EFFECT OF DEHYDROASCORBIC ACID ON THE

ISLETS OF LANGERHANS OF THE RAT PANCREAS

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ABSTRACT

Rat pancreatic islets have been studied following successive daily administration of dehydroascrobic acid (DHA) and during the recovery phase following 3 daily injections. One injection of DHA produces degranulation of B cells seen in the light microscope as a loss of aldehyde fuchsin positivity. In the electron microscope the B cells appear to have secretory granules accumulated subjacent to the plasma membranes. Following 2 and 3 daily injections, B cells evidence alterations in the organization of the granular endoplasmic reticulum and mitochondria, and secretory granules are scant but when present are subjacent to the plasma membrane. After 5 to 7 days' recovery few secretory granules remain in B cell cytoplasm, but the cells have prominent granular ER and a Golgi apparatus with numerous prosecretory granules. The primary effect of DHA is an exaggerated secretory response of B cells, which is intensified with subsequent injections. Necrosis of B cells as produced by alloxan is not seen.

INTRODUCTION

The diabetes produced by dehydroascorbic acid (DHA) in the rat has been attributed by Patterson (22-25) to an action similar to that suggested by Lazarow (12, 13) for alloxan; i.e., an inhibition of essential sulfhydryl enzymes in B cells of the pancreatic islets. Since this interpretation for alloxan has not received support from the studies of our laboratory (15-17), the hypothesis has been advanced that the diabetogenic syndrome following the administration of DHA results from a complex pharmacological action of the drug involving the autonomic nervous system (18). As a matter of fact, in spite of the chemical similarities of the two drugs stressed by Patterson (23), there are fundamental differences in their biological activity, since DHA-induced diabetes requires daily repeated injections of sufficient doses of drug, and a susceptible species; e.g., the rat.

Other differences of biochemical and histological nature have been described in our previous research (18). The histological changes induced by DHA in the pancreatic islets provide some clues as to differences in the pathogenic mechanisms of the two substances. These studies have been extended to the level of the electron microscope and are described in the present report.

MATERIALS AND METHODS

Adult white male Wistar rats (five sets of litter mates) of 120 to 150 gm body weight were injected daily, by the intravenous route, with 0.7 gm dehydroascorbic acid (DHA) per kilogram body weight. The dehydroascorbic acid was prepared according to Moll and Wieters (19). The injection schedule, which followed that given by Patterson (23), produced 75 per cent survival.

All animals studied were killed by decapitation 5 hours after the last injection, at which time they had completely recovered from the acute effects of the

drug. One or two rats were killed after the successive injection periods of 1, 2, and 3 days. Three rats treated with 3 daily injections were killed when the blood sugar returned to normal levels, 7 days after the last injection. Control animals were injected daily with an identical dose of ascorbic acid (0.7 gm/kg), and two animals were killed after each of 3 injection periods of 1, 2, and 3 days. The pancreas was flooded with fixative prior to removal, then cut into small blocks and fixed for 1 hour at room temperature in osmium tetroxide in White's saline according to Bauer (1) and Richardson (26). The tissue was dehydrated through graded ethanols and embedded in Epon 812. Thin sections were cut on a Porter-Blum microtome with glass knives and mounted on copper grids without supporting film. Unstained sections as well as uranyl acetate and lead acetate stained sections were examined. Electron micrographs were obtained with Philips 100 and RCA EMU 3C and 3G microscopes.

The blood sugars on the above animals were determined daily by Nelson's method (21), using tail vein blood. For light microscopy, portions of the pancreas were fixed in Bouin's solution with formol, embedded in paraffin, sectioned, and the sections stained with hematoxylin and eosin, aldehyde fuchsin (8) for B cell granulation, and the phosphotungstic acid–hematoxylin (PTAH) of Mallory for A cell granulation.

For purposes of comparing alterations produced by dehydroascorbic acid and alloxan, 7 animals were injected once intravenously with 40 mg/kg alloxan and killed $\frac{1}{2}$, 2, 5, and 24 hours later. Pancreatic tissue was prepared in an manner identical to that of the animals administered dehydroascorbic acid.

OBSERVATIONS

Normal and Ascorbic Acid–Injected Control Rat Pancreatic Islets

The cytology of the normal rat pancreatic islets has been described in detail by Lacy (10, 11), Ferreira (7), and Caramia (4). Islets from ascorbic acid–injected animals could not be distinguished from the islets of normal animals. B cells are located centrally in the islet and demon-

strate a characteristic shrinkage artifact between the limiting membrane and dense portion of the secretory granules (Fig. 1). The mitochondria are elongated and scattered throughout the cytoplasm, with a somewhat heavier concentration of them on one side of the nucleus. The Golgi apparatus is prominent, and prosecretory granules are near the Golgi apparatus. Occasionally secretory granules can be seen fusing with the plasma membrane, presumably representing release of their contents (insulin) by the cell. The A cells can be easily distinguished from B cells (9, 20) by their granules, which are more electron-opaque than those of the B cells and lack the shrinkage artifact between the granule and its limiting membrane. Their nucleus is more electron-opaque and homogeneous than that of the B cell. D cells characterized by secretory granules of low electron opacity, identical with those described by Caramia (4), are present and usually associated with A cells.

Dehydroascorbic Acid–Injected Rats

PHYSIOLOGIC RESPONSE

The blood sugar of rats 6 hours after the 1st injection of DHA is at hypoglycemic levels (60 to 80 mg per cent). These animals evidenced a marked stimulatory response with acute excitement, especially after the 1st injection, as has been described by Patterson (23) and Merlini (18). At 24 hours after the first injection the blood sugar is normal. The 2nd and subsequent injections produce less excitatory response in the rats. The rats are hypoglycemic 6 hours after every injection. All the animals used in this phase of the study were killed in the hypoglycemic phase (5 to 6 hours after the injection).

1 INJECTION OF DHA

By light microscopy the B cells appear degranulated with no evidence of nuclear or cytoplasmic alteration other than the loss of aldehyde fuchsin–positive material. The A cells are normal in all respects.

FIGURE 1 Normal rat pancreatic islet. B cells are to the right of the capillary containing portions of three red cells. Many granules are scattered throughout the cytoplasm of the B cells. The A cells (A) with very electron-opaque granules are at the margin of the islet. Portions of D cell cytoplasm, characterized by secretory granules of lower electron opacity, are present between the A cells and the capillary. X 4,400.
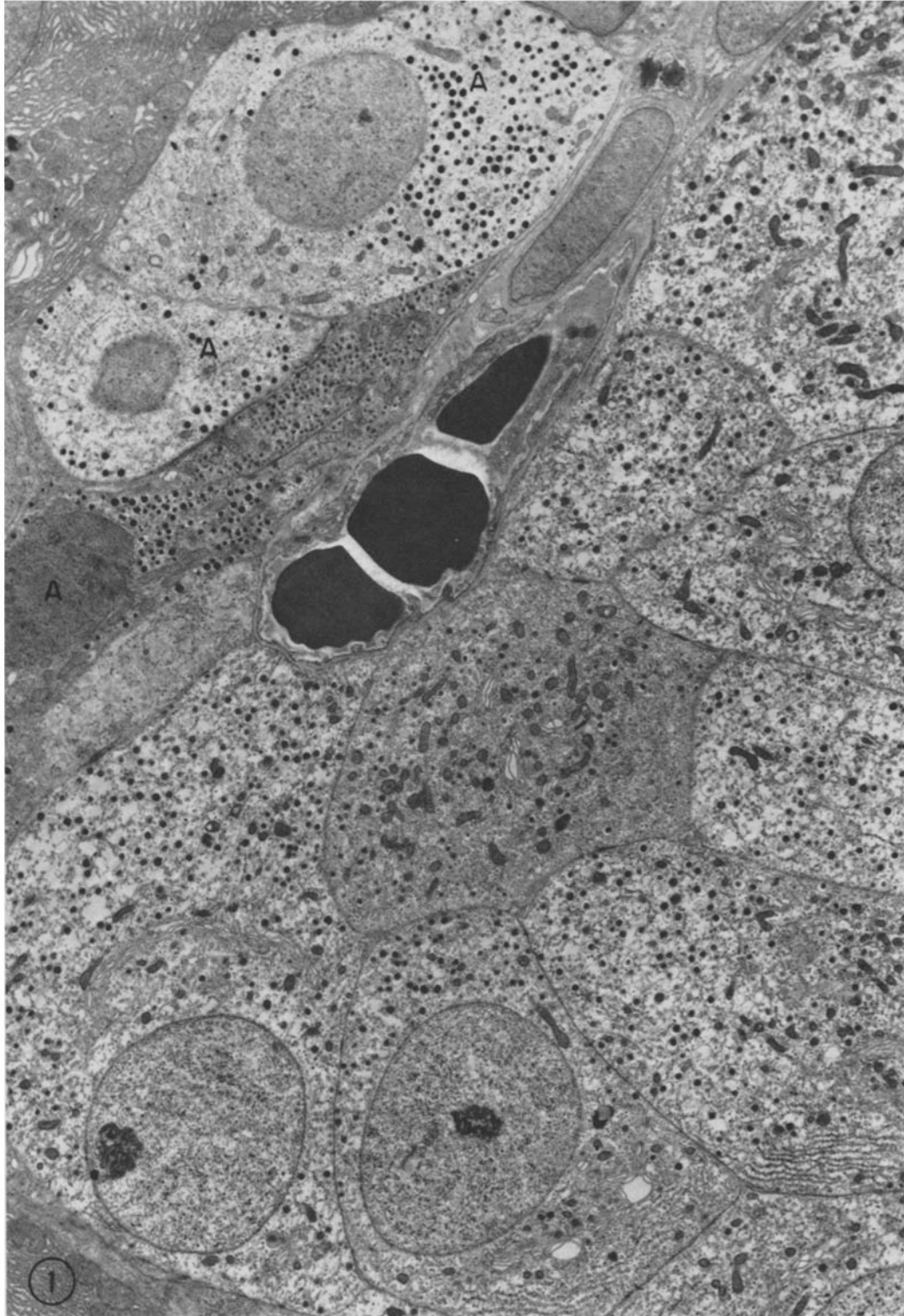
In the electron microscope the cytologic appearance of individual B cells varies somewhat; however, degranulation is extensive. Residual secretory granules in the degranulated cells are located subjacent to the plasma membrane (Fig. 2). The limiting membranes of the granules are often fused with the plasma membrane. Within the cytoplasm the granules and prosecretory granules are associated with the Golgi apparatus.

Cytologic changes other than degranulation are variable. The mitochondria are occasionally enlarged and clumped within the cytoplasm (Fig. 2). Some cisternae of the granular ER are dilated. These changes can be found together or singly within a B cell.

The A cells and D cells are well granulated and appear cytologically unchanged.

2 DAILY INJECTIONS OF DHA

By light microscopy the islets still appear aldehyde fuchsin–negative. PTAH-positive A cells are completely normal.

By electron microscopy the B cells still show a scant number of granules confined to the periphery of the cell (Fig. 3). These granules frequently demonstrate fusion of their limiting membrane with the plasma membrane of the cell (Fig. 5). Occasionally the granules within the cytoplasm have a wide clear space between the dense portion of the granule and its limiting membrane. Such granules frequently appear less electron-opaque than do neighboring granules. Tangential sectioning of the core of an electron-opaque granule is unlikely to account for this lessened opacity since the diameter of the less-opaque granule is as large as that of the neighboring granules.

The mitochondria at this stage are most striking in appearance. They are increased in number and accumulated in a mass at the side of the nucleus where the cytoplasm is more abundant (Figs. 3 and 4). They appear larger in diameter than normal mitochondria, and elongated forms predom-

inate. Their cristae are arranged both cross-wise and longitudinally. Occasionally, electron-opaque tubules within the cristae similar to those that will be described in the following section are observed.

The sacs of the Golgi apparatus are dilated with the result that their agranular membranes are widely separated (Fig. 4). Prosecretory granules are not prominent. The membranous component of the ER is less abundant than normal, and free RNP particles are scattered throughout the cytoplasm (Figs. 3, 4, and 5). A and D cells do not show any cytologic change.

3 DAILY INJECTIONS OF DHA

The light microscopic appearance after 3 daily injections is identical with that after 2 daily injections.

Secretory granules within B cells appear to be virtually absent by electron microscopy. When present, they are close to the plasma membrane (Fig. 6). Mitochondria with fewer cristae than normal are predominantly spherical profiles that appear to be swollen. When the mitochondria are large they occasionally evidence vesiculation of their limiting membrane, and occasionally "open forms" can be seen similar to those described in the adrenal gland (Fig. 7). Electron-opaque tubules are seen within some mitochondrial cristae (Fig. 7). At times more than one tubule is present within a single crista as is best seen in cross-sections of the tubules (Fig. 7). In longitudinal sections these tubules appear as elongated opaque rods within a crista (Fig. 7). Although in most cells the mitochondria are swollen, in some cells they remain normal.

The Golgi apparatus is similar to that seen after 2 daily injections. In general, the cytoplasm is vacuolated to varying degrees with many of the vacuoles rimmed by RNP particles (Figs. 6 and 8). In some cells laminated membranous onion-like configurations of the ER can be seen (Fig. 9). Adjacent membranes of cisternae of the ER are closely

FIGURE 2   Pancreatic islet after 1 injection of dehydroascorbic acid. B cells which comprise the bulk of the tissue evidence varying degrees of degranulation. When degranulation is present the secretory granules line the plasma membrane (arrows). Focal accumulations of mitochondria are present in some of the degranulated B cells. In some B cells an exaggerated clear space is present between the limiting membrane and dense portion of the granule. The A cells (A) appear normal. × 3,600.
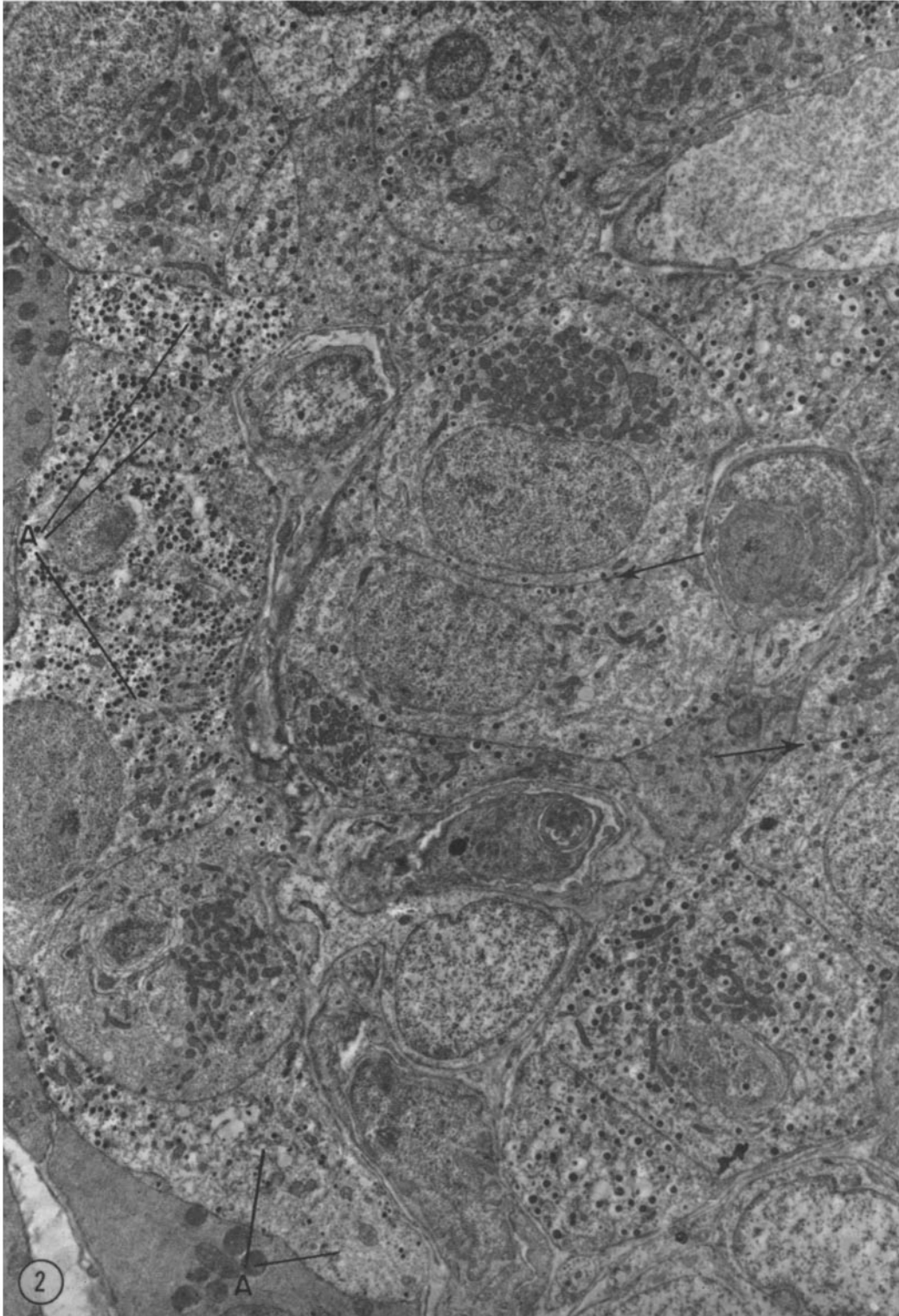
approximated (Figs. 10 and 11) and more electron-opaque than the normal membranes of the ER. The RNA particles between the approximated membranes become difficult to identify. The A and D cells are entirely normal (Fig. 8).

RECOVERY

Three rats, hyperglycemic after their third injection, were killed when the blood glucose had returned to normal after 5 to 7 days. These animals had no aldehyde fuchsin–positive granules in their pancreatic islets.

By electron microscopy the B cells evidenced increased size with a prominent lamellar granular ER (Figs. 12 and 13). Secretory granules and prosecretory granules are prominent in the center of the cell and associated with the Golgi apparatus (Fig. 13), and some secretory granules are also closely applied to the plasma membrane. The mitochondria are normal in both number and size.

Alloxan-Treated Rats

The general appearance of the B cells in the pancreatic islets of rats injected with alloxan in most respects resembles that described by Williamson and Lacy in the rabbit (29). B cells of rats do not show degranulation, but rather vacuolization and condensation of the cytoplasm without loss of granules (Fig. 14). The mitochondria become indistinct, and by 24 hours the nuclei are pycnotic and cell dissolution occurs. The striking difference between the rabbit, as described by Williamson and Lacy (29), and the rat is that 24 hours after alloxan administration the B cells of rats still contain many secretory granules.

DISCUSSION

The present study has shown that dehydro-ascorbic acid given to rats produces marked degranulation of their pancreatic islet B cells without degenerative alterations. Degranulation of B cells, already evident after 2 injections, becomes more evident after 3 injections when almost all B cells

are severely degranulated. Correlated with the cytologic evidence of degranulation is the low blood sugar in these animals, presumably the result of insulin release. The liberation of the granules by the B cells; *i.e.*, margination of granules along the plasma membrane, fusion of their limiting membrane with the plasma membrane, and rupture of the point of contact, follows the normal sequence as described by Ferreira (7). A similar mechanism of release has been described by Williamson and Lacy (30) in tolbutamide and glucose-stimulated animals. Thus, dehydroascorbic acid appears to have stimulated B cells to secrete.

If direct or indirect stimulation of these cells explains the liberation of granules, then the other cytologic alterations are difficult to interpret with certainty. The reactions of mitochondria and the endoplasmic reticulum are the most equivocal in this regard, since it is impossible to separate the direct cytologic effect of dehydroascorbic acid from secondary effects related to the presumed stimulated state of the cells. Swelling of mitochondria and enlargement of the Golgi apparatus could be the result of an intensive effort of the cells to form new secretory granules. The close apposition of membranous sacs of the granular endoplasmic reticulum has been seen in one insuloma described by Bencosme, Allen, and Latta (2). Presumably these cells are also secreting at a rapid rate. A direct cytologic effect of dehydroascorbic acid on B cells is possible, however, since this substance has been shown to be a mitochondrial swelling agent *in vitro* (14). Thus the possibility of a direct intracytoplasmic effect of dehydroascorbic acid cannot be ruled out at the present time.

On the basis of the present study it is apparent that dehydroascorbic acid produces cytologic alterations entirely different from those caused by alloxan. These results in some respects are not surprising since after repeated doses compatible with survival about 80 per cent of the animals recover from the hyperglycemic state, even if this

FIGURE 3   B cells after 2 injections of DHA. This low magnification electron micrograph shows B cells with the majority of their secretory granules lining the plasma membranes. Many mitochondria can be seen accumulated on one side of the cell. Some mitochondria are larger than normal and have indistinct cristae. The cisternae in the Golgi apparatus (*G*) appear dilated. × 6,000.
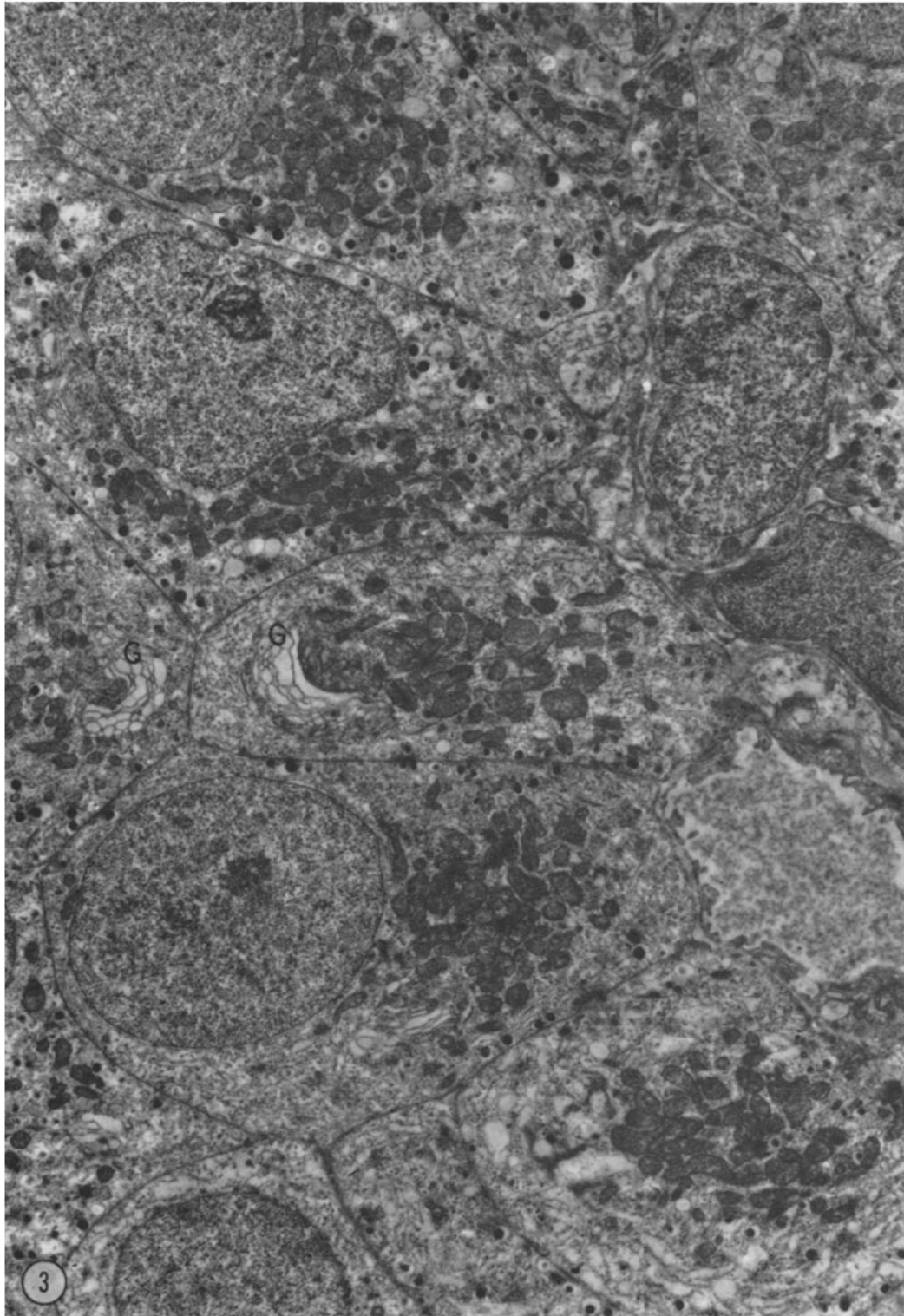
hyperglycemic state lasts for 10 to 15 days. But dehydroascorbic acid, like alloxan, has a specificity for producing alterations in B cells of pancreatic islets without affecting A or D cells. The difference between dehydroascorbic acid and alloxan is especially marked in the rat where, as noted by Lacy and Cardeza (11), as well as in this study, alloxan has been seen to produce a vacuolization of the cytoplasm and clear signs of nuclear necrosis, whereas the secretory granules are apparently well preserved. However, DHA produces degranulation of B cells with minimal alteration of other cytoplasmic organelles. After the 3rd injection of DHA some B cells show dilatation of the ER and swelling of mitochondria that possibly represent a secondary effect.

The mechanism of the diabetogenic activity of dehydroascorbic acid is not easy to explain. As described in detail by Merlini (17, 18) it may well be related to the complex pharmacological action which includes marked motor activity, marked stimulation of the sympathetic and parasympathetic nervous system, transitory vasoconstriction, and more persistent vasodilation resulting in general circulatory collapse. It is impossible to prove whether the circulatory collapse or the stimulation of sympathetic and parasympathetic system *per se* is responsible for the hyperglycemic

response and for the alterations of the B cells. In this respect it is interesting to note that cytologic changes in the pancreatic islets have been produced by direct stimulation of the sympathetic nervous system (27, 28) as well as by the action of sympathetic and parasympathetic drugs (5). Furthermore, atropine and ergotamine inhibit the diabetogenic action of DHA (3). Some role for the autonomic nervous system appears implicated in an understanding of the pathogenesis of the action of DHA.

The suggestion that dehydroascorbic acid, like alloxan, produces diabetes by an inhibition of SH groups (25) is no longer tenable. In fact, Cooperstein and Lazarow (6) have recently demonstrated that alloxan does not enter the B cells. The present study has pointed out the differences in the cytologic alterations produced by the two drugs, and any biochemical similarity would be purely hypothetical.

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FIGURE 4   B cells after 2 injections of DHA. A central accumulation of mitochondria is present as in Fig. 3. Free RNP particles are numerous, but the granular ER is scant in the cytoplasm. The Golgi apparatus is dilated. × 32,000.

FIGURE 5   B cells after 2 injections of DHA. Higher magnification of the same case as Fig. 3. The granules in the B cells are marginated along the plasma membrane. Fusion of the limiting membrane of the granules with the plasma membrane is evident at the arrow. × 23,000.
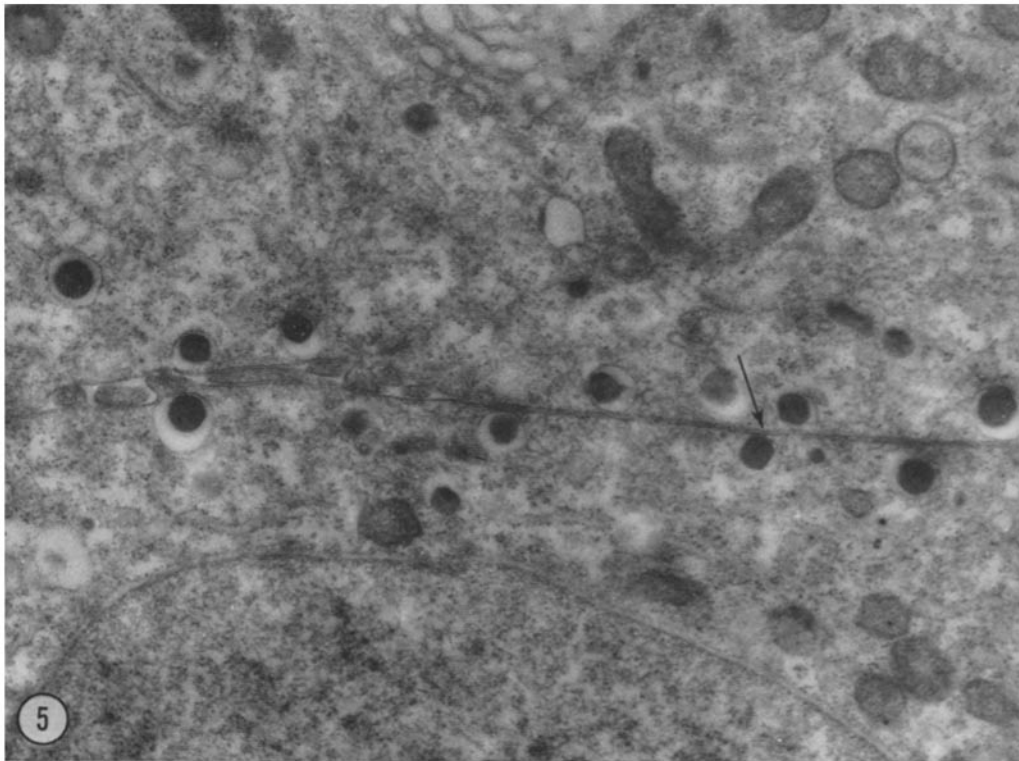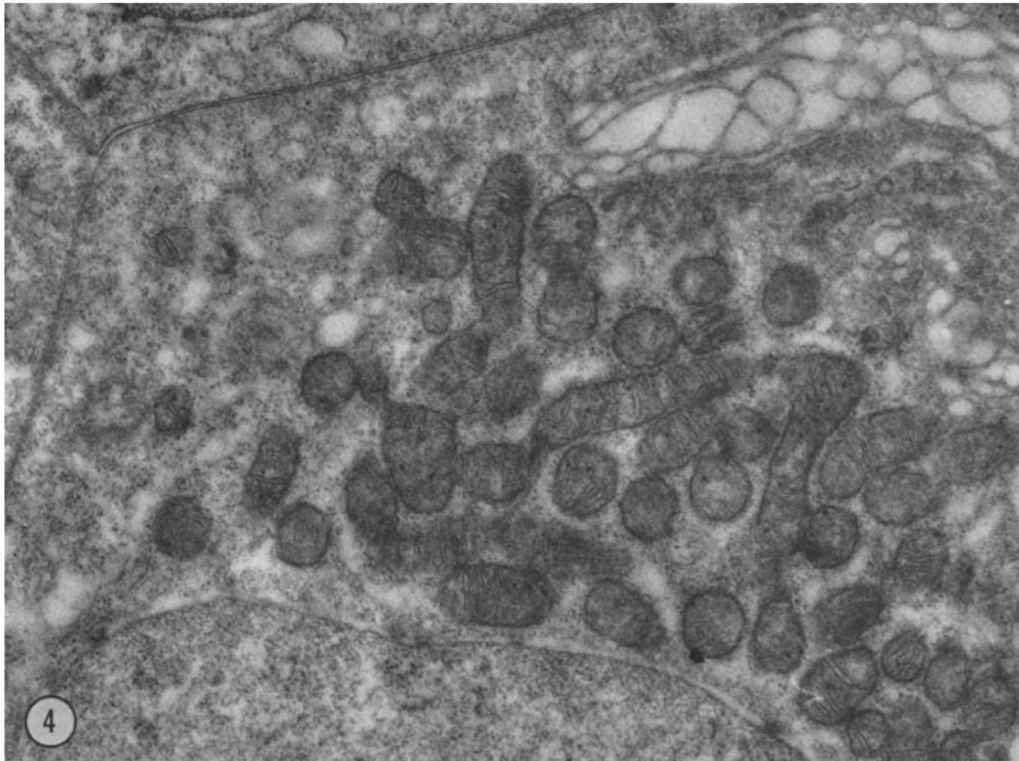
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FIGURE 6 B cells after 3 injections of DHA. The granular endoplasmic reticulum is dilated in the cell to the right, and in the other cells free RNP particles predominate. Swelling of mitochondria is seen in the lower two cells, whereas the upper two cells contain normal mitochondria. The Golgi apparatus (G) is prominent. Electron-opaque material, presumably lipid, is present in the cell to the left. × 19,000.
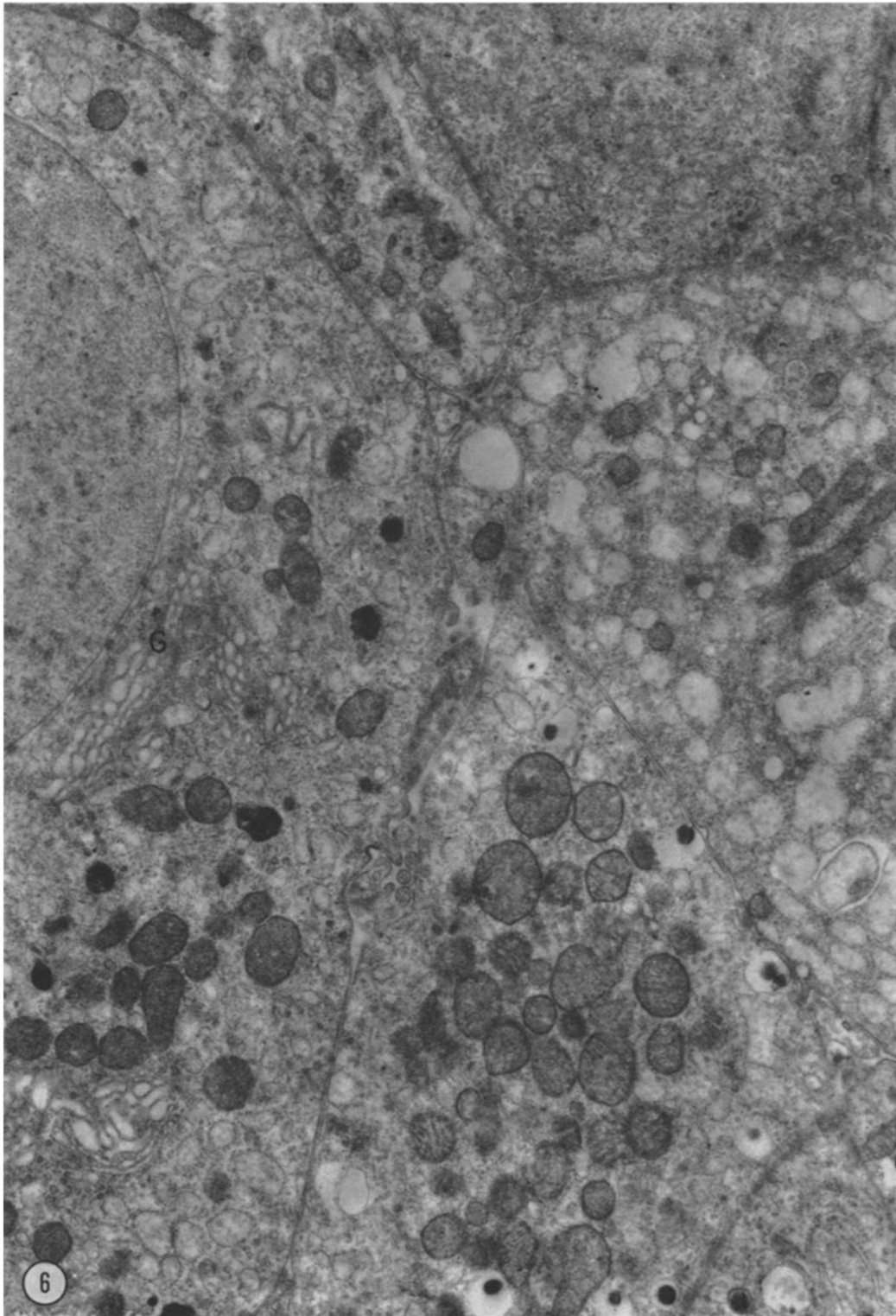
FIGURE 7 Same specimen as Fig. 6. Mitochondrial swelling is shown in addition to open forms (O) in this B cell. Small tubules within mitochondria can be seen in cross- (X) and longitudinal (Y) sections. The cristae cannot be seen within some mitochondria, possibly due to the plane of section. $\times$ 29,000.

FIGURE 8 Islet cells after 3 injections of DHA. Portion of a vacuolated B cell (left) and a normal A (A) and D (D) cell can be seen. The vacuolization within the B cell is confined to cisternae of the granular endoplasmic reticulum. A single granule (B) is present in B cell. $\times$ 20,000.

FIGURE 9 B cell after 3 injections of DHA. A circular configuration of the granular endoplasmic reticulum is present. $\times$ 20,000.
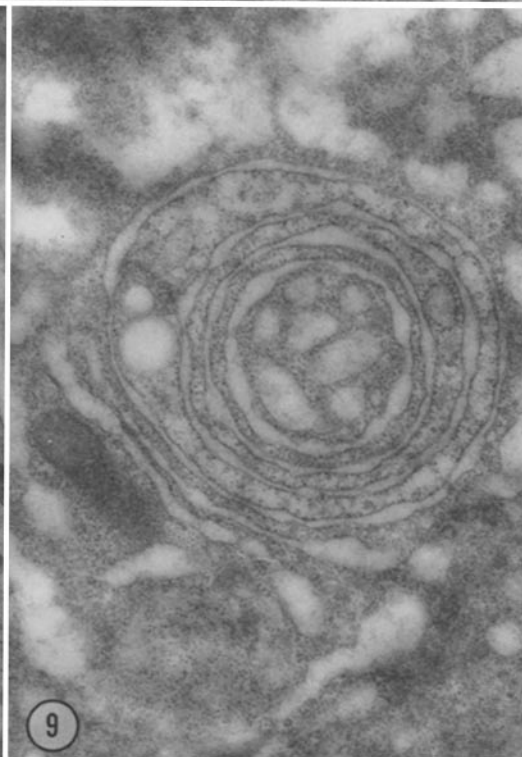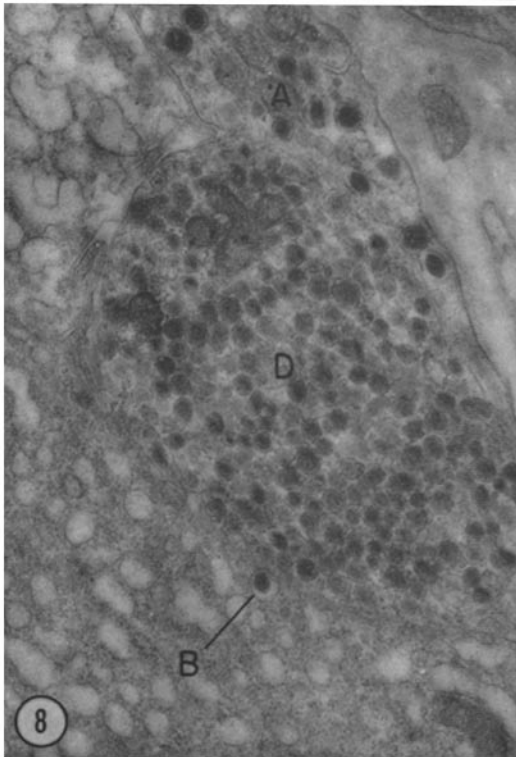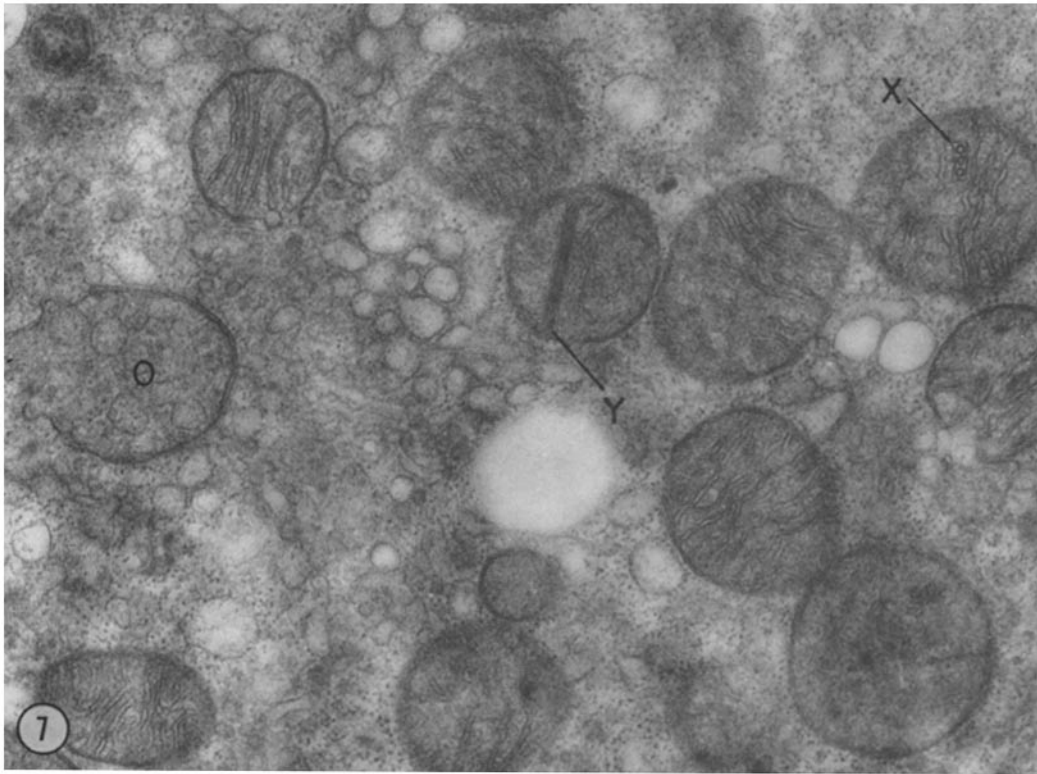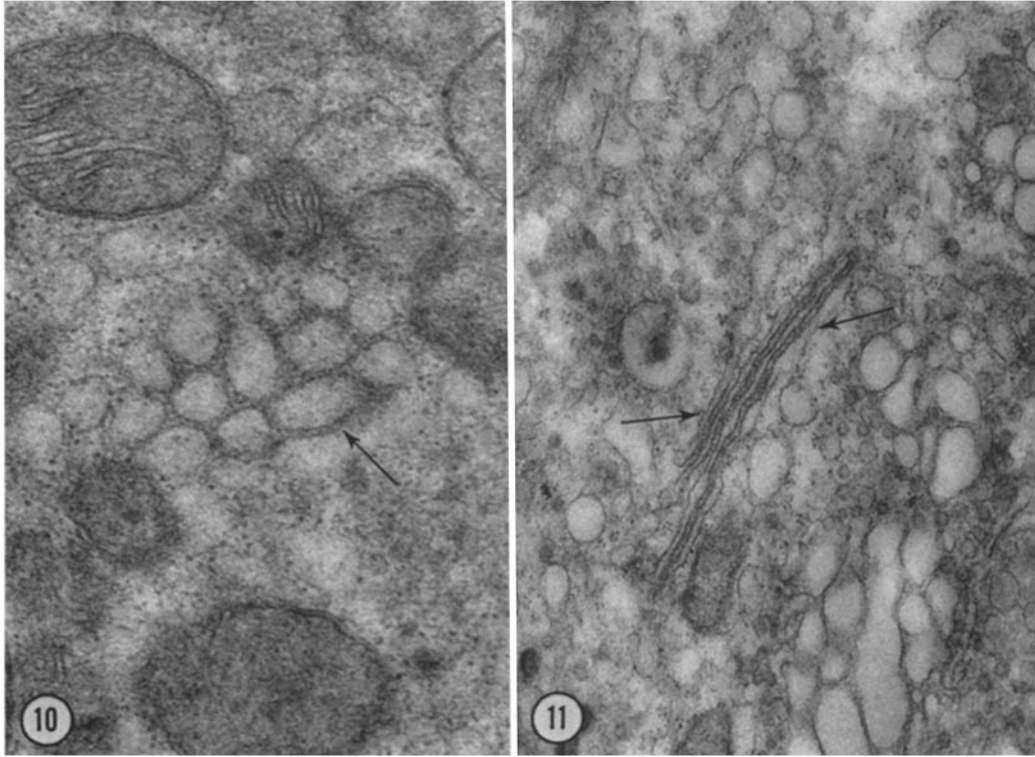
FIGURE 10   B cell after 3 injections of DHA. The structure at the arrow can be interpreted as a cross-section of the approximated membranes of the granular endoplasmic reticulum similar to those seen in Fig. 11. × 46,000.

FIGURE 11   B cell after 3 injections of DHA. Apposition of membranes of the granular endoplasmic reticulum results in a series of parallel electron-opaque lines (arrows). × 27,000.
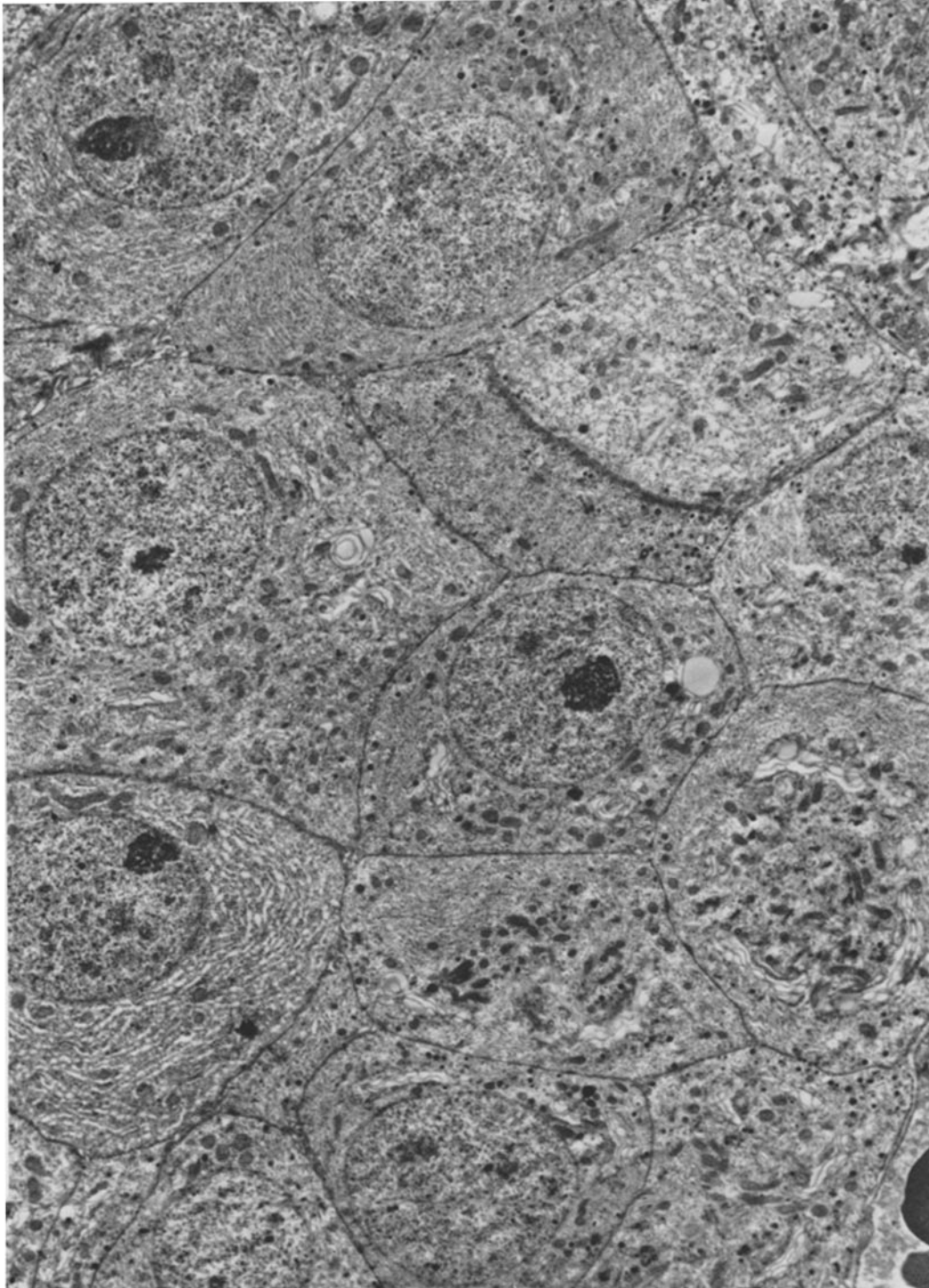
FIGURE 12 B cells 5 days after 3 injections of DHA. The B cells have secretory granules concentrated near the plasma membranes and near the Golgi apparatus. The granular endoplasmic reticulum is abundant, and in some cells it is organized into concentric lamellae. × 3,600.
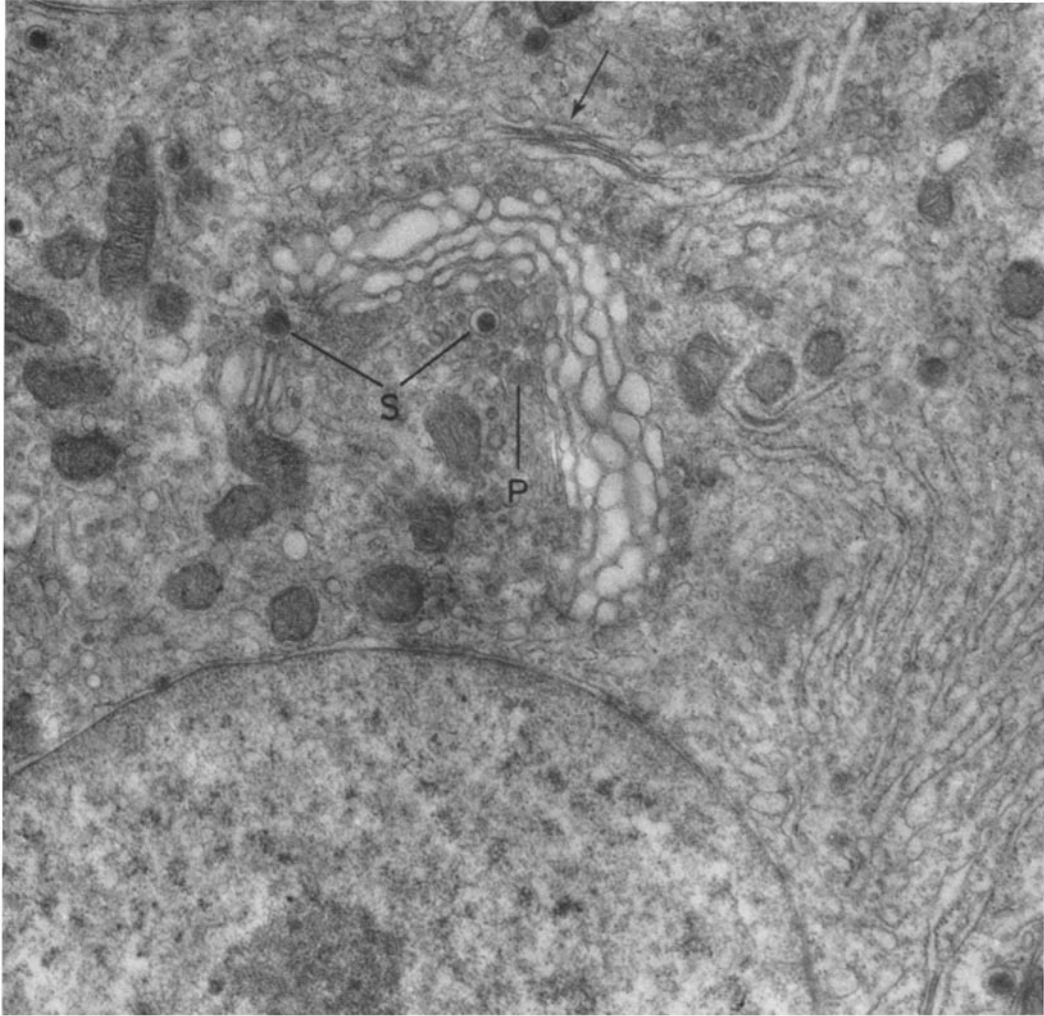
FIGURE 13 Same specimen as Fig. 12. Prosecretory (P) and mature secretory granules (S) are associated with a prominent Golgi apparatus. The granular endoplasmic reticulum is highly organized. Approximations of adjacent membranes of the ER can be seen (arrow) similar to those of Figs. 10 and 11. × 23,000.
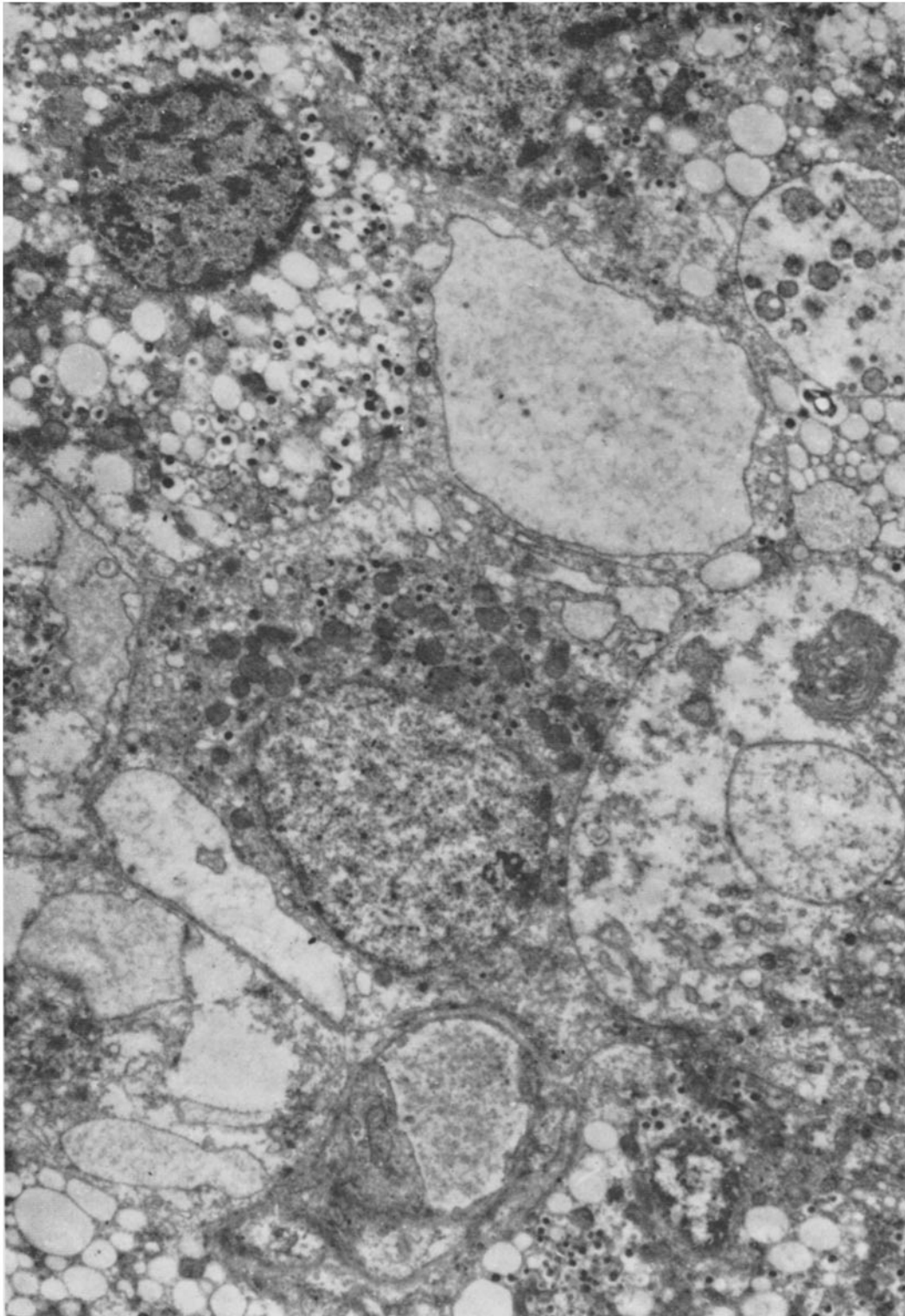
FIGURE 14 B cells 24 hours following alloxan administration. The B cells are markedly disorganized with nuclear and cytoplasmic alterations consistent with necrosis. Secretory granules are present in large numbers throughout the cells. × 6,500.