ELECTRON MICROSCOPE OBSERVATIONS ON FROZEN-DRIED CELLS

SATIMARU SENO and KŌYŌ YOSHIZAWA

From the Department of Pathology, Okayama University Medical School, Okayama, Japan

ABSTRACT

To explore the problem of artefacts which may be produced during usual fixation, dehydration, and embedding, the authors have examined pancreas, liver, and bone marrow frozen at about −180°C., dried, at −55 to −60°C., embedded in methacrylate, sectioned, and floated on a formol-alcohol mixture. By these treatments the labile structure of living cells can be fixed promptly and embedded in methacrylate avoiding possible artefacts caused by direct exposure to chemical fixatives. Cell structures are ultimately exposed to a fixative when the sections are floated on formol-alcohol, but at this stage artefacts due to chemical fixation are expected to be minimized, as the fixatives act on structures tightly packed in methacrylate polymer. In the central zone of tissue blocks so treated, the cells are severely damaged by ice crystallization but at the periphery of the blocks the cell structure is well preserved. In such peripherally located cells, elements of the endoplasmic reticulum (ER), Palade's granules, homogeneously dense mitochondria, and nuclear envelopes and pores, can be demonstrated without poststaining with OsO_4. The structural organization in the nucleus is distorted by vacuolization. The mitochondrial membranes and cristae, cellular membrane, and the Golgi apparatus, however, are detected only with difficulty. The Golgi region generally appears as a light zone, in which some ambiguous structures are encountered. After staining the sections with OsO_4 or Giemsa solution, an inner mitochondrial structure which resembles the cristae seen in conventional OsO_4-fixed specimens appears, but the limiting membrane is absent. Treatment with OsO_4 or Giemsa solution also renders more distinct the membrane of the ER and Palade's granules but not the Golgi apparatus and cellular membrane. Treatment with ribonuclease results in the disappearance of Palade's granules. On the strength of these observations the authors conclude that OsO_4 fixation gives a satisfactory preservation of such cell structures as the nuclear envelope, endoplasmic reticulum, and Palade's granules, though it may induce slight swelling of these cell components.

INTRODUCTION

Recently a wealth of structural details has been revealed in animal cells by the combined use of thin sectioning and electron microscopy of high resolution. So far, however, the observations have been primarily made on cells fixed by chemical reagents. Osmium tetroxide (OsO_4) fixation, for instance, proved to be an excellent method for the preservation of cellular fine structures as shown by Palade (1). But so long as chemical fixatives alone are used, there is no guarantee that we are observing the real structure of the living cell rather than artefacts formed during fixation, dehydration, and embedding. The examination of frozen-dried cells may give a clear answer to this important

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question, or at least may yield more reliable information (13). Several authors who had earlier examined frozen-dried cells under the electron microscope reported disappointing results (2–5). Recently other authors tried again this method under more favorable conditions (6–10, 13, 14). Gersh and his associates, for instance, obtained electron micrographs of frozen-dried hepatic cells (9–12), but in their case the tissue was dehydrated at a rather high temperature and treated before embedding with 95 per cent ethanol followed by OsO_4, platinic tetrabromide or lead acetate treatment. In their material, the fine structure of the cells seems to be severely distorted most likely by ice crystallization during dehydration, and also by subsequent treatment of unembedded dried tissue with 95 per cent ethanol. Ninety-five per cent ethanol is a poor fixative for dried protein. More recently electron micrographs of frozen-dried pancreatic exocrine cells were published by Sjöstrand and Baker (13) and micrographs of chloroplast grana by Müller (14). In Sjöstrand's material, which was not treated before embedding with chemical fixatives, certain structural elements were distinguishable, but the distortion of the structural organization was still marked and therefore critical interpretation of the fine structures became quite difficult. The most satisfactory results have been obtained by Müller who studied the structure of chloroplasts in the leaves of *Eucharis grandiflora*. Although his method is the best available at present, dehydration may still be insufficient and as such may prevent the penetration of embedding material into the tissue. Our experiments have been conducted independently from those of Müller, but in principle our method is almost the same as his, the only differences concern the terminal stages of dehydration and polymerization of methacrylate. The results show that it is possible to preserve the fine structure of the cytoplasm without vacuolization (15, 18).

MATERIALS AND METHODS

Pancreatic and hepatic cells of normal adult mice and bone marrow of normal adult rats served as materials. After decapitation, small pieces of 0.5 $mm.^3$ in size were obtained by cutting the fresh tissue in a moist chamber with a knife, prepared by setting two razor blades sandwiching a steel plate ~0.5 mm. thick. Three to five tissue fragments were put separately in a holder made of metal mesh (Fig. 2, *h*) and frozen by immersion in liquid propan, cooled at about −180°C., in a liquid nitrogen bath.

Liquid propan is prepared, as reported by Gersh, by perfusing propan gas from a propan tank into a flask immersed in a liquid nitrogen bath (6). About 30 cc. of the liquid propan obtained is transferred to a 50 cc. beaker kept in a liquid nitrogen bath. The tissue holder carrying tissue fragments is immersed into the liquid propan. After ~30 seconds, the specimens are transferred with the holder to the tissue chamber (Fig. 2, *a*) of the freezing-drying apparatus previously fitted out for the operation.

A general view of the apparatus is presented in Fig. 1 and its detailed structure is shown diagrammatically in Figs. 2 and 3. The apparatus, which is constructed mainly of pyrex glass, consists of a drying chamber (Fig. 1, *A*), and a vapor trap (*B*), and is provided with vacuum pumps (*C*) and a thermometer (*D*). The tissue chamber, 4 cm. in diameter and 16 cm. in length (Fig. 2, *a*), is a glass tube with five openings: the first for a cold finger 3 cm. in diameter (*e*), at the top; the second for the vapor trap; the third for a methacrylate chamber (*b*); the fourth for a thermometer (*d*); and finally the fifth for a Geissler tube (g_1) on the upper side wall as indicated in Figs. 2 and 3. The thermometer is constructed by using a potentiometer (Fig. 1, *D*) and a galvanometer (Fig. 1, *E*) (Fig. 2, *m*), which are connected to a thermocouple as indicated in Fig. 2. The vapor trap (Fig. 1, *B*), inserted between the drying chamber (*A*) and the vacuum pump (*C*), is constructed of two chambers: one, 7 cm. in diameter and 30 cm. length, having a liquid nitrogen tube 5 cm. in diameter and 25 cm. length (*k*); the other one, 2.5 cm. diameter and 17 cm. length, containing P_2O_5 (*n*) as indicated in Fig. 2. The two chambers are set parallel with each other. The air is led from the drying chamber through either one and pumped out by a diffusion pump (Shimazu 2AF-40) backed by a mechanical pump (Nomura DN-120). A Geissler tube (Fig. 2) is set in between the vapor traps and the diffusion pump to indicate the water content of the air to be led into the diffusion pump.

For operation, P_2O_5 is put in *n* and the methacrylate chamber (*b*) is filled with methacrylate monomer (one volume of methyl methacrylate mixed with nine volumes of *n*-butyl methacrylate) to an appropriate volume and connected to the drying chamber (*a*) with a thick-walled rubber tube. After assembling the apparatus, the vapor trap *k* is filled with liquid nitrogen and the methacrylate mixture in the chamber is frozen in a liquid nitrogen bath. Then the air in the apparatus is pumped out with the stop-cocks *c*, i_1, i_2, t_1, and t_2 open. When the pressure reaches about 10^{-4} mm. Hg, the stop-cocks *c*, t_1, and t_2 are closed and the liquid nitrogen bath of the methacrylate chamber is removed. Then, after

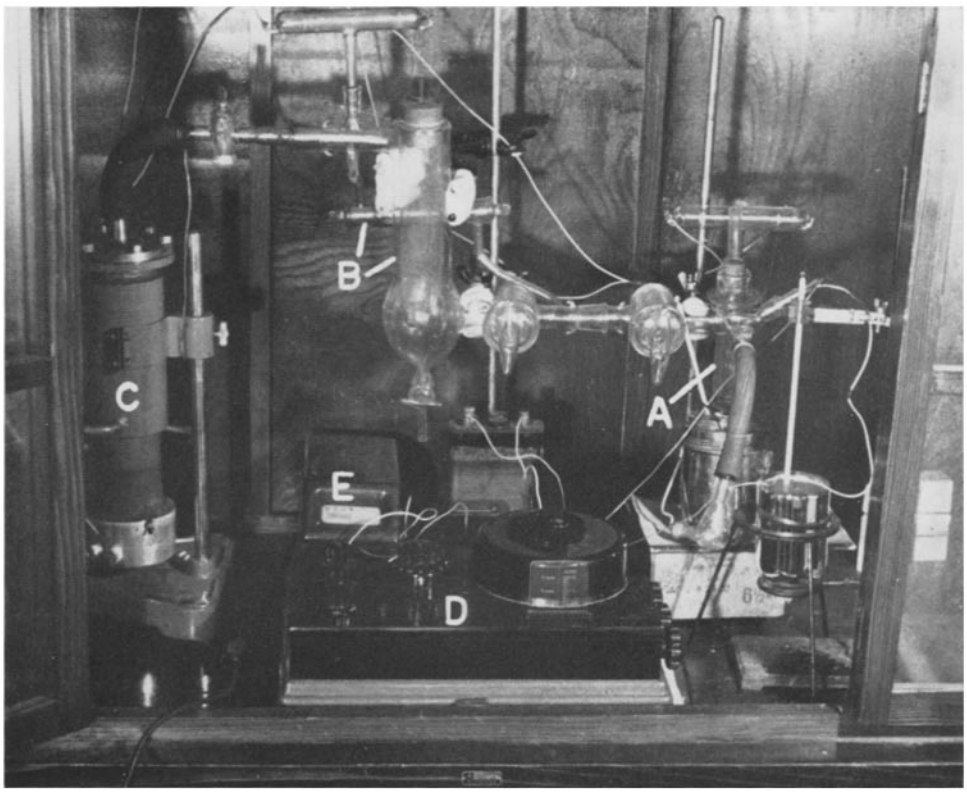


FIGURE 1

The freezing-drying apparatus used for this experiment.

A, Tissue chamber; *B*, vapor traps containing liquid nitrogen and P_2O_5; *C*, oil diffusion pump connected to mechanical pump; *D*, potentiometer; *E*, galvanometer.

closing the stop-cock i_1, the vacuum in the tissue chamber is broken by rotating and loosening *d* or g_1 and the cold finger *e* is removed. The tissue chamber is cooled in the meanwhile to −60°C. in an acetone dry-ice bath (*s*).

At this point the tissue frozen in liquid propan is introduced within its holder *h* into the tissue chamber along the shortest passway, the constantan-copper thermocouple *d* is set as indicated in Fig. 2, and the tissue chamber is closed by reinserting the tube *e*. Then the stop-cock i_1 is opened and the air pumped out by the rotary pump only. When after about 1 hour the vacuum reaches about 10^{-3} mm. Hg, the oil diffusion pump is switched on and the inner tube *e* is filled with liquid nitrogen and closed with a gum plug provided with a glass capillary. The P_2O_5-containing bypath (*n*) is left closed and in this state the drying is performed at −55° to −60°C. for 60 hours at a vacuum of less than 10^{-4} mm. Hg. The distance between the bottom of *e* and the tissue fragments in the holder *h* is as short as ~0.5 cm. Liquid nitrogen must be added from time to time into both chambers *k* and *e* to compensate for losses by evaporation. After 60 hours, the bypath containing P_2O_5 is opened and the temperature of the tissue chamber is gradually raised up to room temperature during the following 15 hours, by adding acetone of about 10°C. to the acetone-dry-ice bath and finally by removing the bath when the temperature reaches 0°C. Then the liquid N_2 in *e* is allowed to evaporate completely. After closing the stop-cock i_1 and checking the complete drying of the materials by the Geissler tube g_1, and then closing the stop-cocks i_1, t_1, and t_2, the methacrylate chamber *b* is raised to the position b_1, the stop-cock *c* is opened gradually, and the methacrylate mixture is poured into the tissue chamber slowly until the materials are submerged. About 5 minutes later the vacuum is broken by loosening *d* and the inner tube *e* is removed.

The tissue fragments are transferred to gelatin capsules which contain an adequate amount of methyl methacrylate and *n*-butyl methacrylate mixed in proportions of 1:9 to 2:8, and containing 3 per cent benzoyl peroxide. Polymerization is carried

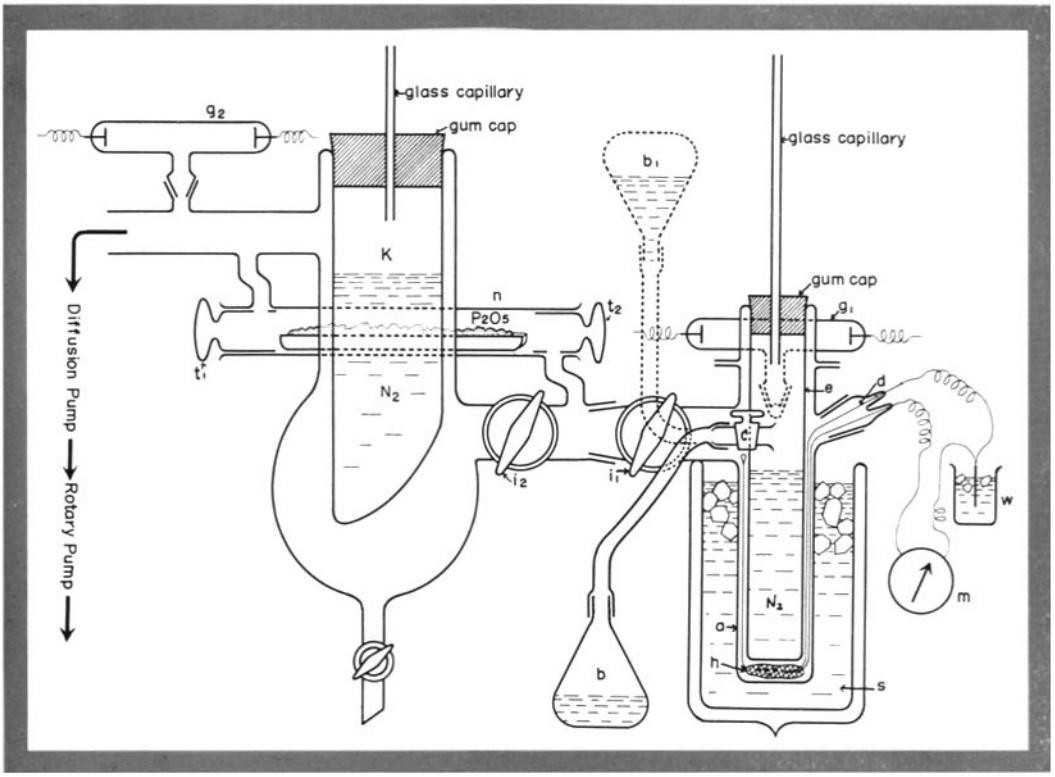


FIGURE 2

The diagram of the freezing-drying apparatus appearing in Fig. 1.

a, tissue chamber; *b*, chamber for methacrylate; *c*, stop-cock; *d*, thermocouple; *e*, cold finger of liquid nitrogen; g_1, g_2, Geissler tubes; i_1, i_2, stop-cocks; *h*, tissue holder; *k*, vapor trap of liquid nitrogen; *m*, thermometer (galvanometer and potentiometer); *n*, vapor trap of P_2O_5; *s*, aceton-dry-ice bath; t_1, t_2, stop-cocks; *w*, ice water.

out in an incubator at 37° to 40°C. for 12 hours. Sections, cut at room temperature with a Shimazu microtome provided with a glass knife, are floated on 50 per cent ethanol containing 10 per cent formol. They are mounted on grids covered with a collodion film, and finally examined in a Hitachi HU-10A electron microscope under a low electron beam, at 20 μA and 75 kv.

A number of specimens were examined after being treated with OsO_4 or stained with a Giemsa solution. Other specimens were treated with ribonuclease (RNase) followed by staining with the Giemsa solution, with or without removing the embedding plastic. For OsO_4 treatment, the sections mounted on grids were floated for 2 hours at room temperature on a 1 per cent OsO_4 solution in veronal buffer, pH 7.4. For staining with Giemsa the grids were floated for 30 minutes at room temperature on a 1 per cent Giemsa solution (Merck) in 0.1 M phosphate buffer at pH 7.4. All sections were washed with distilled water several times, dried, and examined.

For RNase treatment, the specimens were floated at 55°C. for 30 minutes on 0.1 per cent RNase solution buffered with 0.1 M acetate buffer, pH 5.0. After washing with distilled water, the specimens were stained with the Giemsa solution as mentioned above. The RNase was extracted from bovine pancreas by McDonald's method in the laboratory. For control, sections from the same tissue block were treated with the buffer solution containing no RNase.

To remove the methacrylate the tissue sections were mounted and dried on a glass slide. The latter was immersed for 2 minutes at room temperature in pure amylacetate, and then dried and coated with 0.5 per cent collodion in amylacetate. After drying again, the film containing the specimens was stripped by floating on water and then mounted on grids.

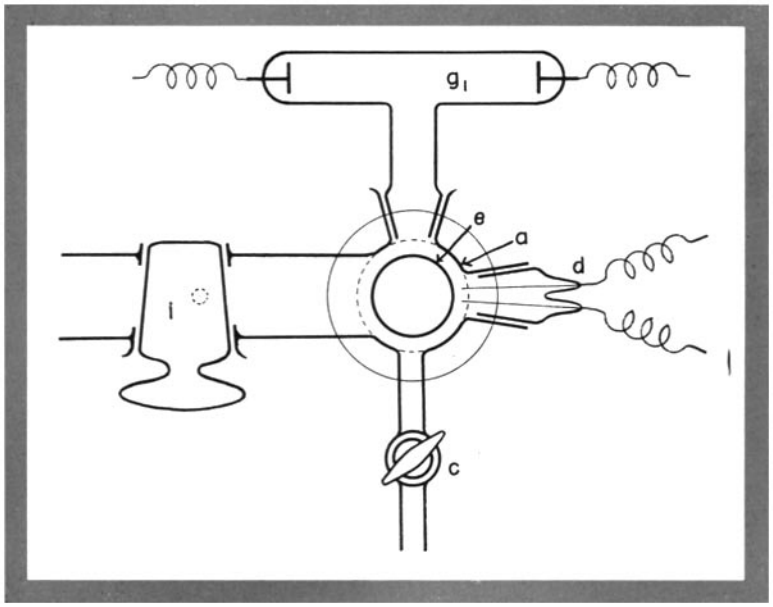


FIGURE 3

Diagram of the tissue chamber seen from upside. For indication see the legend of Fig. 2.

RESULTS

In tissue blocks prepared by this method, three zones can be distinguished according to the degree of ice crystal formation; (*a*) a central zone in which the cell structure is markedly distorted by extensive ice crystal formation; (*b*) an outer layer in which the cytoplasm is well preserved but crystal formation in the nucleus is marked; (*c*) and a peripheral layer less than a cell diameter, in which there is no ice crystallization in the cytoplasm but a few ice crystal spaces are still found in the nucleus. Light microscopic observations of thick sections prove that the cells can be stained well without removing the methacrylate both by OsO_4 and Giemsa, although after methacrylate removal the staining is more rapid and intense. Under the electron microscope the increase in relative contrast due to staining is almost the same in both cases the removal of the plastic making little difference. Therefore, most of our electron microscopic observations have been carried out without removing the methacrylate.

In pancreatic exocrine cells lying near the central zone of the tissue block, the endoplasmic reticulum (ER) appears in the form of dense filamentous structures or as broken or dotted lines. No Palade's granules are seen, while the mitochondria appear as dense masses with several crevices reminiscent of cristae (Fig. 4). In cells lying in the outer layer, the cytoplasmic structures are well preserved. There are no ice crystal spaces and no vacuolization. Elements of the ER appear as paired membranes with a fairly constant spacing and bearing Palade's granules (Figs. 5 to 8). In most cases the spacing of the two membranes in each pair is almost constant, 220 to 400 A (therefore smaller than in OsO_4-fixed specimens) but sometimes vacuolated elements are encountered (Fig. 17). The membranes of the ER usually appear smooth, not uneven and not ruffled as after OsO_4 fixation. Palade's granules appear as dense particles but their density varies from place to place (Fig. 6). They are found lying on the membrane of the ER. Their diameter measures $\sim$200 A and is therefore greater than in OsO_4-fixed specimens. Some elements of the ER without Palade's granules can be seen in the pancreatic exocrine cells but in such specimens the granules can be demonstrated by treating the sections with OsO_4 or Giemsa solution. Treatment

with OsO_4 or Giemsa solution brings about an increase in the relative contrast of both ER membrane and associated granules (Figs. 7, 8). Treatment with RNase results in the complete removal of Palade's granules. The ER membranes, mitochondria and nuclear dense material are not affected (Figs. 9, 10). The specimens treated with the buffer solution containing no RNase retain Palade's granules (Fig. 11).

In the untreated specimen the mitochondria appear as dense homogeneous masses. Neither membrane nor cristae can be seen therein (Figs. 5, 12), though marked differences in density can often be recognized in each mitochondrion. After treating with OsO_4 or Giemsa structures reminiscent of cristae can be distinguished (but not the limiting membrane), especially in the mitochondria of low density (Figs. 13, 14). In some mitochondria a structure reminiscent of the limiting membrane is seen in some parts of the surface but its occurrence is unusual.

The Golgi apparatus which after OsO_4 fixation generally appears as a pile of elongated elements with associated vacuolar structures, can not be recognized in frozen-dried cells. The region corresponding to the Golgi apparatus appears always as a less dense area in which some ambiguous structures can be seen (Figs. 12, 15). In

FIGURES 4 TO 8

Electron micrographs of mouse pancreatic exocrine cells frozen-dried and embedded in methacrylate without prefixation by chemical fixatives.

FIGURE 4

A cell lying near the central part of the block of frozen-dried tissue. Micrograph taken without staining. The fine structure is severely distorted by minute ice crystallization. Mitochondria (*M*) show some crevices reminiscent of cristae. The endoplasmic reticulum (*ER*) can be recognized but its double membrane and the Palade's granules can not be distinguished. *N*, Nucleus, *Z*, Zymogen granules. × 26,000.

FIGURE 5

A cell lying in the outer layer of the tissue; photograph taken without staining. The cytoplasm shows well preserved cytoplasmic fine structure, only the nuclear structure (*N*) is distorted by ice crystal formation. *ER* elements appear as elongated structures limited by membranes which bear Palade's granules. The mitochondria (*M*) appear as electron-dense homogenous bodies. The Zymogen granules (*Z*) are generally surrounded by light spaces. The area, where the zymogen granules are located, corresponds to the Golgi area in OsO_4-fixed cells (refer to Figs. 12, 15, 16.) × 14,000.

FIGURE 6

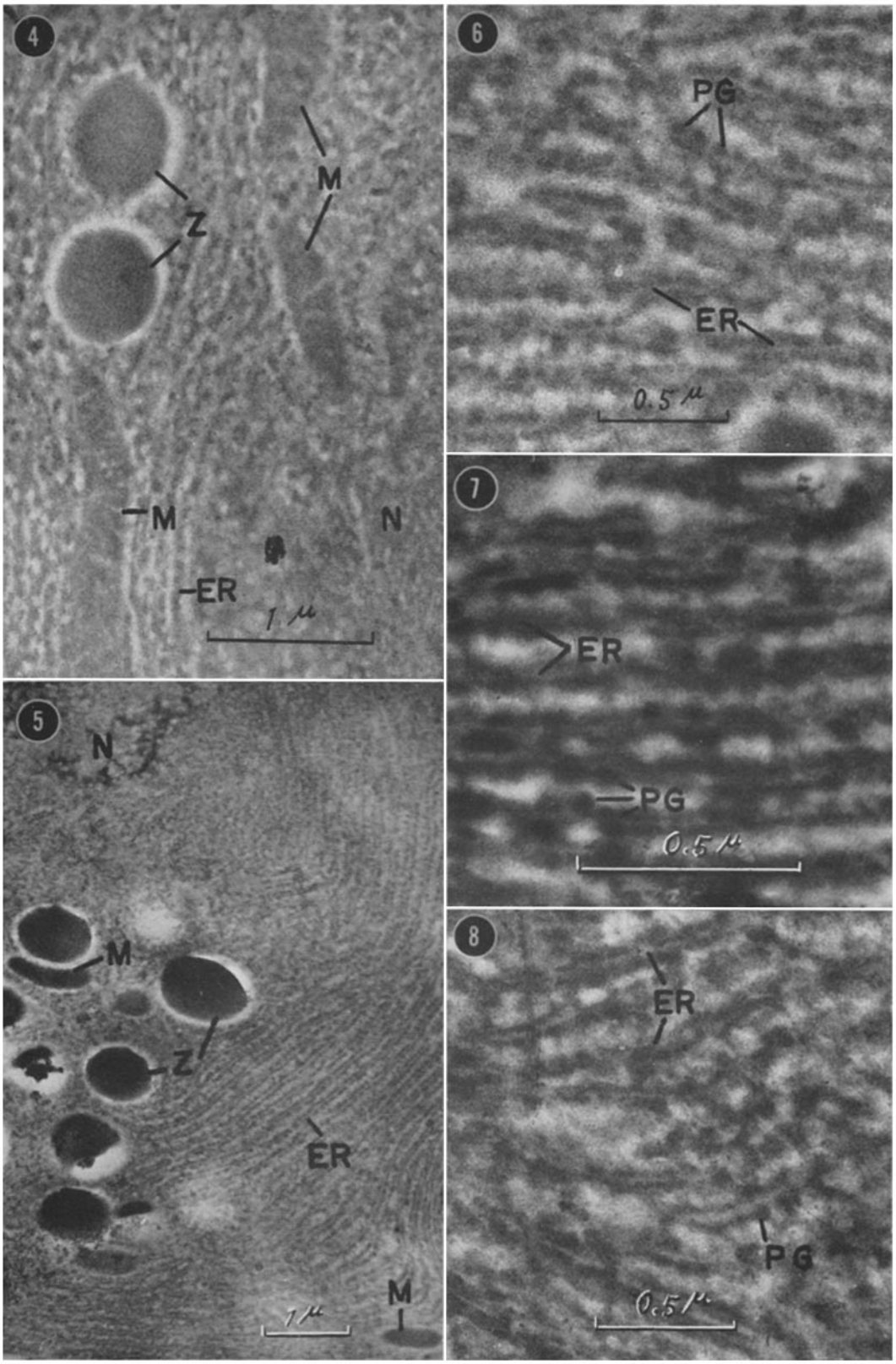
An enlarged picture of a part of the cytoplasm of a cell from the same specimen as in Fig. 5. *ER* elements with attached Palade's granules (*PG*) and less opaque central spaces are seen. × 40,000.

FIGURE 7

An enlarged picture of the ER, taken from the same specimen as in Fig. 5, but photographed after treating with 1 per cent OsO_4 for 2 hours. The contrast of the *ER* membrane and Palade's granules (*PG*) has been markedly increased. × 70,000.

FIGURE 8

ER elements in a specimen stained with Giemsa solution. Moderately increased density of *ER* membranes and attached granules (*PG*). × 45,000.



some cells, dense homogeneous masses of irregular shape (Fig. 15) were found in the Golgi region. In a cell lying in the central zone of the tissue block and having severely distorted cytoplasmic structures, the Golgi apparatus appears as a vacuolated structure (Fig. 16), reminiscent of that seen by Baker in the epithelial cells of mouse intestine (16, 18). However, in specimens with well preserved cytoplasmic elements, OsO_4-staining does not reveal a structure comparable to that shown in Fig. 16 or to the Golgi apparatus seen in OsO_4-fixed material. In the Golgi area the elements of the ER appear irregular in their orientation and zymogen granules are densely distributed. Some of the latter seen just near the less opaque or vacuolated area are small in size and less dense. Such appearances (Figs. 12, 15, 16) suggest the formation of zymogen granules from the opaque material seen in the less dense Golgi area.

In general, the cell membrane cannot be seen clearly but sometimes it appears as a less dense line at the border of the cell (18).

Zymogen granules can be recognized as round, dense bodies (Figs. 4, 5, 12, 15, 16) surrounded by less opaque spaces. Treatment with OsO_4 seems to increase their density. Generally they are found to be concentrated around the area corresponding to the Golgi apparatus.

The fine structure of the nuclei lying in the central zone and even in the outer layer of the tissue block is distorted by ice crystal formation, but the nuclear envelope, an intranuclear network of opaque material and the nucleolus can be easily recognized. Especially, the nuclear envelope shows clearly its detailed structure (Figs. 17, 18).

In well preserved cells, narrow spaces can be seen surrounding the nucleus. The outer membrane bears Palade's granules like the membrane limiting the ER, whereas the inner membrane is hardly recognizable due to close contact with masses of dense nuclear material. The pores or discontinuities of the nuclear envelope can also be demonstrated, the picture being almost identical to that seen in OsO_4-fixed specimens (Figs. 17, 18). In the cells found in the outer layers of the tissue blocks, the inner structure of the nuclei is still distorted, but in the cells lying in the outermost layer the fine structures of the nuclei seem to be well preserved. An unequal

FIGURES 9 AND 10

Mouse pancreatic exocrine cells frozen-dried and methacrylate-embedded. Sections exposed to RNase, stained thereafter with Giemsa solution and finally micrographed in the electron microscope.

FIGURE 9

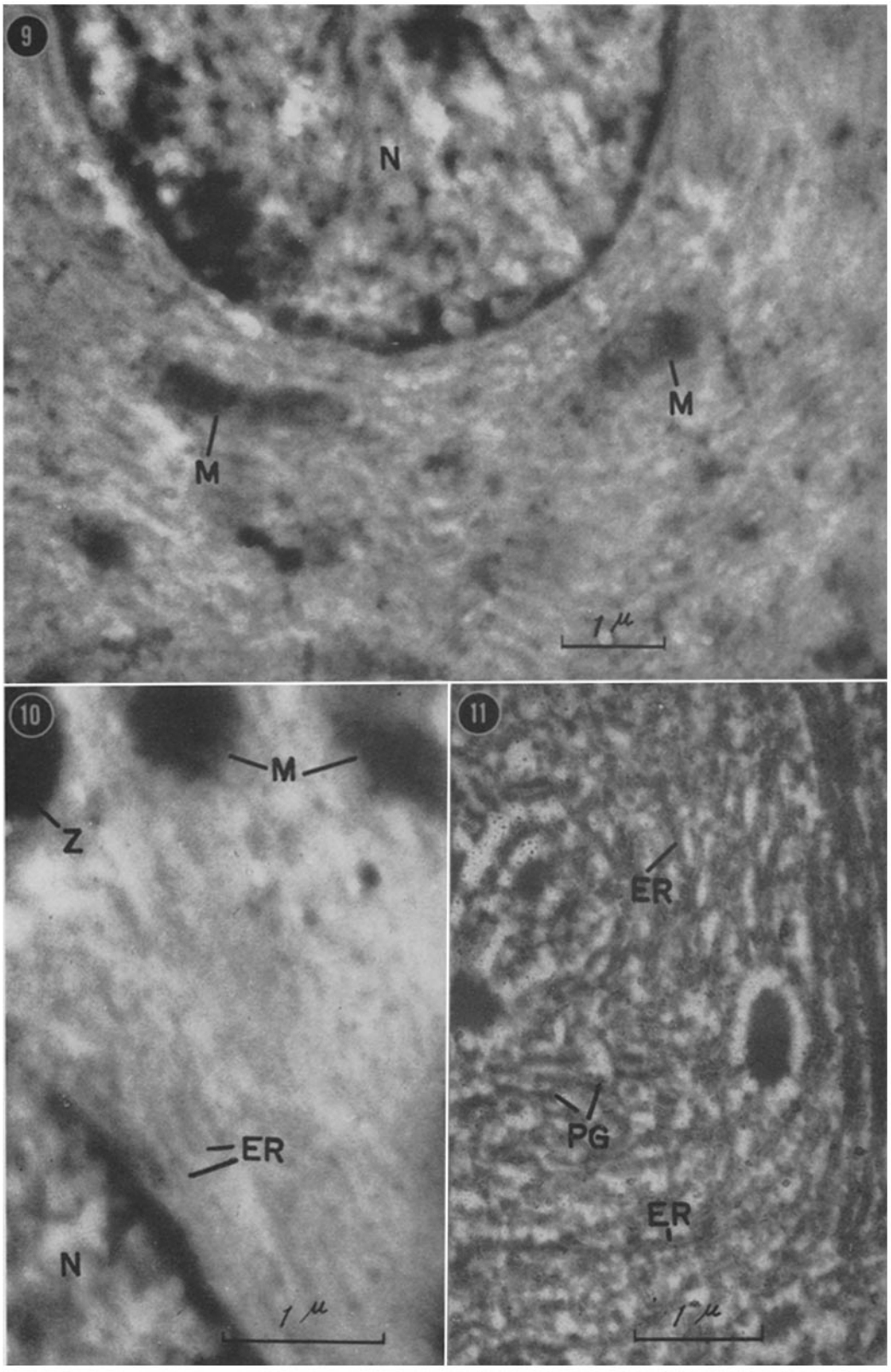
A cell exposed to 0.1 per cent RNase solution, pH 5.0, for 30 minutes at 55°C. and stained thereafter with Giemsa. Palade's granules have disappeared but ER, mitochondria and nuclear dense material are still recognizable. *M*: Mitochondria: the one to the right shows some indication of inner structure similar to the cristae seen in OsO_4-fixed specimens. *N*: Nucleus. × 16,000.

FIGURE 10

A part of a cell seen in the same specimen as in Fig. 9, moderately enlarged. The contrast of the *ER* elements is rather low but the double membranes can be faintly distinguished in a few places (*ER*). *M*: Mitochondria; *Z*: Zymogen granule; *N*: Nucleus. × 25,000.

FIGURE 11

The control. A part of the cytoplasm in a cell from the same material as in Figs. 9 and 10. The specimen was exposed to buffer solution containing no RNase. The rest of the treatment was the same as for the section in Fig. 9. Palade's granules (*PG*) are visible. × 20,000.



distribution of opaque masses can be seen clearly. The nuclear image is closer to that seen under the light microscope in cells fixed with formol than to that shown in the electron microscope by the nuclei of OsO_4-fixed cells. Masses of dense material occur in close contact with the inner nuclear surface and the nucleoli; the former will correspond to the chromatin nets and the latter probably to the nucleolus-associated chromatin (Fig. 19). In the areas which separate the dense masses, fibrous elements measuring 400 to 700 A in diameter are encountered. Nucleoli consist of granular or fibrous elements, comparable to the nuclear fibrous elements in diameter and density (Fig. 19), but packed more tightly than the latter. No membrane is found around the nucleolus.

In lymphocytes, elements of the endoplasmic reticulum are sparse (Fig. 20) while in the plasma cells a well developed ER associated with Palade's granules can be recognized (Fig. 21). Mitochondria appear in each cell as oval or rod-like dense masses (Fig. 20). In these cells, too, the mitochondrial cristae and the limiting membranes can hardly be seen. The Golgi zones appear as less dense areas in which vacuolated structures can be discerned (Figs. 20, 21), a situation similar to that encountered in pancreatic exocrine cells. The nuclear images are also similar to that described in pancreatic exocrine cells.

In hepatic cells, ice crystal formation is usually marked, but it can be reduced by injecting the animals (mice) with glucose prior to specimen collection. The animals receive 2 ml. of 10 per cent glucose solution after 24 hours' starvation and the tissue is taken 1.5 hours after the glucose injection. In such a material, the elements of the ER appear as filamentous structures since their cavities are difficult to distinguish (Fig. 22). The mitochondria are usually seen as round or rod-like bodies, though long filamentous ones are occasionally encountered. Generally, they appear homogeneous but frequently there are marked differences in local density within each mitochondrion. In some cells, elements of internal structure reminiscent of mitochondrial cristae can be seen without staining (Fig. 23) whereas in pancreatic exocrine cells the mitochondria appear always homogeneous. After treatment with OsO_4, the elements of the ER and some small granules show up more clearly (Fig. 24). In the cytoplasm there are two kinds of vacuoles. The small and sharply outlined ones, which appear also in the

FIGURES 12 TO 14

Electron micrographs of the cytoplasm of mouse pancreatic exocrine cells frozen-dried and embedded in methacrylate.

FIGURE 12

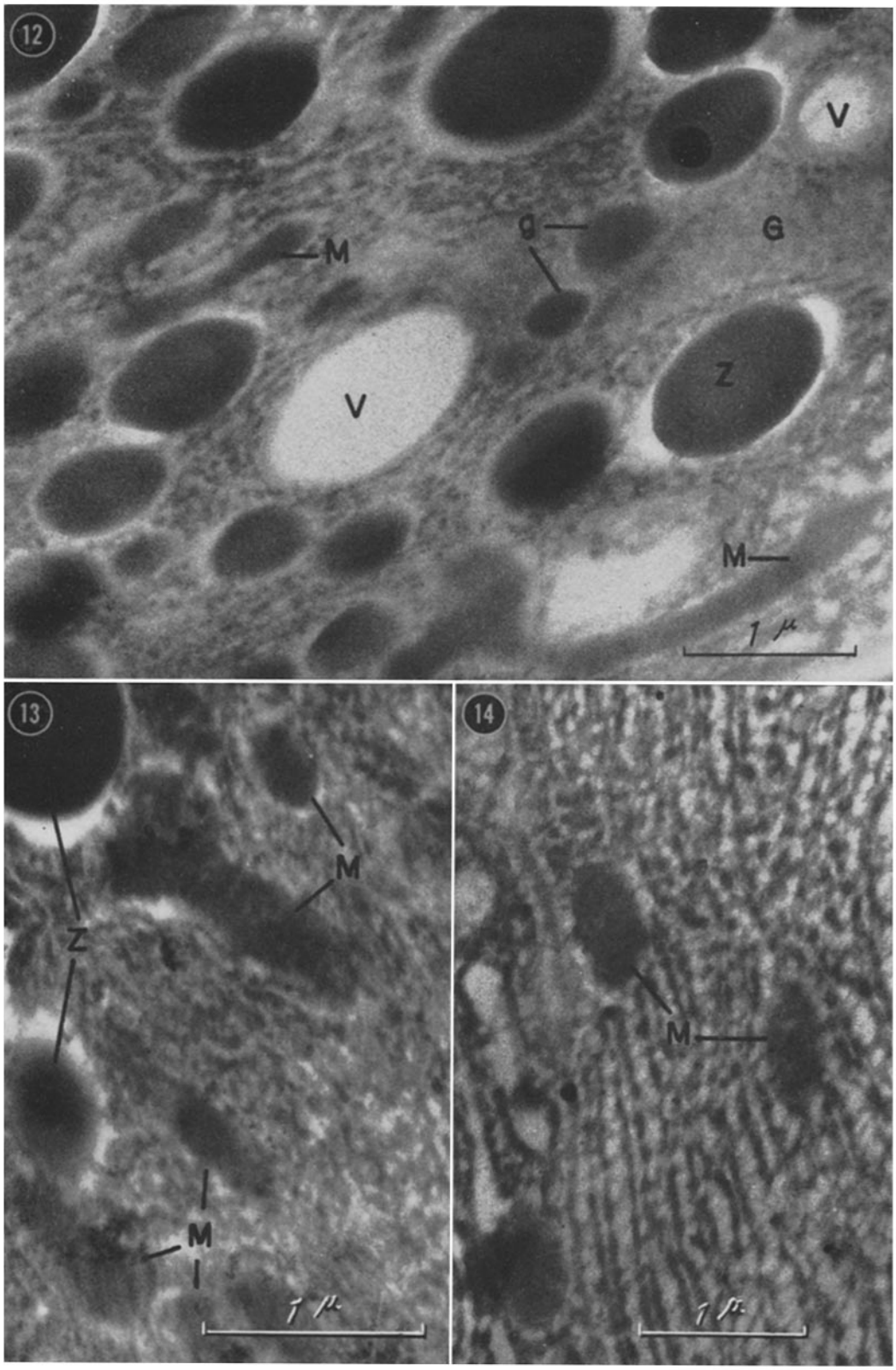
Micrograph taken without previous staining. Filamentous dense homogenous mitochondria (*M*) and zymogen granules (*Z*) can be seen. In the area where zymogen granules are located a number of ER elements generally appear distributed at random and often seem to form meshworks. The right upper area corresponds to the Golgi apparatus of OsO_4-fixed cells. It always appears as a less dense area in which some ambiguous structures (*G*) can be seen together with some vacuoles (*V*). This area is rich in small and less opaque zymogen granules (*g*). × 27,000.

FIGURE 13

Micrograph taken after treating the section for 2 hours with 1 per cent OsO_4. Mitochondrial inner structure is revealed by osmium treatment (*M*), but the limiting membrane can not be seen. *Z*: Zymogen granules. × 35,000.

FIGURE 14

Micrograph taken after treating the section with 1 per cent Giemsa solution for 30 minutes. This treatment also makes the mitochondrial inner structure visible but not the limiting membrane. *M*: Mitochondria. × 26,000.



nucleus (Figs. 25, 26 *V*), are ice crystal spaces. The larger round or oval vacuoles (Figs. 24, 25 *L*) are often surrounded by a thin moderately opaque membrane and can be distinguished from ice crystal vacuoles by their morphological features and the unequal distribution in the cytoplasm. These are presumed to mark the location of extracted lipid droplets. Besides these, we encounter in the cytoplasm quite large, less opaque areas (Figs. 24 to 26 *Gl*), which are absent in blood cells or pancreatic exocrine cells. Light micrographs of thick sections obtained from the same material and stained with PAS or Best's carmine show irregularly distributed masses of glycogen (Fig. 27), indicating that the glycogen is retained throughout the process of floating the sections on 50 per cent ethanol containing formol. A comparison of such pictures with those taken with the electron microscope suggests that the less opaque areas (Figs. 24 to 26 *Gl*) are regions of glycogen deposition. The low opacity of these areas may be attributed to a high water content *in vivo*.

COMMENT

Several attempts have already been made to prepare sections of frozen-dried tissue for electron microscopy but almost all have yielded disappointing results because one or several important requirements of this procedure have not been satisfied.

The first problem is how to freeze successfully the cell in a completely vitreous state without including any ice crystallization. Concerning this point, Stephenson has recently published precise observations and theoretical considerations (7). Calculating from his data, the temperature needs to be brought within $^{2}/_{100}$ sec. to a point under −140°C., the temperature of the transition from the vitreous to the crystalline state of water, in order to freeze water in a vitreous state. He followed the decrease in temperature of a thin slab of copper (0.03 x 1.0 cm.) inserted in liquid nitrogen (−194°C.), isopentan (−150°C.), or liquid propan (−150°C.) cooled in a liquid nitrogen bath, and showed the last one is the most efficient for a prompt lowering of the temperature (7), because of its good heat conductivity, relatively high boiling point, −44.5°C. (14), and lower solidifying temperature (19). These results, however, show that it is almost impossible to freeze a tissue in a completely vitreous state even when cut into very small fragments. According to Stephenson it requires more than $^{8}/_{100}$ sec. to lower the temperature of the thin copper slab below −100°C. in liquid propan cooled at −150°C. (7). Yet the heat conductivity of tissues is very poor as pointed out by Luyet (20), though the transition temperature

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FIGURES 15 AND 16

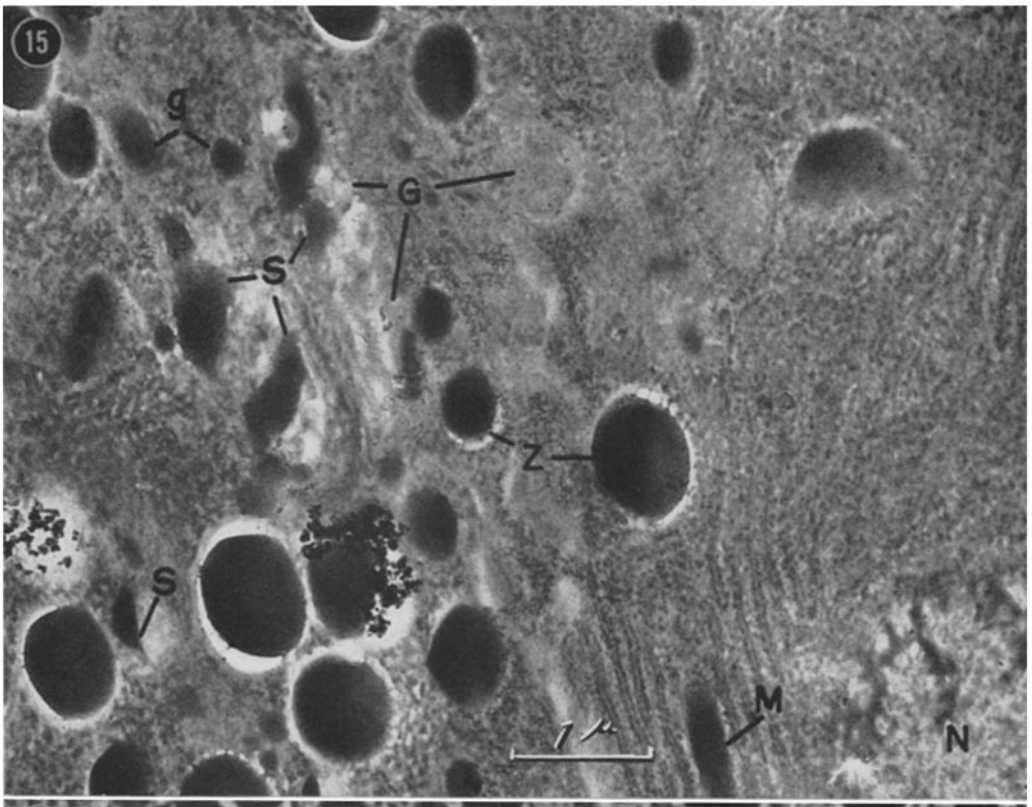
Mouse pancreatic exocrine cells, frozen-dried and embedded in methyacrylate, micrographed without staining. The micrographs show the area corresponding to the Golgi apparatus in OsO_4-fixed cells.

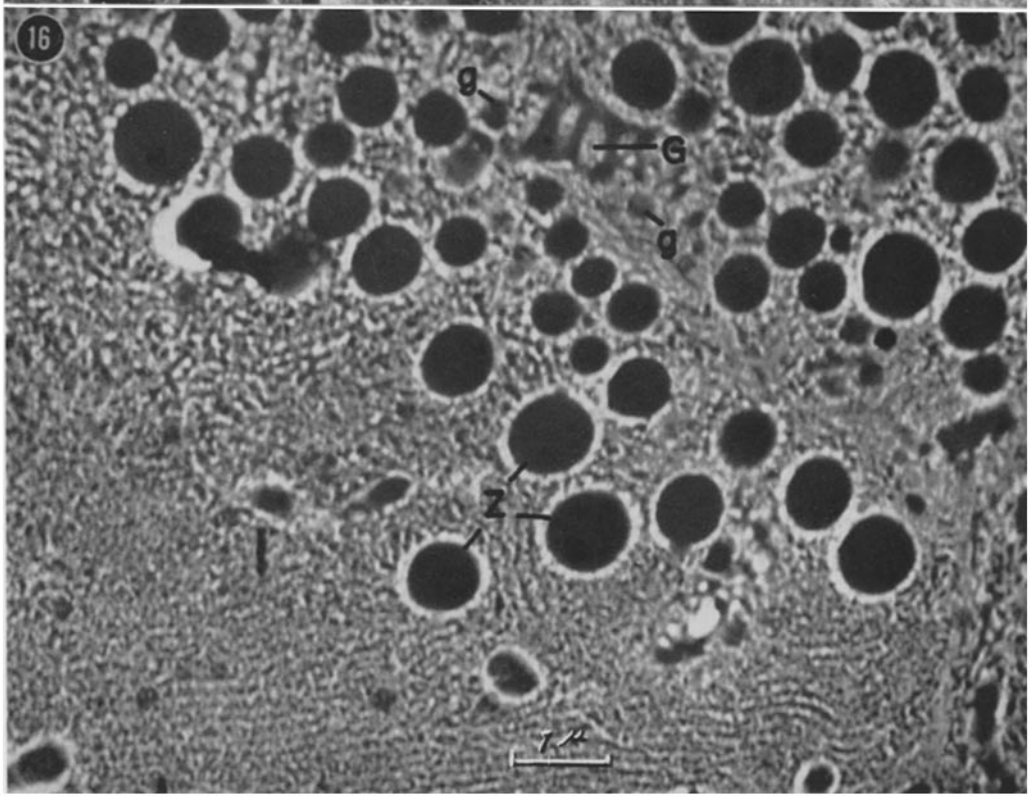
FIGURE 15

The less dense area marked (*G*) corresponds to the Golgi apparatus in OsO_4-fixed material. In pancreatic exocrine cells, this area is generally surrounded and sometimes penetrated by numerous zymogen granules (*Z*). In some parts of this area irregular masses (*S*) of dense material can be seen with small zymogen granules (*g*) in their immediate vicinity. The association suggests that the zymogen granules are formed at the expense of the irregular masses of dense material. *M*: Mitochondria. *N*: Nucleus. × 19,000.

FIGURE 16

The area corresponds to the Golgi zone in a cell whose fine structure has been severely distorted by ice crystallization. The vacuolated structure (*G*) may be an artefact, but it suggests again a process by which small zymogen granules (*g*) form at the expense of the irregular mass of dense material. × 13,000.





from vitreous to crystalline state may be rather high in cytoplasm, about −100°C. according to Stephenson (7). Not withstanding these obvious difficulties, it is reasonable to assume that some areas in the outer layer of the tissue fragments should be frozen in a vitreous state and it is advisable to select these areas for observation. As mentioned, our specimens showed that the cytoplasm of cells lying at the outer layer of the tissue fragments retains its fine structure without distortion by ice crystal formation, though in the central part cellular structures are severely distorted by vacuolization. The nucleus shows marked vacuolization even in the area in which the cytoplasm appears satisfactorily preserved. This could be due to a difference in colloidality between the cytoplasm and the nucleus. Ice crystallization is markedly influenced by the colloidality of the substance (17).

Ice crystallization in water frozen to a vitreous state also occurs in the course of dehydration and this is the most troublesome situation encountered in freezing-drying. According to Asahina, ice crystallization already occurs when the temperature rises up to −120° to −130°C. in the case of pure water (21), and is most marked at −30° to −45°C. in our experience (22). It is desirable to dry the frozen tissue below −130°C., but at this temperature the evaporation pressure is nearly zero and consequently it is very difficult to dry out the tissues at this theoretically optimal temperature. It is known, however, that the recrystallization point goes up when the colloidality increases, for example, in the case of a molar glucose solution the recrystallization does not occur even at −35°C. according to Luyet (23). The devitrifying temperature of cytoplasm is not accurately known and it is expected to be different from cell to cell according to their colloidality. Bell indicated a drying temperature near −55°C. on the basis of the results known in glucose solutions and silica sols (17). Therefore, it seems that tissues must be dried at least at a temperature lower than −55°C.

Another thing to be noted is the crystallization of salts during dehydration as pointed out by Müller (14). One of our collaborators, Kanda proved that basophilic cytoplasm contains a quantity of Ca (24). Accordingly, it is necessary to dry the tissue at least below −54.5°C., which is the melting point of a saturated $CaCl_2$ solution.

FIGURES 17 TO 19

Nuclei of mouse pancreatic exocrine cells, frozen-dried and embedded in methacrylate. Micrographs taken without staining.

FIGURE 17

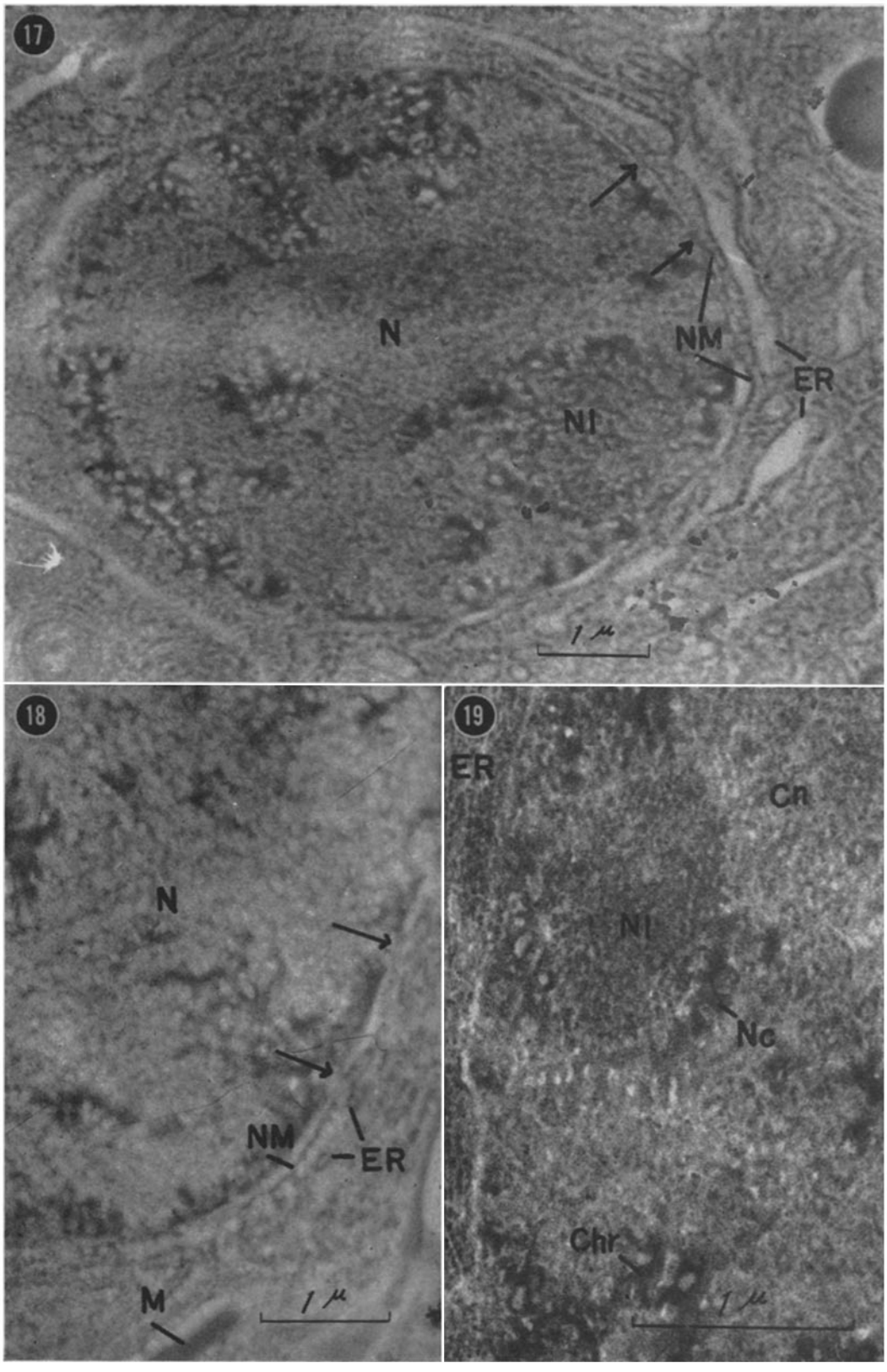
In this cell the cavities of the *ER* are more or less distended. In the nucleus (*N*) an uneven distribution of dense and less dense materials can be seen. The nucleolus (*Nl*) can be distinguished but its structure is distorted by ice crystallization. The inner nuclear membrane clearly visible in OsO_4-fixed cells can hardly be distinguished, but the outer membrane (*NM*) interrupted by a few pores (arrows) can be seen clearly. × 17,500.

FIGURE 18

A nucleus (*N*) showing the perinuclear space, nuclear membrane (*NM*) and pores (arrows) next to which the nucleoplasm appears less oqapue. The membrane (*NM*) bears Palade's granules and thus corresponds to the outer nuclear membrane of OsO_4-fixed material. × 20,000.

FIGURE 19

A part of the nucleus in a cell found in the peripheral layer of the tissue block. The micrograph shows the nucleolus (*Nl*) and the dense nuclear material (*Nc*) surrounding the nucleolus. Fibrous structures (*Cn*) can be distinguishable both in the nucleus and in the nucleolus (*Nl*). *Chr*: Dense nuclear material. × 35,000.



For these various reasons we adopted a temperature of −55° to −60°C. for dehydration. In this case the vapor pressure of water is still low and the dehydration time is prolonged. To shorten the dehydration period we set a big finger cooled with liquid nitrogen at a distance of only 0.5 cm. from the tissue fragments. Under these conditions the tissue fragments can be dried in about 60 hours. Müller proved that at such a low temperature the residual water is still high in percentage (about 10 per cent of the mass) after 80 hours' dehydration using his apparatus, but it reaches about 2 per cent after about 5 hours by drying with P_2O_5 at 20°C. (14). After the termination of drying at −60°C. we raised the temperature of the tissue chamber gradually to room temperature over the following 15 hours, with the P_2O_5 vapor trap opened. In this way the amount of residual water in the tissue was minimized and the penetration of methacrylate made easier. Judging from the results obtained, the gradual rise in the temperature after 60 hours' dehydration does not bring actual distortion of minute cell structures.

The next problem is to fill up the spaces, which had been occupied by water, with embedding material without distorting the fine structural organization. Sjöstrand calls attention to the vacuolization and destruction of cellular structure which occur during tissue embedding in methacrylate (13), but in our experience embedding without vacuolization can be attained by immersing the dried tissue in methacrylate monomer without breaking the vacuum. Even a temporary break of he vacuum must be carefully avoided, as the dehydrated tissue is very hydrophilic and its fine structure may be distorted by rehydration. Immersion in methacrylate, which is extremely hydrophobic, will not result in the distortion of a structure composed of protein whose bound water is extremely low, supposedly less than 2 per cent judging from Müller's (14) data. In our case immersion was carried through at room temperature, about 20°C., and polymerization at 40°C. for 12 hours with added benzoyl peroxide. The results are satisfactory but the micrographs must be interpreted with due consideration to lipid loss as pointed out by Müller (14) and to a slight shrinkage during polymerization (26).

In floating the sectioned tissues it is to be noted that their proteins are not fixed. Protein leakage from the sections can be prevented by using 50 per cent ethanol containing 10 per cent formol as the floating medium. Some swelling or shrinkage of the structure may result by exposing the sections to the formol alcohol, however, proteins will be fixed perhaps without causing any marked structural distortion, as the structures are firmly packed with plastic. It may be argued that certain water-soluble substances like glycogen could be lost by floating the sections on such a mixture. Indeed the areas of glycogen deposition in liver cells appear bright under the electron microscope. But cytochemical tests carried out on thick sections cut from the same material demonstrated the glycogen was well preserved throughout this treatment. Therefore, the low density of the glycogen-depositing areas is probably due to the high local water content.

FIGURES 20 AND 21

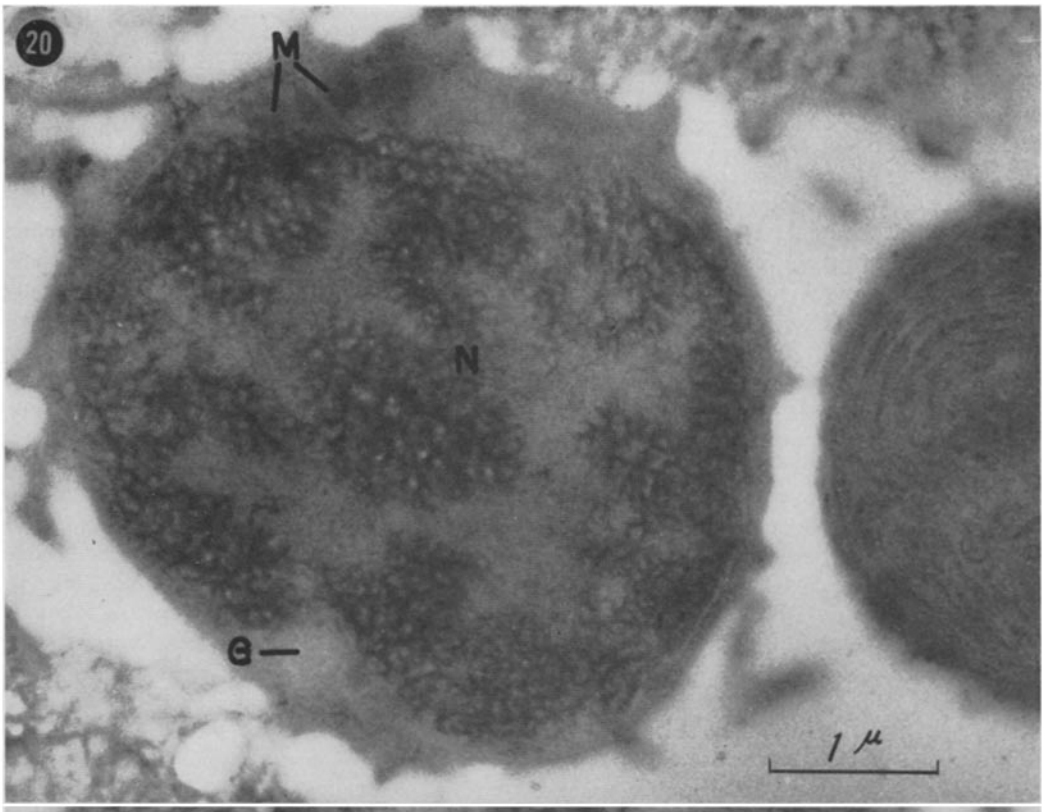
Blood cells from rat bone marrow frozen-dried, and embedded in methacrylate. Micrographs taken without staining the sections.

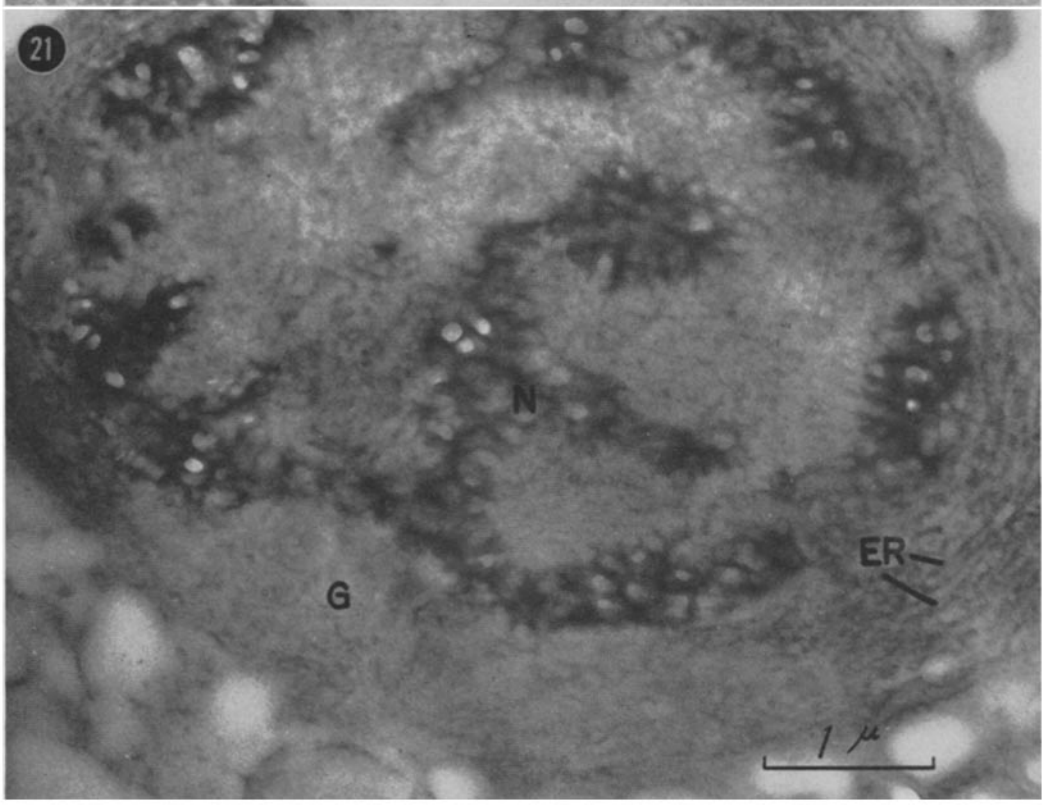
FIGURE 20

A lymphocyte found in the bone marrow. Cytoplasm is poor in ER. Mitochondria (*M*) appear as homogenous dense masses. The Golgi area is less opaque (*G*) than the rest of the cytoplasm. Part of the cytoplasm of a plasma cell appears to the right. *N*: Nucleus. × 22,500.

FIGURE 21

A plasma cell found in the same specimen as the cell in Fig. 20. The double membranes of the *ER* elements can be distinguished. The less dense area (*G*) corresponds to the Golgi apparatus and contains some vacuolated structures. *N*: Nucleus. × 22,500.





As the proteins have been fixed and the sections are very thin, subsequent drying will not cause any severe structural changes, especially in sections from which the plastic has not been removed.

The staining or the digestion with RNase can also be done without removing the methacrylate. These findings show that the fixatives, dyes, or RNase penetrates into the sections through the cut surface of the structural organization embedded in methacrylate.

To keep the nucleus free from vacuolization, a more rapid freezing or dehydration at a deeper temperature than currently possible is required. With the results available at present, a discussion on the detailed structural organization of the nucleus is, therefore, unwarranted.

From the reasons mentioned above cytoplasmic structures composed primarily of protein are considered to be preserved close to their original state by the present method. Therefore, it can be said that the limiting membrane of ER and the small granules are structures present *in vivo* and not artefacts formed by precipitation or agglutination caused by chemical fixatives. These structures are probably composed of proteins different from those of the cell membrane and limiting membrane of mitochondria. The latter are absent in frozen-dried cells supposedly because they are composed mainly of lipids. The disappearance of the granules after RNase treatment indicates that these granules contain RNA or are ribonucleoprotein (RNP) particles. Staining with Giemsa solution increases moderately the relative contrast of these particles. Under the light microscope, the cytoplasm of the pancreatic exocrine cells appears deep blue after staining with Giemsa and the basophilia is completely lost by RNase treatment. The finding suggests that the increase in the relative contrast of the granules by Giemsa staining is mainly due to the binding of basic dye to the RNA. This binding may increase the density of the particles or prevent the loss of their substance under the electron beam. The effect of OsO_4 treatment on the granules and ER membrane is most probably due to its binding to proteins since the bulk of the lipids has been removed by immersion in methacrylate and since OsO_4 does not react with RNA (25). For these reasons we also used Giemsa solution to test the effect of RNase by electron microscopy. As in control sections treated only with the buffer solution the granules were retained and their density could be increased by staining with Giemsa, it is reasonable to conclude that the granules contain RNA.

The ER membrane has a smooth outline which

FIGURES 22 TO 24

Mouse hepatic cells frozen-dried, and embedded in methacrylate.

FIGURE 22

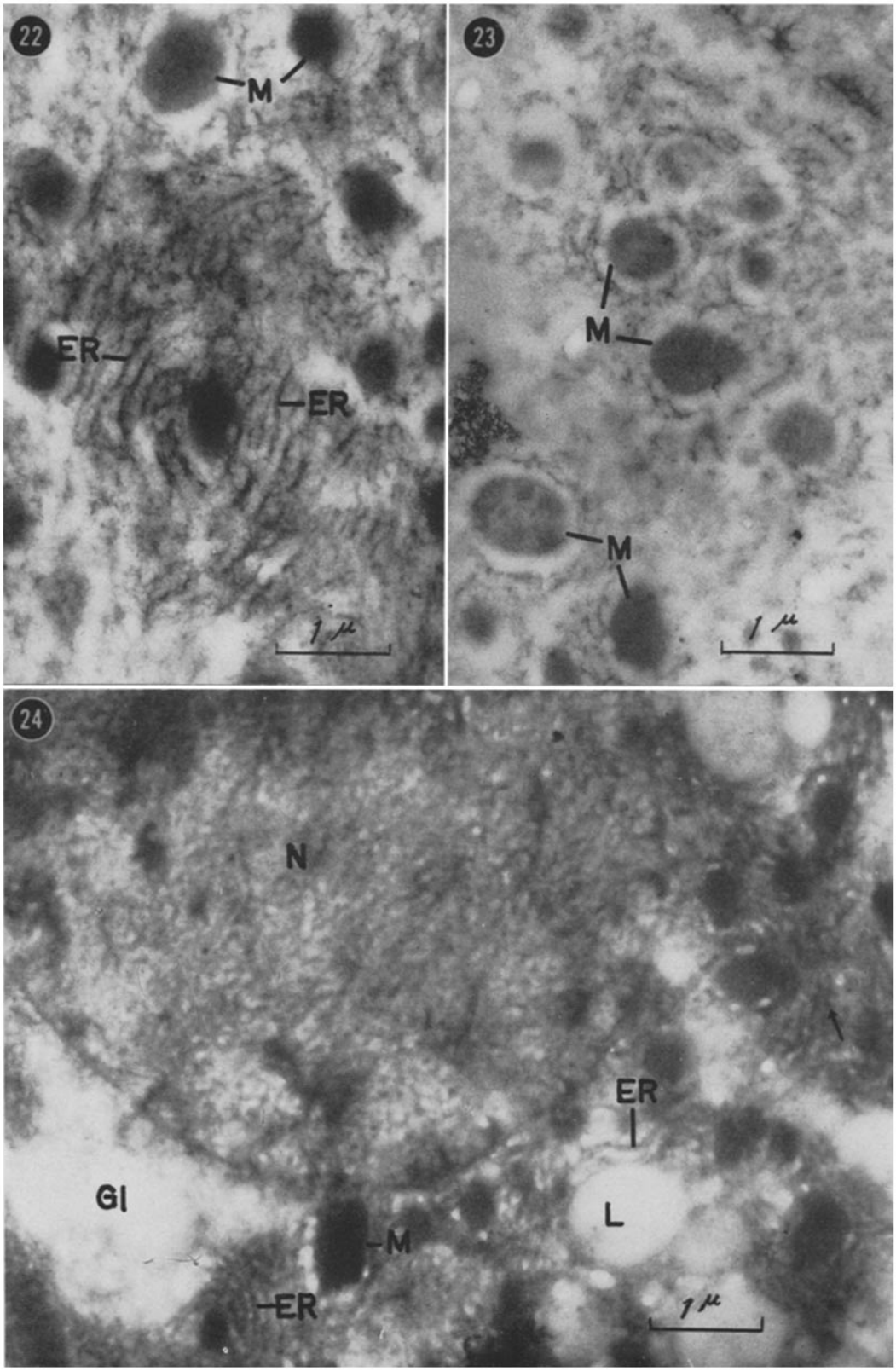
Cytoplasmic area of a hepatic cell micrographed without staining. Oval or round mitochondria (*M*) and fibrous *ER* elements can be seen, the inner space in the latter is hardly visible. × 18,000.

FIGURE 23

Cytoplasmic area of a hepatic cell from the same tissue block as Fig. 22. In this area mitochondria (*M*) show an inner structure similar to the cristae seen in OsO_4-fixed cells. × 18,000.

FIGURE 24

A hepatic cell from a mouse. The liver was frozen 1.5 hours after the subcutaneous injection of 2 cc. of 10 per cent glucose solution after 24 hours' starvation. Micrograph taken after staining the section with OsO_4. The cell structure is better preserved than in Figs. 22 and 23. The ER elements appear distinctly (*ER*). Some Palade's granules can be distinguished (arrow). *N:* Nucleus; *M:* Mitochondria; *L:* Supposedly lipid vacuole; *Gl:* Glycogen-depositing area. × 18,000.



is retained after RNase treatment and the spacing of the double membranes is almost constant, though sometimes local dilatations are encountered. Therefore, the ruffling of the membrane generally seen in OsO_4 fixation is probably an artefact resulting from non-uniform swelling at fixation and from subsequent shrinkage during dehydration as suggested by Bahr's experiment (26).

In frozen-dried specimens, the mitochondria appear as homogeneous dense masses. Except for shape and general dimensions, their appearance is rather different from that found in OsO_4-fixed material. Treatment with OsO_4 or Giemsa demonstrates structures reminiscent of the cristae. As the limiting membrane seen after OsO_4 fixation is not recognized in frozen-dried cells, it may be argued that in frozen-dried specimens we see a negative picture of OsO_4-fixed structures as suggested by Sjöstrand and Baker (13). But dense lines similar to those seen in OsO_4-fixed specimens appear in the mitochondria of some hepatic cells (Fig. 23) and in those of pancreatic exocrine cells after staining with Giemsa (Figs. 9, 14). It seems, therefore, that the cristae are more resistant to methacrylate extraction than the limiting membrane, but the point requires further observations.

The structure of the Golgi apparatus is not similar to that seen after OsO_4 fixation, the organella appearing as a less opaque poorly defined, sometimes vacuolar structure located in the expected area. The finding suggests that the Golgi apparatus is composed mainly of lipids. The masses of dense material observed in the Golgi area in some pancreatic exocrine cells may be the centrioles but it is more likely that they represent developing zymogen granules. As already pointed out, zymogen granules are frequently encountered in the Golgi zone and smaller and less opaque granules are situated nearer the center of this area. The findings suggest the formation of zymogen granules from the opaque irregular masses lying in the less opaque area (Figs. 5, 12, 15, 16). Most zymogen granules and some mitochondria are surrounded by a light halo. Its significance is uncertain: it may be a space formed by the unequal swelling of the individual cell components during freezing or by their shrinkage during the polymerization of the methacrylate.

The inner nuclear membrane well outlined after OsO_4 fixation is hardly visible in frozen-dried specimens, but the outer membrane is easily recognized, like the limiting membrane of the ER. The finding suggests that the outer nuclear membrane is similar to the ER membrane but rather different from the cell membrane.

By comparison with OsO_4 fixation, freezing-drying generally gives a rather faint, low contrast image of the structural organization of the cell.

FIGURES 25 TO 27

Cells from the same tissue block as in Fig. 24. Except for Fig. 27, the micrographs were taken without staining.

FIGURE 25

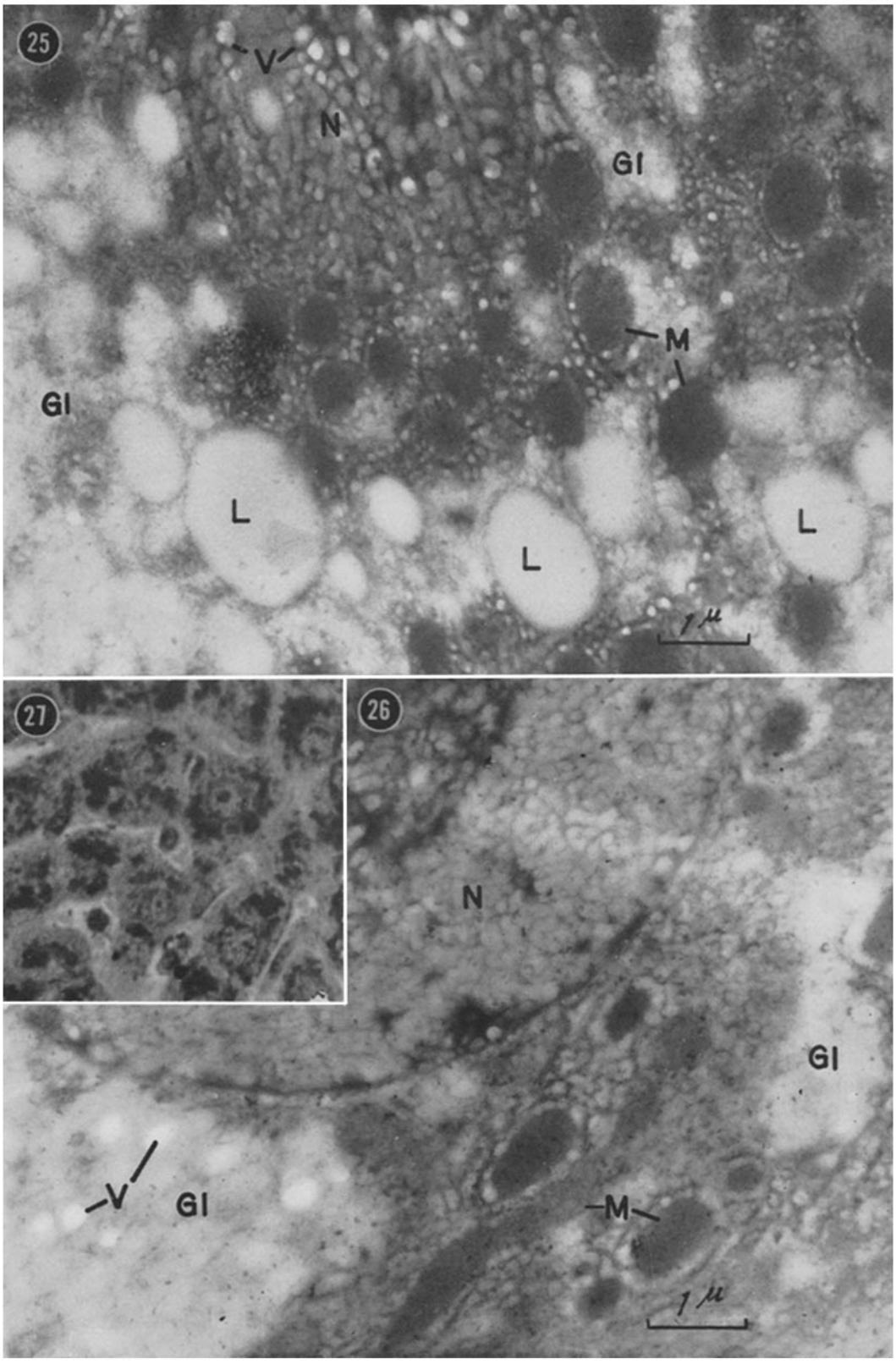
In this cell two kinds of vacuoles can be clearly seen. The small and sharply demarkated ones (*V*) are ice crystal spaces: the large vacuoles (*L*) probably represent extracted lipid droplets. The large less opaque area (*Gl*) corresponds to a zone of glycogen deposition. *N:* Nucleus; *M:* Mitochondria. × 14,000.

FIGURE 26

This cell has a large glycogen-depositing area (*Gl*). The area is not so sharply demarkated from the adjacent cytoplasm as the vacuoles of extracted lipid droplets (*L* in Fig. 25), and some vague structure can be seen therein. *V:* Ice crystal vacuoles; *N:* Nucleus; *M:* Mitochondria. × 15,000.

FIGURE 27

A light microscope picture of a thick section stained by the PAS procedure. Glycogen deposits appear as dark masses and granules.



This may reflect lipid losses during embedding and a loose binding of other molecules which may permit some evaporation under the electron beam. OsO_4 fixes most lipids and may bind more firmly other molecules thus preventing their destruction or evaporation in the microscope. In addition, the bound Os increases the density of fixed structures and thus gives images of high relative contrast. The treatment of frozen-dried sections with OsO_4 increases the relative contrast to some degree but does not make it as high as in the case of OsO_4 fixation. This finding also may reflect the lipid losses incurred by frozen-dried cells during embedding.

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