ULTRASTRUCTURAL CYTOCHEMISTRY

Enzyme and Acid Hydrolysis of Nucleic Acids and Protein

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ABSTRACT

Selective extraction of specific cell components by enzyme or acid hydrolysis is possible from ultrathin sections for electron microscopy and parallel 2 μ sections for light microscopy of tissues fixed in formalin and embedded in a water-soluble polyepoxide, product X133/2097. Normal rat tissues fixed 15 minutes in formalin at 3°C are more rapidly digested by proteinases than those fixed for the same length of time at 20°C. Trypsin selectively attacks the nuclear chromatin and the ribonucleoprotein particles of the ergastroplasm, whereas mitochondria and zymogen granules resist tryptic digestion. Pepsin rapidly attacks the mitochondria and zymogen granules. The ergastoplasm and nucleus at first resist peptic digestion, but in time the entire cytoplasm and interchromatinic portion of the nucleus are attacked. Ribonuclease abolishes cytoplasmic basophilia in 2 μ sections, but parallel ultrathin sections, stained with uranyl acetate and examined in the electron microscope, show no change in the ribonucleoprotein particles of the ergastoplasm. Desoxyribonuclease alone had no effect, but after pretreatment of the sections with pepsin or hydrochloric acid, desoxyribonuclease specifically attacked the nuclear chromatin. Nucleic acid-containing structures in the sections are gradually disintegrated by perchloric acid or hydrochloric acid.

INTRODUCTION

The application of histochemical techniques in electron microscope studies of cell ultrastructure has already yielded impressive results (1, 8, 19) but efforts in this direction have been hampered by the use of osmium tetroxide for the preservation of fine structures. Even brief exposure of tissues to this fixative limits or inhibits the subsequent demonstration of endogenous enzyme activity or utilization of enzymes to digest specific cell components. The introduction of water-soluble embedding media, first by Gibbons (10) and then by Stäubli (25), however, made possible the use of formalin (a fixative of proven advantage in histochemistry) for good preservation of electron microscopical specimens.

The aim of our project was to apply histochemical methods to ultrathin sections of tissues fixed in formalin and embedded in the medium introduced by Stäubli, then to examine them with the electron microscope. The methods selected were those which should specifically attack nucleic acids and proteins, in order that we might ultimately utilize such techniques in the study of the ultrastructure of chromosomes and viruses. The reactions were carried out directly on the sections so that several techniques might eventually be applied even to a single cell. We have found that enzymatic and acid hydrolysis of such ultrathin sections of formalin-fixed tissues is possible, and that specific subcellular components are attacked.

MATERIAL AND METHODS

The pancreas, liver, and thymus of 10 normal rats, 50 to 150 grams in body weight, were used.

Small blocks of these tissues, roughly 1.0 mm^3, were fixed in 10 per cent formalin (3.0 per cent formaldehyde)[1] buffered at pH 7.3–7.5 with Michaelis' veronal acetate buffer. Fixation times were: (1) 15 minutes at 3°C; (2) 15 minutes at 20°C; (3) 50 minutes at 20°C. Other blocks of the same tissues were fixed in 2.0 per cent osmium tetroxide, similarly buffered, for 30 minutes at 3°C.

The embedding medium is an experimental, water-soluble aliphatic polyepoxide, designated as "product X133/2097,"[2] which was introduced for this purpose by Dr. W. Stäubli (25). The fixed tissues were progressively impregnated with the embedding resin by carrying them through a series of 50 per cent, 70 per cent, and 90 per cent of X133/2097[3] in distilled water, 30 minutes for each step, followed by two changes of the undiluted compound, 1 hour each. This infiltration of tissues can be carried out either in the cold room (3°C) or at room temperature. The time in each bath is not critical. We avoided using a 30 per cent bath since tissues tend to swell there. In the 90 per cent and 100 per cent solutions the tissues become very hard. From the pure polyepoxide the tissues were transferred into the following mixture:

X133/2907[2]	5 ml
Hardener 964[2]	11.7 ml
Accelerator 964[2]	1.0–1.2 ml
Plasticizer[2]	0.2–0.4 ml

The use of the plasticizer is not essential. Too little accelerator produces blocks that are too soft to section; too much, blocks that are too brittle. The mixture becomes orange or amber in color. It is very viscous and, to ensure its penetration through the block of tissue, the mixture, plus tissues, was placed on an agitator in the cold room overnight. They were then transferred to gelatin capsules and polymerization was allowed to proceed at 37°C; at this temperature it may take 3 to 4 days. More rapid polymerization occurs at higher temperatures. It is essential that the mixture be free of moisture for polymerization to take place. All formalin-fixed blocks and some osmium-fixed blocks were so embedded, and other osmium-fixed blocks were embedded in methacrylate by the routine procedure.

Ultrathin sections for electron microscopy and 2μ sections for light microscopy were cut with a Porter-Blum microtome and glass knives. Sectioning is somewhat more difficult than with methacrylate and chattering often occurs.

The 2 μ sections can be stained without removal of the embedding medium either after they are dried onto a slide or by floating on the staining solutions. We stained 30 minutes in 1 per cent aqueous methylene blue to reveal cytoplasmic basophilia and applied the routine Feulgen method for nuclear DNA. Unstained ultrathin sections of formalin-fixed tissues have very little contrast in the electron microscope (25); hence all such sections were stained 1 hour in 5 per cent uranyl acetate (30).

The hydrolyzing solutions were as follows:

1. Ribonuclease,[4] dissolved in distilled water at concentrations of 0.1 per cent, 0.4 per cent, 0.7 per cent, and 1.0 per cent, and adjusted to pH 6.8 with 0.01 M NaOH, was employed at both 37°C and 54°C for 1, 6, 16, 24, and 48 hours.

2. Desoxyribonuclease,[4] 0.1 per cent, 0.4 per cent, and 1.0 per cent, was employed in two different aqueous solutions, (*a*) $MgSO_4$, 0.003 M, adjusted to pH 6.2 with 0.01 M NaOH (29), and (*b*) $MgCl_2$, 0.02 M, adjusted to pH 7.4 with 0.01 M NaOH (17). The incubation was carried out at 37°C for 6, 24, and 48 hours.

3. Pepsin,[5] 0.5 per cent, in 0.1 N hydrochloric

[1] This was a dilution of the 30 per cent formaldehyde solution available in France, instead of the 38 per cent solution available in the United States.

[2] Produced by CIBA, Basle, and sold as "Durcupan" by Fluka, A. G. Buchs, St. Gallen (Switzerland).

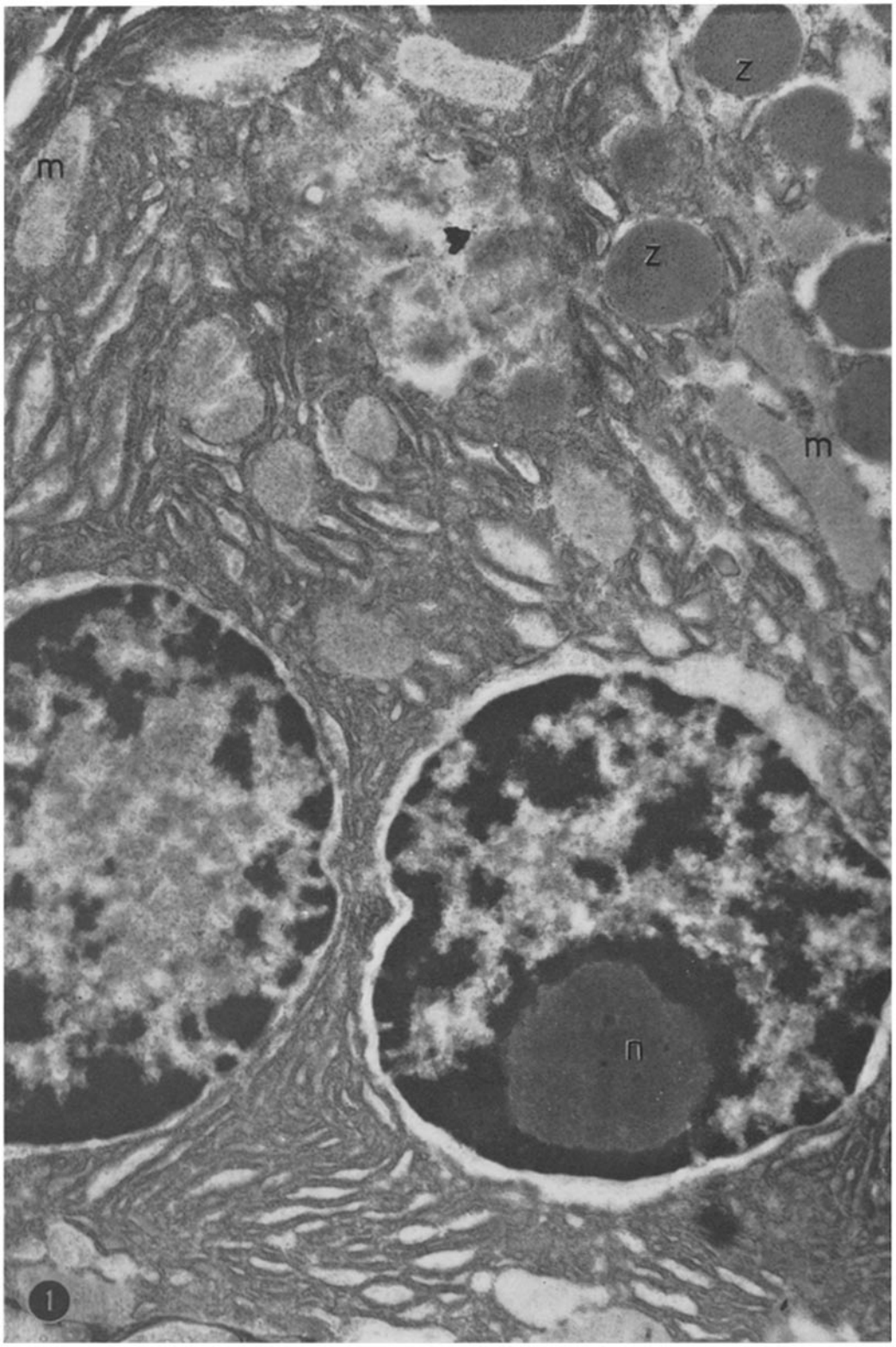
[3] Product X133/2097 is toxic and after repeated contact can produce a severe skin reaction; the polymerized plastic does not have this effect.

[4] Worthington Biochemical Corporation, Freehold, New Jersey.

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.

FIGURE 1

Rat pancreas. Control fixed 50 minutes in formalin 10 per cent at 20°C. Typical aspect of exocrine cells after polyepoxide embedding and uranyl acetate staining. Two nuclei with very dense chromatin closely applied to the inner surface of the nuclear membrane and surrounding the nucleolus (*n*) (nucleolus-associated chromatin). The nucleolus is composed of a homogeneous mass of medium density. Ergastoplasmic lamellae in the cytoplasm, enclosing mitochondria (*m*) and zymogen granules (*z*). The shrinkage of the nucleus, a frequent artifact of this technique, is clearly visible in this picture. × 24,000.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acid at pH 1.2, was employed at 37°C for 5, 10, 20, 30 minutes and 1, 2, 4, 6, and 23 hours.

4. Trypsin,[5] 0.3 per cent, in distilled water adusted to pH 8.0 with 0.01 M NaOH was employed at 37°C for 1, 2, 4, 6, 16, and 23 hours.

Control solutions at the same pH levels but minus the enzymes were always employed.

5. Perchloric acid, 10 per cent, (17) was employed at 37°C for 15 and 30 minutes, 1, 2½, 5 and 15 hours.

6. Hydrochloric acid, 0.1 N and 1.0 N, was employed at 37° and 57°C for 15 and 30 minutes and 1, 2, 4, 6, and 24 hours.

One ml of the hydrolyzing solution was placed in a watch glass and this, in turn, was placed in a Petri dish lined with moistened filter paper. The sections were transferred from the trough of the knife, on the end of a smooth wooden toothpick, to the surface of the hydrolyzing solution or control solution where they floated throughout the period of incubation. They were then transferred in the same way to a rinsing bath of distilled water, then picked up on a formvar-coated grid and stained with uranyl acetate. Parallel 2μ sections were similarly incubated, then floated on staining solutions and mounted on glass slides. The embedding material around the tissue section was not dissolved by any of the treatments described. No adequate solvent is available to remove the polymer of X133/2097 from sections.

Photographs were made with a Siemens Elmiskop I electron microscope.

OBSERVATIONS

Control, Undigested Tissues: The appearance of rat pancreas, liver, and thymus after fixation in formalin and inclusion in the polymer of product X133/2097 has been described by Stäubli (25), but will be reviewed briefly here as a point of departure for our enzyme-incubated sections. Unstained sections are barely visible on the fluorescent screen of the microscope and in electron micrographs only faint outlines of the organelles can be seen. Several stains were tried, of which uranyl acetate was by far the best; phosphotungstic acid and gallocyanine-chromalum were fair and lead acetate failed completely. Therefore, all descriptions in this paper are based on ultrathin sections which have been stained with uranyl acetate to improve their contrast in the electron microscope.

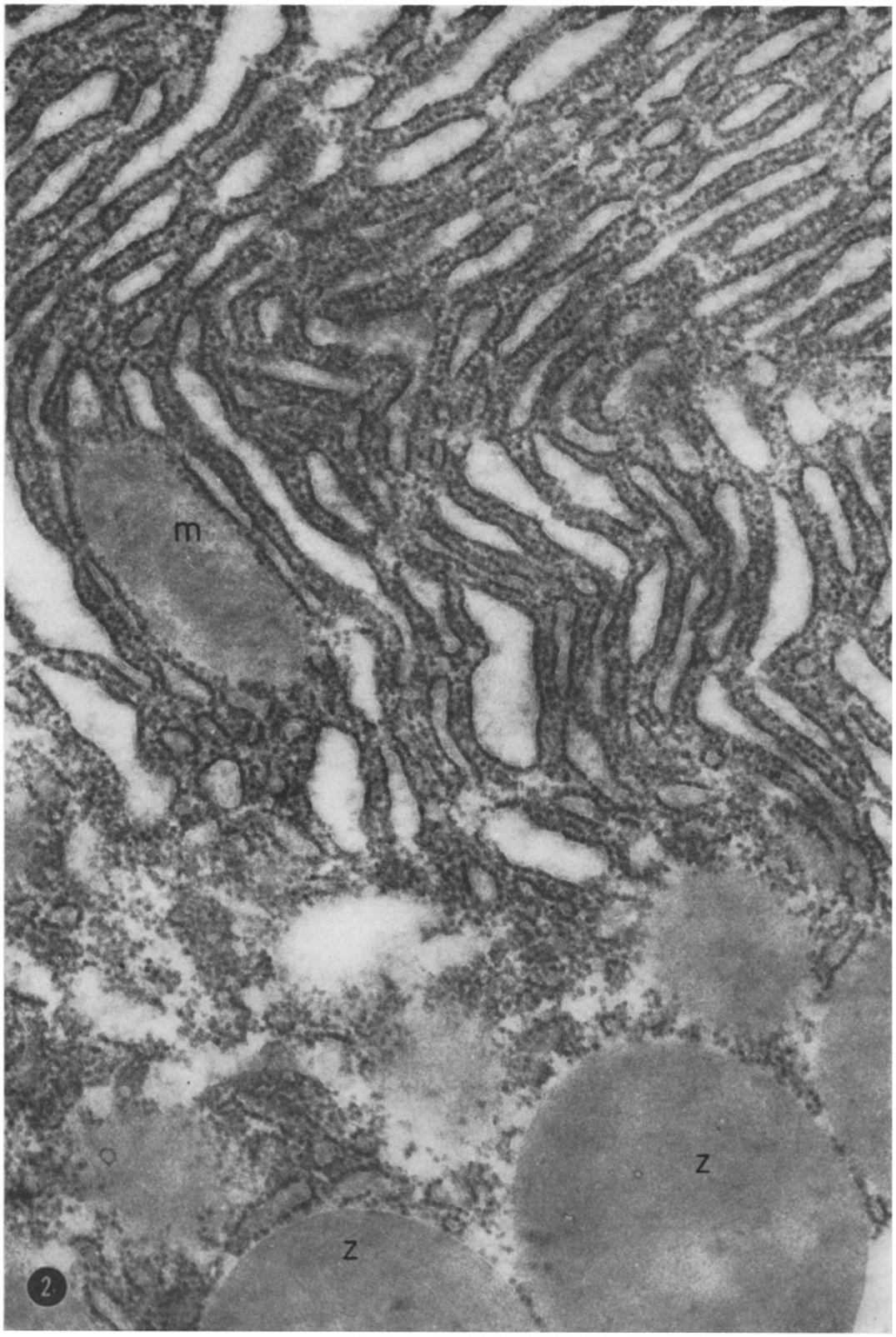
Chromosomes of mitotic figures and the chromatin of interphase nuclei, closely applied to the inner surface of the nuclear envelope and surrounding the nucleolus, consist of masses of very dense, granular material (Fig. 1). The nucleolus has the appearance of a more finely textured, almost homogeneous substance which exhibits only moderate density (Fig. 1). Dispersed between the chromatin masses and nucleoli is a complex "interchromatinic material" of unknown composition which is variously reticular or coarsely granular.

Cell membranes, unlike those in tissues fixed with osmium tetroxide, do not appear dense in the electron beam; conversely, they appear as negative images against a darker background. Hence, the membranes forming the nuclear envelope, cristae mitochondriales, walls of the ergastoplasm, and outer cell membrane appear as thin, light lines against the darker cytoplasm and the matrices of the ergastoplasmic sacs (Fig. 2) or mitochondria (Fig. 15). The ribonucleoprotein (RNP) particles, on the other hand, are sharp and dense as in osmium-fixed tissues, and their association with the ergastoplasmic lamellae is clear (Fig. 2). Zymogen granules of the pancreas are uniformly homogeneous and moderately dense (Figs. 1 and 2).

Formalin-fixed tissues embedded in the polymer of X133/2097 suffer from a certain amount of shrinkage, as seen by the irregular contours of the nuclei (Fig. 1). OsO_4-fixed blocks embedded in this polymer are not contracted in this way, hence they are probably more resistant to the dehydrating effects of product X133/2097 than are formalin-fixed blocks.

FIGURE 2

Rat pancreas. Fixed 15 minutes in formalin 10 per cent at 3°C. Section stained 1 hour in uranyl acetate. The fine structure of the ergastoplasm is very similar to the classic OsO_4–methacrylate picture. RNP granules clearly visible on the lamellae. Mitochondrion (*m*) and zymogen granules (*z*). The negative images of the mitochondrial membranes may not show in the final reproduction but will be visible in Fig. 15. × 60,000.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Pepsin Digestion: Ultrathin sections of liver and pancreas fixed in formalin for *15 minutes* at *3°C* were attacked by pepsin very rapidly. In the *liver* after only 5 minutes of incubation there was a diminution in density of the matrix of the mitochondria and small holes were scattered within them (Fig. 4). After 10 minutes the holes were larger and becoming confluent and the mitochondria were markedly swollen (Fig. 5). After 20 minutes the mitochondria were completely destroyed. Meanwhile, through 30 minutes of treatment the ergastoplasm and nuclei remained unchanged. After 1 and 2 hours the chromatin persisted but the interchromatinic substance of the nuclei became filled with holes and the entire cytoplasm was disrupted. In the *pancreas* the same changes occurred and, in addition, the zymogen granules were digested. After 5 minutes, only the periphery of the zymogen granule was affected; after 10 minutes, only traces of material persisted at the center (Fig. 6), and after 30 minutes the entire zymogen granule had disappeared, leaving a hole in the section. Parallel sections of liver and pancreas incubated 2 hours in the control solution or vehicle for the pepsin, 0.1 N HCl, showed no changes whatsoever in form or density of ultrastructural components.

Tissues fixed in formalin for *15 minutes* at *20°C* were considerably more resistant to pepsin digestion in that, although the same changes occurred, they took place much more slowly. In the pancreas after 30 minutes only the edge of the section of the zymogen granule was digested. After 2 hours the periphery of the granule was further digested and the mitochondrial matrix was peppered with small holes, an effect much like 5 minutes' digestion of tissues fixed at 3°C. After 4 and 6 hours the holes in the mitochondria were larger, the zymogen granules had almost completely disappeared, but the ergastoplasm and nuclei were still unchanged; in other words, these preparations resembled those of pancreas fixed at 3°C and incubated only 10 to 30 minutes. Pancreas fixed for 50 minutes at 20°C was still more resistant. The mitochondria were unchanged through 8 hours, denser after 12 hours but not digested even after 23 hours, and the zymogen granules, ergastoplasm, and nuclei were unaffected.

The same livers and pancreases fixed in 2 per cent osmium tetroxide for 30 minutes at 3°C and embedded in methacrylate or in the X133/2097 polymer were similarly incubated with pepsin for periods up to 48 hours. No changes in intracellular components were observed (15).

Trypsin Digestion: In liver and pancreas fixed in formalin *15 minutes* at *3°C* neither mitochondria nor zymogen granules were digested by trypsin; indeed, these structures became increasingly dense. Instead, it was the ergastoplasm and nuclear chromatin which were attacked. The RNP particles of the ergastoplasm became indistinct after 1 hour, and after 2 hours rows of small holes appeared along the ergastoplasmic lamellae (Fig. 8). These holes were enlarged after 4 and 6 hours (Fig. 9) and became confluent after 23 hours. In the nucleus the interchromatinic material, which had been more susceptible to pepsin digestion, remained unaffected, but a reduced density of the chromatin became evident

FIGURES 3 TO 5

Rat liver, after 15 minutes of formalin fixation at 3°C and polyepoxide embedding. Uranyl acetate stain.

FIGURE 3

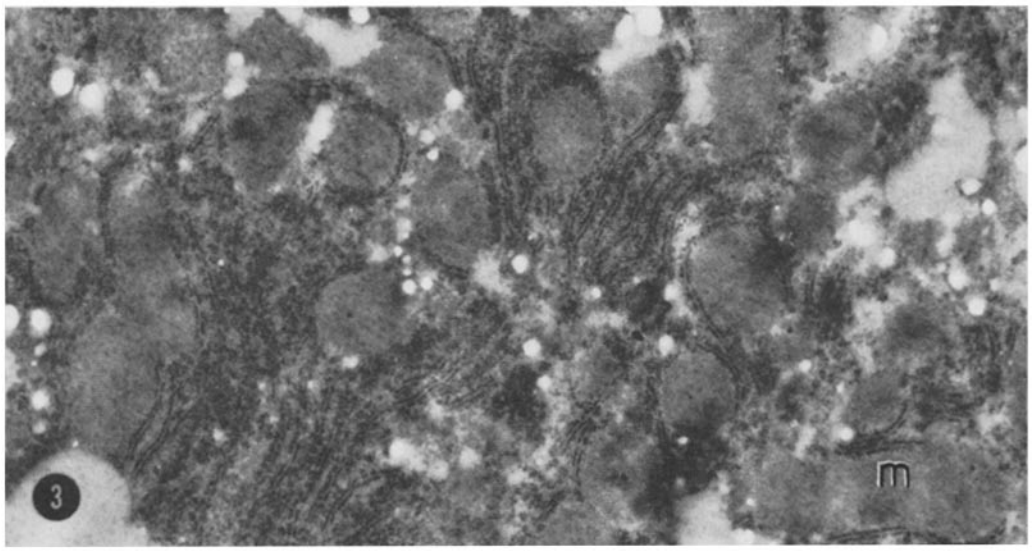
Untreated control. Cytoplasm with mitochondria (*m*) and ergastoplasm. × 24,000.

FIGURE 4

Treated with pepsin 5 minutes. Mitochondria become clear. × 24,000.

FIGURE 5

Treated with pepsin 10 minutes. Mitochondrial bodies almost completely digested. Ergastoplasm intact. × 24,000.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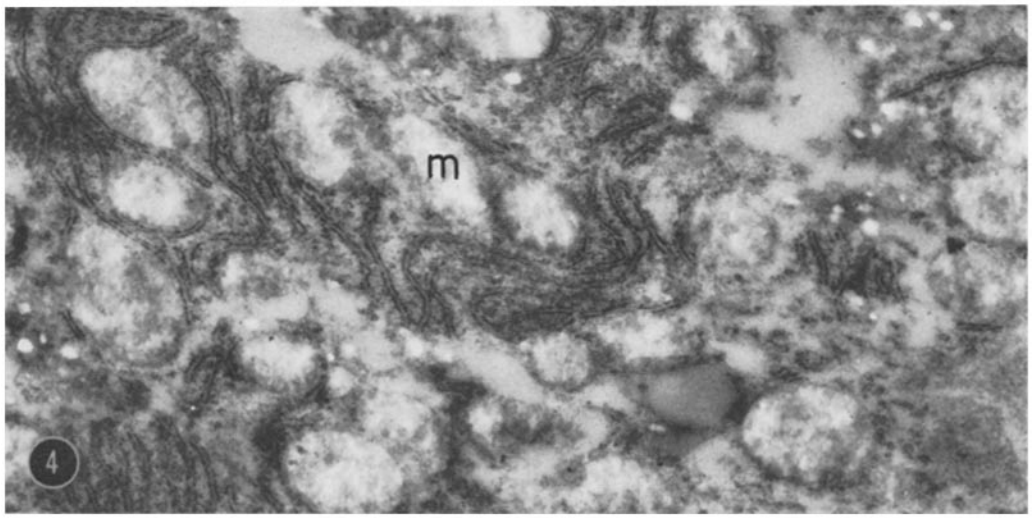
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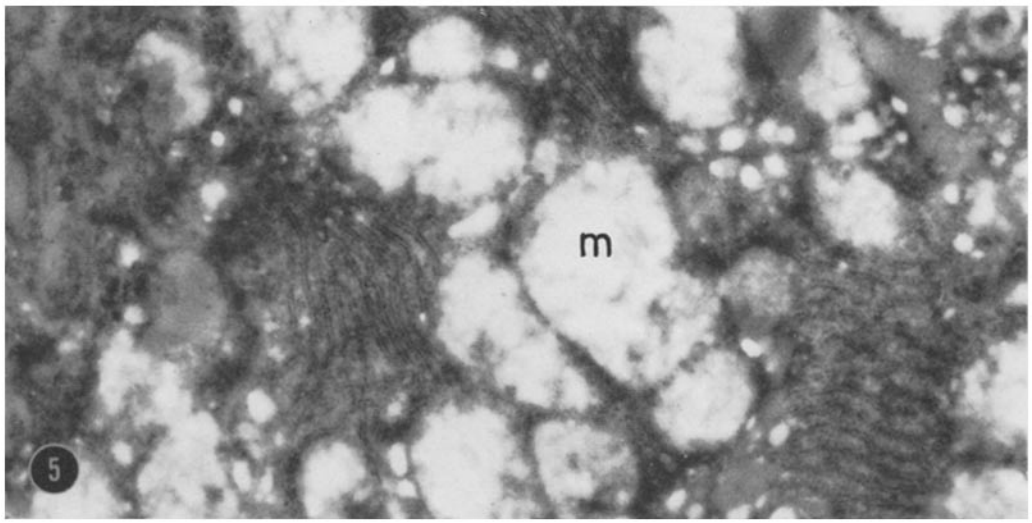
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after 4 hours (Fig. 9). After 6 hours there was a complete reversal of the chromatin from the darkest to the palest portion of the nucleus, and after 23 hours the chromatin was completely digested away, leaving large holes in the sections. The nucleolus-associated chromatin and the moderately dense, homogeneous substance of the nucleolus disappeared, but a very dense network within the nucleolus was revealed and persisted (Fig. 10).

Fixation of the tissues in formalin at *20°C* instead of 3°C also retards trypsin digestion (Fig. 7). For example, 23 hours of incubation of tissues fixed at 20°C were required to obtain the same effects that were obtained after 4 to 6 hours with tissues fixed at 3°C. Incubation of sections in the control solution, distilled water plus NaOH to attain pH 8.0, had no effect on the ultrastructure of any of these tissues. Sections of the same tissues fixed with osmium tetroxide were incubated with trypsin up to 48 hours but no effect was observed (15).

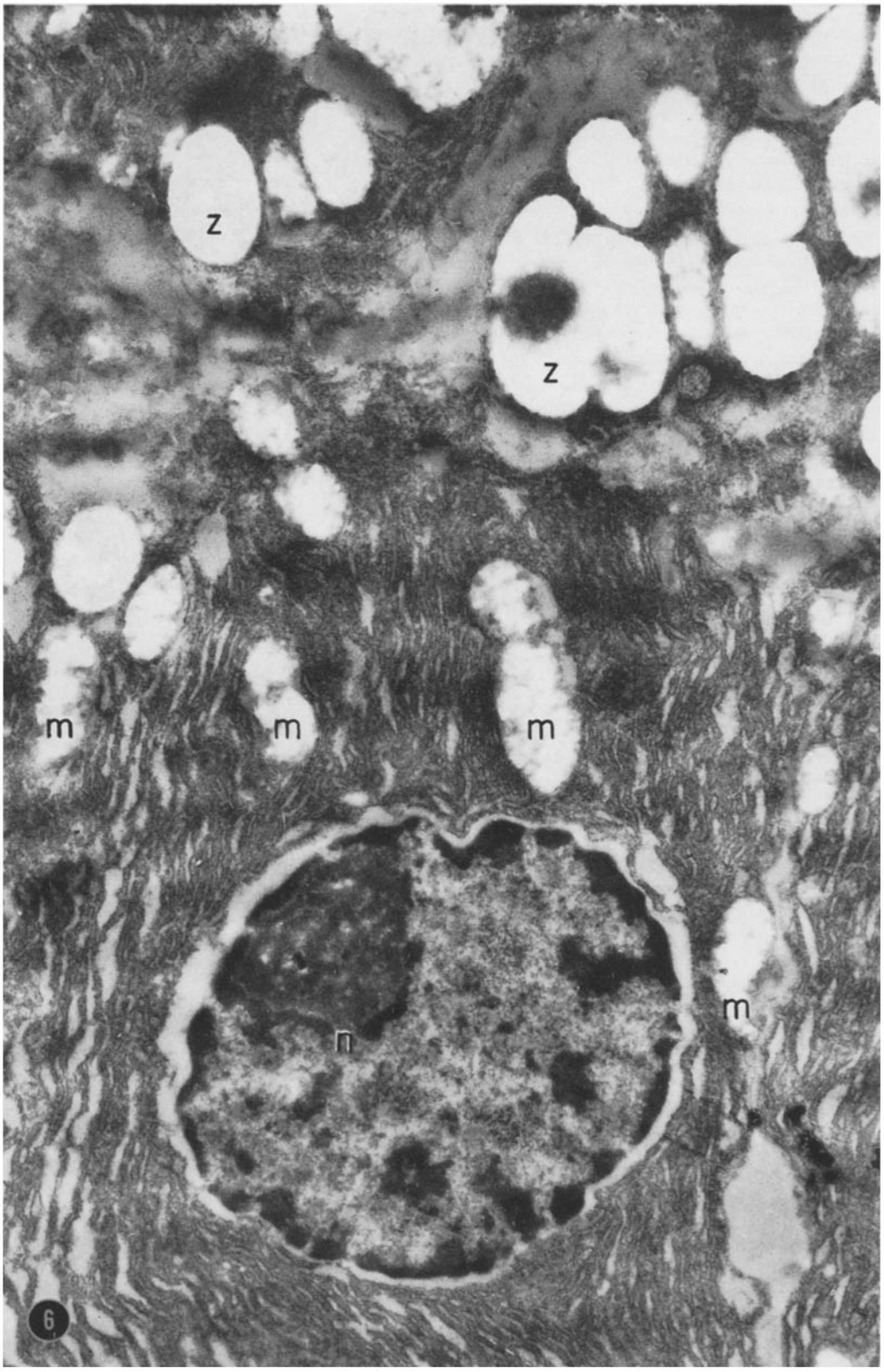
Ribonuclease: Sections 2 μ in thickness, stained with methylene blue and examined with the light microscope, were employed to monitor the effects of ribonuclease digestion. Enzyme concentrations of 0.1 per cent and 0.4 per cent were insufficient to affect the cytoplasmic basophilia of livers and pancreases fixed in formalin for 15 minutes at 3°C (3). With concentrations of 0.7 per cent and 1.0 per cent, cytoplasmic basophilia could be completely abolished after 24 hours at 54°C, but it was usually irregularly or incompletely removed at 37°C (16). The basophilia of controls incubated in distilled water alone was unaffected. In ultrathin sections, simultaneously incubated, then stained with uranyl acetate and examined in the electron microscope, the RNP particles of the ergastoplasm were always clearly visible both in the controls and in the sections incubated with ribonuclease, even when the basophilia had completely disappeared from the parallel 2 μ sections (Fig. 11). This led us to believe that the uranyl acetate might stain the protein component of the RNP particle and, therefore, that loss of the nucleic acid component could not be detected this way. Attempts to utilize another stain, gallocyanine-chromalum, that might combine specifically with the ribonucleic acid, were not successful (15).

Desoxyribonuclease: Concentrations of 0.1 per cent, 0.4 per cent, and 1.0 per cent of this enzyme, at pH 6.2 and pH 7.4 with 0.003 M and 0.02 M, respectively, magnesium activators, were employed at 37°C for 6, 24, and 48 hours. The Feulgen reaction for desoxyribonucleic acid was never abolished from 2 μ sections, and ultrathin sections examined with the electron microscope revealed no change whatsoever in nuclear chromatin of tissues fixed 15 minutes at 3°C. On the basis of suggestions by Peters and Stoeckenius (21), therefore, we pretreated sections with pepsin and then exposed them to the desoxyribonuclease. Pancreas and thymus tissues that had been fixed in formalin for 50 minutes at 20°C were employed since after such fixation pepsin digestion alone for up to 23 hours did not visibly affect the nuclear chromatin. Ultrathin sections were incubated in 0.5 per cent pepsin for 4 hours, rinsed, then incubated with 0.1 per cent and 0.4 per cent desoxyribonuclease for 4 to 16 hours. Under these conditions the density of nuclear chromatin and of the chromosomes of dividing cells became greatly reduced until the formerly chromatinic regions appeared almost in negative image (13). This clarification of the chromatinic area was more rapid with 0.4 per cent than with 0.1 per cent desoxyribonuclease solutions. The cytoplasm was unaffected (Fig. 12).

The same results were obtained with pretreatment of sections with 1.0 N HCl at 57°C for 30 minutes, followed by 0.1 per cent desoxyribonuclease for 5 hours (13). The hydrochloric acid alone (see below), for 30 minutes, did not completely abolish the Feulgen reaction in 2 μ sections but the density of the chromatin in ultrathin sections was already greatly reduced. Subsequent incubation of control sections in the vehicle or control solution minus the desoxyribonuclease brought about a further slight clarification of the

FIGURE 6

Rat pancreas. Formalin fixation 15 minutes at 3°C. Pepsin treatment 10 minutes. Zymogen granules (*z*) and mitochondria (*m*) almost completely digested. Rest of the cell organelles intact. × 24,000.


z
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m
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6

chromatinic areas, but a reversal of chromatin density or development of a negative image of the chromosomes did not occur as it did when desoxyribonuclease was present.

Whenever nuclear chromatin was destroyed, the relatively homogeneous component of the nucleolus also disappeared, leaving a dense, coarse network.

Hydrochloric Acid: Ultrathin sections and parallel 2 μ sections of tissues fixed at both 3° and 20° were incubated in 0.1 N and 1.0 N HCl at both 37°C and 57°C. With increasing concentrations of the acid and with increasing temperature, the changes occurred more rapidly. The effects on pancreas fixed 15 minutes at 20°C may be taken as examples. Incubation in 0.1 N HCl at 37°C had no effect through 5 hours. At 57°C, there was no effect through 2½ hours, but after 5 hours cytoplasmic basophilia had diminished greatly in 2 μ sections and the RNP particles in ultrathin sections were no longer distinct. After 15 hours all basophilia and all RNP particles had disappeared. With 1.0 N HCl at either 37° or 57°C, cytoplasmic basophilia was greatly reduced after 30 minutes and abolished after 1 hour. Concomitantly, in ultrathin sections stained with uranyl acetate the RNP particles were ill defined after 30 minutes and gone after 1 hour; after 5 to 15 hours holes appeared in the ergastoplasm which gradually increased in size and suggest a negative image of the control. The basophilia in 2 μ sections and density in ultrathin sections of the nuclear chromatin were similarly affected. In 1.0 N HCl at 57°C, they were reduced after 30 minutes, still more so after 1 hour, and after 2 to 5 hours the chromatin was completely digested away, resulting in a reversal of the image (13). The nucleolar network and interchromatinic substance persisted (Fig. 13).

Perchloric Acid: This is sometimes employed in place of the nucleases to hydrolyze RNA and DNA (17). A 10 per cent solution at 37°C produced a more gradual series of changes than observed with 1.0 N HCl hydrolysis in the ergastoplasm of pancreas fixed 15 minutes at 20°C. There was no effect after 15 and 30 minutes; after 1 hour the RNP particles appeared fainter, and after 2 hours their outlines were less distinct. No loss of basophilia occurred in 2 μ sections up to this point. After 4 to 6 hours the individual particles were still less distinct; they seemed to fuse to form continuous beaded, stainable substance or "pseudo-membrane" outlining the ergastoplasmic lamallae. All traces of RNP particles disappeared after 15 hours treatment and tiny holes appeared along the ergastoplasmic lamellae (Fig. 14). It was characteristic in all types of digestion, and especially pronounced with perchloric acid, that the structures that were not digested actually increased in density. Thus the matrix within the ergastoplasm (Fig. 14) and within the mitochondria (Fig. 15) became increasingly dense in these preparations. The effect on the nucleus was slower. The chromatin remained very dense through 2½ hours and became pale after 5 hours; a complete reversal to form a negative image occurred after 15 hours. Even at this time some substance remained in the

FIGURES 7 TO 9

Rat pancreas. Fig. 7, fixed in formalin 50 minutes at 20°C, Figs. 8 and 9, fixed 15 minutes in formalin at 3°C.

FIGURE 7

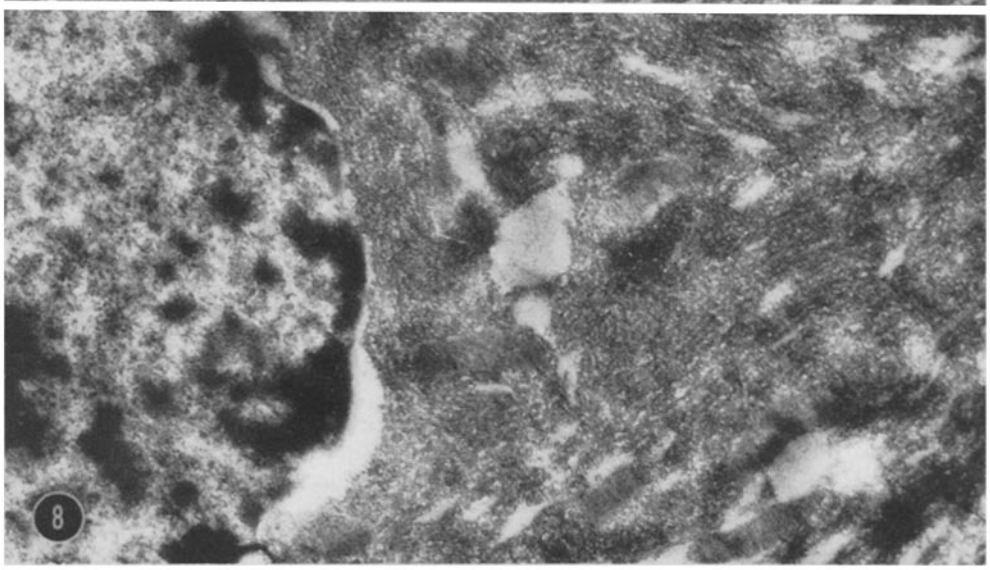
Digestion with trypsin 5 hours. Only slight action visible on RNP granules. Lamellae intact. × 36,000.

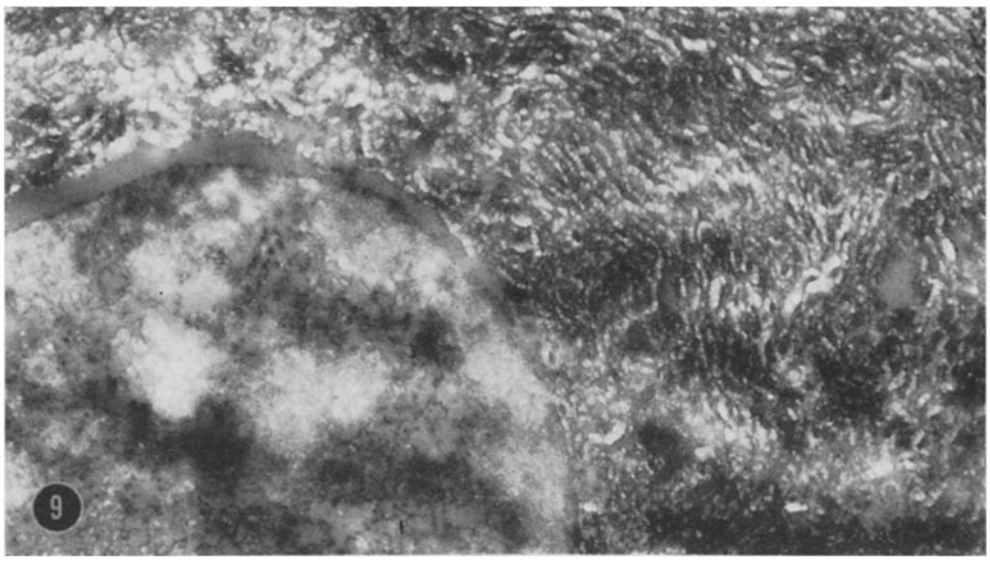
FIGURE 8

Digestion with trypsin 2 hours. Enzyme action visible both on RNP granules and on the lamellae. Nucleus appears normal. × 24,000.

FIGURE 9

Digestion with trypsin 4 hours. Strong action both on ergastoplasm and the chromatin of the nucleus (in the lower left corner). ×24,000.





formerly chromatinic areas and this material was peppered with very abundant, tiny holes (14). The interchromatinic substance and nucleolar network persisted.

DISCUSSION

The water-soluble polyepoxide employed in these experiments has two significant effects on tissues. On the one hand, it acts as a dehydrating agent. In fact, Stäubli (26) recently has employed it to dehydrate tissues which were subsequently embedded in methacrylate. In the second place, it probably has some fixative action, as suggested by Gibbons (10) for other such media, and by producing additional intermolecular cross-linkages in proteins and nucleic acids (9, 24), it may render these tissue components more resistant to enzyme digestion. In spite of this, we have shown that extraction of nucleic acids and proteins from sections occurs more readily in tissues fixed 15 minutes in formalin at 3°C than at 20°C, and that more prolonged exposure to formalin renders the tissue still more resistant to enzyme or acid hydrolysis.

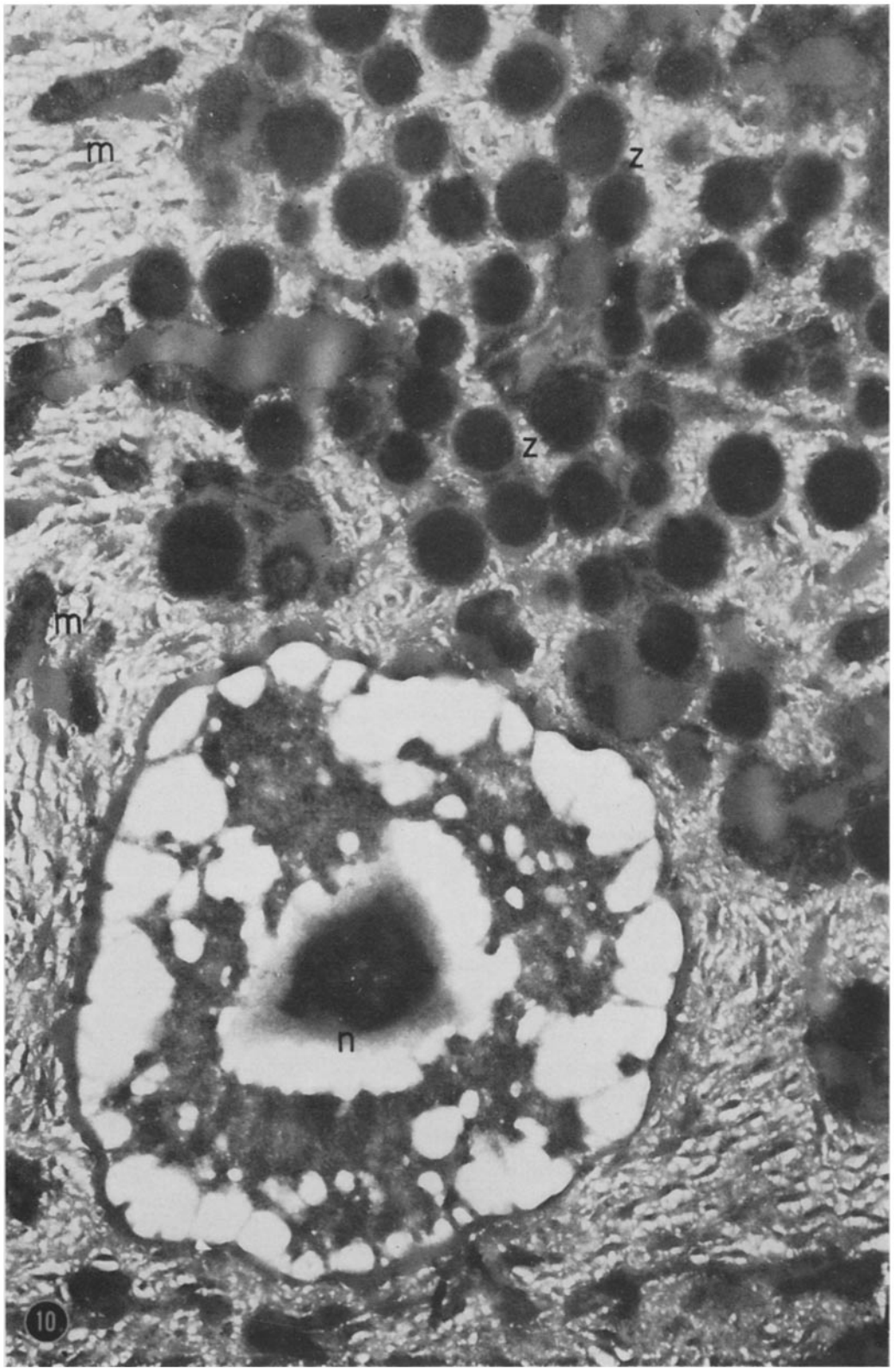
It was not possible to dissolve the embedding medium out of the sections, yet its presence did not inhibit the activities of pepsin, trypsin, and ribonuclease. The concentration of ribonuclease and the duration of incubation required to abolish cytoplasmic basophilia, however, were much greater than those employed with deparaffinized sections fixed according to Brachet (6). Desoxyribonuclease failed to react alone. Even in deparaffinized sections this enzyme reacts very slowly (7, 28) and sometimes incompletely (22, 27) after formalin fixation. Its molecular weight is approximately twice those of the proteinases and four times that of ribonuclease, hence it may have difficulty in penetrating into the sections. In our material its activity may have been prevented by firm bonding between DNA and proteins brought about by both the fixing and embedding procedures. This seems very likely in view of the success of desoxyribonuclease digestion after pretreatment of the sections with pepsin.

The removal of DNA from ultrathin sections by the pepsin-desoxyribonuclease sequence or by hydrochloric acid or perchloric acid was traced in electron micrographs as a gradual change in the chromosomes or the chromatin of interphase nuclei; these nuclei, being previously the most densely stained portion of the cell, became the least dense portion and produced a virtually negative image of the control nuclei. With prolonged perchloric acid hydrolysis and the HCl-desoxyribonuclease sequence, these formerly chromatinic regions also became peppered with small holes and resembled the chromosome regions irradiated by micro spots of DNA-destroying, ultraviolet light as demonstrated by Bloom (5). With prolonged hydrochloric acid hydrolysis all components of chromatin disappeared. According to the review by Pearse (20), under the conditions in which it is employed in the Feulgen reaction (*i.e.*, 1.0 N HCl at approximately 60°C) hydrochloric acid first rapidly removes the purine bases of DNA; further hydrolysis removes both the histones and the nucleic acids from nuclei. Under the conditions of fixation employed with our material, the extraction of DNA revealed no hidden ultrastructure within the chromosome.

Our failure to detect any change whatsoever in the ribonucleoprotein particles of the ergastoplasm after ribonuclease digestion, even when the cytoplasmic basophilia of parallel 2 μ sections was destroyed, might be explained in one of two ways. On the one hand, most RNA may have been removed but the protein component persisted and stained with the uranyl acetate. This, however, is inconsistent with the report by Huxley (11) that uranyl acetate combines with isolated nucleic acids but not with proteins. Huxley's material, however, was not subjected to a fixative and embedding medium as were our tissues. In our own material the uranyl acetate certainly imparted some density to proteins such as those in zymogen

FIGURE 10

Rat pancreas, fixed in formalin 15 minutes at 3°C and digested with trypsin during 23 hours. The chromatin of the nucleus and the homogeneous substance of the nucleolus have completely disappeared. The ergastoplasm is heavily attacked. Zymogen granules (*z*), mitochondria (*m*) interchromatinic substance in the nucleus, and a dense, irregular network (*n*) in the nucleolus are not digested. × 24,000.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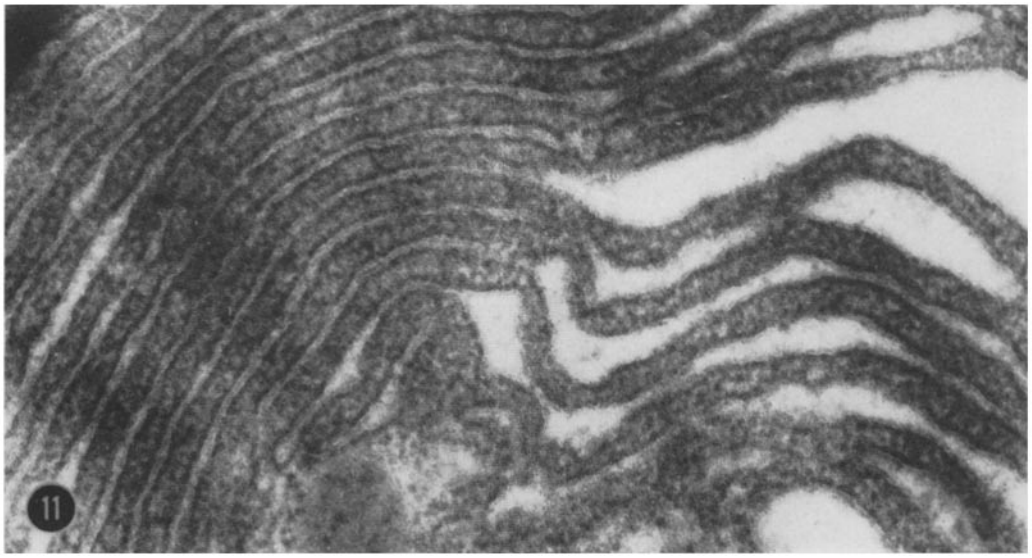

FIGURE 11

Rat pancreas. Formalin fixation 15 minutes at 3°C, digestion 24 hours in 1 per cent ribonuclease at 54°C. Uranyl acetate stain. In spite of the complete disappearance of the cytoplasmic basophilia in the light optical controls, persistence of the RNP particles. × 60,000.

granules. On the other hand, it is possible that the ribonuclease attacked only a portion of the RNA, being sufficient to abolish basophilia at the light microscope level but insufficient to abolish uranyl acetate binding at the electron microscope level. It is noteworthy that Siekevitz and Palade (23) recently extracted approximately 80 per cent of the RNA and 25 per cent of the protein from isolated, unfixed RNP particles; after subsequent OsO_4 fixation and lead hydroxide staining, the particles were still intact, although swollen. Our problem can be resolved only when other stains are available that are specific for nucleic acids and which can be used with polymers of water-soluble embedding media.

In undenatured proteins pepsin typically splits the peptide linkages between the α-carboxyl group of a dicarboxylic amino acid, such as aspartic or glutamic acid, and the amino group of the aromatic amino acids, tyrosine and phenylalanine; and trypsin acts at the carboxyl end of the basic amino acids, lysine and arginine (2, 20). The difficulties involved in the interpretation of the effects of these proteinases on fixed tissues is well recognized by all histochemists, yet experience indicates that in fixed tissues trypsin does, in fact, attack structures rich in basic amino acids, *i.e.*, the histone-rich chromosomes, and that pepsin digests more acidic proteins (12, 18, 20). The effects of these proteinases under the conditions of our experiments are in line with those reported by Mazia *et al.* (18). They found in isolated salivary gland chromosomes, both fresh and fixed in acetic acid, that the entire chromosome was disintegrated by trypsin but not by pepsin. The latter caused chromosome shrinkage, which was greatest in the interband region, but the chromatinic bands remained intact. In our material trypsin attacked the densely chromatinic portion of the nucleus very precisely, so that after long periods of digestion all components of chromatin disappeared, leaving a hole in the section, while the interchromatinic material remained relatively unaffected. Thus, not only did specific products of tryptic digestion diffuse away into the incubating medium but eventually there was a disappearance of all associated substances, and even the embedding medium gave way at these sites. The extreme thinness of the sections, their lack of any supporting film while floating on the incubating medium, and, possibly, a relatively high proportion of trypsin-digestible histones in

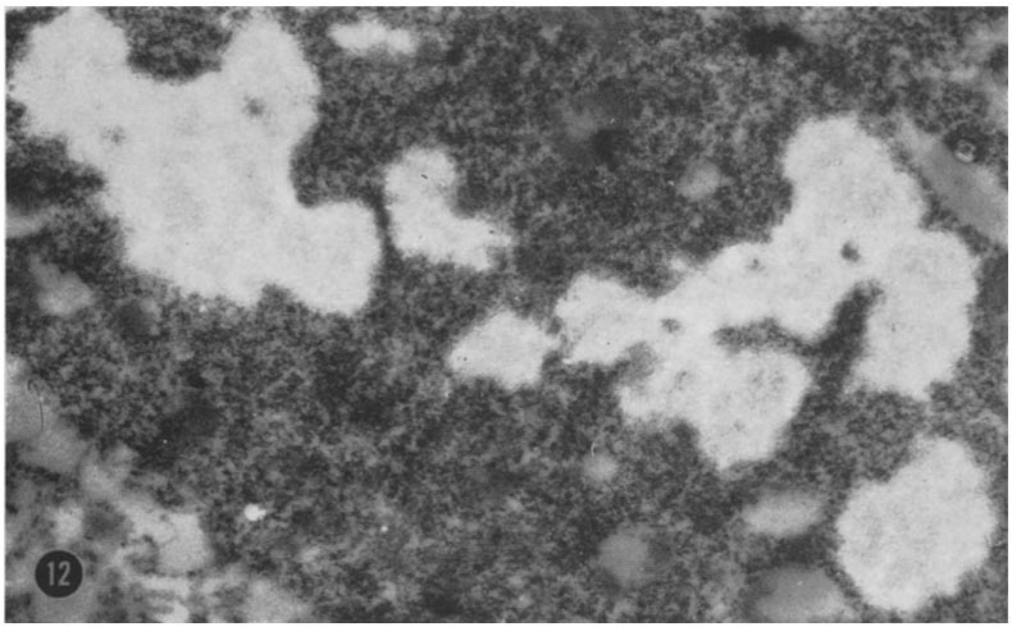

FIGURE 12

Rat thymus. Fixed 50 minutes in formalin at 20°C. Treatment with pepsin 4 hours, then, with DNAse 6 hours 30 minutes. Portion of a cell in mitosis. The chromosomes, which have lost completely the chromatin substance, appear as negative figures in the relatively intact cytoplasm. × 24,000.

the chromatin could account for this complete loss of substances after a portion has been selectively extracted.

Tryptic digestion of the ergastoplasm brought about a rapid dispersal of the ribonucleoprotein particles followed by the appearance of double rows of small holes. Because the cell membranes and mitochondrial membranes, in negative image as always, remained intact during trypsin treatment, we believe that the double rows of holes do not represent alterations of the membranous component of the ergastoplasm. The alternate interpretation is that the holes are, in fact, negative images of the ribonucleoprotein particles which have been completely digested. Perchloric acid brought about a very slow dispersal of the ribonucleoprotein particles which eventually resembled the early changes induced by trypsin; thus perchloric acid, unlike ribonuclease, may eventually attack protein structure as well as the nucleic acids.

On the basis of current concepts of the histochemical action of pepsin (20) one might conclude that mitochondria and zymogen granules, which are first attacked, are richest in proteins containing dicarboxylic amino acids (2). Since the fixative reached the periphery of the zymogen granule first, one would expect this zone to be most firmly fixed, yet it was the periphery that was digested first in ultrathin sections where all parts of the granules were equally exposed to the enzyme. A difference in composition or in physical organization between the center and the edge of the granule might account for this. In contrast, the entire mitochondrion was attacked at once.

All procedures which attacked nuclear chromatin also changed the appearance of the nucleolus. The uniformly distributed, finely granular and moderately dense substance of the unextracted nucleolus became replaced by an irregular, dense network. It is tempting to equate the extractable homogeneous component with the pars amorpha and the fibrous network with the nucleolonema. The latter constituent of the nucleolus was extremely resistant to all the extractive procedures employed in this study.

As a rule those structures which were not digested away gradually increased in density;

for example, the mitochondria and zymogen granules during trypsin digestion. The increased density most likely represents an increased avidity for the stain, possibly through the liberation of more binding sites by the action of the enzyme or acid.

This approach to the study of the ultrastructure of tissues will become increasingly rewarding as more gradual or more precisely controlled extraction procedures are employed and as new methods of fixation, embedding, and staining become available. Successful application of these techniques to the study of viruses has already been attained by Bernhard *et al.* (4).

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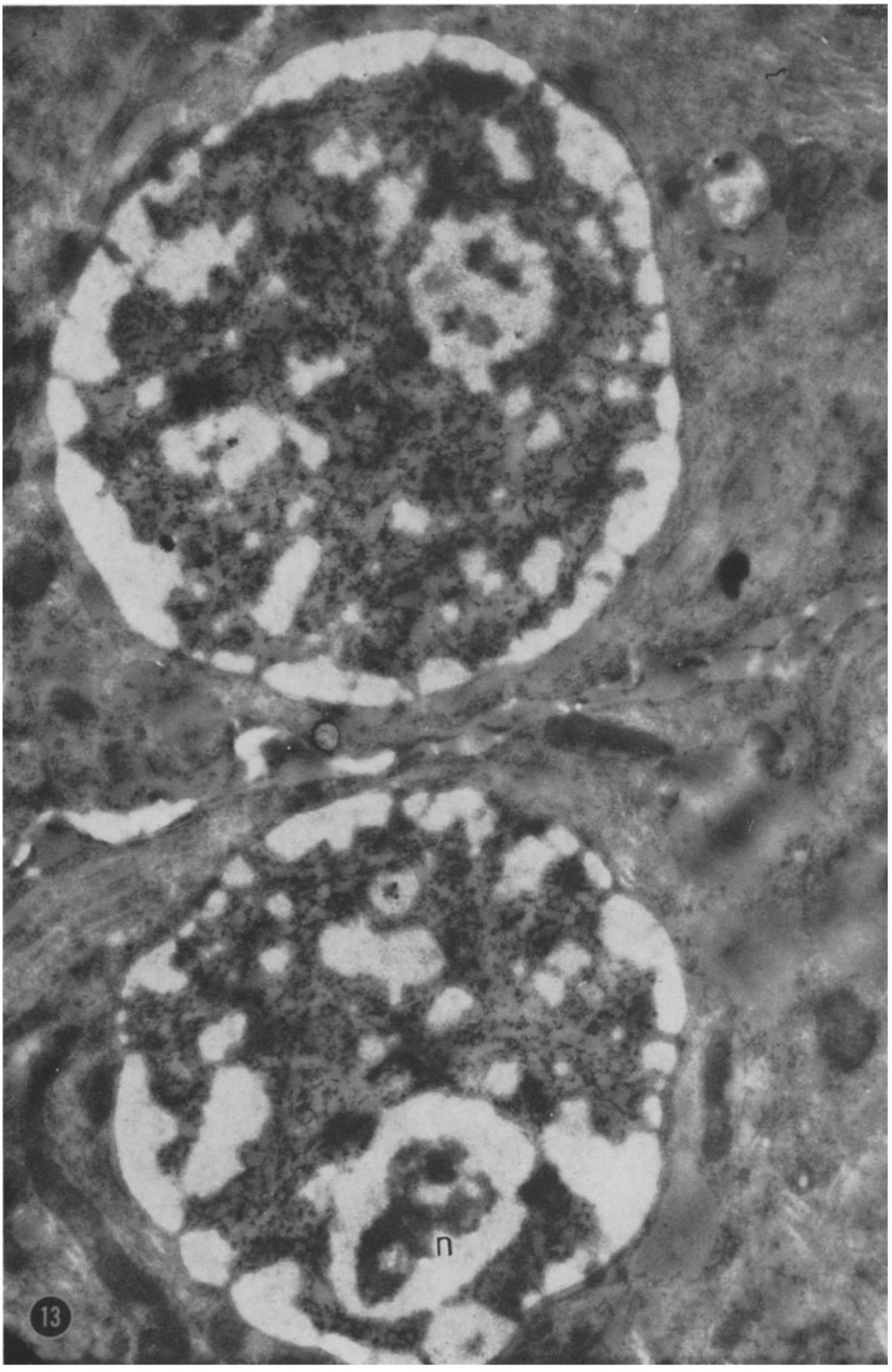
FIGURE 13

Rat pancreas, formalin fixation 15 minutes at 3°C. Section treated during 5 hours with 1.0 N hydrochloric acid at 58°C. Uranyl acetate stain. "Inversion" of the two nuclei, visible on this picture. All the chromatin is gone. Dense, interchromatinic substance remains. In the nucleolus (*n*) of the lower nucleus, a dense network has resisted the treatment. The ergastoplasm is partially destroyed. × 26,000.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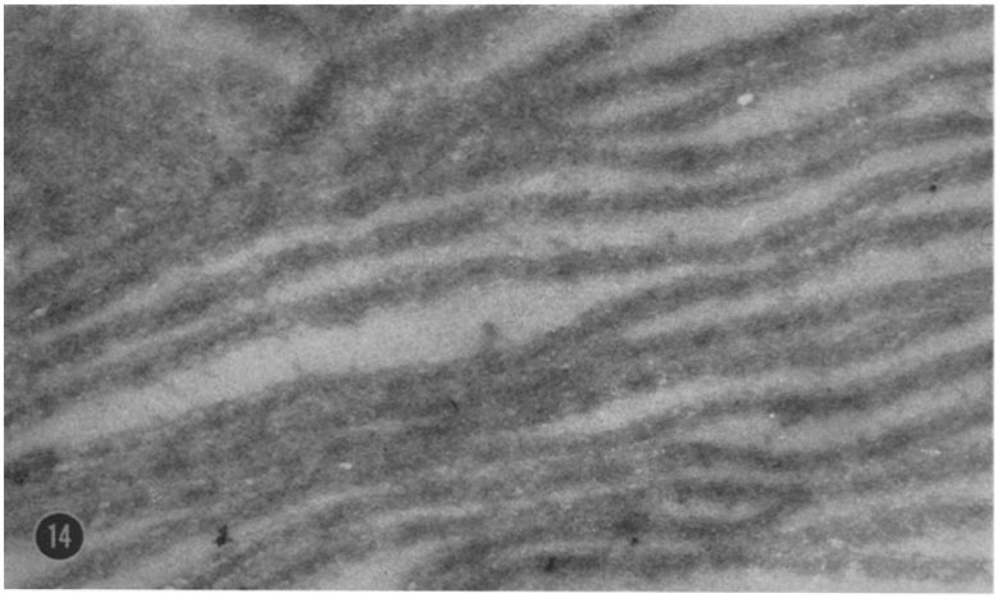

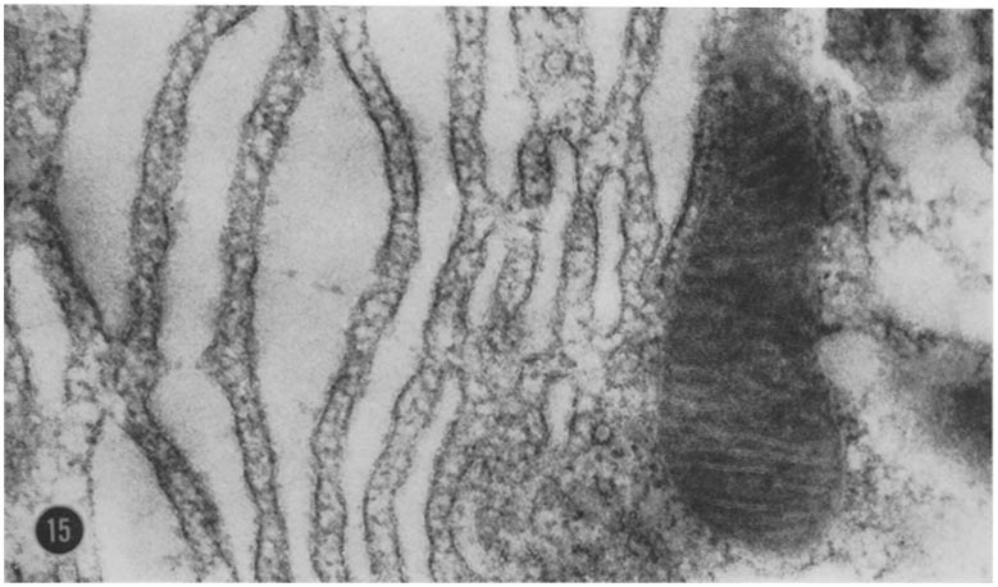

FIGURE 14

Rat pancreas. Formalin fixation 50 minutes at 20°C. Section treated 18 hours with 10 per cent perchloric acid at 37°C. Uranyl acetate stain. The ergastoplasmic lamellae are still intact, but the RNP granules have disappeared. × 36,000.

FIGURE 15

Rat pancreas. Formalin fixation 50 minutes at 20°C. Section treated 2 hours with 10 per cent perchloric acid at 37°C. Uranyl acetate stain. Greatly increased staining of mitochondrial matrix reveals negative images of cristae and external mitochondrial membranes. × 60,000.

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