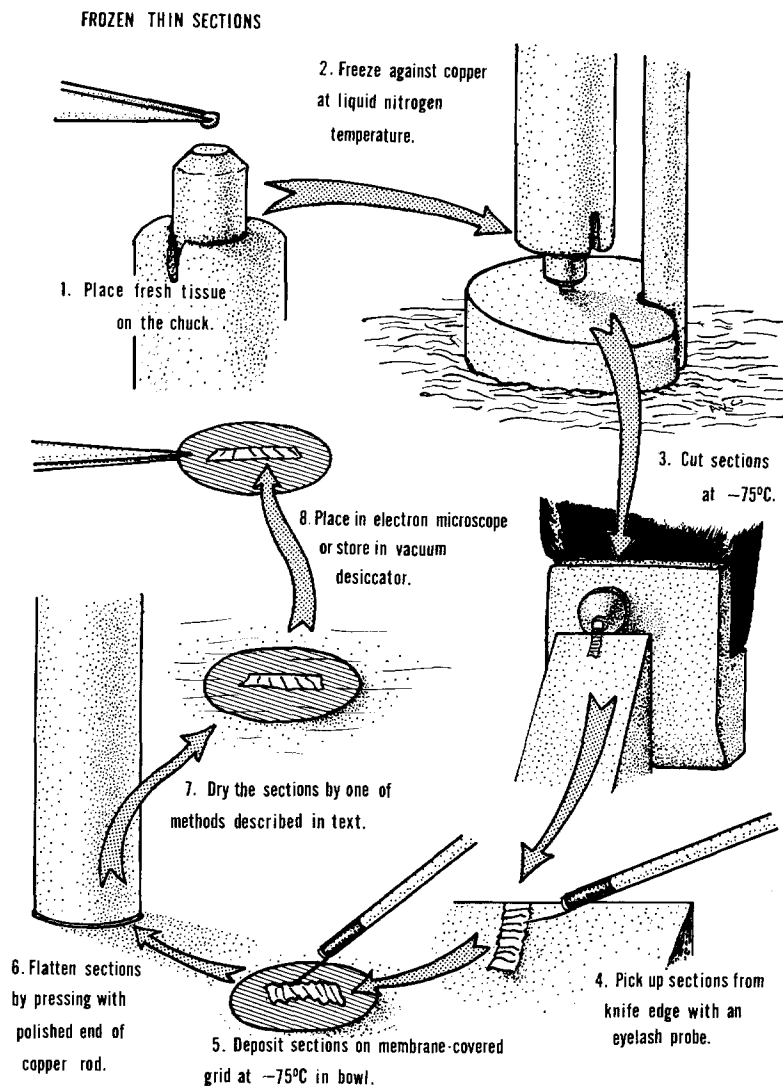


FIGURE 3 Summary of the technique for freezing the tissue, and cutting, flattening, and drying the sections. See text for a detailed description of the steps. The tissue is untreated, except for freezing and drying, and the whole procedure can be carried out at low temperature without any solvent ever coming in contact with the tissue. It is thus possible to obtain dried sections that contain the native materials and in which diffusible substances remain essentially in place.

gen gas line has been expanded to $\frac{3}{4}$ inch inside diameter, so the cold gas slows down somewhat before entering the bowl. This cuts down breezes within the bowl, and thus makes it much easier to handle sections. The temperature is measured by an iron-constantan thermocouple (Fig. 1), read with a potentiometer (Leeds and Northrup Co., Philadelphia, Pa., model 8686) against an ice-water standard (Fig. 2).

The open bowl can be cooled in this manner for

hours without any appreciable internal accumulation of frost, since the cold nitrogen gas is heavy and excludes atmospheric moisture. The cold gas is anhydrous by virtue of its origin from liquid nitrogen. Approximately 10 liters of liquid nitrogen are boiled away during an afternoon's cutting at -75°C . The cutting temperature can be set anywhere between -160°C and -30°C by means of the Variac, and remains quite constant. The optimal temperature

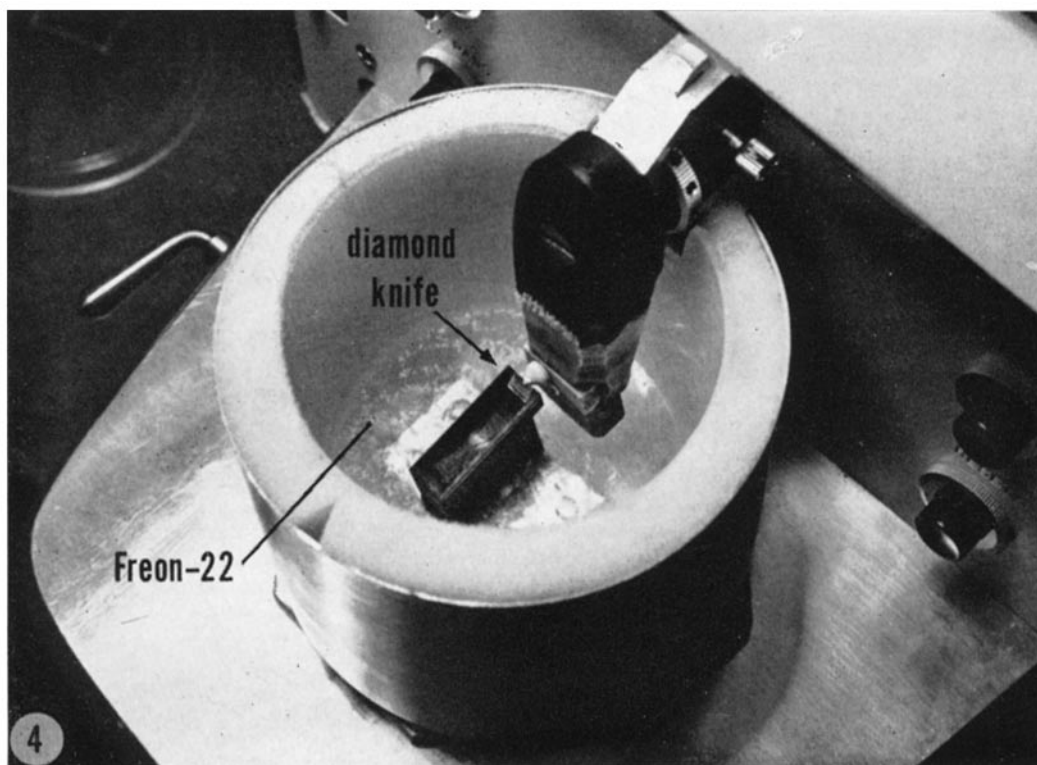


FIGURE 4 As an alternative means of cooling the bowl, a liquified gas, in this case Freon-22 (-41°C), has been poured into the bowl to a level well below the knife edge. The bowl fills with cold gas as the Freon boils, thus cooling the bowl and eliminating atmospheric moisture. A diamond knife is in use here.

range for cutting frozen thin sections is approximately -65° to -90°C ; at temperatures much colder than this only section fragments are obtained.

Another means of cooling that is more simple but less flexible and accurate than the preceding is to treat the bowl as a Dewar flask, filling it to a level well below the knife edge with a liquified gas. As the coolant boils, cold gas fills the bowl, keeping the components within at low temperature and relatively free from atmospheric moisture. To prepare for this type of cooling, the gas entry tube is removed and the hole in the wall is plugged from inside with a cork stopped with a little water around its margin to insure the seal.

Fig. 4 shows this mode of cooling, with Freon-22 (CHClF_2 , which boils at -41°C) as the coolant. The use of liquid nitrogen (-196°C) as the coolant has been illustrated in an earlier paper (reference 13, Fig. 24). Other coolants that have been employed in this study are Freon-14 (CF_4 , -128°C), Freon-13 (CClF_3 , -81°C), and Freon-12 (CCl_2F_2 , -30°C).⁵

⁵ Freons are obtained from Du Pont's Freon Products Division. A simple way of getting Freons 12 and 22

The ambient temperature in the vicinity of the chuck and knife edge (measured with the iron-constantan thermocouple) is about -175°C for liquid nitrogen, about -90°C for Freon-14, about -50°C for Freon-13, about -20°C for Freon-22, and about -10°C for Freon-12. Tissues seem brittle when cut over liquid nitrogen and only small section fragments are obtained (which nevertheless show the same tissue fine structure as sections obtained at warmer tem-

out of the cylinders in liquid form is to invert the cylinder and allow the gas to escape until the nozzle cools, at which time liquid will emerge and can be collected in a precooled Dewar flask. The liquid form of Freons 13 or 14 can be obtained by passing the gas rapidly through a copper coil immersed in liquid nitrogen. For critical work it may be desirable to distill Freon-22, since it commonly contains an oily residue that is deposited on structures in the bowl when the Freon evaporates. Freons are excellent lipid solvents and therefore must be used with care around diamond knives, since they can dissolve the mounting medium that holds the diamond in place.

peratures). Freon-22 is inexpensive and is effective for routine cutting of thin sections; Freon-13 is more expensive, but also yields good sections and can be used when a colder temperature is desired.

Cutting and Handling Sections

Chucks that have been stored in a nitrogen refrigerator are poured out into a glass Petri dish full of liquid nitrogen, where a chuck is selected and the rest are returned to the refrigerator. If the chuck bears a coating of ashes, this must be scraped off with a razor blade under liquid nitrogen. The chuck holder is kept cold in the same Petri dish. After the chuck is screwed tightly into the chuck holder by means of cold long-nosed pliers,⁶ a cold razor blade (or cold fingernail file) can be used to fashion a suitable block face. The assembly is then transferred quickly to the bowl and attached tightly to the cutting arm. Before cutting commences the chuck must warm to the temperature of the bowl, which takes about 15 min when the bowl is cooled with cold nitrogen gas. Otherwise, the expansion of the chuck as it warms will yield much thicker sections than are anticipated.

Glass knives made with an LKB Knifemaker and bearing a 45° cutting edge have been effective in the device described here. Large knife angles (2) yield sections that are more compressed than those cut with the usual angle. A diamond knife can be used and does not seem to be damaged appreciably by thin sectioning.

To initiate cutting, the knife edge is brought very near the tissue by moving the bowl, which is then clamped tightly in place. The microtome, set for about 2000 Å thickness, is then turned on, and gradually advances the tissue toward the knife. When sectioning begins, the cutting thickness is reduced to a setting between 540 and 1000 Å and the cutting speed is set to about 1.0 mm/sec. If cutting is intermittent, then sections are only used when at least two or three have been produced in consecutive passes.

As sections are cut (Fig. 3, step 3), they appear either translucent or white at the edge of the knife (Fig. 6) when viewed with the microtome's dissecting microscope (fitted with a long focal length attachment). Translucent sections are more favorable than white ones, since they indicate less ice-crystal damage in the tissue. Occasionally, the sections come off in a

⁶ In the prototype described in footnote 1, the chuck holder is left in the bowl and the chuck is merely slipped into it and clamped. The more cumbersome procedure outlined here is necessary with my original model because the chuck is screwed into the chuck holder, which can only be accomplished outside the bowl.

ribbon (Fig. 6). Usually, however, the sections are separate and may curl, bunch on one side, or double back over the knife edge. When this happens, an eyelash probe, consisting of an eyelash mounted on the end of an applicator stick (Fig. 1 and Fig. 3, step 4), can be used to tease the sections into a more favorable position.

An eyelash probe is used to pick up the sections from the knife edge and transfer them to grids lying nearby on the plexiglass shelf (Fig. 3, steps 4 and 5). A second eyelash probe may be useful in positioning the sections optimally on the grid. The 200-mesh copper grids have a Formvar membrane, with or without a carbon coat.

Another method of placing sections on a grid is to grasp the grid with stainless-steel jeweler's forceps, cool both the grid and forceps tips in the cold atmosphere of the bowl, then carefully touch the membrane surface of the grid to the sections on the knife edge with a gentle upward movement. The sections will usually stick to the membrane and are often flattened somewhat in the process. Although simpler than the preceding method, this approach does not offer the same degree of control over the exact position of the sections on the grid.

Flattening and Drying the Sections

To flatten sections against the grid membrane, the highly polished end of a cold $\frac{1}{8}$ inch copper rod (Fig. 3, step 6) is brought down firmly on the sections and grid, and is rocked slightly back and forth. The grid bars bear the brunt of the pressure, but the sections are pressed with sufficient force against the supple membranes that they generally flatten and adhere. The polishing of the rod is carried out with metal polish (Wenol, Ladd Research Industries, Inc., Burlington, Vt.) and a power buffer. Once the rod is cold, the polished end must be kept in the bowl to protect it from frost. The other end bears a cork to facilitate handling (Fig. 1). After flattening, the grids can be left on the plexiglass shelf, or stored in small snap-cap vials (BEEM capsules, BEEM, Inc., Bronx, N. Y.) in the bowl.

The sections are dried in any one of several ways. The comparative advantages of the methods will be considered in the Discussion. A very simple means of drying (and further flattening) the sections is to close a snap-cap vial containing a grid and bring it out of the bowl while warming it with the hands. The sections thaw briefly, are pulled flat against the membrane by the water's surface tension, and then dry in the atmosphere of anhydrous nitrogen gas present within the vial. Figs. 11, 12, and 14 show sections dried by this means.

Another simple method of drying also involves a brief thaw, but is more rapid and may thus allow less time for movement of soluble materials and other

changes in the tissue before drying. A grid with sections on it is grasped with cold jeweler's forceps and is then flushed with room-temperature nitrogen gas (Matheson extra dry) as it is brought out of the bowl. Figs. 15, 16, and 17 show tissue dried in this manner.

If freeze-drying is advisable, this can be done easily within the bowl by placing the grid on some surface below the plastic shelf and leaving it there for about an hour (see Discussion). After drying, the grid must be flushed with room-temperature nitrogen gas (extra dry) as it is removed from the bowl in order to avoid condensation of moisture, and should then be transferred immediately to a vacuum desiccator. The section in Fig. 13 was dried by this procedure.

After the sections are dry, the grids are either placed directly into the electron microscope, subjected to further treatment, or stored. Dry sections are extremely hygroscopic and must be protected from moisture. They may be stored in open BEEM capsules (one grid per capsule) over phosphorus pentoxide in a vacuum desiccator evacuated to about 10^{-3} torr. Whenever the desiccator is to be opened, it is first brought to atmospheric pressure with extra-dry nitrogen gas. BEEM capsules should be closed before they are removed from the desiccator and transported to the microscope room or elsewhere.

The sections in this study were examined either in an RCA EMU-3F electron microscope (Figs. 11-14 and 17) operated at 50 kv and fitted with a $30\ \mu$ French foil objective aperture, in a Philips EM-200 electron microscope (Figs. 15 and 16) at 60 kv with a $20\ \mu$ aperture, or in a Siemens 1A electron microscope (Fig. 17, inset) at 60 kv with a $30\ \mu$ aperture. For taking electron micrographs, the beam intensity was raised judiciously to a level necessary for a 3 sec exposure, and focusing was carried out on holes, if these were available in the field. Sections could only withstand the greater beam intensity required for high magnification if they were stabilized under low beam for a minute or so, or were carbon coated before being put into the microscope.

Sections for Light Microscopy

Thicker sections for light microscopy are cut at warmer temperatures, for example -40° to -55°C for $1\text{-}2\text{-}\mu$ sections. Temperatures above -40°C should be used for still thicker sections; the range from -20° to 0°C can be attained by warming the tube between the Dewar and bowl with a heating tape, or by using Freon-22 or Freon-12 as coolants in the bowl. The sections are picked up from the edge of the knife with an eyelash probe and are transferred either to a BEEM capsule for storage, or are placed on a cover slip lying on the plastic shelf. The sections are flattened on the cover slip by gentle pressure with the polished copper rod. The cover

slips are placed in improvised holders and stored in liquid nitrogen.

Sections that are mounted on cover slips can be dried by being flushed with extra-dry nitrogen gas (Matheson) at room temperature as the cover slip is removed from the bowl. Alternatively, to obtain optimal morphology or for the localization of diffusible substances, the sections are freeze-dried as described by Stumpf and Roth (67),⁷ with the following modifications. The freeze-drying chamber is first brought to atmospheric pressure with extra-dry nitrogen gas and is then cooled with acetone-dry ice. Sections are transported from the bowl to the chamber in the improvised holders (for sections mounted on cover slips) or in BEEM capsules (for unmounted sections), carried in a small Dewar of liquid nitrogen. Caps must be removed from the BEEM capsules by gentle twisting with long-nosed pliers before the capsules are placed in a receptacle within the drying chamber.

Dry sections are stored over phosphorus pentoxide in a vacuum desiccator. To observe untreated tissue, cover slips bearing the dried sections are mounted on glass slides with mineral oil and the material is viewed with phase contrast. Unmounted sections can be used for dry-emulsion autoradiography to localize diffusible substances, as described by Stumpf (64).

The following method of fixing and staining the sections yields routine permanent preparations in which mitochondria and other organelles are shown to good advantage. Sections $1\text{-}3\ \mu$ thick are cut and flattened on a cover slip within the bowl as described above. The cover slip is removed from the bowl and transferred directly to 70% ethanol in a Columbia staining jar for 3 min. The cover slip is then passed successively through 1 min in distilled water, 30 sec in "Mallory A" (0.2% acid fuchsin in distilled water), 30 min in "Mallory B" (0.5% aniline blue, 2% orange G, and 1% phosphomolybdic acid in distilled water), rinsed briefly in distilled water, dipped in 95% ethanol, then left 3 min in absolute ethanol. After a few minutes in xylene the cover slip is mounted on a glass slide with Permunt (Fisher Scientific Company, Pittsburgh, Pa.). A light micrograph of tissue prepared by a similar but somewhat more lengthy version of this technique has already been published (reference 10, Fig. 5).

RESULTS

The light and electron micrographs presented here show frozen sections of rat pancreas and liver that

⁷ Available from Delmar Scientific Glass Products Div. Coleman Instruments, Maywood, Ill. 60153. A more recent version is being produced by Thermovac

are unfixed, unembedded, unstained, and have come in contact with no solvents. Tissues that have been fixed or treated in other ways can also be frozen and cut by this method, but the fresh-frozen material presents more technical difficulties and is also of greater interest.

Section Quality

This approach yields frozen thin sections that are often large and even, and if the knife is sharp, show little chatter, although there may be some compression. Glass knives furnish sections of good quality, but a sharp diamond should yield sections that are thinner and more regular. The actual thickness of the sections is uncertain and probably irregular, since slight temperature changes in the bowl during cutting may give rise to expansions or contractions that influence section thickness at a given setting.

It is important to know whether true sections are being produced by the present method, or whether the knife is merely fracturing along structural planes in the tissue, as occurs in the technique of freeze-etching (45). To investigate this question, sections about 2000 Å thick cut with a glass knife were mounted on membrane-coated grids, dried in a jet of dry nitrogen gas, and examined in a JEOLCO JSM-2 scanning electron microscope.⁸ Fig. 7 is a scanning micrograph taken in secondary electron mode with a 25 kv beam, and shows a section lying across grid squares of a copper mesh grid. The section is generally quite even in thick-

ness, although there are some drying cracks. The appearance was similar throughout the material examined, and indicates that the tissue is being cut uniformly by the knife.

Ice Crystals

A very serious problem in any morphological study of frozen tissue is the ice-crystal damage that occurs during initial freezing or subsequent recrystallization (see Discussion). The rapid freezing used here should minimize the size of ice crystals, but most cells are permeated with them even in the best areas. The most favorable freezing occurs in a surface layer of tissue, which may correspond to a translucent layer about 30 μ thick present at the surface of the frozen block. Deeper regions show increasing damage to a point where large ice crystals fill the cells, obliterating cellular structure.

The morphology of holes left by ice crystals in frozen thin sections depends on the method of drying. In cells that are freeze-dried, the crystal outlines are maintained clearly, and the intervening cytoplasm appears to shrink somewhat during drying. The result is a considerable distortion of cellular structure which can obscure the organelles if the holes are extensive. On the other hand, a drying procedure that includes a brief thaw permits the cytoplasm to spread back into areas previously occupied by ice crystals, with a consequent general improvement in the morphology. However, it must be borne in mind that even a momentary thaw may allow movement of diffusible substances and other changes to take place in the tissue.

Occasional cells or groups of cells come through freezing with little or no evidence of ice crystals, perhaps because of partial dehydration before freezing (53, 60). Most of the cells illustrated in the

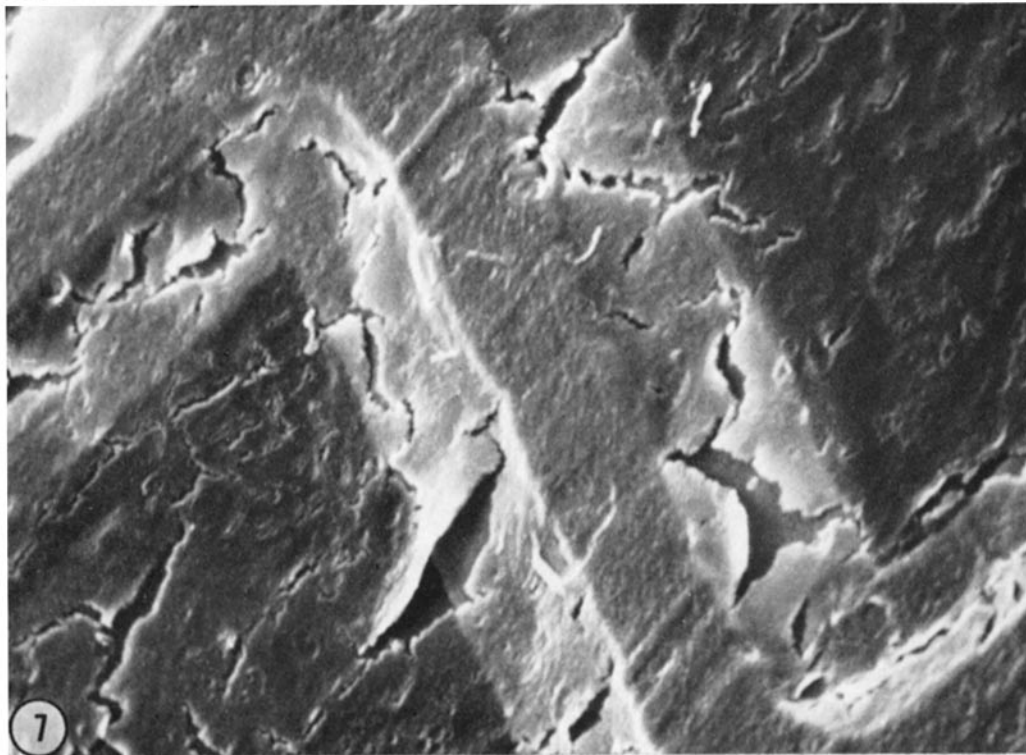
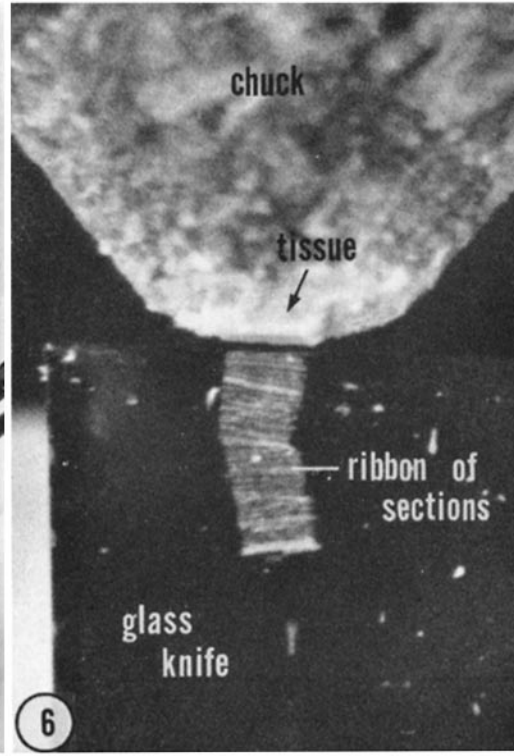
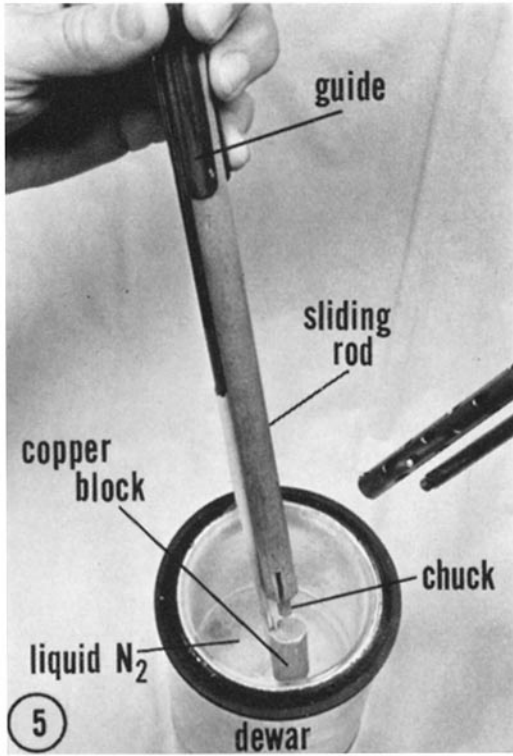
Industries Corp., Sub. Tod Mfg. Corp., Copiaque, N. Y. 11726.

⁸ I would like to thank the Japan Electron Optics Laboratory Co., Ltd., Medford, Mass., for taking the scanning electron micrograph shown in Fig. 7.

FIGURE 5 Tool for freezing the tissue. A chuck, bearing a piece of fresh tissue, is mounted in the end of a sliding rod that can be brought rapidly but gently against a copper block precooled to the temperature of liquid nitrogen (-196°C). The path of the sliding rod is controlled by a guide. At right are the lower ends of a modified guide and sliding rod used for freezing at liquid helium temperature (-268°C).

FIGURE 6 Ribbon of frozen thin sections. This photograph, taken through the dissecting microscope of the ultramicrotome, shows a ribbon of 1000 Å sections of fresh-frozen liver cut with a glass knife at about -75°C . The bowl was cooled with cold nitrogen gas.

FIGURE 7 Scanning electron micrograph of a 2000 Å frozen thin section at very low power. The section lies across a grid bar and the adjoining carbon-coated membranes. Although there are drying cracks, the section is clearly a fairly uniform slice through the tissue, and not just the product of fractures along irregular tissue planes, as happens in freeze-etching. $\times 1700$.



electron micrographs of the present paper are of this type, although Figs. 14 and 16 show some areas of ice-crystal damage. All were melt-dried except for that in Fig. 13 which is from a frozen-dried preparation. Further technical details on the figures precede the legend to Fig. 11.

In addition to ice-crystal holes, melt-dried preparations also exhibit holes that arise during drying (Figs. 11, 14, and 16). These drying holes (*h*) have very distinct outlines, in contrast to the very indistinct margins of ice-crystal holes (*i*) seen in such material. Often a drying hole is present within the boundary of an ice-crystal hole (upper right in Fig. 14). Lines of small, clear-cut drying holes, too small to be due to ice crystals, often lie between cells (e.g., Fig. 11).

Light Microscopy

Figs. 8–10 are light micrographs of fresh-frozen pancreas and liver sectioned at 1–2 μ , freeze-dried and photographed under phase contrast. They illustrate the degree of structural integrity seen in the best material obtained by this method. Although the ice-crystal damage is particularly low in the fields shown here, the sections themselves are of a quality that can be obtained routinely. A description of the morphological features shown in the figures, as well as further details on methodology, are provided in the figure legends.

Electron Microscopy

Electron micrographs of fresh-frozen rat pancreas and liver are shown in Figs. 11–17. The sections show relatively little ice damage and are even, with no obvious chatter, although there are signs of compression in some cases. The material in Figs. 11–16 was cut with the microtome set at 540–1000 Å thickness with glass knives, at a tem-

perature of -70° to -85°C maintained by cold nitrogen gas. Further details of preparative technique are specified in the figure legends. Similar morphology is seen in material sectioned at other temperatures, such as with Freon-22 at a cutting temperature of about -20°C (Fig. 17, cut with a diamond knife) or, at the other extreme, in section fragments cut over liquid nitrogen at about -175°C .

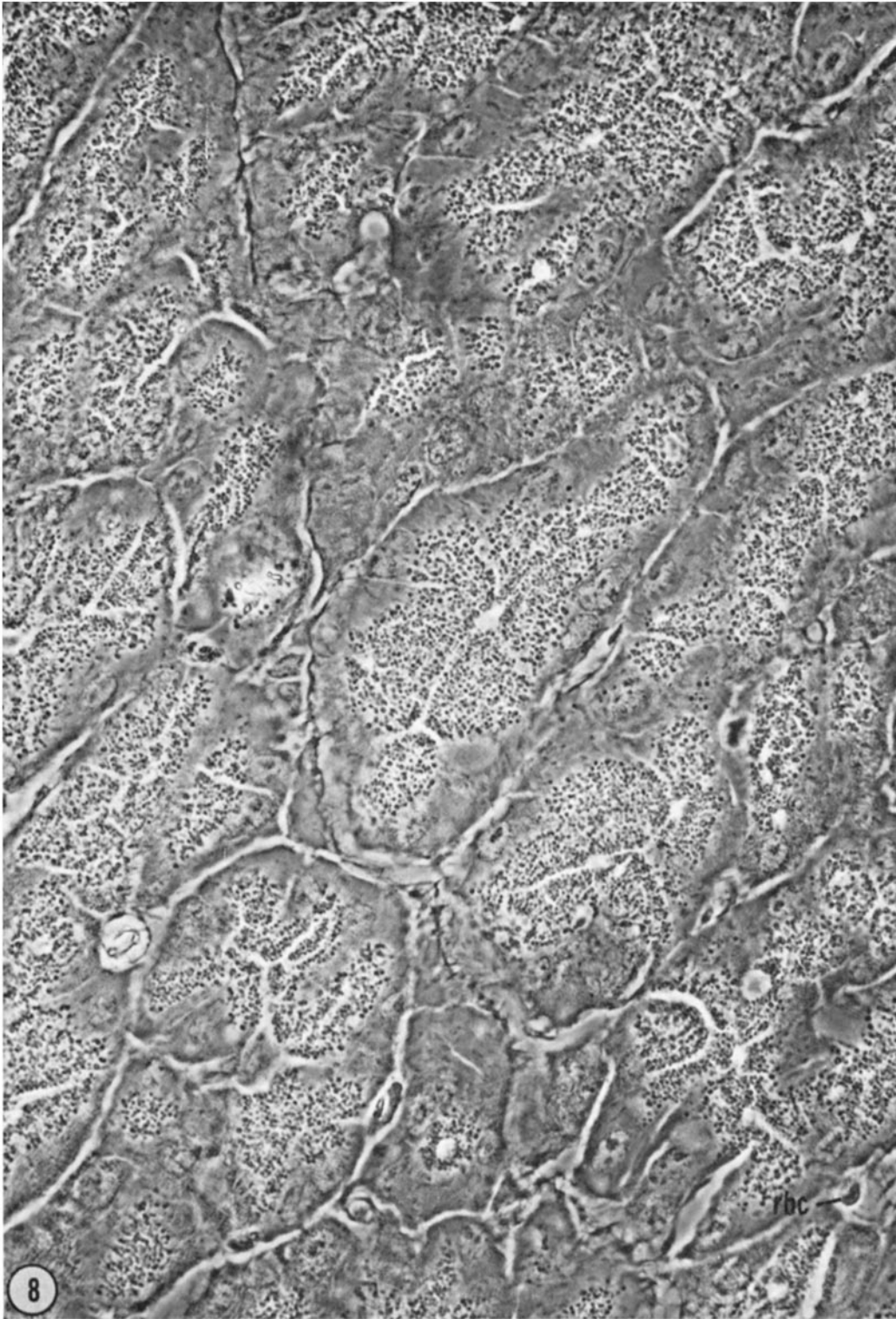
The figure legends provide a basic description of the electron micrographs illustrating rat pancreas (Figs. 11–13) and liver (Figs. 14–17). The morphology is generally similar to that seen in conventional fixed and embedded tissue (rat pancreas, 20; rat liver, 6), with which it will be assumed that the reader is familiar.

The individual organelles will now be described as they appear in frozen thin sections of these two tissues. Since the material is seen by its inherent contrast without staining, many features are poorly defined. The nature of image contrast is considered in the Discussion.

The nuclei (*n*, Figs. 11, 12, 14, and 15) in frozen thin sections have much the same appearance as those seen in material prepared by conventional means. The condensed chromatin (*cc*), or heterochromatin, is often dense in appearance (Fig. 15) although in other micrographs it may appear lighter than the background nucleoplasm (Figs. 11, 12, and 14). At higher magnification the chromatin appears finely granular but shows no interpretable detail. Rather dense boundaries are seen around the condensed chromatin in Fig. 12 but these may be artifacts (see end of Results section). Near the chromatin are occasional perichromatin granules (*pg*, Fig. 15) about 200 Å in diameter that are surrounded by halos. Similar granules about 500 Å in diameter occur in the nucleus at upper right in Fig. 12, but since they

FIGURES 8–10 Light micrographs of frozen thick sections of fresh-frozen tissue viewed under phase contrast. Sections were cut with a glass knife, flattened on a cover slip in the bowl at about -55°C , freeze-dried at about -76°C in the Stumpf-Roth unit (67), then mounted in mineral oil on a glass slide and viewed with a Zeiss 40 \times (Fig. 8) or 100 \times (Figs. 9 and 10) planapo-oil phase objective. The material is thus unfixed, unembedded and unstained, and comes in contact with no solvent during cutting, flattening or freeze-drying.

FIGURE 8 Frozen thick section (2 μ) of rat pancreas. The section is even and is relatively free from compression or chatter. Several acini appear in the field, each consisting of secretory cells arranged around a lumen. The acinus in the center is shown at higher magnification in Fig. 9. Capillaries, often containing red blood cells (*rbc*, at lower right), lie between the acini. $\times 980$.



lack halos and do not seem associated with the chromatin, it is doubtful that they are perichromatin granules. Areas of euchromatin between the condensed chromatin have a faintly mottled appearance which in favorable areas gives the impression of being fibrillar. Clusters of interchromatin granules about 150 Å in diameter occur here and there in the euchromatin (indicated by arrowheads in Fig. 15; also present in Fig. 12 although very low in contrast). The nucleolus (*nu*, Fig. 15) appears pale even when the surrounding condensed chromatin is dense. The texture of the nucleolus appears granular in some areas, although detail is difficult to discern.

The membranes of the cell are not usually visible in these unstained sections. An exception is the rough endoplasmic reticulum (*rer*, Figs. 11–17) which consists of the usual parallel arrays of flattened cisternae. The cisternae are somewhat irregular in thickness, varying between 300 and 500 Å, with a space of about 300–600 Å between cisternae. The content of the cisternae is usually paler than the background cytoplasm (for example, Fig. 12). In most preparations (Figs. 12, 14; and some areas of Fig. 16), the ribosomes seem to flatten out on the membranes to a varying degree, and are then less easily distinguished individually although they may contribute appreciably to the contrast of the membranes. In favorable areas of some sections, ribosomes retain their particulate form (Figs. 13, 16, and 17; places where they are seen particularly well are indicated by arrowheads). They are usually round in outline, with a diameter of approximately 120–150 Å, but in some orientations appear oblong, measuring about 160×120 Å.

The nuclear envelope (*ne*, Figs. 12 and 15) can be distinguished in favorable sections, since its outer layer resembles the rough ER, and the inner layer either has intrinsic contrast (Fig. 12) or is defined by the periphery of the condensed chro-

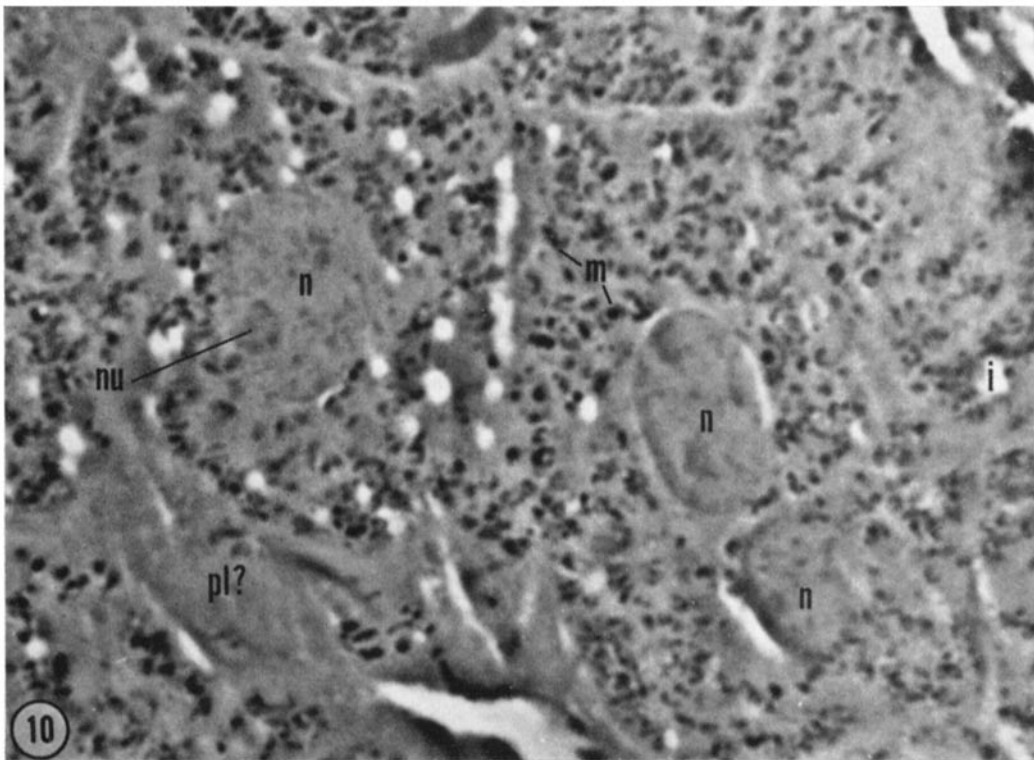
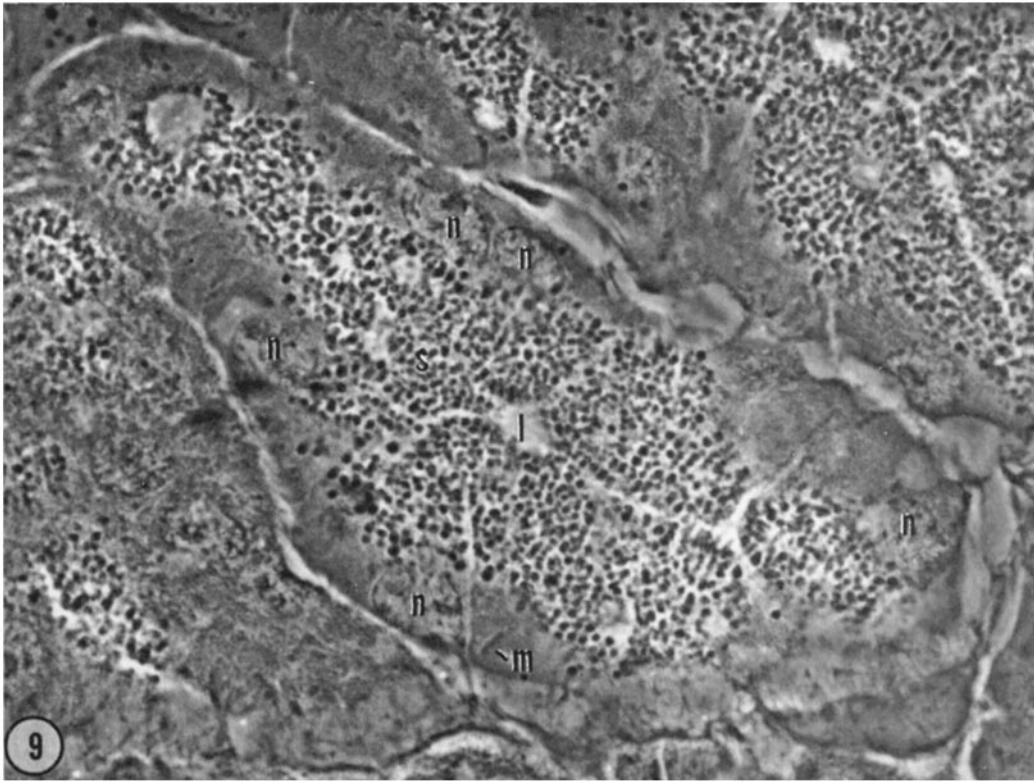
matin (Fig. 15). The outer and inner membranes are about 200–300 Å apart. A probable nuclear pore (*np*) is present in Fig. 12 as a discontinuity in the nuclear envelope and in the associated condensed chromatin.

From conventional electron microscopy, areas of cytoplasm that lack rough ER in liver cells would be expected to contain primarily smooth endoplasmic reticulum and interspersed glycogen, as well as occasional elements of the Golgi complex. When such areas are viewed in frozen thin sections of liver, the findings vary. Sometimes profiles are seen that resemble the anastomosing tubules seen in conventional micrographs of smooth ER, but without distinct membranes. This configuration has been illustrated in an earlier paper (reference 12, Fig. 2). In other micrographs of frozen thin sections, these areas show scattered dense spots having a size and irregular shape that suggest they could constitute material lying within the smooth ER. This suggestion and an electron micrograph showing such an area have been published elsewhere (reference 13, Fig. 23). However, this interpretation is open to question (see end of Results section). In still other electron micrographs the areas of presumed smooth ER show ice-crystal damage to a degree that prevents any interpretation of the fine structure (*i*, Fig. 14). No glycogen clusters were discerned in the areas of smooth ER; their density may match that of the background material.

The Golgi complex seems particularly susceptible to ice-crystal damage, and its undistorted structure in this material remains to be elucidated. The area labeled *g* in Fig. 12 is probably part of the Golgi complex, and is similar in morphology to that seen in other micrographs where the Golgi complex could be identified unequivocally by its characteristic position in the pancreatic acinar cell. The zone consists of a pale, homogeneous area through which run scattered strands of dense

FIGURE 9 Light micrograph at higher magnification of the pancreatic acinus in the center of the previous figure. Cells, some of whose nuclei (*n*) are visible, surround the central lumen (*l*), into which they empty their secretory granules (*s*). Mitochondria (*m*) are seen in the basal cytoplasm. $\times 1600$.

FIGURE 10 Two rat liver cells in a 1μ frozen thick section. The nucleus (*n*) at left contains a nucleolus (*nu*) and scattered patches of condensed chromatin. The cell at right is binucleate. Mitochondria (*m*) are the most prominent feature of the cytoplasm. A space between the hepatocytes, presumably a sinusoid, contains a homogeneous material that is probably plasma (*plf*). White spots mark the position of ice crystals (*i*); there are cracks in the section below and at upper right. $\times 3200$.



material about 150–400 Å in thickness. This morphology, however, is common in areas of severe ice-crystal damage followed by melt-drying, and probably does not give an accurate view of Golgi organization.

Mitochondria (*m*, Figs. 11–14, 16, and 17) are easily recognized by their number, shape, and mitochondrial granules, even though their membranes usually cannot be discerned; thus it is impossible to see cristae. In Fig. 12, each mitochondrion is outlined by a dark line which may correspond to the outer membrane, or may be an artifact. Pancreatic mitochondria (Figs. 11, 12, and 13) are about 0.25–0.4 μ in diameter, while those of the liver (Figs. 14, 16, and 17) measure 0.25–0.6 μ . Mitochondria seldom show ice crystals even when surrounding areas are extensively damaged. However, they may become badly shriveled during suboptimal freezing, since water is withdrawn from them into nearby ice crystals.

The matrix of the mitochondrion appears homogeneous, except for the presence of dense mitochondrial granules (*mg*, Figs. 13, 14, and 16). The granules are considerably more abundant and pleomorphic in frozen thin sections than in the usual fixed and embedded tissue. They vary in number and size (up to 600 Å in diameter) from one mitochondrion to another, or may be absent. In the liver cells of Fig. 14, the granules tend to lie at the periphery of the mitochondria. The granules were present in most of the liver tissue

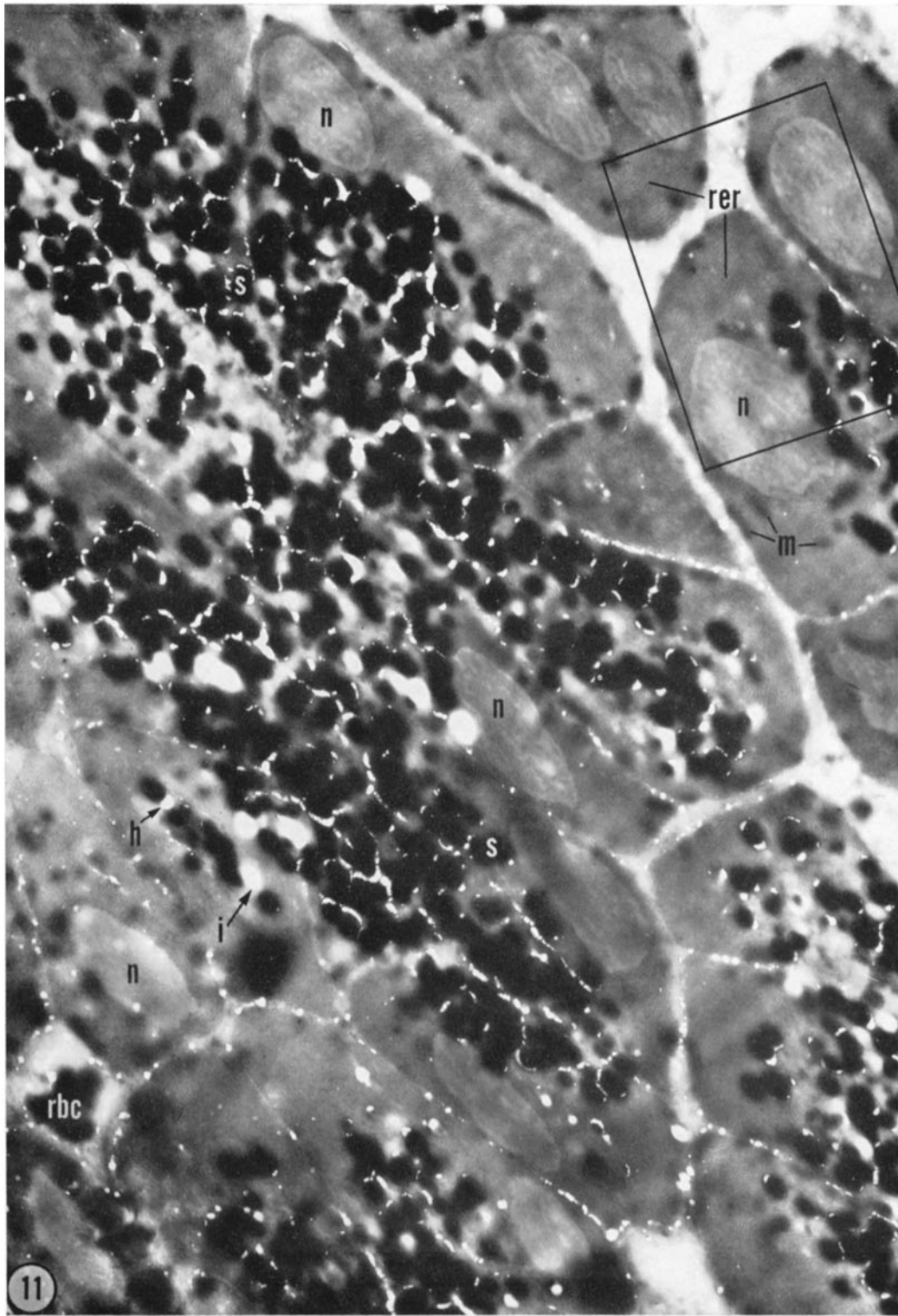
examined, but their presence in pancreas varied from animal to animal. There are indications that this was not due merely to differences in the technical procedure used in preparing the frozen thin sections. The pancreas in Figs. 11 and 12, which lacks mitochondrial granules, was prepared at about the same time and by the same procedure as was utilized for the liver in Fig. 14, where granules are abundant. Mitochondrial granules are numerous in the pancreatic acinar cell of Fig. 13, and were also present in all the pancreatic tissue examined from that animal, even though some of the frozen thin sections were freeze-dried in the bowl, while others were melt-dried. The nature of the granules will be considered in the Discussion.

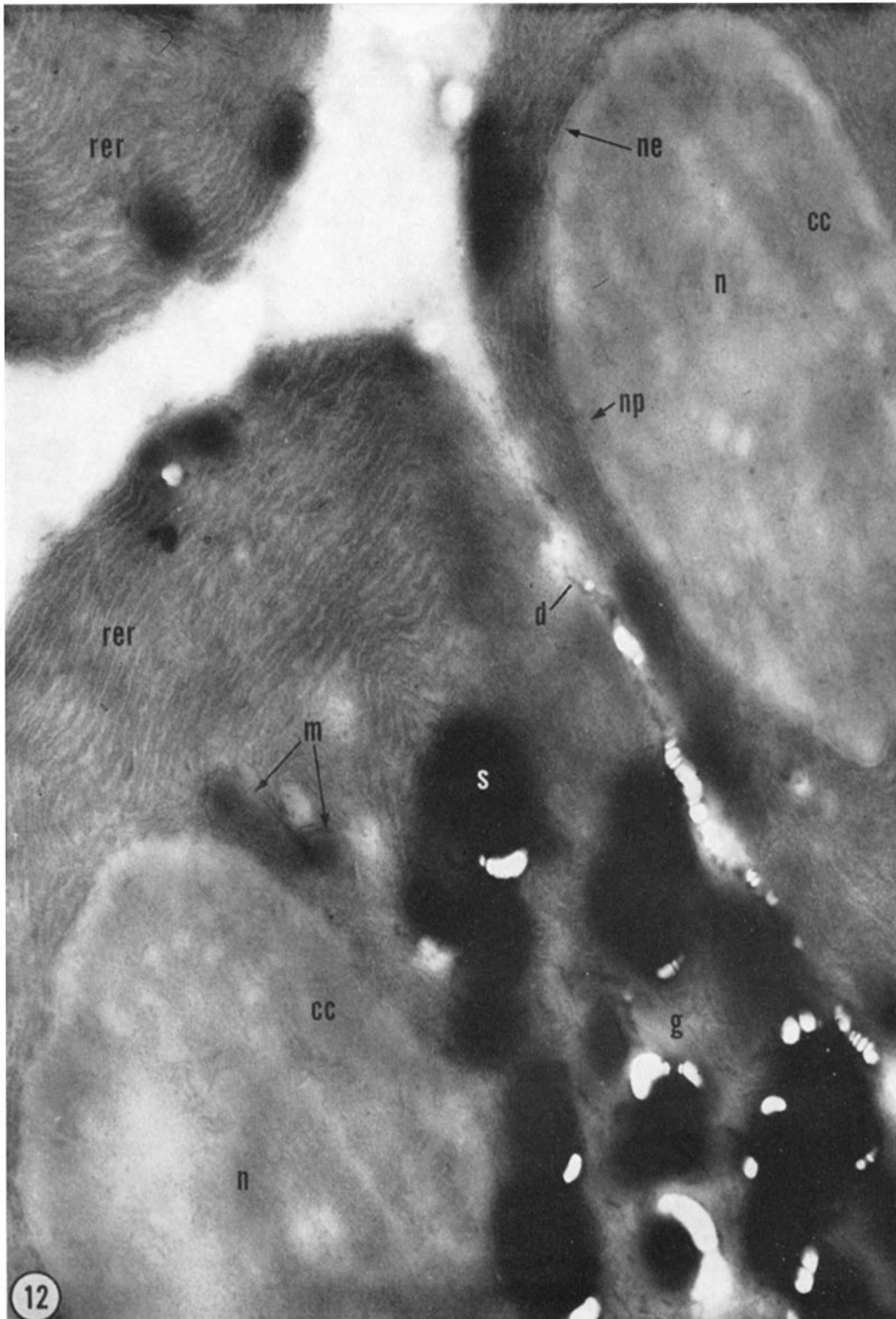
So far it has not been possible to distinguish peroxisomes (microbodies) and lysosomes from mitochondria with any confidence in frozen thin sections. Some of the oval structures in Fig. 14 lack typical mitochondrial granules, although they have a few indistinct granules about 250 Å in diameter around their periphery. These could be peroxisomes (*p*), although there is no indication of a uricase crystal.

Secretory or zymogen granules (*s*, Figs. 11 and 12) are 600–1000 Å in diameter, and appear black in these unstained sections, probably reflecting the high concentration of protein within them. Their boundaries are somewhat vague, which suggests a slight migration of material, perhaps

FIGURES 11–17 Electron micrographs showing frozen thin sections of fresh-frozen tissue. Except for Fig. 17, the sections were cut with a dry glass knife at a temperature of -70° to -90°C and with the microtome set for a cutting thickness of 540 Å (Fig. 13), 700 Å (Figs. 11, 12, and 14) or 1000 Å (Figs. 15 and 16). The sections were flattened against grid membranes by pressure from the polished end of a cold copper rod, and were then either freeze-dried in cold nitrogen gas (about -90°C) under the shelf of the bowl for 2 hr (Fig. 13), dried under a jet of room-temperature dry nitrogen gas (Figs. 15 and 16), or dried simply by being brought out of the bowl to room temperature within a BEEM capsule full of cold nitrogen gas (Figs. 11, 12, and 14). The tissue in Fig. 17 was cut with a diamond knife at a microtome setting of 850 Å, and with the bowl cooled by Freon-22 as shown in Fig. 4. The sections were picked up from the knife edge by bringing the grid face down on them, and they were then dried under a jet of room-temperature nitrogen gas. All of the material was viewed unstained in the electron microscope.

FIGURE 11 Frozen thin section of rat pancreas. The area shown in this electron micrograph is roughly comparable to that seen in a previous light micrograph (Fig. 9). Secretory granules (*s*) are prominent in the apical portions of the acinar cells, and occasional nuclei (*n*) are seen more basally. The cytoplasm at the base of the cells is filled with rough endoplasmic reticulum (*rer*) and scattered mitochondria (*m*). Spaces that contained ice crystals (*i*) have indistinct borders, while drying-holes in the section (*h*) have sharp boundaries. Many of the acinar cells are outlined by rows of small drying-holes in the section. A capillary at lower left contains a red blood cell (*rbc*). The area enclosed in the rectangle at upper right is shown at higher magnification in Fig. 12. Melt-dried. $\times 5400$.





12

FIGURE 12 Pancreatic acinar cells in a frozen thin section, enlarged from the region outlined by a rectangle in the previous figure (Fig. 11). The nuclei (*n*) contain condensed chromatin (*cc*) that is seen here in negative contrast. The nuclear envelope appears to consist of two layers, although the membranes are not seen clearly. What is probably a nuclear pore (*np*) appears as a gap in the nuclear envelope and peripheral condensed chromatin. In the cytoplasm is an abundant rough endoplasmic reticulum (*rer*), on which ribosomes are not discernible in this preparation. The mitochondria (*m*) are homogeneous in appearance, without visible cristae. Between the secretory granules (*s*) is an area that seems to have suffered greater ice-crystal damage and may represent part of the Golgi complex (*g*). A few disconnected dense lines (*d*) are all that is seen where one would expect the plasma membrane. Melt-dried. $\times 21,000$.

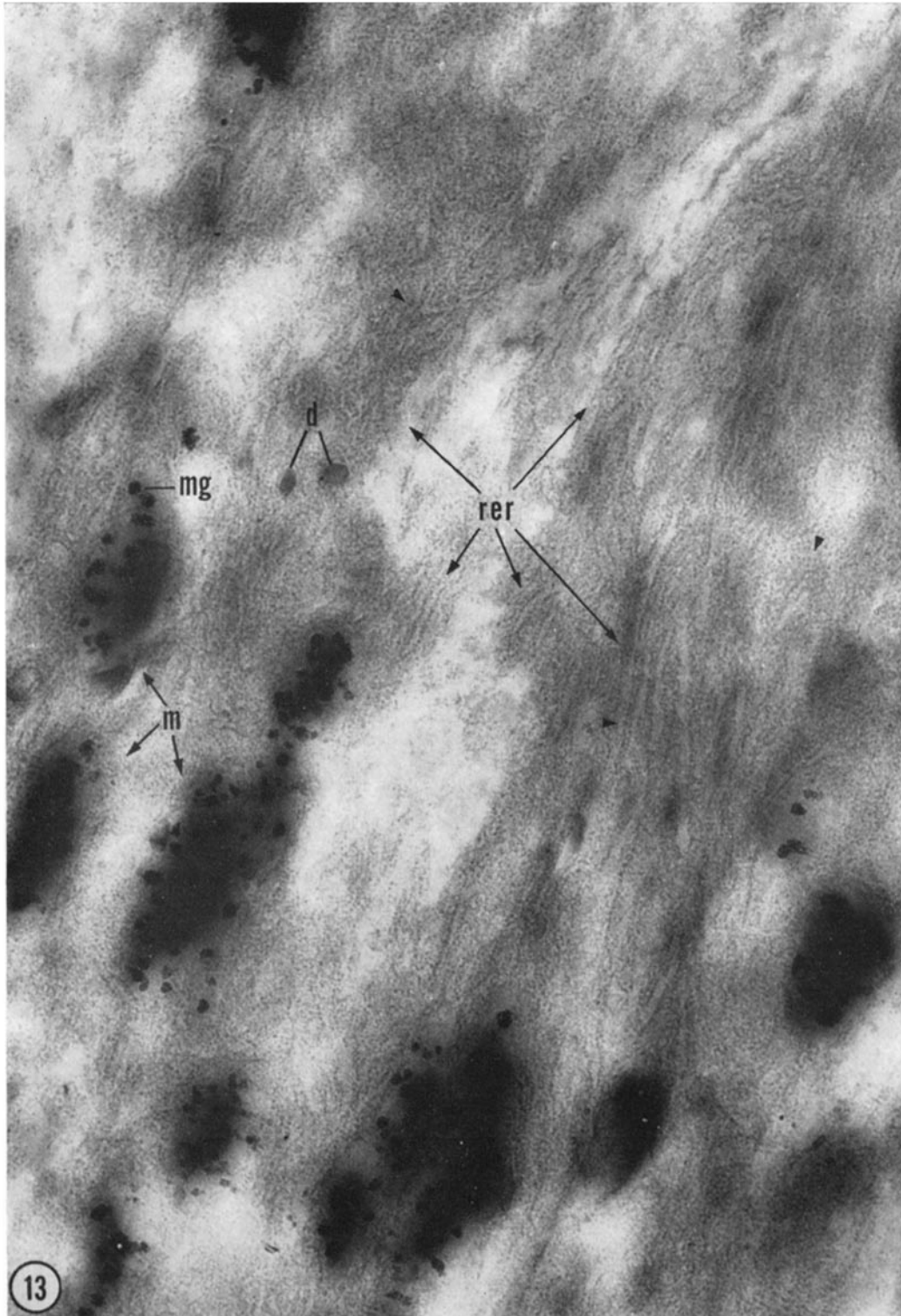


FIGURE 13 Cytoplasm of a pancreatic acinar cell in a frozen thin section. In this frozen-dried preparation, distinct ribosomes are abundant on the surface of the rough endoplasmic reticulum (*rer*) and in between its cisternae (arrowheads indicate sample areas where ribosomes are seen especially clearly). The ribosomes are about 120–150 Å in diameter. Mitochondria (*m*) contain large and abundant mitochondrial granules (*mg*). It is not clear why ribosomes and mitochondrial granules are visible here but not in Fig. 12 (see text). Small accumulations of dense material (*d*) of unknown nature are seen in the cytoplasm. Freeze-dried. $\times 43,000$.

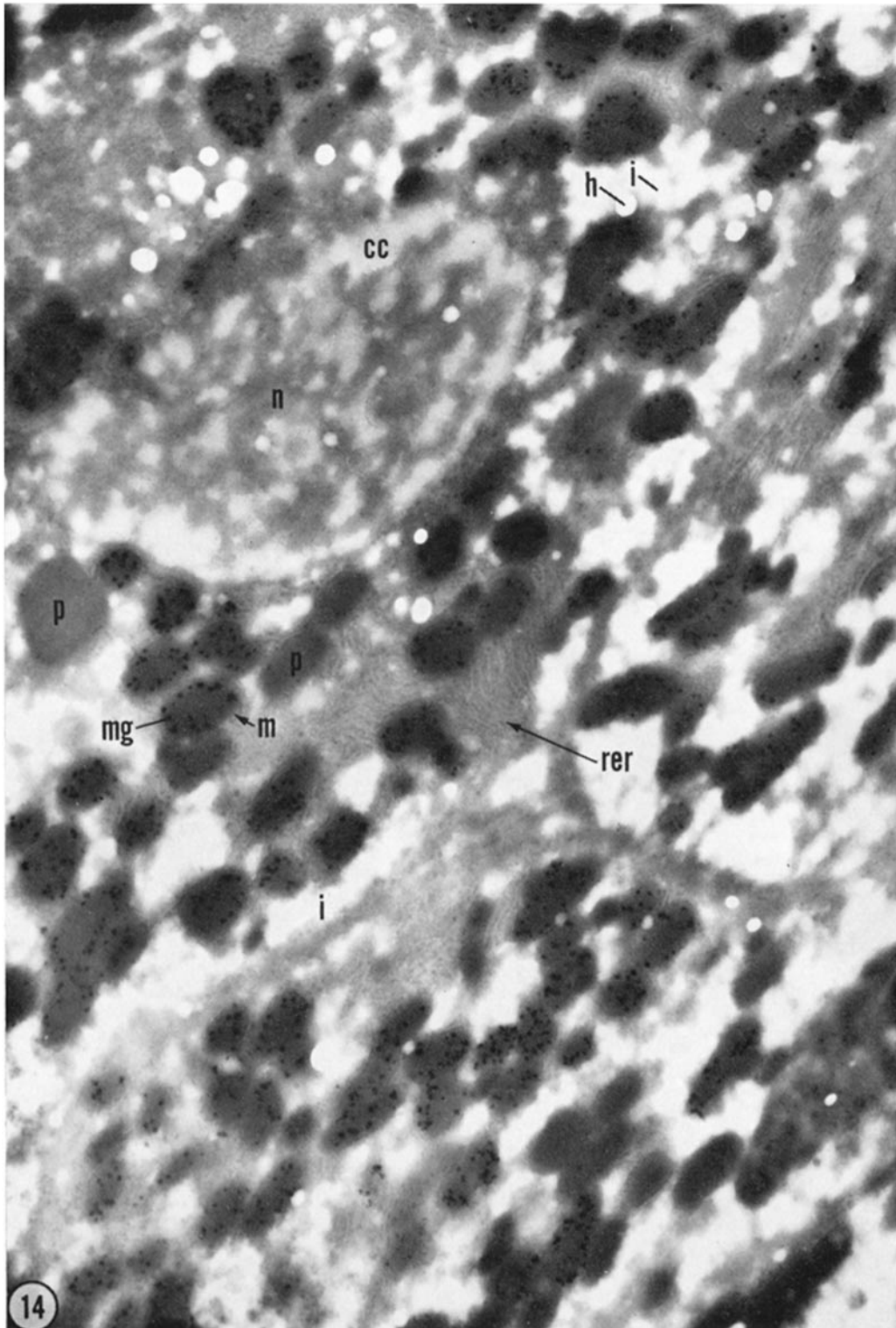


FIGURE 14 Frozen thin section of rat liver. Parts of three cells are visible. The one at upper left includes the nucleus (*n*), in which the condensed chromatin (*cc*) appears in negative contrast. In the cytoplasm are numerous mitochondria (*m*), exhibiting abundant mitochondrial granules (*mg*), much more numerous here than in conventional material. Some of the structures resembling mitochondria lack the granules, and may be peroxisomes (*p*). Patches of rough endoplasmic reticulum (*rer*) are well maintained, but other areas of cytoplasm, presumably containing smooth endoplasmic reticulum, seem more subject to ice-crystal damage (*i*). Drying holes (*h*), with distinct outlines, are also present in this melt-dried preparation, occasionally appearing at the margin of ice-crystal holes (upper right). $\times 17,500$.

during the brief thaw that preceded drying in the tissue for Figs. 11 and 12.

Ferritin molecules (*f*, Fig. 17) with cores about 60 Å in diameter are abundant in liver cells of Sprague-Dawley rats from our colony in the Anatomy Department at Stanford. The ferritin is prominent in frozen thin sections because of low contrast in the background cytoplasm. It does not occur in mitochondria or within cisternae of the rough ER, although a moderate amount is present in the nucleus. At high magnification (*f*, inset to Fig. 17), some of the particles show the characteristic substructure that is diagnostic for ferritin (55, Fig. 18). There are occasional dense clusters of ferritin about 0.4 μ in diameter in the cytoplasm.

In no case was a plasma membrane clearly distinguished at the surface of cells, although short, dense lines of uncertain nature were sometimes present where a plasma membrane would be expected (for example, *d* in Fig. 12).

One of the puzzling aspects of fine structure in frozen thin sections is a dense material that sometimes occurs around mitochondria and other structures. Fig. 17 shows the dense material (*d*) as it is seen typically. The material partially surrounds a mitochondrion (*m*) at lower left, forming a clear-cut layer 550 Å at its thickest point. In places the layer is broken into segments (breaks are indicated by white bars). In the micrograph the mitochondrion at lower right has a thin layer of dense material on one side and a slight indication of it on the other side. Elsewhere, dense material lies against rough ER in two places, and various lesser accumulations are also visible (although unlabeled). In some frozen thin sections in this study the dense material has been still more widespread, while in others it is virtually absent. Similar material could be responsible for the local densities (*d*) in Fig. 13, which were very abundant in occasional electron micrographs of pancreas in this study. Dense material is associated with the periphery of most secretory granules in Figs. 11 and 12, although it is masked by the extreme opacity of the granules (but is clearly visible on the negatives). All such densities could be artifact, perhaps arising as a bunching of material during cutting or as a preferential contamination within the electron microscope. However, recent reports of a dense component surrounding mitochondria between their outer and inner membranes, as shown by special staining in liver cells and cardiac

muscle (48) or by experimental treatment of skeletal muscle (E. J. Blanchette-Mackie, personal communication), suggest the possibility that at least some of this dense material may have functional significance.

DISCUSSION

A relatively simple means is described in this paper for producing frozen thin sections of tissue that is unfixed, unembedded, comes in contact with no solvents, and is maintained at -75°C or lower throughout the process of cutting, flattening, and freeze-drying. These sections easily satisfy the criteria specified by Stumpf and Roth (66) as necessary to keep diffusible substances in place for light microscope autoradiography; whether they will be adequate for such work at the electron microscope level remains to be determined. Cutting frozen thin sections involves no appreciable modification of the ultramicrotome, which operates at room temperature and remains available for other uses. A change from frozen sectioning to the cutting of ordinary plastic sections or back again can be made in a matter of minutes. The open top of the cutting bowl provides ready access to the sections and clear visibility with a dissecting microscope. The equipment is relatively inexpensive and should make frozen thin sections routinely available to any laboratory. Thicker frozen sections are easily obtained for light microscopy, and the device has the advantage that it can cut thinner sections than conventional cryostats. The flexibility of this approach therefore recommends it for a variety of uses.

Work of Others

Several workers attempted to cut frozen thin sections in the late 1940s and early 1950s when sectioning techniques were being developed for electron microscopy. It proved very difficult to obtain usable frozen thin sections even of fixed tissue, and so the cutting of plastic embedded material, which offered a great many advantages, eventually prevailed.

Fernández-Morán did early work with frozen thin sections (22, 23) and has continued to be an important figure in this area. More recently he has claimed that "thin frozen sections of native unfixed tissue can also be examined after ultrathin sectioning (20–100 Å) with a diamond knife in microtomes operating in nitrogen or helium

cryostats" (26). To my knowledge, electron micrographs of such sections have not yet been published, and a detailed description of the apparatus and how the sections are cut and handled is not available, although articles alluding to the technique have appeared (24, 25). His primary goal over the last few years has been to study hydrated tissue in the frozen state with electron microscopes fitted with superconducting lenses.

Bernhard has been influential in the development of frozen thin sectioning. He placed a Sorvall MT-1 ultramicrotome in a deep freeze at -35°C (3), and by this means obtained frozen thin sections of fixed tissue (5). By a supplemental use of dry ice, the tissue could be maintained at about -60°C , but the sections came to -35°C as they were cut. The method (4) was developed primarily for work with labeled antibodies (57) and for certain cytochemical techniques (35) for which fixation and other treatment of the tissue is permissible. Tissues are fixed in glutaraldehyde, partially embedded in gelatin, soaked briefly in glycerol, and frozen; sections are cut with a glass knife, floated on 40% dimethyl sulfoxide, washed with distilled water, and stained. Although this approach is not suitable for localizing diffusible substances or delicate enzymes, it does allow cytochemical incubations for antigens and hardy enzymes to be performed directly on thin sections, thus avoiding the penetration problems that hamper the usual incubations done on thicker slices.

Over the last few years, three main types of apparatus and method have emerged for cutting frozen thin sections: (a) the one described here, (b) that of Appleton, and (c) a third exemplified by the device of Dollhopf and the method of Hodson and Marshall. These three lines had independent

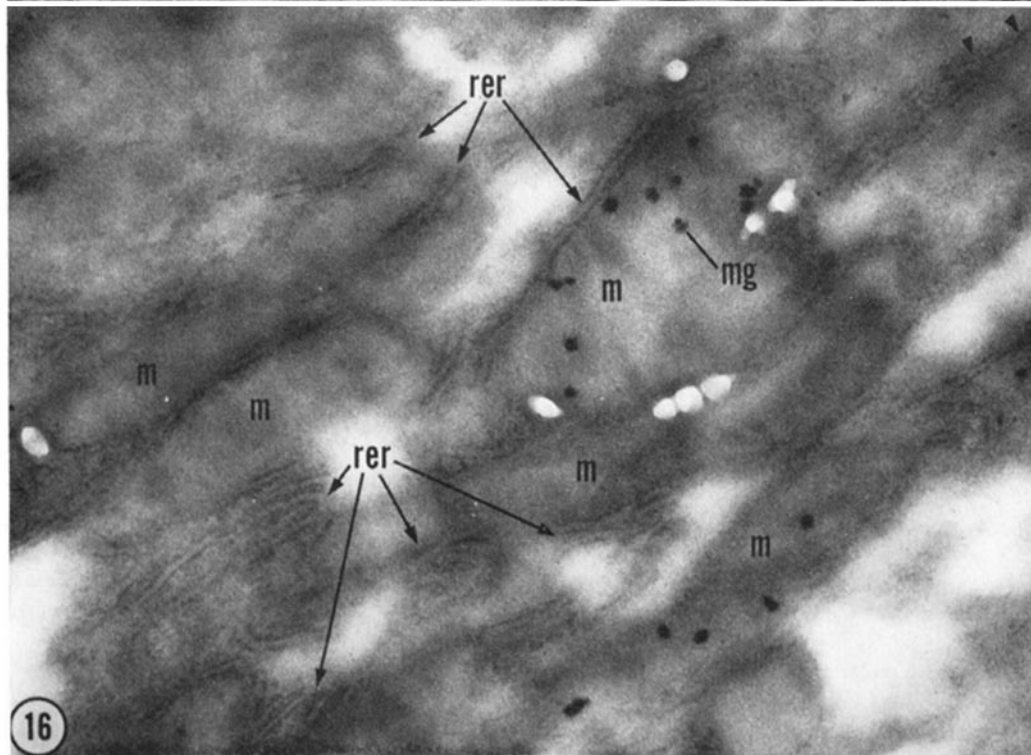
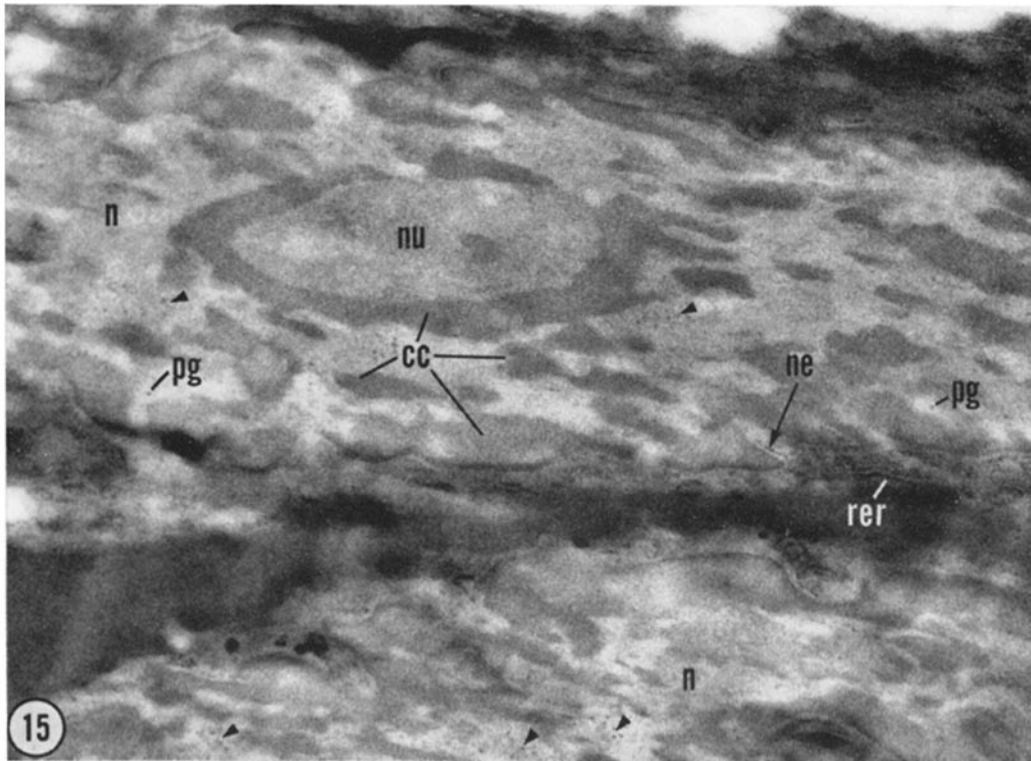
beginnings at approximately the same time in early 1966, and they have been developed without significant interplay until fairly recently.

The system developed by Appleton (2) is a refinement of Bernhard's approach, and the light microscope method of Stumpf and Roth (65). Appleton uses an LKB ultramicrotome mounted within a SLEE refrigerated chest capable of temperatures down to -85°C . Although he has published preliminary electron micrographs of fresh-frozen tissue (1, 2), his main published figures are of tissue fixed in glutaraldehyde (2). More recently he has obtained frozen thin sections of fresh-frozen pancreas similar to those of the present study (personal communication). He cuts sections with a dry glass knife, and orients them on the knife face with a small suction probe. The sections are picked up by pressing the face of a membrane-coated grid (held in a special magnetic holder) against them; they adhere best if the grid is at -20°C . Freeze-drying is accomplished in the Stumpf-Roth unit (67), or more recently by leaving sections in a special container in the cold chest for about 2 hr.

The third recent approach to frozen thin sectioning has evolved along at least three distinct lines, characterized by the work of Dollhopf (17, 18), of Hodson and Marshall (29, 30, 31), and of Kolehmainen-Sevéus (32, 59). The instruments developed in the three cases are quite similar, and their mutual genealogy is difficult to untangle (it is in fact the subject of some dispute). Dollhopf has gone farthest in the development of the instrument itself, while Hodson and Marshall have evolved a technique for frozen thin sectioning of fresh-frozen material based on their version of the unit. Essentially, in each case the instrument consists of a box enclosing the cutting components on

FIGURE 15 The nuclei (*n*) in these two rat liver cells show condensed chromatin (*cc*) that appears electron opaque rather than having the pale appearance seen in the nuclei of Figs. 11, 12, and 14. The reason for this difference is unclear. Near the chromatin are occasional perichromatin granules (*pg*). The euchromatin areas between the chromatin contain clusters of interchromatin granules (indicated by arrowheads) and indistinct filaments. The nucleolus (*nu*) is light, in contrast to the dense nucleolar-associated chromatin that surrounds it. The nuclear envelope (*ne*) and rough endoplasmic reticulum (*rer*) are discernible in some places. Melt-dried. $\times 36,000$.

FIGURE 16 Cytoplasm of a rat liver cell. Two of the mitochondria (*m*) contain mitochondrial granules (*mg*), but no internal membranes can be distinguished. Ribosomes generally are flattened on the rough endoplasmic reticulum (*rer*), but sometimes appear as individual particles, for example at upper right (between the two arrowheads). Melt-dried $\times 48,000$.



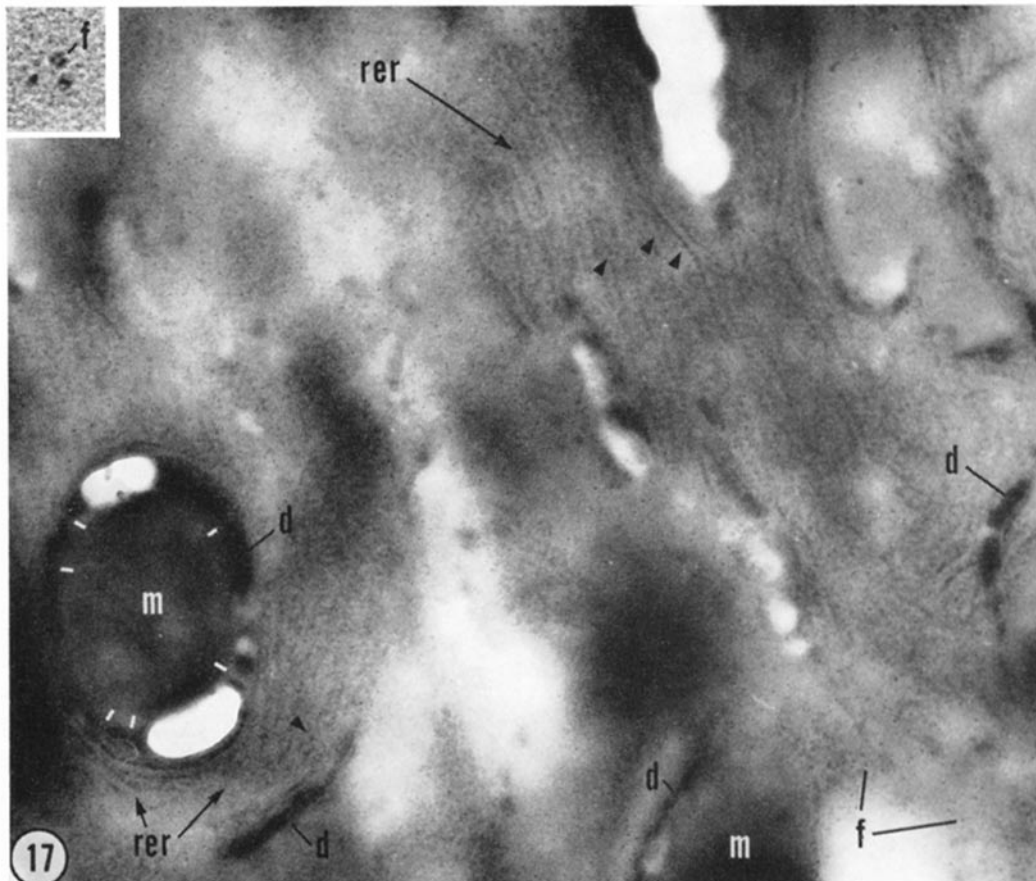


FIGURE 17 This section of rat liver was cut at about -20°C in 1967, at an earlier stage in the development of the method (see further technical information preceding the legend to Fig. 11). Despite the warmer temperature, the fine structure is essentially similar to that seen in material cut since mid-1968 at lower temperatures. The two mitochondria (*m*) lack mitochondrial granules, which is not unusual. The rough endoplasmic reticulum (*rer*) bears ribosomes (arrowheads indicate ribosomes seen rather clearly). Two features are shown here which, though not unusual, are not illustrated in the other figures. Ferritin (*f*) is abundant in the liver cell cytoplasm of rats from our Stanford colony. The *inset* at upper left shows the typical ferritin substructure in these particles at high magnification. In addition, a dense material (*d*) outlines the mitochondrion at left, and also appears elsewhere in the field. Breaks in the dense material are indicated by white bars. The occurrence of this material in frozen thin sections is rather capricious, and its nature is unknown (see end of Results section). Melt-dried. $\times 45,000$; *inset* $\times 310,000$.

the stage of a commercial ultramicrotome. The box has a transparent lid, which must be heated to prevent frosting over. The specimen holder and knife holder each contains a heating coil and a small reservoir for liquid nitrogen. The desired low temperature (anywhere from -150° to 0°C in Dollhopf's unit) is maintained by a balance between the addition of liquid nitrogen to the reservoir and heating with the coil. The liquid

nitrogen is supplied from nearby Dewars by way of insulated lines. The specimen and knife can be set at different temperatures, which may be useful for some applications. This unit is less convenient for the dry-knife sectioning described in the present paper, since sections must be manipulated continuously and the components within the box of this unit are subject to serious frost if the lid is left up for an appreciable time. In Hodson and

Marshall's (31) method the specimen is purported to be maintained at liquid nitrogen temperature, although this is open to some question (see Discussion). As sections are cut they pass into liquid cyclohexane at -120°C , and are retrieved from the fluid onto grids by electrostatic attraction, after which they are freeze-dried at -70°C and 10^{-4} torr.

Manufacturers of ultramicrotomes are already producing devices for frozen thin sectioning based on these various approaches. I have cooperated with Ivan Sorvall, Inc. in the development of an improved version of the instrument described in this paper (see footnote 1). Appleton's unit is available from LKB-Produkter AB (Bromma, Sweden). Dollhopf's apparatus was developed under the auspices of the Shandon Scientific Co., London and C. Reichert A. G., Vienna, and is now available from Reichert. LKB has produced a similar device, the "Cryokit," which Kolehmainen-Sevéus has used in addition to the Appleton unit.

State of the Tissue in Frozen Thin Sections, and a Comparison with Usual Fixed and Embedded Material

Electron microscopists have long been aware of the extensive chemical alterations that customary methods of fixation and embedding bring about in cells. Glutaraldehyde and osmium tetroxide react chemically with a variety of substances during fixation, and probably inactivate a majority of the cell's enzymes. The succession of polar and non-polar solvents used in fixation, dehydration, and embedding extract great quantities of material from the cells, and many of the substances that remain may be shifted in position. As a result of these and other changes, the cells seen after fixation and embedding are, from a chemical point of view, mere shadows of their former selves.

We might expect changes in cellular morphology to accompany these chemical alterations. Although some structural distortions undoubtedly occur, there are indications that the main features of fine structure seen with the best conventional techniques of fixation and embedding reflect fairly well the structural organization of intact cells. One evidence is furnished by the technique of freeze-etching (45), which involves neither fixation nor embedding, although the tissues are commonly soaked for several minutes in 20%

glycerol or other cryoprotectant before freezing. The results of this method have generally corroborated the main outlines of cellular fine structure seen in fixed and embedded material. Since the end result of freeze-etching is a shadowed replica of cellular structures, the material is not amenable to autoradiography or other cytochemical procedures.

In contrast to conventional fixed and embedded material, where cellular morphology is maintained fairly well but there are vast chemical alterations, the tissue in frozen thin sections is relatively intact chemically, but has undergone some morphological distortion. Most of this structural change is the result of ice-crystal formation during the initial freezing or during later recrystallization. It is therefore essential, in considering the state of tissues in frozen thin sections, to understand how biological freezing takes place. For detailed information, the reader is referred to two extensive works on cryobiology (42, 71), and three useful review articles (40, 43, 8). Unless otherwise specified, the following brief description is based on these sources. Later in the discussion, it will be shown how the method described in this paper is tailored to minimize changes that occur during freezing and subsequent steps.

When a cell undergoes rapid freezing, minute ice crystals (sites of nucleation) arise here and there throughout the cell and begin to grow by taking up water from the surrounding protoplasm. The growing crystals emit heat (latent heat of crystallization) which warms the surrounding area slightly and inhibits the formation of further sites of nucleation in the vicinity. Only if freezing is very rapid can this heating effect be overcome to yield numerous very small ice crystals, rather than a few larger ones.

As water accumulates in ice crystals, the adjacent protoplasm becomes increasingly dehydrated until it reaches a concentration and temperature where solutes and the remaining water solidify into a common mass. This completes the freezing process and the cells now contain numerous ice crystals between which is partially dehydrated, congealed protoplasm. A more rapid freezing yields a less extensive dehydration, since less water has time to leave the cytoplasm before it solidifies. The increase in concentration of electrolytes and other substances during protoplasmic dehydration seems to be the principal cause of whatever damage

is sustained by proteins and other macromolecules during freezing.

It is conceivable that cooling might take place so rapidly that the protoplasm would congeal before appreciable water could migrate to form ice crystals. The water would then freeze in place as "vitreous ice" without crystallizing. This ideal freezing is apparently impossible to attain by usual methods because the low thermal conductivity of water or ice in the tissue limits the speed of freezing. Moor (46, 47) has calculated that even in an ideal freezing situation, with optimal temperature gradient and infinite thermal conductivity around the tissue, the layer of vitreous ice formed would be only 2-3 μ thick, and deeper levels would show ice crystals of progressively larger size.

Ice crystal damage can still increase after the tissue is frozen if the storage temperature is inadequate. Frozen tissue is stable at liquid nitrogen temperature (-196°C), but at more elevated temperatures the water molecules in the ice crystals become progressively more mobile, which may allow crystals to grow and change their shapes. This "recrystallization" can produce damage comparable to faulty initial freezing.

Biologists working with frozen tissues need to know the temperature range where recrystallization becomes rapid enough to be of practical concern. This question can be approached at several levels. Often quoted are studies describing the ice crystal types obtained when water vapor is condensed on metal at various temperatures, and the changes these crystals undergo with warming. Dowell and Rinfret (19) found that vitreous ice was formed below -160°C , cubic ice predominated between -160° and -130°C , while above -130°C , the ice was hexagonal. When vitreous ice was warmed it recrystallized into cubic ice at a rate which became irruptive at about -130°C . Cubic ice transformed into hexagonal ice with increasing rate as the temperature rose, becoming irruptive at about -60°C . These very accurate results with frozen water vapor are of limited relevance to biological freezing, however, since the authors reported that no way could be found to make vitreous or cubic ice from liquid water. Only hexagonal ice resulted, as would presumably be true in the freezing of tissues. The recrystallization of hexagonal ice made from pure water proceeds very slowly at temperatures below -100°C , but can take place in a few seconds above -50°C (41). Still higher tempera-

tures are necessary for recrystallization of hexagonal ice in frozen tissue, since the movement of water molecules between ice crystals is strongly impeded by the congealed cytoplasm. There is little exact information on the rate of this recrystallization at different temperatures in tissues. Studies by Luyet and coworkers on model systems consisting of 25-50% solutions of various solutes (38; 42, p. 115; 71, p. 27) indicate that the irruptive recrystallization temperature depends more on the molecular weight of the solute than on its concentration. This is illustrated by some sample values: starch (-5°C), albumin (-5.3°C), gelatin (-11.5°C), sucrose (-30.5°C), dextrose (-38°C), and glucose (-40.6°C). These results, along with observations by Meryman (42, p. 1), suggest that frozen cells, with their high content of free protein, may undergo little recrystallization damage during moderate periods in the -50° to -30°C temperature range. However, for long term storage, a temperature of -70°C or colder is advisable to minimize the gradual migration of water molecules between crystals, the so-called "migratory recrystallization."

Tissue may undergo some changes during the cutting of frozen thin sections. Thornburg and Mengers (68) proposed a model for frozen sectioning in which a zone of "micro-melting" accompanies the knife through the tissue. If the model is correct, then the melting could cause surface distortion of the sections. This might account for some of the densities occasionally seen around structures (see end of Results section).

The theory and technique of freeze-drying has been reviewed by Meryman (42, p. 609), and some of the pitfalls and potential artifacts of the method are summarized by MacKenzie (39). The morphological appearance of ice-crystal damage in frozen thin sections after freeze-drying, as described in the Results section, resembles that seen in earlier studies (reviewed in reference 8) where tissues were frozen-dried, vapor-fixed, and then vacuum-embedded in plastic (see especially reference 60), or were freeze-substituted with acetone- OsO_4 or other medium, and embedded in plastic (further references in 53). Early disputes over the identity of ice-crystal holes are now generally settled (53) and the present results support the conclusion in material that is not freeze-substituted. The apparent improvement in the morphology of cells described here after melt-drying is well known in the light microscope literature, and more recently

has been assessed at the electron microscope level in frozen tissue that is thawed and then fixed and embedded in the usual way (reviewed in references 69 and 8).

Beam damage could alter frozen thin sections somewhat as electron micrographs are taken, especially at beam intensities necessary for work at higher magnification. In general, however, the main structural features do not change noticeably during electron microscopy if reasonable care is taken.

Rationale behind the Present Method

Having discussed some of the factors that alter tissue during the preparation of frozen thin sections, I will now describe how the present method is designed to minimize these difficulties and yield sections that depart as little as possible from the structural and chemical organization of intact cells. The approach will also be compared to that used by others in this field.

Although rapid freezing has its problems, it should maintain cellular constituents in place more effectively than slow freezing. Water withdrawal from the cells during slow freezing leaves them drastically shrunken and deformed. The most rapid freezing method is desirable to make intracellular ice crystals as small as possible. A tissue's rate of freezing depends on how cold its surroundings are (thermal gradient) and how rapidly the surroundings can conduct heat out of the tissue (thermal conductivity). If tissue is dropped directly into liquid nitrogen, there is an excellent thermal gradient, but violent boiling around the tissue covers it with a shell of nitrogen gas that has very poor thermal conductivity (less than 10^{-4} w/cm °C, see 58, p. 280). The same seems to be true even of tissues plunged into superconducting helium II at a temperature of about 2.2°K (7). To avoid this it is common practice to freeze in a liquified gas (propane, isopentane, Freon-22) of relatively high boiling point that is brought near its melting point with liquid nitrogen. However, the thermal conductivity of these fluids at best is poor (about 0.09 w/cm °C for liquid Freon-22, according to Du Pont sales literature [E. I. Du Pont de Nemours & Co., "Freon" Products Div., Wilmington, Del.]). Still another approach (60) is to lower the temperature of liquid nitrogen until it partially freezes (at about -212°C), then freeze the tissue in the nitrogen that remains liquid. Because of its low temperature this liquid nitrogen

does not boil; however, it has very low thermal conductivity (1.2×10^{-3} w/cm °C, see 58, p. 276).

In the present study the tissues are frozen against a block of copper at either liquid nitrogen or liquid helium temperature. This approach offers the advantage of bringing excellent thermal gradient and conductivity to bear on the part of the tissue that will be used for frozen thin sections. This method of freezing is based on a technique developed by Eränkö (21) for work at the light microscope level. Another modification of it for electron microscopy has been used by Van Harreveld et al. (70) to freeze material which is then fixed by freeze-substitution for 2 days in OsO₄-acetone at -85°C, and then warmed to room temperature for conventional embedding and sectioning. These authors use a highly polished silver block for freezing, and show a layer about 10 μ deep at the tissue surface that is without recognizable ice crystal ghosts. The copper used for the freezing block in the present study is 99.999 + % pure, and is the same as that studied by Powell et al. (51). They found that when properly annealed, this copper has a thermal conductivity of about 6 w/cm °C at liquid nitrogen temperature (-196°C or 77°K), while it exhibits a conductivity peak of 134 w/cm °C at 12°K near the boiling point of liquid helium (4.6°K). This suggests that when the present technique is used with liquid helium, it could offer an exceptional combination of high thermal gradient and conductivity.

The soaking of tissue in a cryoprotectant (such as glycerol or dimethyl sulfoxide) before freezing, customary in freeze-etching (45), might be expected to yield material with little ice-crystal damage. However, these agents make sectioning very difficult in the present method, and there may also be movements of diffusible substances and other changes within the cells during the soaking period. Rebhun has developed a promising approach to freezing without ice crystals, involving partial dehydration of the cells by osmotic or other means before freezing (53). A very different way to attain essentially vitreous ice is to freeze tissue rapidly under a pressure of 2000 atmospheres (46). It has been shown recently (47) that cells can survive this pressure.

It was considered necessary to freeze the tissue while it was on the chuck rather than separately, since the resulting firm attachment of tissue to

chuck insures stability during cutting. However, this has the disadvantage that the part of the tissue in contact with the chuck is frozen less rapidly, since liquid nitrogen cools the chuck comparatively slowly. This difficulty can be reduced by coating the chuck with cigarette ashes, which increases its cooling rate dramatically by inducing "nucleate boiling" around it rather than the usual "film boiling" (15, 37). This was tested in the present study by noting how long boiling continued around a chuck that had been dropped into liquid nitrogen. Boiling lasted 30 sec for a control chuck, 20 sec for a chuck coated with vacuum grease only, and only 3 sec for a chuck coated with cigarette ashes held on with vacuum grease. I have tried a similar experiment in liquid helium, but here the control and coated chucks do not differ significantly in the time required to attain thermal equilibrium.

Temperatures between -70° and -90°C seem most favorable for thin sectioning, and should be adequate to protect against appreciable recrystallization damage or migration of diffusible substances.

Hodson and Marshall (31) report that sections cut at temperatures above -100°C in their apparatus occasionally seemed to melt as they struck the knife, and from this they conclude that the pressure of cutting may cause a 100° rise in the temperature of tissue in the section. Neither in the present study nor in the work of Appleton (2, and personal communication) has this melting been observed at cutting temperatures of -70° to -90°C , and I have obtained fairly consistent frozen thin sections at about -20°C (Fig. 17 is an example). The explanation offered by Hodson and Marshall for the phenomenon is reminiscent of the Thornburg-Mengers model of how frozen sections are cut (68, discussed above), but claims much more. Thornburg and Mengers calculated that the zone of melting, due to pressure in advance of the knife edge, would be about 600 A wide for usual cryostat sectioning, and predicted that it would be considerably narrower at lower temperatures (such as -100°C). The melted layer, less than 300 A thick on the inner face of the section, would refreeze almost immediately after the knife edge passed by, since the pressure would then be relieved. Thus their model would not predict a visible thaw under the conditions described by Hodson and Marshall.

The findings of Hodson and Marshall (31) also

differ from those of the present study in another regard. These authors claim to cut frozen thin sections of appreciable size at liquid nitrogen temperature (-196°C), while in the present study the tissue tends to be brittle at low temperatures, and efforts to cut at about -175°C , using liquid nitrogen as a coolant in the bottom of the bowl, have yielded only clusters of small section fragments. A possible explanation for this difference, and also that mentioned in the previous paragraph, is suggested by an examination of the specimen holder of Hodson and Marshall's device, as illustrated in Fig. 2 *b* of their paper. The tip of the long, tapered specimen is exposed to the ambient temperature of the box, which is not specified but is presumably comparatively warm. It seems likely that the thin layer of tissue about to be cut at the specimen tip will be more strongly influenced by the ambient temperature than by cold from the liquid nitrogen reservoir, which is some distance away, with intervening materials being of very low thermal conductivity (ice and invar steel). There thus may be some question about the actual cutting temperature of the specimen when the thermometer (mounted near the liquid nitrogen reservoir) reads either -196° or -100°C .

Frozen thin sections must be flattened and caused to adhere to the grid membrane before freeze-drying or other procedures can be carried out. If recrystallization and substance migration are to be avoided, the flattening must be done without warming the sections appreciably and without bringing them into contact with any solvent. Koller (33) utilized electrostatic attraction of up to 10,000 v (but very small amperage) to attract frozen thin sections of fixed tissue from the knife edge and flatten them against a grid membrane. In the present study a pressure method of flattening has been developed in which the sections are pressed against the grid membrane either by means of a polished copper rod or by bringing the grid down on the sections as they lie at the knife edge. Independently, Appleton (2) has also developed a pressure method, utilizing a magnetic device to hold the grid as it is brought face down on the sections near the knife edge.

The only value of a vacuum in freeze-drying is to increase the mean free path of water molecules as they leave the tissue, so they will be less likely to return. Meryman (42, p. 634) has pointed out that any other method of accomplishing this aim is also effective, such as blowing an anhydrous gas

rapidly over the surface to carry away the water molecules. He describes an example where an appreciable piece of tissue took 8 hr to freeze-dry with -30°C anhydrous gas blowing across the surface, compared to 6 hr for vacuum freeze-drying at the same temperature. Since the frozen thin sections of the present study are less than 1000 Å in thickness, they should dry rapidly, probably within minutes, at even colder temperatures. Frozen thin sections mounted on grids and placed under the plastic shelf of the cutting bowl will be swept constantly by cold nitrogen gas that is almost completely anhydrous (since it arises from liquid nitrogen). It is impossible to know how long freeze-drying will take under these conditions but an hour at -90°C should be ample. At that temperature there should be little danger of recrystallization or migration of diffusible substances.

As has been discussed earlier, the morphological advantage of melt-drying nevertheless incurs the risk of recrystallization during warming, movements of diffusible substances before and after melting, and distortion due to surface tension effects during drying. The approach to melt-drying described in this paper is intended to minimize these hazards by drying extremely rapidly. A frozen thin section about 1000 Å in thickness should melt and dry almost instantaneously in a jet of room-temperature dry nitrogen gas. However, it must be acknowledged that a great deal might happen at the molecular level during that instant.

Comparison of Fine Structure with that Seen in Conventional Material

Low contrast and ice-crystal damage limit the amount of structural information obtained at present from frozen thin sections. However, the morphology that can be discerned is generally similar to that in conventional electron micrographs, when one makes allowances for changes that could be expected during freezing and drying. It is thus probable that fixed and embedded tissue will remain the principal means of studying the larger scale structural organization of cells, unless dramatic advances in biological freezing can be made in the future. The chief value of the frozen thin section lies in its potential for cytochemistry.

It may seem surprising that there is enough contrast in these very thin slices of unstained material for structure to be perceived. A structure

has greater inherent contrast in frozen thin sections if its substance is more concentrated than surrounding materials and therefore more mass remains after drying, or if its composition includes materials of higher atomic number. The great electron opacity of secretory granules in the pancreas is clearly due to the concentration of material. On the other hand, the high electron opacity seen in mitochondrial granules and in the core of ferritin molecules is presumably due to a combination of the two factors: both concentration of material and higher atomic number. Ribosomes may also derive their electron opacity from both factors. An 80S ribosome contains two RNA molecules of 1.7×10^6 and 0.8×10^6 mol wt respectively, and about 150 proteins of 10,000–30,000 mol wt (62). All of these constituents are concentrated into a particle that is about 150 Å in diameter in the dried state, according to measurements in the present study. It is possible that the somewhat higher atomic number of phosphorus in the RNA still further increases the electron opacity.

Membranes, on the other hand, are not usually seen clearly in frozen thin sections, even though they contain considerable protein and phospholipid. The clarity of membranes in conventional electron micrographs results both from staining and from the extraction of considerable background material during the preparative procedure, causing the membranes to stand out against lighter surroundings. Neither of these morphological advantages is present in frozen thin sections, where the membrane may be masked by materials of similar density that surround it. However, there appears to be appreciable contrast in some membranes, such as the rough ER in Fig. 12. This contrast is difficult to explain, although ribosomal flattening could be a contributing factor.

Efforts to improve contrast in frozen thin sections with vapor staining (osmium tetroxide, chromyl chloride) and by stains in solution have been unsuccessful so far.

In this study the ribosomes were usually flattened on the membranes of the rough endoplasmic reticulum, although in favorable instances they were seen as distinct particles. Similar results were obtained by earlier workers, using freeze-drying and sometimes freeze-substitution, followed by plastic embedding. This finding raised the possibility that the flattened ribosomal material constituted the biologically active form, while particu-

late ribosomes arose as fixation artifacts (28, 60). Evidence against this view came from freeze-substitution studies of ice crystal-free cells (52, 53), in which ribosomal particles were abundant. The extensive biochemical literature now available on ribosomes (see reference 62), together with the demonstration here of particulate ribosomes in frozen tissue that has neither been freeze-substituted nor plastic-embedded, suggests that ribosomal flattening is an artifact of biological freezing. In the present study, particulate ribosomes could be distinguished in some preparations that were dried rapidly under a jet of room-temperature nitrogen gas, as well as in frozen-dried material.

Particulate ribosomes were smaller in frozen thin sections than those illustrated in the literature of conventional electron microscopy. In the present study, ribosomes that were oriented so they appeared oblong were about 160×120 A, while round profiles were usually about 120–150 A in diameter. As a basis of comparison with conventional fixed and embedded material, I have measured the approximate dimensions of ribosomes in published micrographs⁹ of rat liver. To insure consistent orientation, the measurements are limited to ribosomes that are part of polysomes seen in surface views of the rough endoplasmic reticulum. The dimensions are somewhat variable from one paper to another, due perhaps to differences in the mode of preparation or the accuracy with which the authors calculated the magnifications: 280×200 A (16, Fig. 6), 270×180 A (6, Fig. 4), 210×150 A (63, Fig. 26), and 220×150 A (34, Fig. 11). In Sjöstrand and Elfvin's (60) freeze-dried, plastic-embedded sections of mouse pancreas, the ribosomes measured 150–200 A. The small size of ribosomes in the frozen thin sections described here probably results from the

⁹ It is usually difficult to measure small structures in published electron micrographs because the stark background of engraved dots obscures the micrometer scale. These measurements were made with a $7 \times$ magnifier fitted with a scale divided into 0.1 mm divisions (Bausch and Lomb Incorporated, Rochester, N. Y.). A white card was placed across the particle to be measured, occluding half the particle along the axis of measurement. The length on the micrometer scale was read against the white background. Dividing the length by the magnification and placing the decimal point gave the approximate diameter of the particle.

loss of most of their water during drying. This would reduce their size compared to ribosomes in embedded material, where plastic may occupy space in the macromolecules. The problem still remains to explain why these dried ribosomes are smaller than ribosomes that have been isolated and then dried and negatively stained, which measure about 230 A (61). It is likely that ribosomes in frozen thin sections are supported by the surrounding protein in the section as they dry, and thus do not flatten on the membrane, as presumably occurs to some extent during the drying of isolated ribosomes.

Similar considerations may explain why the perichromatin granules here measure about 200 A, rather than the 300–600 A (6) or 400–450 A (44) that has been described for rat liver. Interchromatin granules of rat liver are about 150 A in diameter in the present study, while in fixed and stained material they measure 200–250 A (44).

It is of interest to know the nature of the mitochondrial granules seen in frozen thin sections. In conventional electron micrographs, mitochondria of most cell types contain granules of moderate density about 300–400 A in diameter, which may be termed "normal" mitochondrial granules. It has been suggested that these granules are sites where divalent cations can accumulate (50). When isolated mitochondria are put in a medium that can support respiration and which contains calcium and phosphate ions, the mitochondria actively accumulate the calcium and phosphate ions in a molar proportion characteristic of hydroxyapatite (36). Electron micrographs of these mitochondria show large (500–1000 A) dense granules, presumably composed of hydroxyapatite (27). There is some question whether these accumulations are organized around the normal mitochondrial granules seen in untreated mitochondria. The large (hydroxyapatite) granules are commonly located near the cristae, while the normal granules are usually free in the matrix. The normal granules do not seem to contain a significant amount of the calcium known to be present in mitochondria (49).

The abundant mitochondrial granules seen in the present study resemble the large hydroxyapatite granules (27), rather than the normal matrix granules of untreated animals. It is significant that none of the animals in this study received any treatment, and yet these granules were numerous in a majority of liver mitochondria in

each animal, and were also abundant in pancreatic mitochondria of some animals. This would suggest that the larger granules are more common in mitochondria of normal animals than has been suspected, and that conventional methods of tissue preservation remove some of this granule material. It has not been possible to identify normal granules with certainty in frozen thin sections. Either the granules do not contain enough dense material to stand out, or they are being overlooked among the other granules.

It is conceivable that the presence of numerous large mitochondrial granules in frozen thin sections is an artifact of freezing. If the inner compartment of mitochondria normally contains a considerable level of calcium and phosphate ions in solution, then the lowering of the temperature and partial dehydration of the mitochondria during freezing might well cause significant precipitation of calcium phosphate. This explanation seems inadequate. If freezing produced the granules, then one would expect them to have been present in the many earlier studies where tissue was frozen, freeze-dried, freeze-substituted, and plastic embedded (reviewed in reference 8). However, there is no mention in these studies of especially numerous mitochondrial granules, and since the freezing and drying carried out by these earlier workers was often comparable to that used in the present study, then the granule material must have been removed during the later steps of their procedures. In summary, the likelihood remains that large mitochondrial granules are more plentiful in normal cells than previous methods of preservation would indicate, although the number of granules may be exaggerated somewhat in frozen thin sections as an artifact of freezing.

Possible Uses

The routine availability of frozen thin sections of fresh-frozen tissue for electron microscopy should open up new avenues of investigation. One area of interest is the localization of diffusible substances by autoradiography at the electron microscope level or by other means. It is already possible to localize soluble materials at the light microscope level (64, 66). Work is underway in our laboratory (11, 14) to apply autoradiographic emulsions to frozen thin sections in a way that will allow such localizations with the electron microscope. Although our principal goal is to follow steroid substrates through synthesis and

secretion in cells that make steroid hormones, the method should also be applicable to other diffusible substances of interest in cell biology. The localization of small ions, such as sodium or potassium, will be difficult because of their considerable mobility, but may still be possible. The electron microprobe and related instruments should prove of value in such localizations, especially if the resolution and sensitivity can be improved.

Another possible avenue is the cytochemical localization of delicate enzymes that do not survive present techniques of preservation. As in the work of Leduc and associates (35), this approach offers the advantage that the cytochemistry can be done directly on the sections, rather than on preliminary thick slices where the substrates often must penetrate several membranes to reach the enzyme sites. It may also be possible to localize enzymes and other intracellular antigens by means of labeled antibodies applied to frozen thin sections (57).

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REFERENCES

1. APPLETON, T. C. 1968. The application of autoradiography to the study of soluble compounds. *Acta Histochem.* (Suppl. 8):115.
2. APPLETON, T. C. 1969. The possibilities of

- locating soluble labeled compounds by electron microscope autoradiography. In *Autoradiography of Diffusible Substances*. L. J. Roth and W. E. Stumpf, editors. Academic Press Inc., New York. 301.
3. BERNHARD, W. 1965. Ultramicrotomie à basse température. *Année Biol.* 4:5.
 4. BERNHARD, W., and E. H. LEDUC. 1967. Ultrathin frozen sections. I. Methods and ultrastructural preservation. *J. Cell Biol.* 34:757.
 5. BERNHARD, W., and M. T. NANCY. 1964. Coupes à congélation ultrafines de tissu inclus dans la gélatine. *J. Microsc.* 3:579.
 6. BRUNI, C., and K. R. PORTER. 1965. The fine structure of the parenchymal cell of the normal rat liver. I. General observations. *Amer. J. Pathol.* 46:691.
 7. BULLIVANT, S. 1965. Freeze substitution and supporting techniques. *Lab. Invest.* 14:1178.
 8. BULLIVANT, S. 1970. Present status of freezing techniques. In *Some Biological Techniques in Electron Microscopy*. D. F. Parsons, editor. Academic Press Inc., New York. 101.
 9. CARO, L. G. 1961. Electron microscopic radioautography of thin sections: the Golgi zone as a site of protein concentration in pancreatic acinar cells. *J. Biophys. Biochem. Cytol.* 10:27.
 10. CHRISTENSEN, A. K. 1969. A way to prepare frozen thin sections of fresh tissue for electron microscopy. In *Autoradiography of Diffusible Substances*. L. J. Roth and W. E. Stumpf, editors. Academic Press Inc., New York. 349.
 11. CHRISTENSEN, A. K. 1970. Frozen thin sections of fresh tissue for autoradiography of diffusible substances. Proceedings 7th International Congress Electron Microscopy, Grenoble. 1: 503.
 12. CHRISTENSEN, A. K. 1970. Preparation of frozen thin sections of fresh tissue for electron microscopy. Proceedings 28th Annual Electron Microscopy Society of America, Houston. 294.
 13. CHRISTENSEN, A. K., and S. W. GILLIM. 1969. The correlation of fine structure and function in steroid-secreting cells, with emphasis on those of the gonads. In *The Gonads*. K. W. McKerns, editor. Appleton-Century-Crofts Inc., New York. 415.
 14. CHRISTENSEN, A. K., L. G. PAAVOLA, and C. J. CONNELL. 1971. A method of using frozen thin sections of fresh-frozen tissue for autoradiography of diffusible substances at the electron microscope level. *Anat. Rec.* 169:296. (Abstr.)
 15. COWLEY, C. W., W. J. TIMSON, and J. A. SAWDYE. 1961. Ultra rapid cooling techniques in the freezing of biological materials. *Biodynamica.* 8:317.
 16. DALLNER, G., P. SIEKEVITZ, and G. E. PALADE. 1966. Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in developing rat hepatocyte. *J. Cell. Biol.* 30:73.
 17. DOLLHOPF, F. L., and H. SITTE. 1969. Die Shandon-Reichert-Kühleinrichtung FC-150 zum Herstellen von Ultradünn- und Feinschnitten bei extrem niederen Temperaturen. I. Gerätetechnik. *Mikroskopie.* 25:17.
 18. DOLLHOPF, F. L., G. WERNER, and E. MORGENSTERN. 1969. Die Shandon-Reichert-Kühleinrichtung FC-150 zum Herstellen von Ultradünn- und Feinschnitten bei extrem niederen Temperaturen. II. Anwendungsbeispiele aus Biologie und Technik. *Mikroskopie.* 25:33.
 19. DOWELL, L. G., and A. P. RINFRET. 1960. Low-temperature forms of ice as studied by X-ray diffraction. *Nature (London).* 188:1144.
 20. EKHOLM, R., T. ZELANDER, and Y. EDLUND. 1962. The ultrastructural organization of the rat exocrine pancreas. I. Acinar cells. *J. Ultrastruct. Res.* 7:61.
 21. ERÄNKÖ, O. 1954. Quenching of tissues for freeze-drying. *Acta Anat.* 22:331.
 22. FERNÁNDEZ-MORÁN, H. 1952. The submicroscopic organization of vertebrate nerve fibers. An electron microscope study of myelinated and unmyelinated nerve fibers. *Exp. Cell Res.* 3:282.
 23. FERNÁNDEZ-MORÁN, H. 1952. Application of the ultrathin freezing-sectioning technique to the study of cell structures with the electron microscope. *Ark. Fys.* 4:471.
 24. FERNÁNDEZ-MORÁN, H. 1962. Cell-membrane ultrastructure: Low-temperature electron microscopy and X-ray diffraction studies of lipoprotein components in lamellar systems. *Circulation.* 26:1039.
 25. FERNÁNDEZ-MORÁN, H. 1964. New approaches in correlative studies of biological ultrastructure by high-resolution electron microscopy. *J. Roy. Microsc. Soc.* 83:183.
 26. FERNÁNDEZ-MORÁN, H. 1966. High resolution electron microscopy of biological specimens. Proceedings 6th International Congress Electron Microscopy, Kyoto. 2:13.
 27. GREENAWALT, J. W., C. S. ROSSI, and A. L. LEHNINGER. 1964. Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria. *J. Cell Biol.* 23:21.
 28. HANZON, V., and L. H. HERMODSSON. 1960. Freeze-drying of tissues for light and electron microscopy. *J. Ultrastruct. Res.* 4:332.
 29. HODSON, S. A., and J. MARSHALL. 1969. A device for cutting ultra-thin unfixed frozen

- sections for electron microscopy. *J. Physiol. (London)*. 201:63P. (Abstr.)
30. HODSON, S., and J. MARSHALL. 1969. A device for trimming frozen biological tissue preparatory to ultra-thin sectioning. *J. Microsc.* 89:373.
 31. HODSON, S., and J. MARSHALL. 1970. Ultracryotomy: a technique for cutting ultrathin sections of unfixed frozen biological tissues for electron microscopy. *J. Microsc.* 91:105.
 32. KOLEHMAINEN-SEVÉUS, L. 1970. Frozen, ultrathin sections. Proceedings 7th International Congress Electron Microscopy, Grenoble. 1:423.
 33. KOLLER, T. 1965. Mounting of ultrathin sections with the aid of an electrostatic field. *J. Cell Biol.* 27:441.
 34. LAFONTAINE, J. G., and C. ALLARD. 1964. A light and electron microscope study of the morphological changes induced in rat liver cells by the azo dye 2-Me-DAB. *J. Cell Biol.* 22:143.
 35. LEDUC, E. H., W. BERNHARD, S. J. HOLT, and J. P. TRANZER. 1967. Ultrathin frozen sections. II. Demonstration of enzymic activity. *J. Cell Biol.* 34:773.
 36. LEHNINGER, A. L., C. S. ROSSI, and J. W. GREENAWALT. 1963. Respiration-dependent accumulation of inorganic phosphate and Ca^{++} by rat liver mitochondria. *Biochem. Biophys. Res. Commun.* 10:444.
 37. LUYET, B. 1961. A method for increasing the cooling rate in refrigeration by immersion in liquid nitrogen or in other boiling baths. *Biodynamica*. 8:331.
 38. LUYET, B. 1967. Various modes of recrystallization of ice. In *Physics of Snow and Ice*. H. Oura, editor. Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan. 1(Pt. 1):51.
 39. MACKENZIE, A. P. 1965. Factors affecting the mechanism of transformation of ice into water vapor in the freezing-drying process. *Ann. N. Y. Acad. Sci.* 125:522.
 40. MAZUR, P. 1970. Cryobiology: the freezing of biological systems. *Science (Washington)*. 168:939.
 41. MAZUR, P., and J. J. SCHMIDT. 1968. Interactions of cooling velocity, temperature, and warming velocity on the survival of frozen and thawed yeast. *Cryobiology*. 5:1.
 42. MERYMAN, H. T., editor. 1966. *Cryobiology*. Academic Press Inc., New York.
 43. MERYMAN, H. T. 1969. Aspects of freezing injury relevant to autoradiography. In *Autoradiography of Diffusible Substances*. L. J. Roth and W. E. Stumpf, editors. Academic Press Inc., New York. 201.
 44. MONNERON, A., and W. BERNHARD. 1969. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* 27:266.
 45. MOOR, H. 1969. Freeze-etching. *Int. Rev. Cytol.* 25:391.
 46. MOOR, H. 1971. Recent progress in the freeze-etching technique. *Phil. Trans. Roy. Soc. London Ser. B Biol. Sci.* 261:121.
 47. MOOR, H., and M. HOECHLI. 1970. The influence of high-pressure freezing on living cells. Proceedings 7th International Congress Electron Microscopy, Grenoble. 1:445.
 48. MUMAW, V. R., and B. L. MUNGER. 1971. Uranyl acetate-oxalate an en bloc stain as well as a fixative for lipids associated with mitochondria. *Anat. Rec.* 169:383. (Abstr.)
 49. PASQUALI-RONCHETTI, I., J. W. GREENAWALT, and E. CARAFOLI. 1969. On the nature of the dense matrix granules of normal mitochondria. *J. Cell Biol.* 40:565.
 50. PEACHEY, L. 1964. Electron microscopic observations on the accumulation of divalent cations in intramitochondrial granules. *J. Cell Biol.* 20:95.
 51. POWELL, R. L., H. M. RÖDER, and W. J. HALL. 1959. Low-temperature transport properties of copper and its dilute alloys: pure copper, annealed and cold-drawn. *Phys. Rev.* 115:314.
 52. REBHUN, L. I. 1961. Applications of freeze-substitution to electron microscope studies of invertebrate oocytes. *J. Biophys. Biochem. Cytol.* 9:785.
 53. REBHUN, L. I., and G. SANDER. 1971. Electron microscope studies of frozen-substituted marine eggs. I. Condition for avoidance of intracellular ice crystallization. *Amer. J. Anat.* 130:1.
 54. REVEL, J. P., and E. D. HAY. 1961. Autoradiographic localization of DNA synthesis in a specific ultrastructural component of the interphase nucleus. *Exp. Cell Res.* 25:474.
 55. RICHTER, G. W. 1957. A study of hemosiderosis with the aid of electron microscopy, with observations on the relationship between hemosiderin and ferritin. *J. Exp. Med.* 106:203.
 56. SABATINI, D. D., K. BENSCH, and R. J. BARNETT. 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* 17:19.
 57. SCOTT, G. B., and S. AVRAMEAS. 1968. Intracellular antibody formation demonstrated on ultrathin frozen sections with alkaline phosphatase used as an antigen and as a marker. *Proc. 4th Eur. Reg. Conf. Electron Microsc.* 2:201.
 58. SCOTT, R. B. 1959. *Cryogenic Engineering*. D. Van Nostrand Company, Inc., Princeton, N. J.

59. SEVÉUS, L. 1969. Cryo-ultramicrotomy. *Process Biochem.* 4:35.
60. SJÖSTRAND, F. S., and L. G. ELFVIN. 1964. The granular structure of mitochondrial membranes and of cytomembranes as demonstrated in frozen-dried tissue. *J. Ultrastruct. Res.* 10:263.
61. SLAYTER, H. S., J. R. WARNER, A. RICH, and C. E. HALL. 1963. The visualization of polyribosomal structure. *J. Mol. Biol.* 7:652.
62. SPIRIN, A. S., and L. P. GAVRILOVA. 1969. The ribosome. Springer-Verlag New York Inc., New York.
63. STEINER, J. W., and C. M. BAGLIO. 1963. Electron microscopy of the cytoplasm of parenchymal liver cells in α -naphthylisothiocyanate-induced cirrhosis. *Lab. Invest.* 12:765.
64. STUMPF, W. E. 1970. Localization of hormones by autoradiography and other histochemical techniques. *J. Histochem. Cytochem.* 18:21.
65. STUMPF, W. E., and L. J. ROTH. 1965. Frozen sectioning below -60°C with a refrigerated microtome. *Cryobiology.* 1:227.
66. STUMPF, W. E., and L. J. ROTH. 1966. High resolution autoradiography with dry mounted freeze-dried frozen sections. Comparative study of six methods using two diffusible compounds ^3H -estradiol and ^3H -mesobilirubinogen. *J. Histochem. Cytochem.* 14:274.
67. STUMPF, W. E., and L. J. ROTH. 1967. Freeze-drying of small tissue samples and thin frozen sections below -60°C . A simple method of cryosorption pumping. *J. Histochem. Cytochem.* 15:243.
68. THORNBURG, W., and P. E. MENGERS. 1957. An analysis of frozen section techniques: I. Sectioning of fresh-frozen tissue. *J. Histochem. Cytochem.* 5:47.
69. TRUMP, B. F. 1969. Effects of freezing and thawing on cells and tissues. In *Autoradiography of Diffusible Substances*. L. J. Roth and W. E. Stumpf, editors. Academic Press Inc., New York. 211.
70. VAN HARREVELD, A., J. CROWELL, and S. K. MALHOTRA. 1965. A study of extracellular space in central nervous tissue by freeze-substitution. *J. Cell Biol.* 25:117.
71. WOLSTENHOLME, G. E. W., and M. O'CONNOR, editors. 1970. *The frozen cell*. J. & A. Churchill Ltd., London.