ELECTRON MICROSCOPY AND HISTOCHEMISTRY OF RABBIT PANCREAS IN PROTEIN MALNUTRITION (EXPERIMENTAL KWASHIORKOR)

SYDNEY S. LAZARUS, M.D., AND BRUNO W. VOLK, M.D.

From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, N.Y.

In previous work [1] it was shown by light microscopy that rabbits fed a deficient diet, simulating that of poor native Jamaicans, developed pancreatic acinar atrophy with reduction in the number of zymogen granules. In addition to the alterations in the exocrine portion of the pancreas, there was also some suggestion of B cell degranulation. These animals also exhibited marked weight loss, changes in fur, fatty metamorphosis of the liver and eventually cirrhosis. These findings were similar to those which some authors have described in human kwashiorkor.[2-7] The diet utilized was high in carbohydrate, low in fat and low in protein. Low-protein diets as well as methionine deficiency induced by ethionine administration have been shown by various other workers to cause pancreatic acinar alterations.[8-12] This has been borne out by electron microscopic studies [13-16] which have demonstrated reduction in zymogen granule formation as well as various types of degenerative lesions in pancreatic acinar cells.

The purpose of the present study was to investigate, using histochemical methods, the extent of changes, if any, in certain oxidative enzymes and phosphatases in the rabbit pancreas after the deficient diet and to compare these with ultrastructural alterations.

MATERIAL AND METHODS

Thirty-four New Zealand white rabbits of either sex, weighing between 3,000 and 4,000 gm., were divided into 2 groups. Group I consisted of 10 control rabbits which received Purina Rabbit Chow and water *ad libitum*. Group II consisted of 24 rabbits who received the previously described deficient diet.[1] This was administered *ad libitum* for periods up to 21 weeks. Surviving animals were sacrificed by overdosage with sodium Nembutal® at intervals of 3 to 5 months after the beginning of the diet. Portions of pancreas and liver were fixed in formalin as well as in Zenker formol solution and processed as previously described.[17] Sections were stained with hematoxylin and eosin, by the periodic acid-Schiff trichrome method controlled by diastase digestion for glycogen identification,[18] and by the aldehyde fuchsin trichrome

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method.[17,19] In addition, portions of pancreas were fixed in buffered 1 per cent osmic acid with added sucrose [20] and embedded in Epon 812 according to the method of Luft.[21] Thin sections were cut with a Porter Blum microtome with glass knives, and viewed with an RCA EMU 3 electron microscope. Portions of pancreas were also quick-frozen in liquid nitrogen and sectioned in the cryostat at 10 μ. After fixation of the sections for 15 minutes in 6 per cent formol calcium the following enzymes were demonstrated: (a) adenosinetriphosphatase (ATPase) by a modification [22] of the method of Padykula and Herman [23]; (b) acid phosphatase (ACPase) by Gomori's method [24]; (c) glucose-6-phosphatase (G6Pase) by a modification of the methods of Chiquoine and Wachstein [25]; (d) glucose-6-phosphate dehydrogenase (G6PD) and lactic dehydrogenase (LD) by a modification of the method of Hess, Scarpelli and Pearse [26]; (e) triphosphopyridine nucleotide diaphorase (TPND) and diphosphopyridine nucleotide diaphorase (DPND) by a modification of the method of Scarpelli, Hess and Pearse.[27]

Results

Diet-Treated Animals

General. The results of dietary treatment were similar to those previously described.[1] There was marked weight loss which varied with the duration of treatment and ranged from 20 to 50 per cent of the initial body weight. The fur became sparse and had a fuzzy appearance. Occasionally animals also showed paralysis of their hind legs.

Light Microscopy. The lesions in the pancreas varied considerably in severity in different animals but not in type. The pancreatic acinar cells became atrophic and shrunken, showed reduction in zymogen granule content and frequently exhibited hyperchromatic nuclei (compare Figs. 1 and 2). There was also separation of lobules, apparently due to the acinar atrophy. In some instances atrophy was very pronounced, and the acini seemed to lie rather loosely within the supporting connective tissue stroma. There was a loss of their normal compactness and cohesiveness. Acinar cells frequently contained small vacuoles. In instances of severe exocrine atrophy, there was a suggestion that there might be degranulation of B cells. The liver usually exhibited marked fatty metamorphosis and frequently a well-marked cirrhosis was present.

Enzymatic Staining Reactions

Normal Pancreas. The distribution of the various enzymes within the normal rabbit pancreas was similar to that previously reported.[22,25,28–30] In slides stained for SH-dependent ATPase at pH 9.4, the islets could be distinguished with low power magnification, since they appeared somewhat darker than the surrounding acinar tissue. Many islet cells, presumed to be B cells, were heavily stained and opaque because of densely packed cytoplasmic granules and rods and possibly also diffuse cytoplasmic staining. The cytoplasm of other pale

cells, presumed to be A cells, contained only rodlike mitochondria. D cells could not be definitely identified. Acinar cells were pale and contained long, filamentous, evenly stained mitochondria. G6Pase activity was confined to B-cell cytoplasm and appeared as a somewhat granular precipitate. ACPase staining was found throughout the pancreas; its greatest activity was at the secretory poles of acinar cells and at the vascular poles of islet cells (Fig. 3). Stains for alkaline phosphatase demonstrated marked activity in intralobular and intercalated ducts (Fig. 5); little was seen in the islet cells. After incubation for G6PD and TPND, staining was most intense in the B cells and the ductular epithelium, so that they stood out clearly. Stains for LD and DPND yielded a precipitate which was well marked in ductular epithelium, acinar tissue and A cells, but minimal in B cells.

Diet-Treated Pancreas. There were no clear-cut differences in distribution of any of the enzymes except for acid and alkaline phosphatase. ACPase was either no longer distinguishable at the secretory pole of the acinar cells or, when still present, was evident in only very limited amounts. However, large discrete foci of activity were frequently seen at the base of the acinar cells or in periacinar areas (Fig. 4). On the other hand the activity of the enzyme in islet cells did not differ significantly from that seen in normal animals. Alkaline phosphatase activity was maintained in the intralobular and intercalated ducts but was markedly decreased when compared with normal control animals (compare Figs. 5 and 6).

Ultrastructure

Normal Pancreas. The ultrastructure of the normal rabbit islet has been described in detail.[17] The exocrine pancreas (Fig. 7) was similar to that in other species.[31-37]

Diet-Treated Pancreas. The exocrine pancreas exhibited conspicuous alterations which began after 3 to 10 weeks and became increasingly accentuated as treatment was continued. Most of the acinar cells were smaller than those in untreated animals. There was usually a conspicuous reduction in the number of mature zymogen granules and in some instances these were absent from the apex of acinar cells (Fig. 8). Instead, the apical cytoplasm contained rounded structures having a diameter equal to or sometimes greater than that of normal zymogen granules, but usually containing a substance of much less density (Figs. 8 and 9). There was no consistent alteration in the mitochondria, the endoplasmic reticulum or in the smooth-surfaced membranes of the Golgi apparatus. At the base of many acinar cells, vacuoles with varying patterns were frequently manifest. Many were comparatively trans-

lucent; others showed a rim of electron-dense material or exhibited diffusely distributed, moderately osmiophilic content (Figs. 9 and 10). There were also additional lesions with several patterns. Some appeared as vacuolated structures with aggregates of membranes and others as dense bodies containing mitochondria or other cell constituents. Some dense bodies consisted of whorls of densely packed ergastoplasmic membranes with numerous evenly spaced ribosomes. Some large lesions were enclosed by an agranular membrane which surrounded varieties of vacuoles formed by thickened masses of membranes (Figs. 10 to 12).

Discussion

The morphologic alterations in pancreas and liver observed by light microscopy in the present series of rabbits were similar to those reported by us previously.[1] These included pancreatic acinar cell atrophy and vacuolation as well as fatty infiltration and cirrhosis of the liver.

Atrophy of pancreatic secretory acini during malnutrition or inanition has been known for many years.[38] Pellagrins have been noted to show glandular atrophy as well as fat vacuoles within acinar cells.[39] In dogs, Grossman, Greengard and Ivy[40] demonstrated that a high-fat, low-protein diet caused pancreatic atrophy and suppressed enzyme secretion. Friedman and Friedman,[41] also utilizing a low-protein diet, observed acinar cell atrophy in rats. Adams, Fernand and Schnieden,[42] using a diet of cassava, attempted to produce a syndrome similar to that of human kwashiorkor in rats. They also found pancreatic acinar cell atrophy and a decrease in the number of zymogen granules. Methionine deficiency induced by ethionine administration produced somewhat different changes consisting of loss of acinar cell basophilia followed by necrosis and acute pancreatitis.[8] More recently it has been shown[43] that the phenylalanine analog, B-3-thienyl-DL-alanine, caused inhibition of zymogen granule formation and vacuolation of acinar cells.

The electron microscopic studies confirmed the existence of acinar cell atrophy and loss of zymogen granulation. There were also various vacuolar lesions and dense bodies, principally at the vascular poles of acinar cells. Some vacuoles had no definite limiting membrane but appeared as spaces in the matrix of the endoplasmic reticulum. Others showed membranes and contained varying amounts of moderately electron-dense material. There were also many membrane-limited bodies which contained agglomerates of smooth or rough-surfaced membranes, myelin figures, mitochondria, etc. The lesions were almost identical to those reported by Herman and associates[13-15] in ethionine-treated protein-deprived animals.

Pleomorphic inclusions have been observed ultramicroscopically in

the cytoplasm of a variety of cells and have been termed cytosomes, dense bodies, etc.[13-15,44-48] These have been observed in the peribiliary portions of liver cells[49,50] and have been shown to correspond to hemosiderin granules.[51,52] The dense bodies have also been considered to be lysosomes or lysosome derivatives.[53,54] More complex dense bodies in the livers of O_{20}-mice,[55] in livers perfused with glucagon[56] and in the small intestine epithelium of newborn rats,[57] have also been interpreted as lysosomes. Many of the bodies observed at the base of the acinar cells were presumably "autophagic" as indicated by their content of membranes, mitochondria and other cell constituents. How these bodies formed and how the self-engulfment of cell fragments took place was not clear. It could very well be that the morphologic variation in the dense bodies was due to differences in the types of material sequestered in them or to a variety of changes in form induced by digestion.

The alterations in acid phosphatase activity aided in the interpretation of the ultrastructural alterations. This enzyme tended to disappear from the secretory poles of the acinar cells where it is normally present, and large foci of activity appeared at the base of the acinar cells. Apparently the enzyme was localized in the various vacuolar structures observed ultramicroscopically. This would tend to indicate the "lysosomal" character of these structures.[58] The loss of ACPase from the secretory pole was presumably related to a diminished formation of prozymogen granules with which this enzyme has been linked.[59,60] That this was not a nonspecific effect of the dietary regimen but was related directly to an effect on zymogen production was evidenced by the fact that islet cell acid phosphatase was not affected. Moreover, new foci of acid phosphatase activity appeared at the base of the acinar cells. The question also arose as to whether acid phosphatase played a role in zymogen production so that enzyme loss anteceded zymogen loss or was only a concomitant phenomenon associated with malnutrition.

The loss of alkaline phosphatase activity from ductules was presumably also a reflection of a diminution of enzyme production associated with protein malnutrition. On the other hand the integrity of the oxidative enzymes and ATPase reflected the essential role these enzymes play in the cell economy; they are altered only when the cell loses its viability.

Summary

A study of the morphologic and histochemical changes in the rabbit pancreas, induced by a low-protein, low-fat, high-carbohydrate diet was conducted. A kwashiorkor-like syndrome resulted. The pancreas showed acinar atrophy and vacuolation with reduction of zymogen granules by conventional microscopy. Electron microscopy demonstrated a decreased

size of acinar cells and loss of mature zymogen granules. Vacuoles as well as various types of dense bodies frequently containing cytoplasmic constituents (mitochondria, compressed endoplasmic reticulum) appeared at the base of the acinar cells. Histochemically there were no changes in distribution of oxidative enzymes or of adenosinetriphosphatase. On the other hand alkaline phosphatase exhibited a marked diminution in activity. In many instances acid phosphatase had almost entirely disappeared from the secretory poles of acinar cells while large foci of activity appeared at their basal portions. The latter activity was presumably associated with the lysosomal "autophagic" bodies visualized ultramicroscopically. The loss of acid phosphatase at the secretory poles of acinar cells was presumably a fairly specific effect of the diet since neither islet cell nor lysosomal acid phosphatase were affected.

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[*Illustrations follow*]

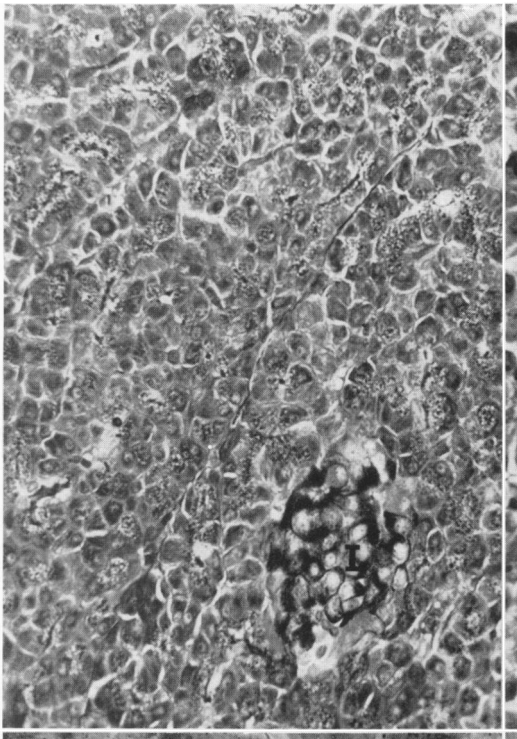
Legends for Figures

Fig. 1. Normal rabbit pancreas. Zymogen granules are abundant in acinar cells, and an islet (I) contains well-granulated B cells. Aldehyde fuchsin trichrome stain. × 375.

Fig. 2. Pancreas, deficient diet for 20 weeks. There is considerable acinar atrophy, marked depletion of zymogen granules and acinar cell nuclear pyknosis. The islet (I) appears somewhat degranulated in comparison with the normal. Aldehyde fuchsin trichrome stain. × 500.

Fig. 3. Normal rabbit pancreas. There is marked acid phosphatase activity at the secretory pole of acinar cells. The black granules (arrows) at the base of the cells represent lysosomes. × 375.

Fig. 4. Pancreas, deficient diet for 20 weeks. A significant decrease of acid phosphatase is evident. Enzyme activity has practically disappeared from the secretory pole while varying sized foci of activity (arrows) appear at the base of the acinar cells. × 960.



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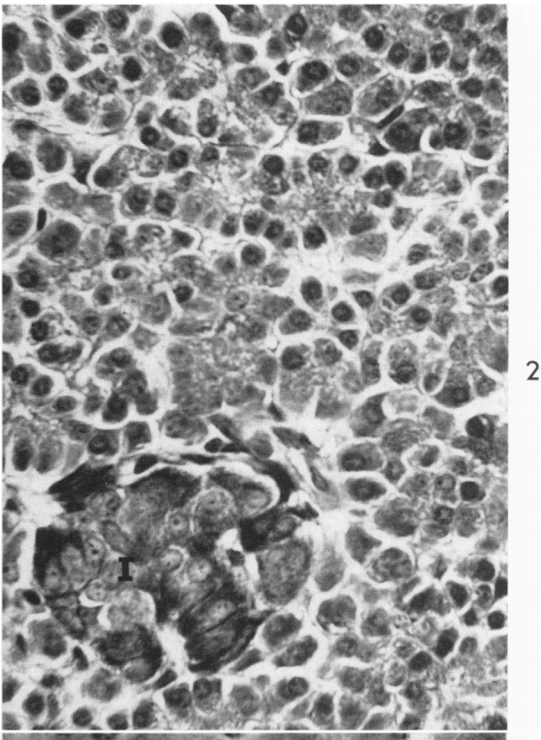

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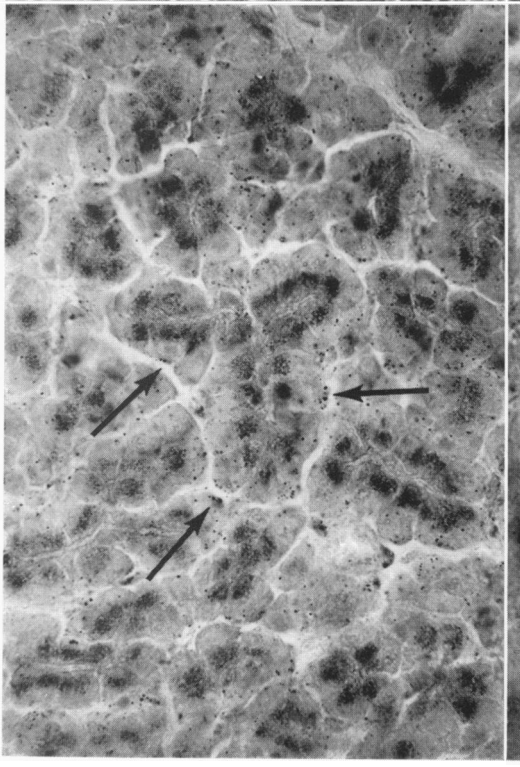

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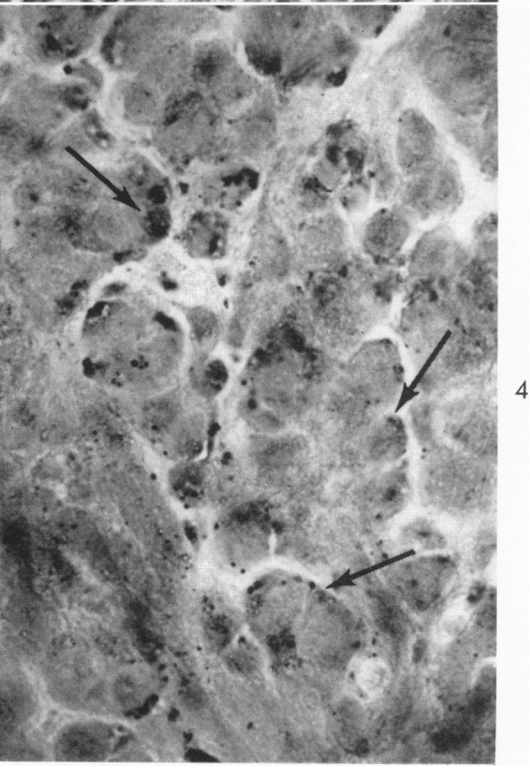

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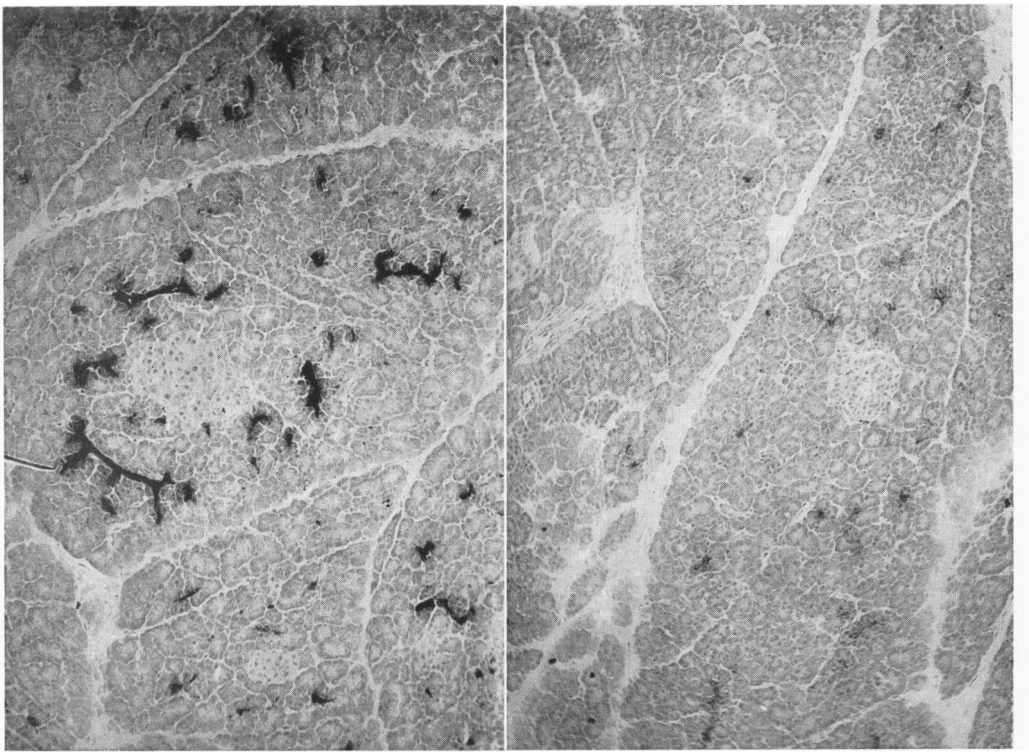

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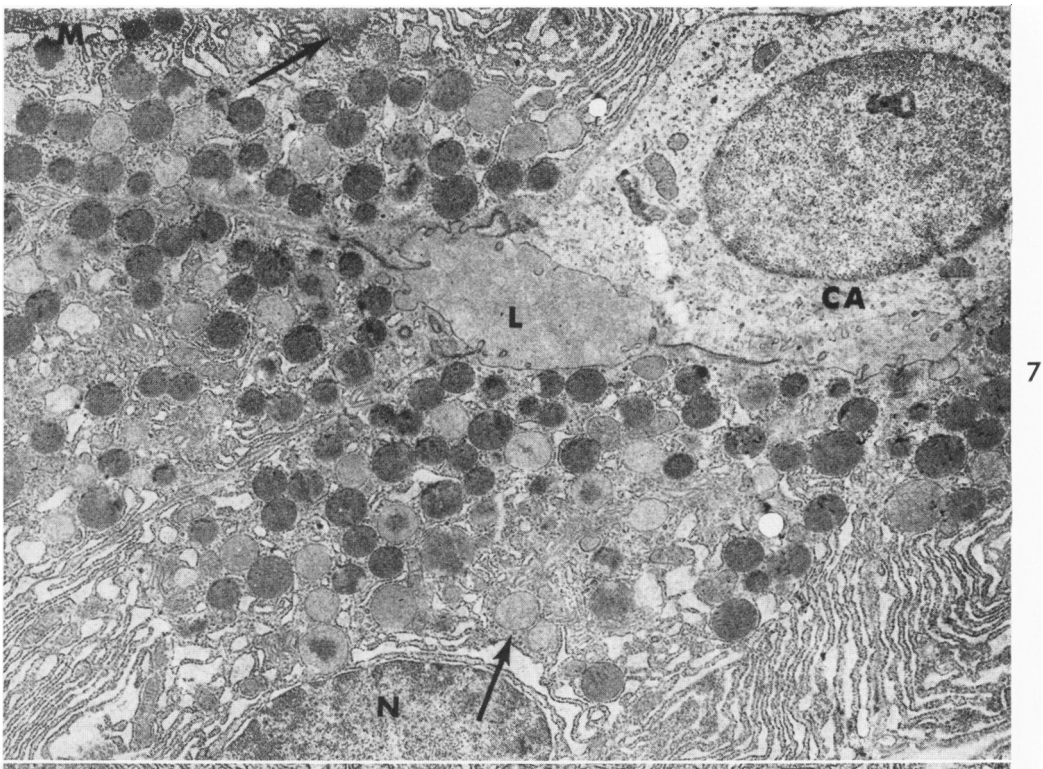
FIG. 5. Normal rabbit pancreas. There is marked alkaline phosphatase activity in intralobular and intercalated ducts. × 95.

FIG. 6. Pancreas, deficient diet for 20 weeks. Activity of alkaline phosphatase is markedly decreased as compared with that of the normal animal (Fig. 5). × 95.

FIG. 7. Centro-acinar area in rabbit pancreas, stock diet. Acinar cells contain many electron-dense mature zymogen granules clustered primarily at the lumen pole. There are also a number of less osmiophilic prozymogen granules (arrows). Centro-acinar cell, CA; nuclei, N; mitochondrion, M; acinar lumen, L. × 4,280.

FIG. 8. Pancreas, deficient diet for 20 weeks. The centro-acinar area exhibits a loss of zymogen granules. There are occasional prozymogen granules (arrows) present. Acinar lumen, L; mitochrondria, M; nuclei, N; Golgi complex, GC; centro-acinar cell, CA. × 4,280.



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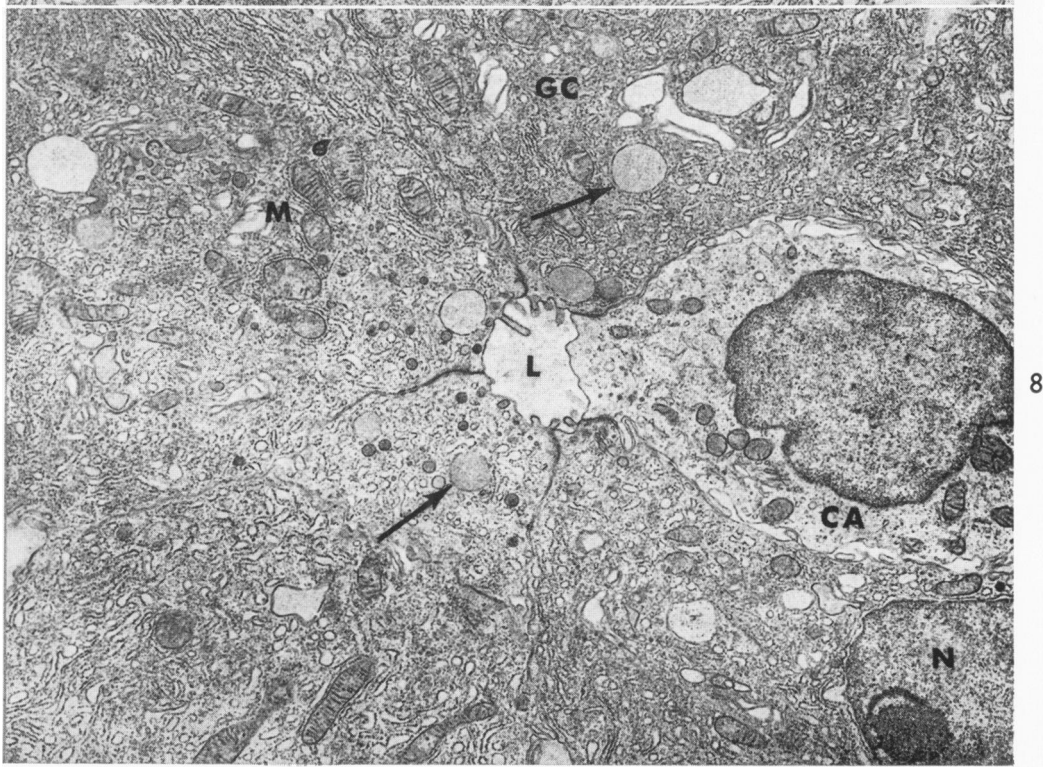

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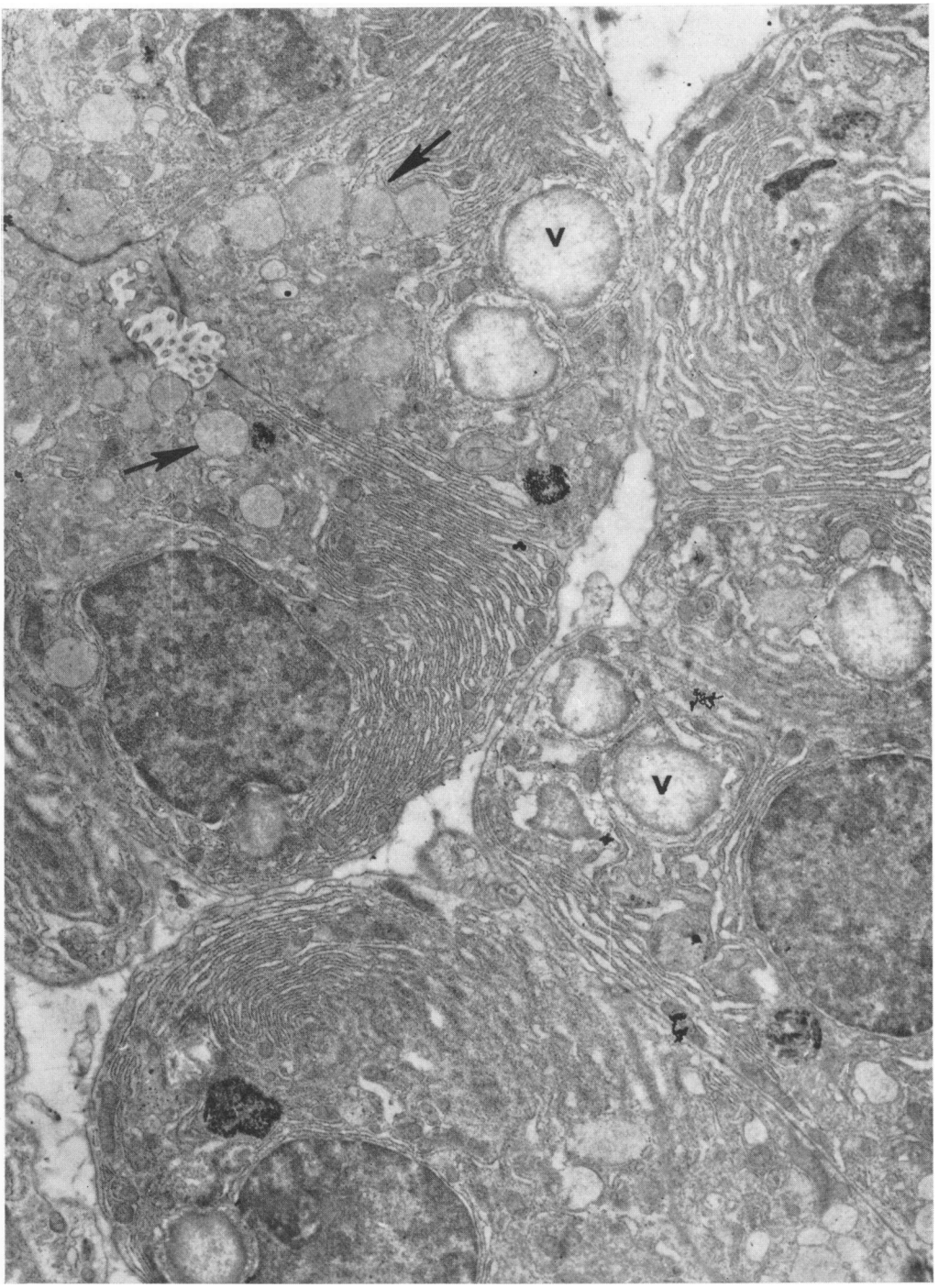

FIG. 9. Pancreas, deficient diet for 22 weeks. There are many prozymogen granules (arrows) at the apexes of acinar cells. Mature zymogen granules are not seen. Numerous vacuolar structures (V) appear at the basal portions of the cells. These exhibit a peripheral osmiophilic rim with less dense central regions. × 4,280.

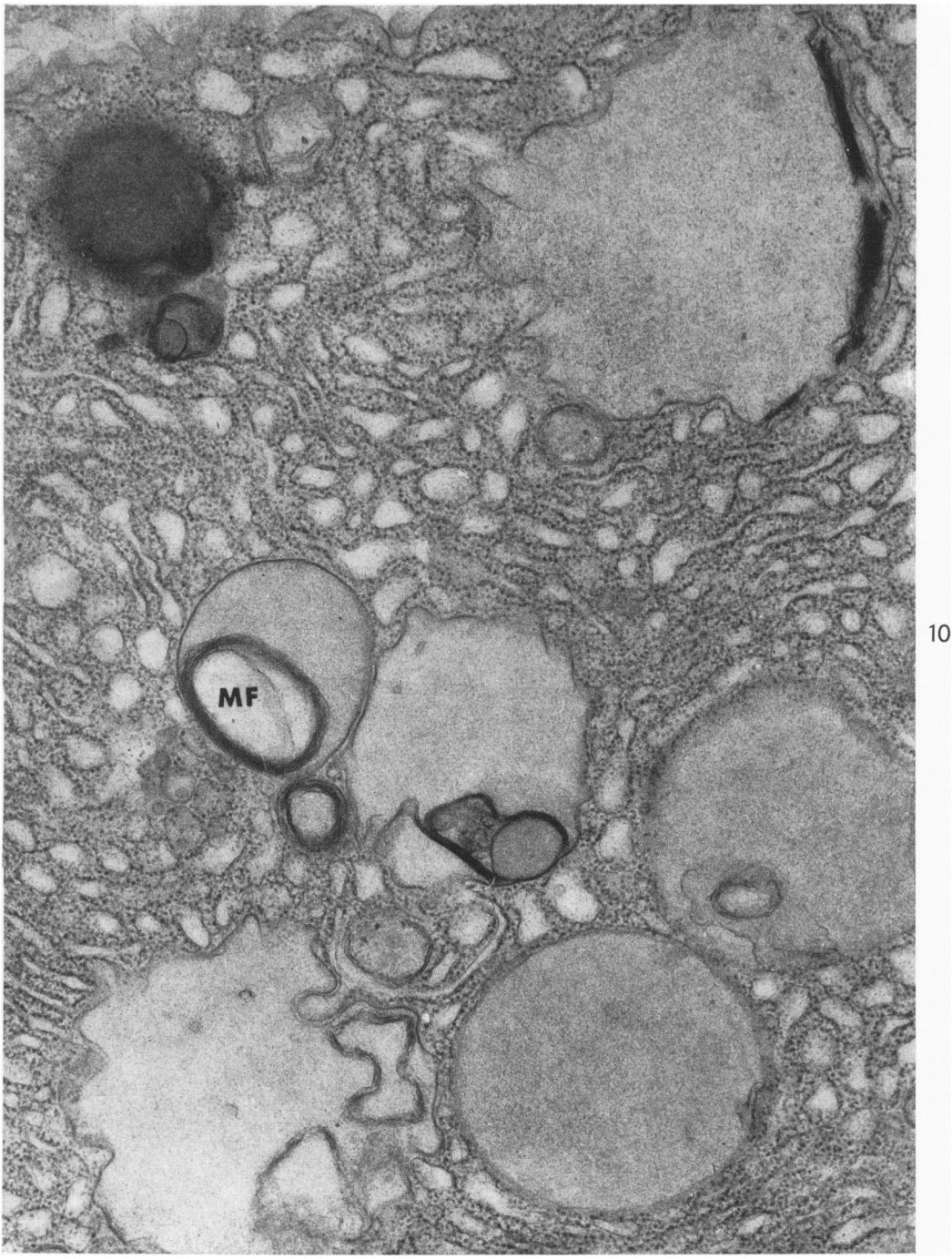

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FIG. 10. Portions of a pancreatic acinar cell, deficient diet for 22 weeks. Varying types of vacuoles contain myelin figures (MF) or aggregates of osmiophilic membranes; others are empty. There is also an almost homogeneously osmiophilic body. × 28,800.

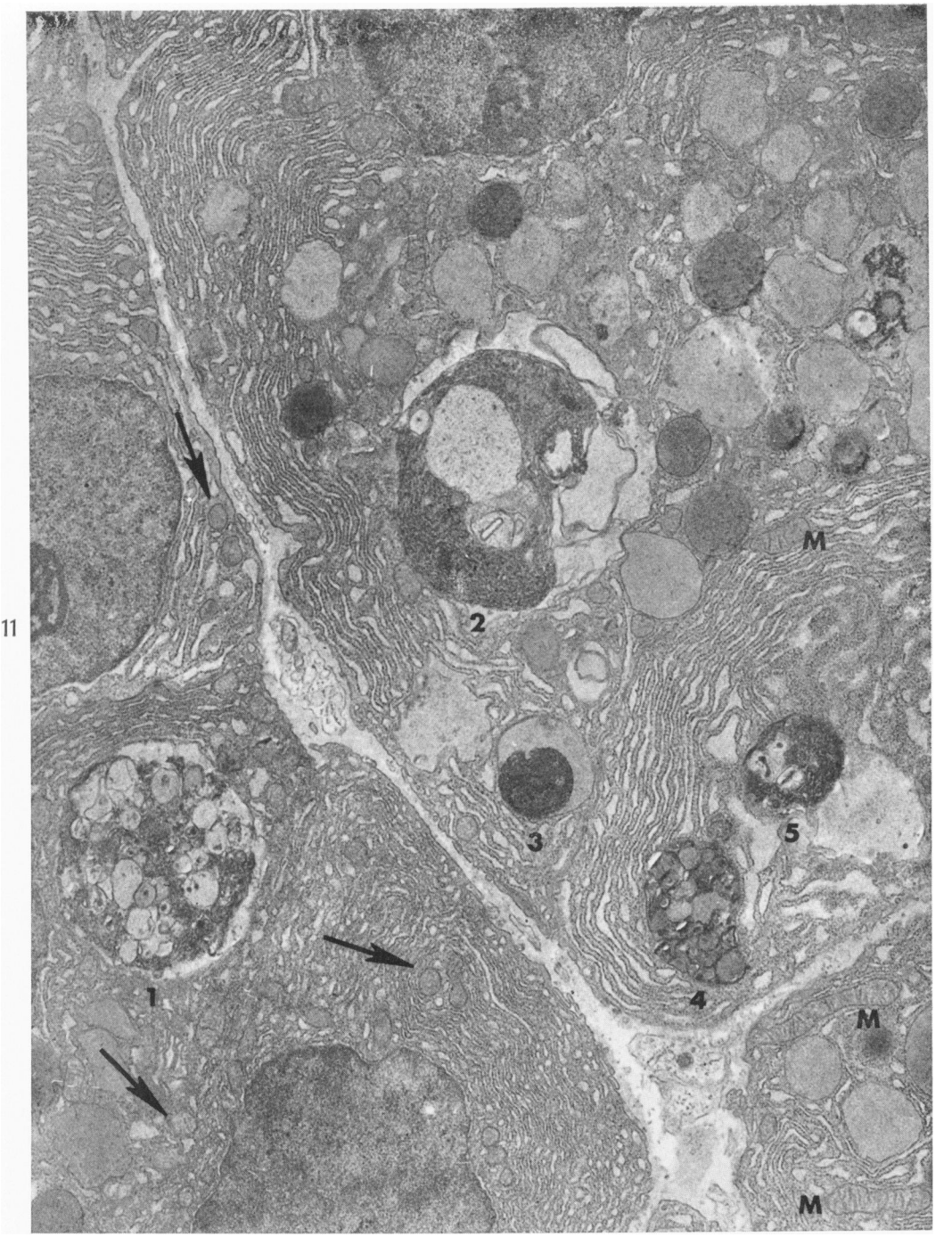
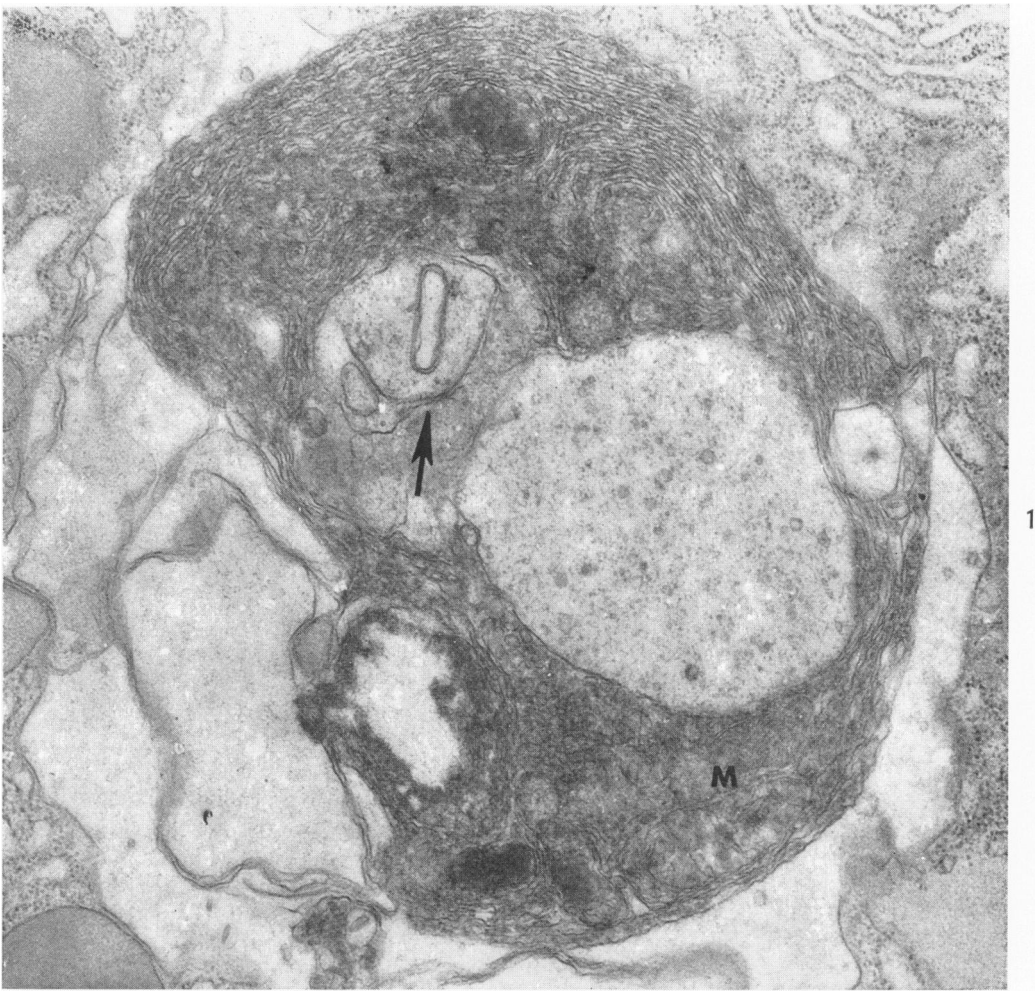


FIG. 11. Pancreas, deficient diet for 20 weeks. Many prozymogen granules (arrows) and several cytoplasmic lesions (1,2,3,4,5) appear. Some of the lesions (1) contain rounded vesicles or osmiophilic granules surrounded by clear halos. Others (2) exhibit whorls of closely packed osmiophilic membranes. Mitochondria, M. × 5,760.



12

FIG. 12. A higher magnification of one of the cytoplasmic lesions seen in Figure 11. Whorls of parallel ergastoplasmic lamellas are denser than normal. The structure contains trapped mitochondria (M), as well as vacuoles, one of which contains rectangular membranous bars (arrows). × 26,200.