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SCHOOL OF DENTISTRY

Technical Report

EFFECTS OF ALTERED RNA METABOLISM IN SALIVARY GLANDS

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SUMMARY PAGE

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(f) Summary statement of the report which follows:

Efforts up to this point have been concerned with experiments aimed at clarifying the fine structural and functional modifications of salivary glands and pancreas in animals that were injected with actinomycin D, hypophysectomized or treated with anoxia. Following actinomycin D administration, the parotid gland cell undergoes a serious structural damage that is characterized by the reduction in size and organization of the Golgi apparatus, appearance of cytosegrosomes, and certain hitherto-undescribed nucleolar changes. Studies on the incorporation of radioactive uridine and leucine indicated that the synthesis of RNA and proteins is significantly suppressed. Quantitative radioautography of the salivary glands and pancreas in hypophysectomized and anoxia-treated animals has revealed a reduction in the capacity of these cells to incorporate tritiated amino acids into proteins. Electron microscopic observation of these glands is being presently carried out.

DETAILED REPORTS

The following are some of the major efforts that have been carried out with respect to experimental works on salivary glands.

A. Studies on the Effect of Actinomycin D

In an earlier light microscopic study of salivary glands of the rat, following an injection of a sub-lethal dose of actinomycin D, it was noted that the body weights as well as weights of the parotid and submandibular glands were significantly decreased during the first 7 to 14 days. The loss in weight was the greatest in the parotid gland. Histological and cytological observations indicated that the changes in secretory cells occurred in the following order: (1) Beginning of nuclear pycnosis with a rapid decrease in number and size of nucleoli by 24 hours after injection; (2) advanced stages of nuclear pycnosis with concomitant decrease in cytoplasmic basophilia and granules by day 3; (3) apparent vacuolization and rarification of cytoplasm by day 7; (4) beginning of nuclear and nucleolar recovery by day 10; and (5) complete cytoplasmic recovery by the end of the experiment, as evidenced by the return of basophilia and disappearance of vacuoles.

Paralleling the weight changes, these cytological damages were greater in the parotid gland than in the submandibular cells. This was thought to be related to differences in the nature and rate of synthesis of secretory products by the two organs. A decrease in number and pronounced irregularity in size of the granules in salivary duct cells of the submandibular gland were observed, but at a later time than the above-mentioned changes in the acini. Following this study a number of experiments were made in order to illuminate the underlying chemical and ultrastructural changes of the rat parotid gland caused by actinomycin D administration.

1. RADIOAUTOGRAPHIC STUDY OF LEUCINE-H^3 INCORPORATION

Adult male Sprague-Dawley rats were paired according to weight and one of each pair was injected with 0.125 μg actinomycin D per gram body weight. Three pairs as a group were sacrificed on days 1, 3, 5, 7, 10, 14 and 21 after the injection. One pair of these were intracardially injected with leucine-H^3 in the amount of 2.5 μc per gram body weight, 10 minutes prior to sacrifice, whereas the remaining two pairs were given the same injection 30 minutes and 4 hours before the sacrifice, respectively. Under ether anesthesia pieces of the parotid gland were dissected and prepared for radioautographic exposure in a routine manner. The slides were coated with Kodak NTB-3 liquid emulsion,

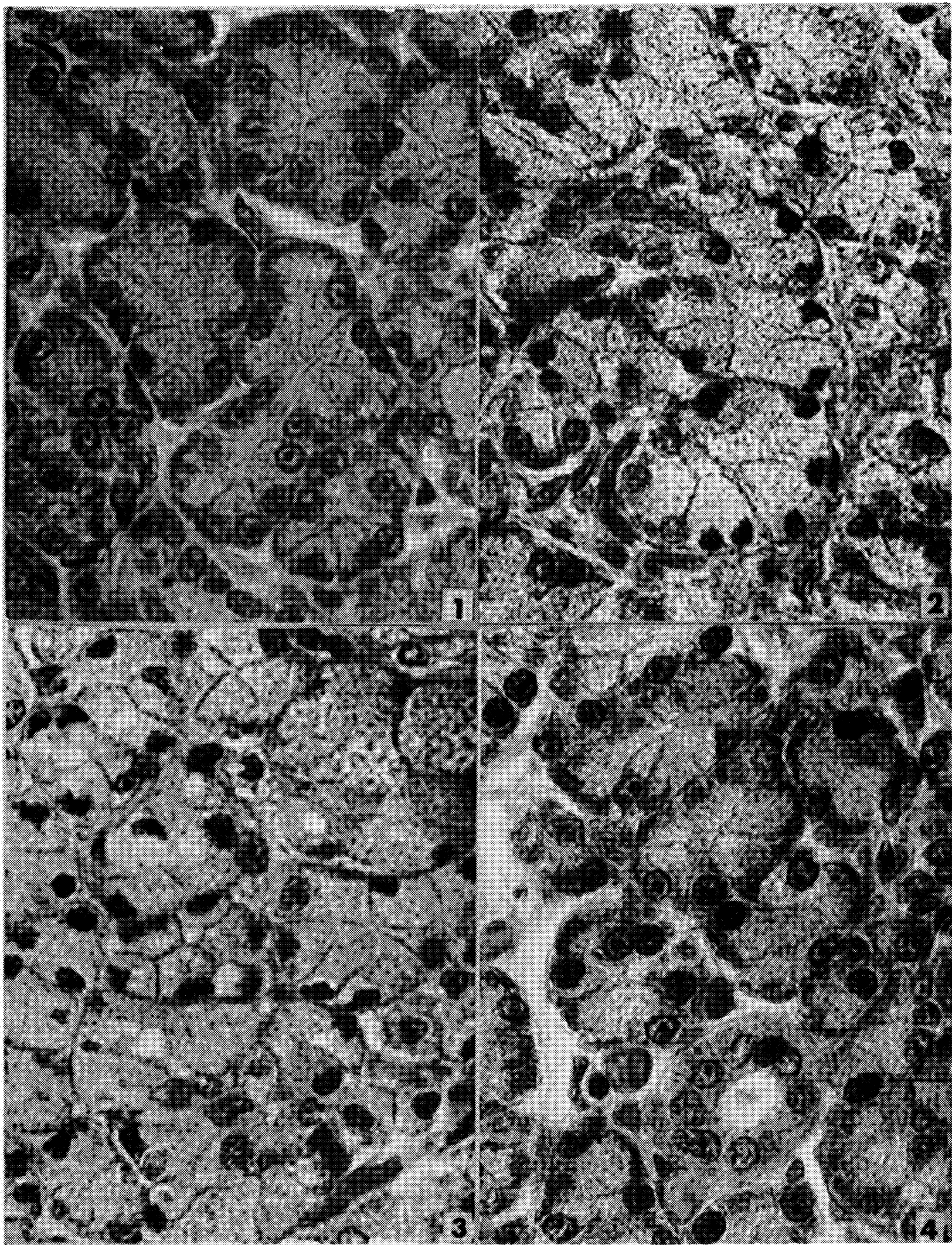
kept in an airtight box containing Drierite, and stored at 4°C. After 4 or 6 weeks of exposure, the slides were developed and stained with hemotoxylin and eosin before observation.

The microscopic appearance of the parotid cells confirmed data from previous studies. In control animals acinar cells were well defined, containing a distinct basophilic region at the base and a supranuclear accumulation of zymogen granules. The nucleus was large, rounded and vesicular in appearance, having one or more prominent nucleoli (Fig. 1). One day after the administration of actinomycin D the pycnosis of acinar nuclei became apparent. By day 3 many acinar cells had condensed pycnotic nuclei with an apparent reduction of the cytoplasmic basophilia (Fig. 2). By the end of the first week the nuclear pycnosis progressed further, and occasional vacuolization was seen in association with the nucleus (Fig. 3). The cytoplasm of the cell was less dense and gave the impression of a generalized rarification. By day 10 the nuclei started to return to normalcy, assuming a rounded contour with the reappearance of nucleoli, and by day 14 many acinar cells showed a fair amount of basal basophilia (Fig. 4).

In addition, radioautographic slides showed the following features: 10 minutes and 30 minutes after the injection of leucine-H^3, silver grains were more-or-less diffusely distributed throughout the gland cells, both in the control and in the experimental animals, although in the former group, more silver grains appeared to be concentrated in the basal and supranuclear region of the cell after 30 minutes of leucine-H^3 injection. By 4 hours after the injection, the number of grains in experimental animals were reduced, and they were distributed evenly throughout the entire cell (Fig. 5). This was most obvious in the day 7 animals. In contrast to this, cells from control glands after 4 hours of leucine-H^3 injection showed a definite concentration of silver grains in the apical cytoplasm, which corresponded to the area of zymogen accumulation (Fig. 6).

Thus the results from radioautographic studies confirm the previous data and provide for further support that actinomycin D does induce serious morphological defects in cells of the parotid gland, which produce proteinaceous enzymes. Furthermore, the differences in appearance of grain distribution observed in radioautographs of the day 7 rat, especially after 4 hours of leucine-H^3 injection, indicate a definite damage done to the synthetic machinery responsible for protein biosynthesis.

The work by Warshawsky and others has also shown that at 4 hours leucine-H^3 accumulates maximally in the zymogen region of pancreatic acinar cells, which are not unlike parotid gland cells, although our recent observations indicate that the pancreatic cells show a more discrete pattern of grain distribution than the parotid cells.



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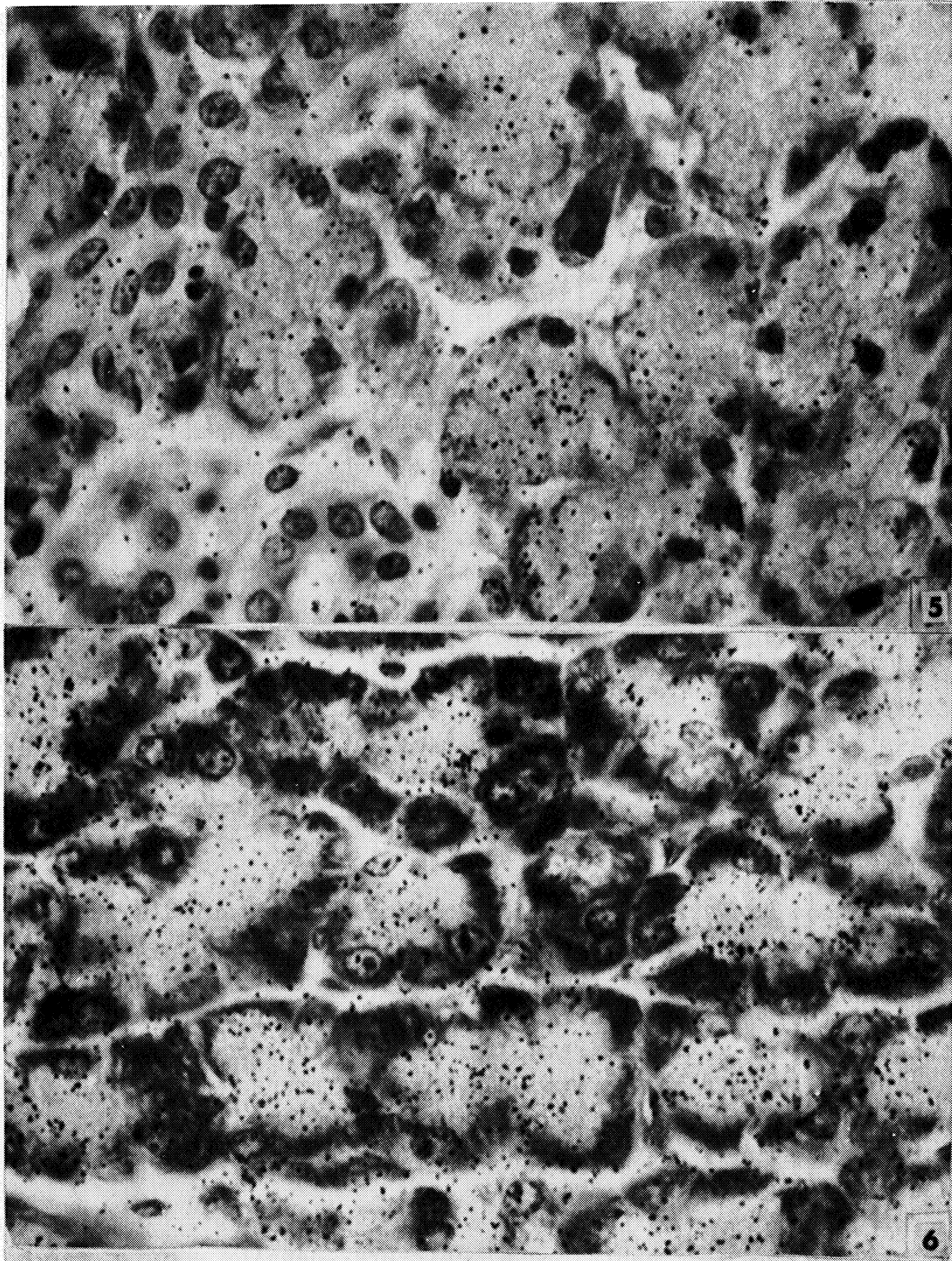
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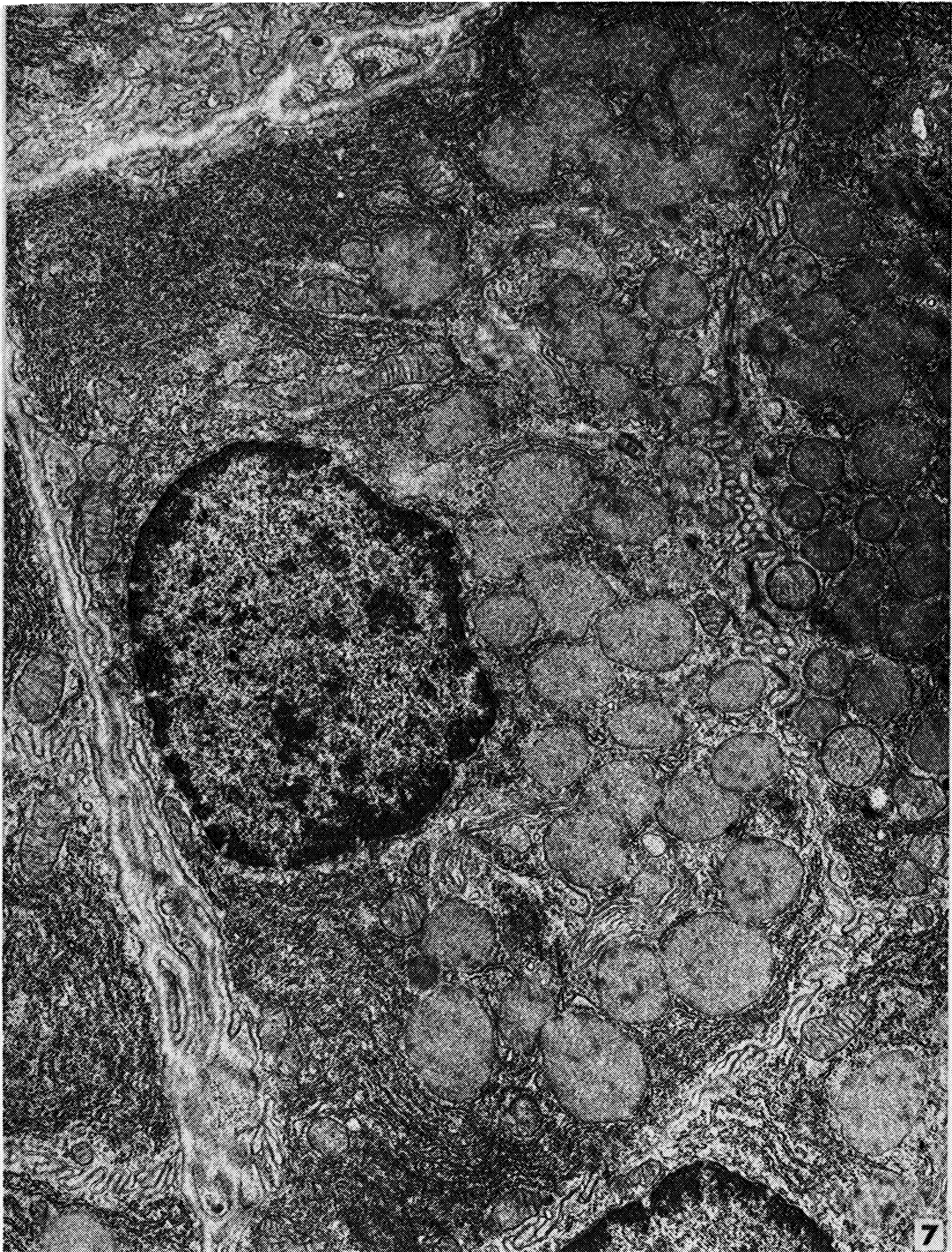
2. ELECTRON MICROSCOPIC OBSERVATIONS OF THE PAROTID GLAND FOLLOWING ACTINOMYCIN D ADMINISTRATION

The materials for electron microscopic observations were taken from rats which were treated in a manner identical to that given to the rats used for radioautographic studies. Small pieces of the parotid gland were dissected and fixed in either 2% OsO_4 in 0.1 M phosphate buffer at pH 7.4, or in 2% glutaraldehyde, followed by post-fixation in OsO_4. Tissues were dehydrated, embedded in epoxy resin, and stained with phosphotungstic acid and uranyl acetate.

For the convenience of description, most of the micrographs presented in this report will be from rats sacrificed 7 days after the actinomycin D administration. As has been shown by previous workers, acinar cells from control animals contained numerous profiles of regularly arranged rough-surfaced endoplasmic reticulum (RER) which were basally located, and occasional mitochondria being interposed between membranes of the RER (Fig. 7). Supranuclearly, a large Golgi complex was located. The Golgi complex was made up of 4 or 5 units, each one of which was composed of several stacks of flattened membranes and a large number of small vesicles and a few vacuoles (Fig. 9). Elsewhere, the apical cytoplasm was filled with zymogen granules. Under conditions of OsO_4 of fixation, zymogen granules had a somewhat homogeneous, finely granular interior of moderate electron density, and the surrounding membrane often showed irregularities and possible breakages. Only occasional granules showed the bizonal appearance; that is, a darker outer zone with a lighter interior.

In comparison to this, the cells from experimental animals were usually smaller, containing fewer profiles of the RER in the basal region (Fig. 8). Despite the reduction in total number of ribosomes in experimental animals, a fair number of polyribosomes was seen whenever the RER was oriented in a favorable plane. The Golgi apparatus was definitely reduced in its degree of organization, and only 1 or 2 units of stacked membranes were visible in any single cell at a given plane of section (Fig. 8). Individual units of the Golgi complex were small and were devoid of or drastically reduced in number of the vesicles (Fig. 10).

Many zymogen granules contained a peripheral density showing the bizonal appearance, while others have lost the clarity of their outer limiting membrane (Figs. 8 and 10). In certain cells, zymogen granules formed aggregates of various sizes by fusion of several to a dozen or more granules (Figs. 11 and 12). Although the preservation of membranes surrounding the circumference of such aggregates was questionable in osmium-fixed preparations, clear-cut limiting membranes were observed following glutaraldehyde fixation (Figs. 13 and 14). Occasionally the aggregates contained other cytoplasmic organelles, such as ribosomes, RER, mitochondria, and what appeared to be lipid droplets. In many instances the individual zymogen granules contained within the aggregate body maintained their own limiting membrane. They were similar in appearance to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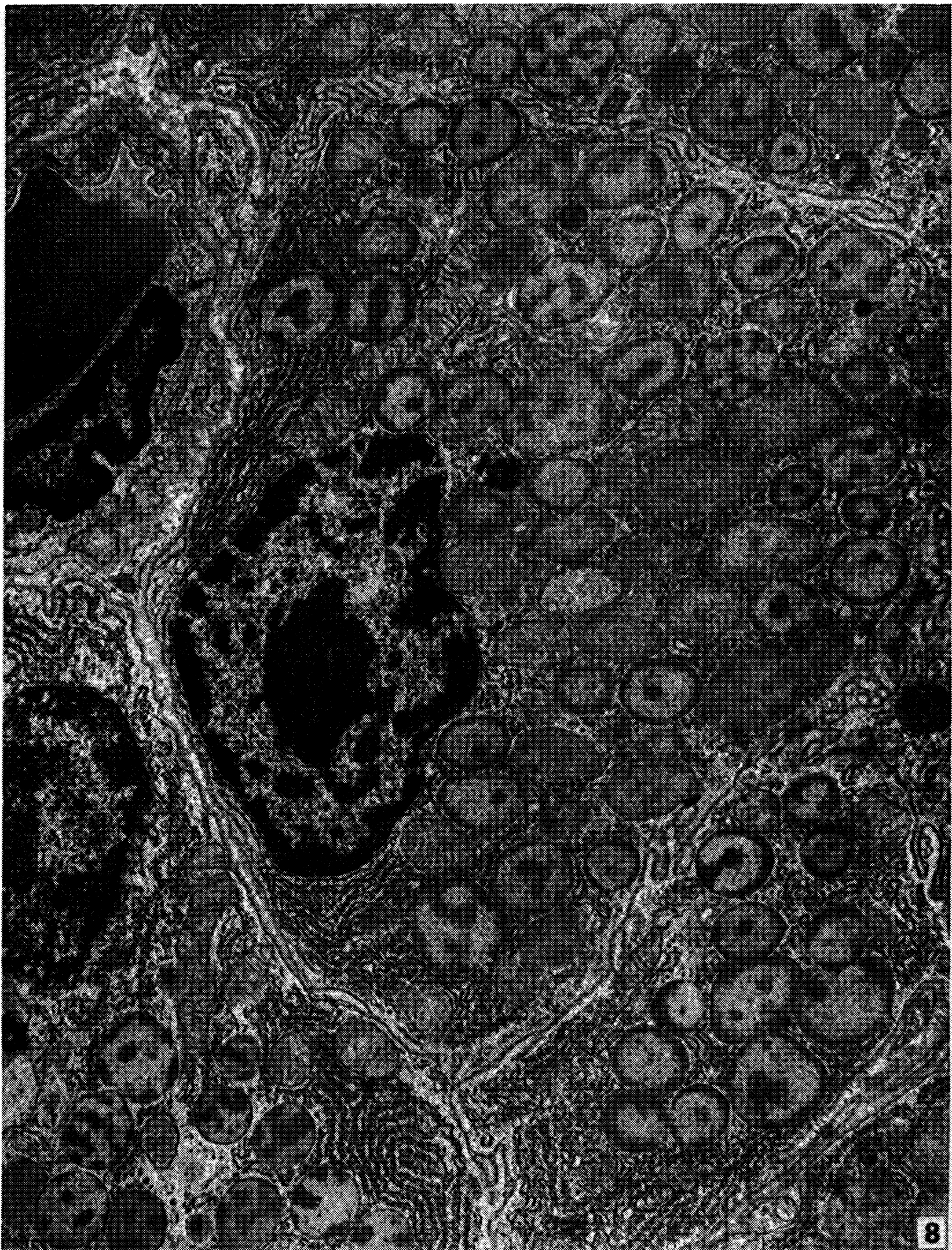
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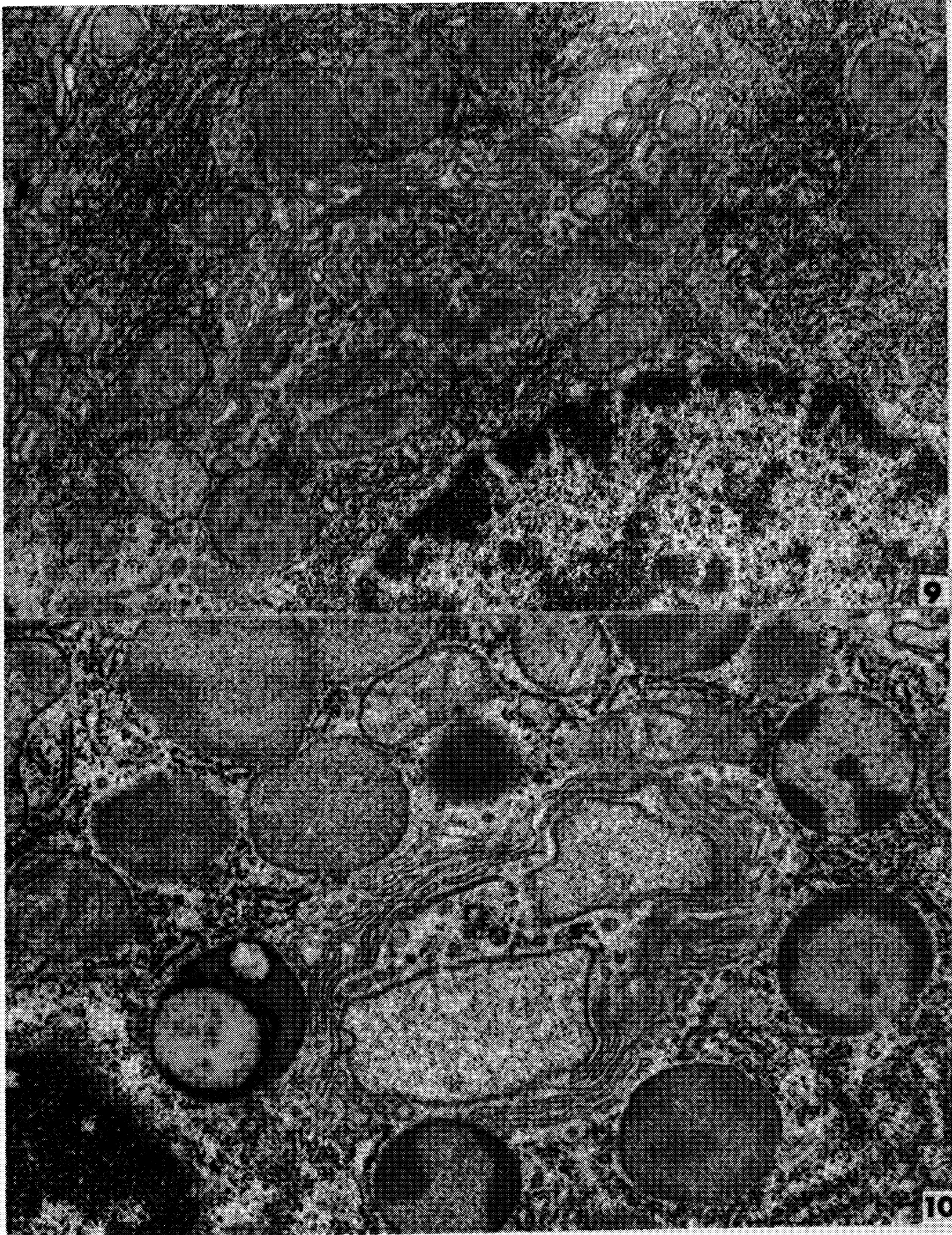
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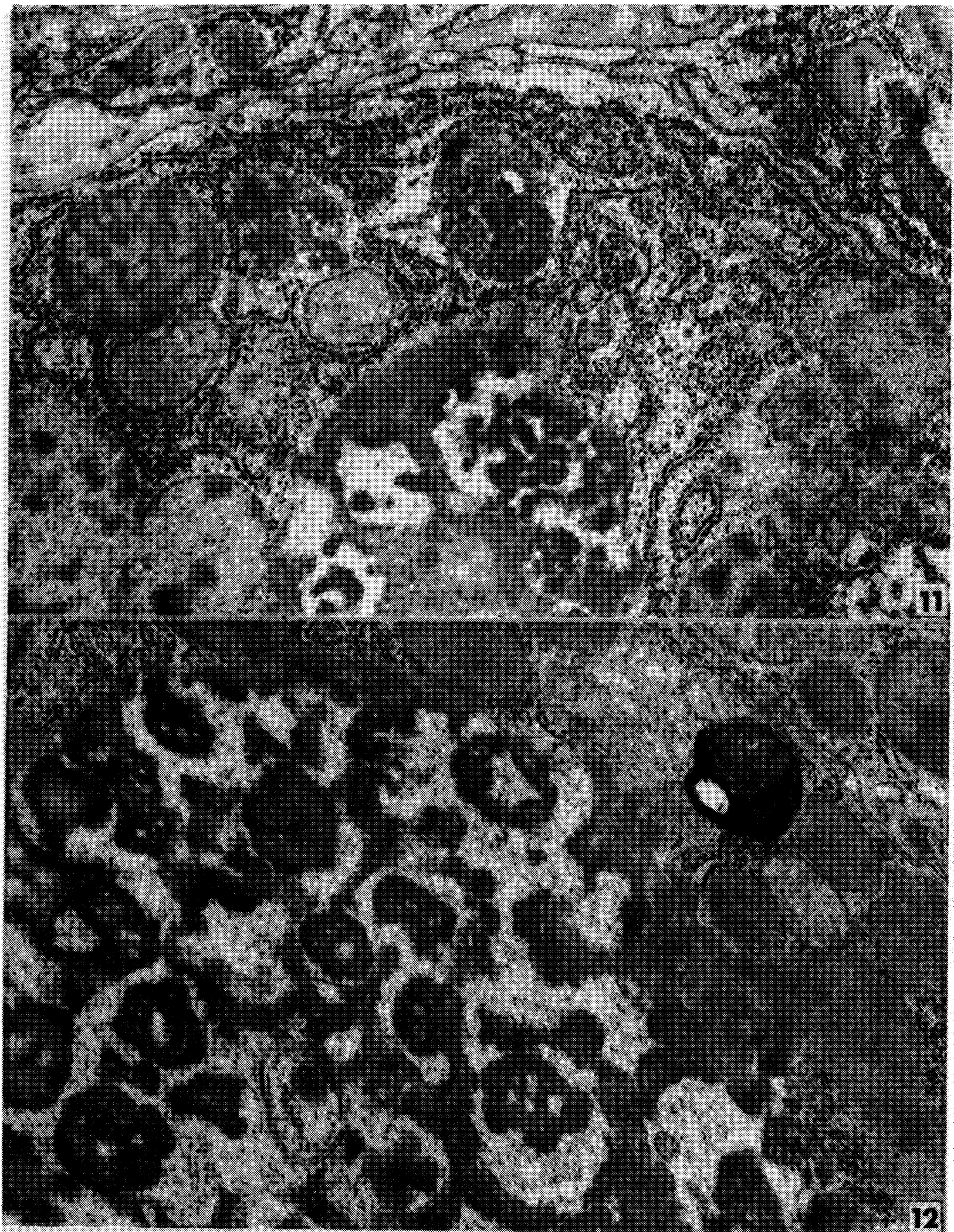
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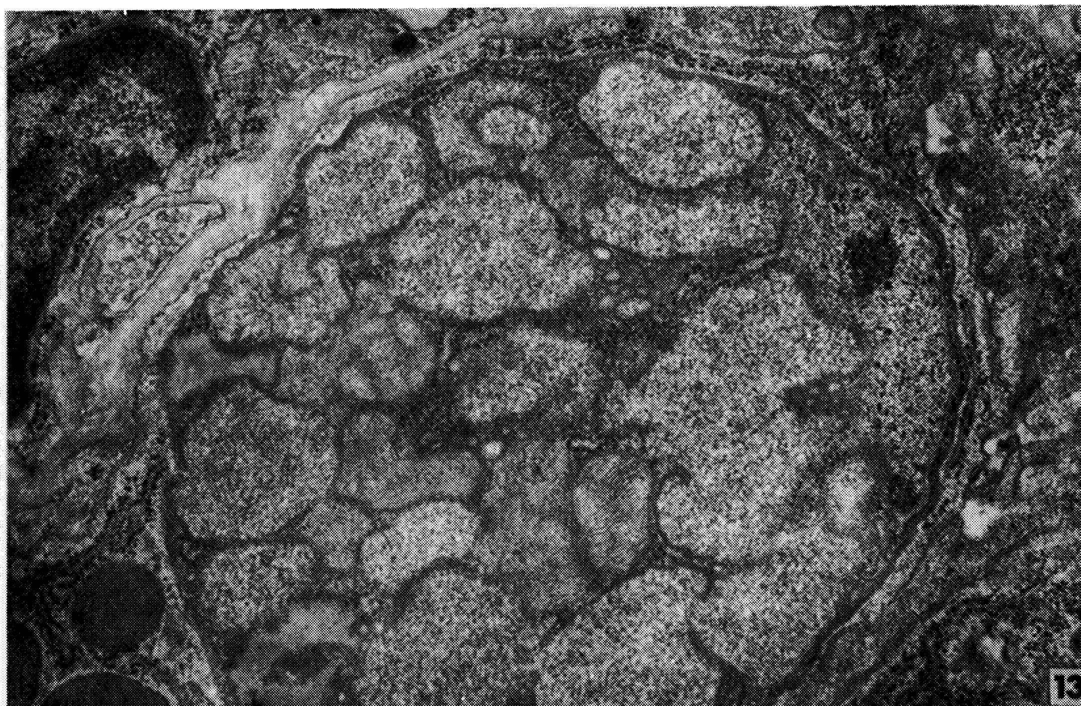
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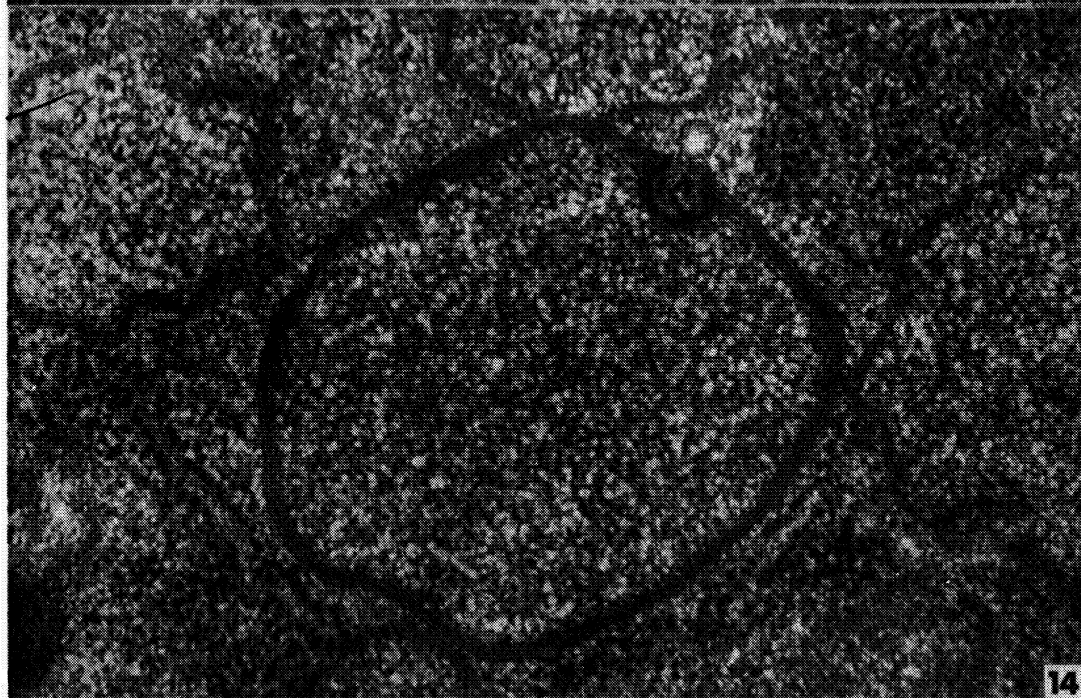
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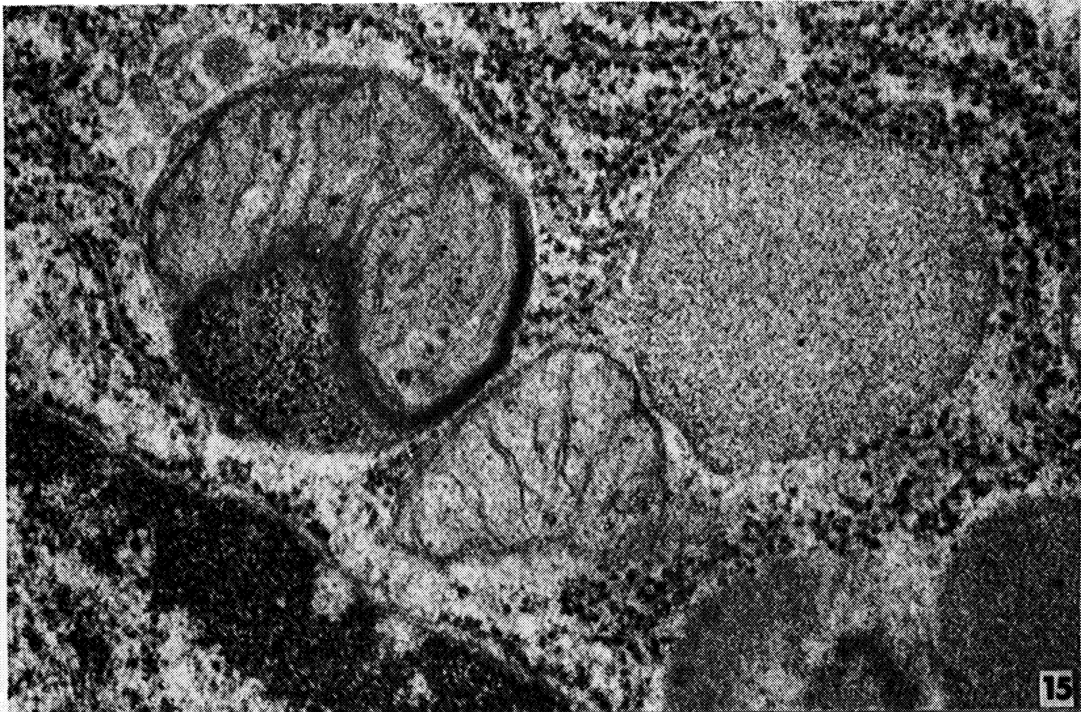
typical cytolysomes, which primarily contained mitochondria and degraded products of the RER (Figs. 15 and 16), and increased in number towards the end of the first week. A number of lipid droplets appeared at this time, especially in the basal region of the cells, some of them in close proximity to the nuclear membrane (Fig. 17). Large crystalloid structures were often present in acinar cells (Fig. 18).

Modifications of the nuclear structure induced by actinomycin D in the parotid acini were of the following two types: first, there was a remarkable reduction in size of the nucleoli, which appeared to have lost most, if not all, of the 150Å units granules associated with the structure (Figs. 19 and 20). The second change was the formation of small encapsulated areas of nucleoli (Figs. 21 through 24). It appeared that a finally-filamentous substance developed around pieces of degenerating nucleoli, which, as judged by observations on a large number of sections, would eventually surround and separate the portion of the nucleoli from the rest of the cytoplasm, forming a spherical body. Such encapsulated bodies contained ribosome-like granules as well as dense homogeneous portion of the nucleoli (Figs. 23 and 24).

Essentially no alterations were observed in the structure of intercalated duct cells, which appeared to maintain a normal complement of the RER, ribosomes, a small Golgi apparatus, and mitochondria (Fig. 25). No apparent changes were observed in the intercellular relationship. Some of the cells making up the secretory duct, however, showed the following differences (Figs. 26 through 28). The appearance of mitochondria, basal infoldings of the plasma membrane, and intercellular interdigitations were largely unchanged, although mitochondria often became smaller and denser than those of control animals. More significantly, small vacuoles of irregular shape, which characterized the apical cytoplasm of secretory duct cells, were increased in number. They were also larger, and many of them showed an irregular stellate contour containing a fairly electron dense matrix (Fig. 28).

These electron microscopic observations provide definitive support for the conclusion derived from the radioautographic data. Thus, all intracellular structures identified so far to be parts of the synthetic apparatus for proteins showed serious reduction in their degree of organization or in their number. In addition, the appearance of other structures that have been observed in situations of altered glandular metabolism, such as the formation of crystalloids or fat droplets, add to the evidence that the physiology of the acinar cells is altered. Of interest to note is the persistence of polysomes in insulted cells, which might be due to the small dose employed in this study. However, it is also possible that they represent more resistant polyribosomes, as implied by previous biochemical studies of Staehelin and others, which showed only 50 to 80% breakdown of polyribosomes, even after a massive amount of actinomycin D capable of total inhibition of RNA synthesis.

It is difficult to assess, although highly desirable, exactly what is the primary morphological effect of any insult on the cell. In electron microscopy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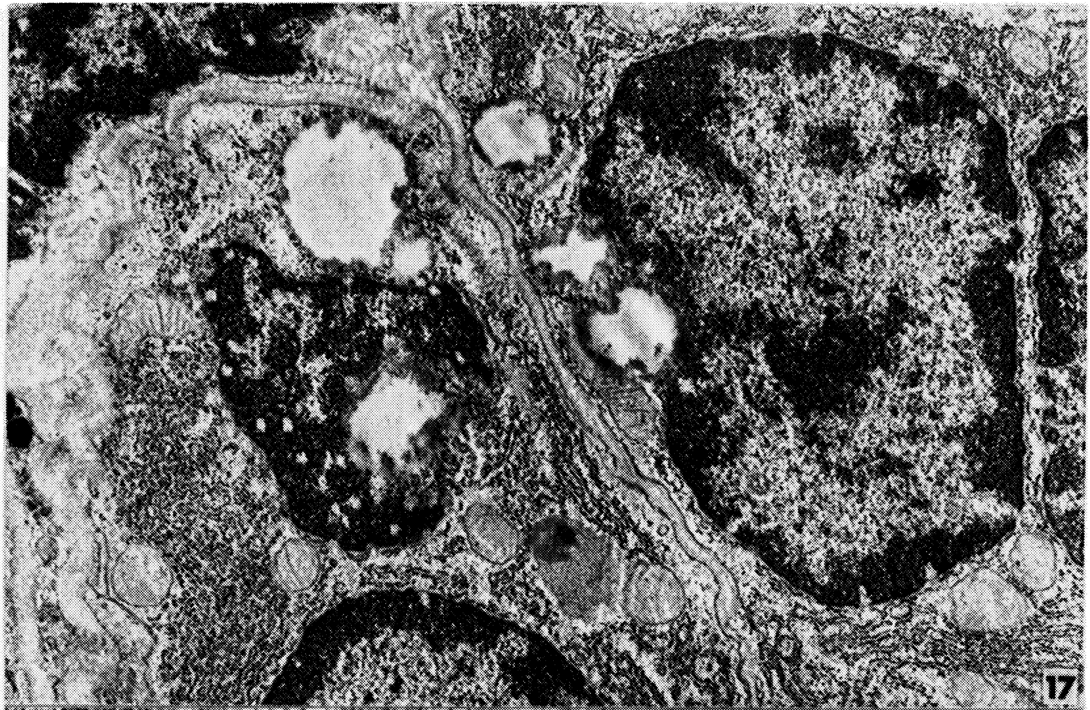
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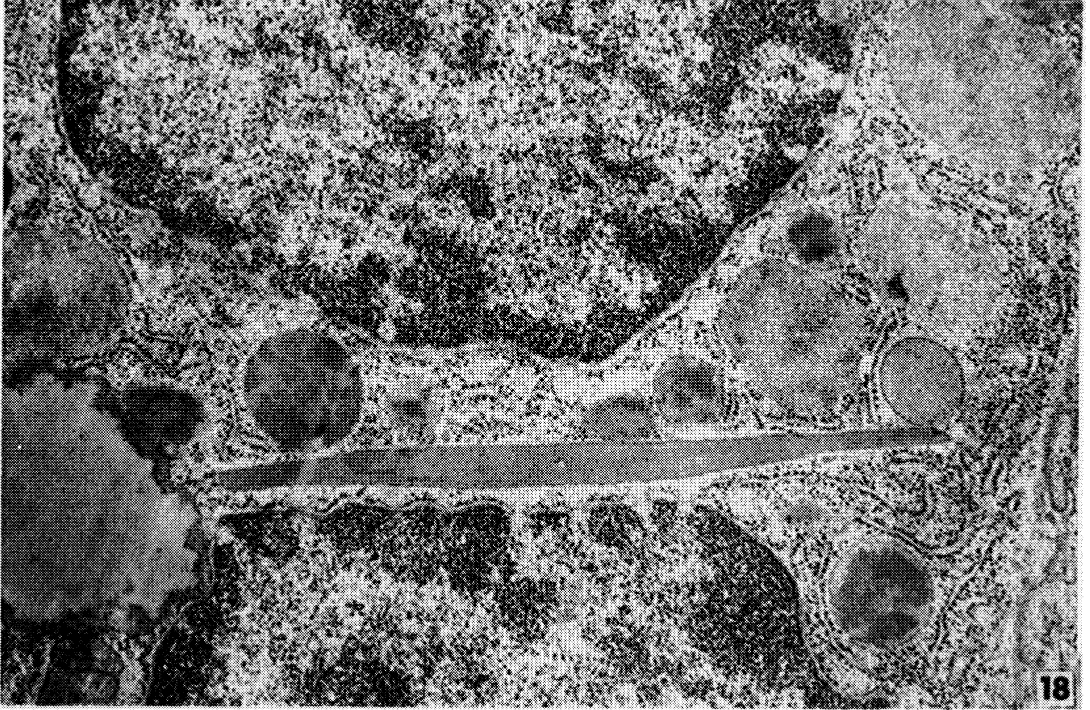
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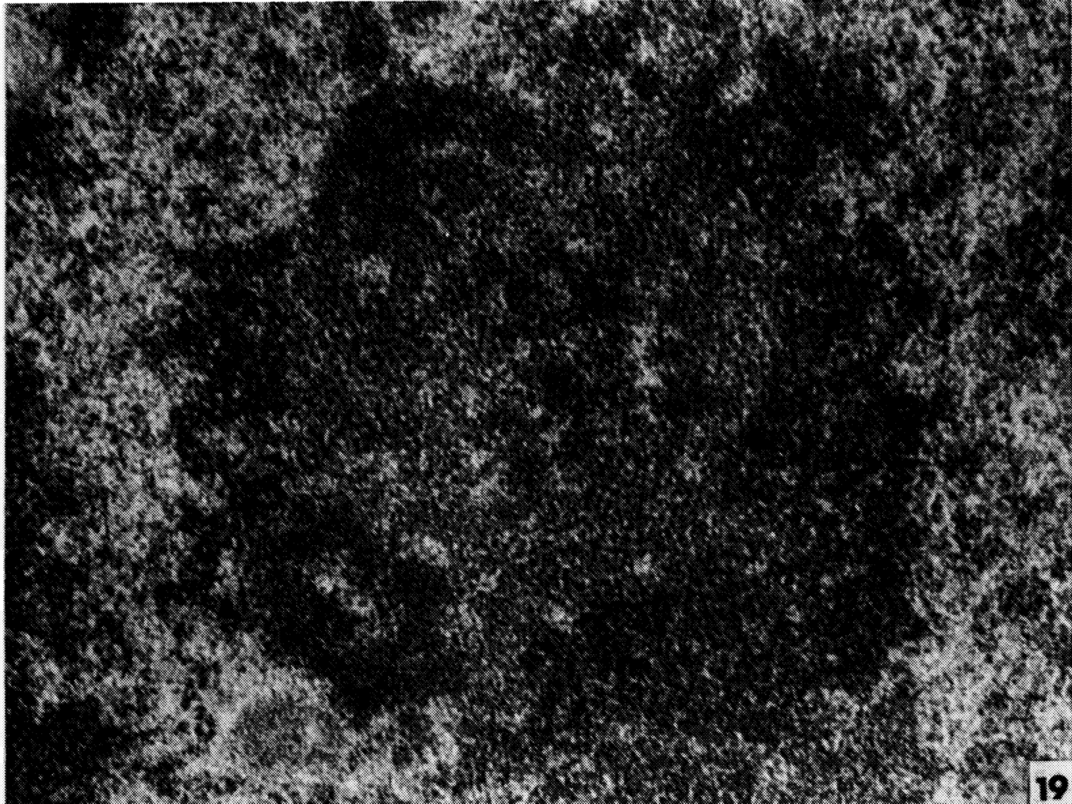

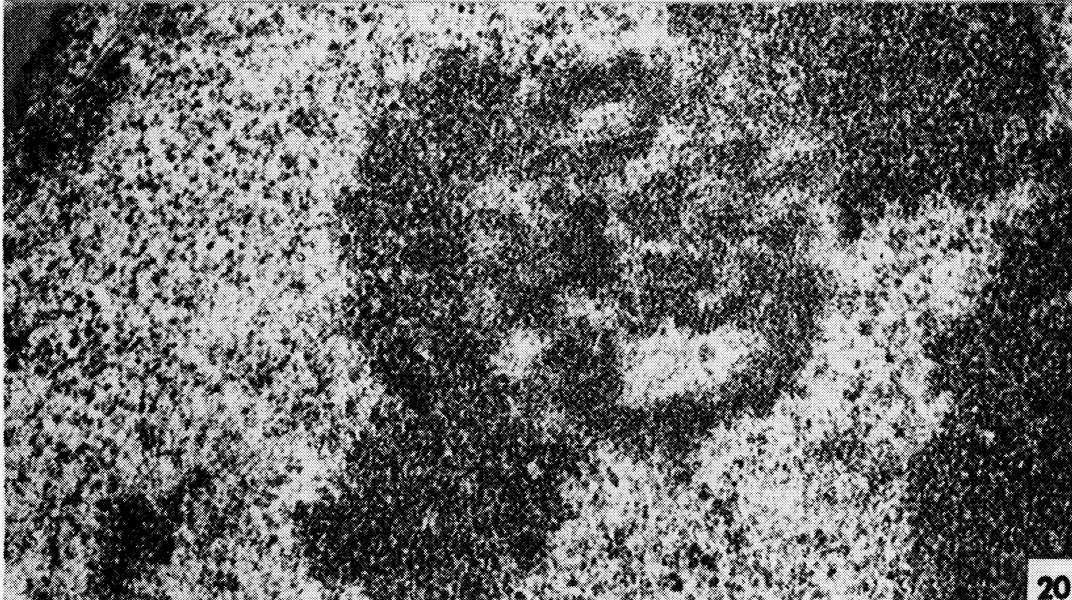

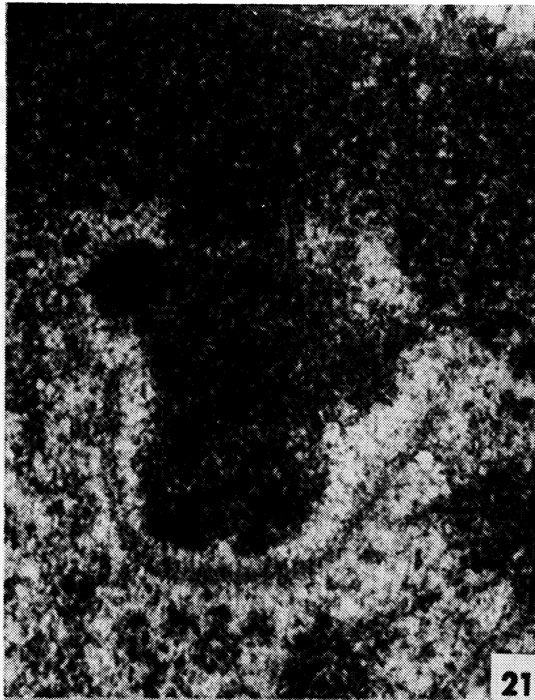
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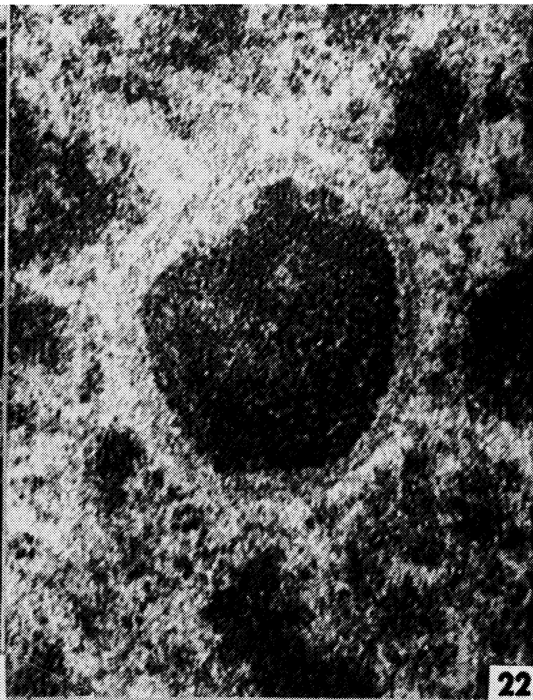
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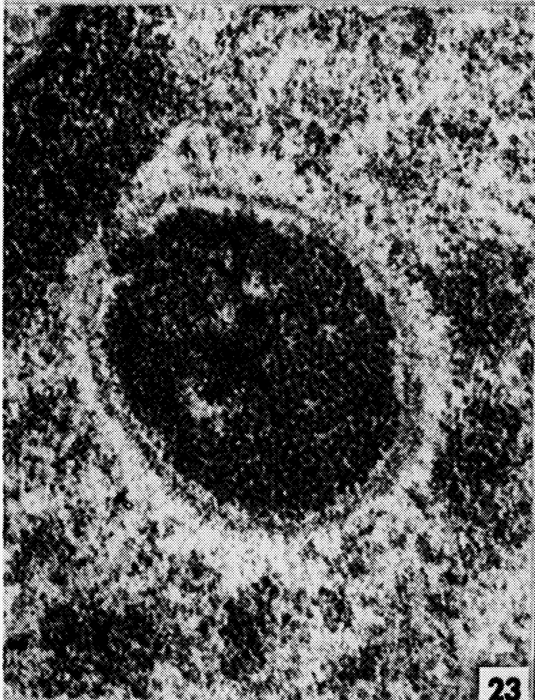
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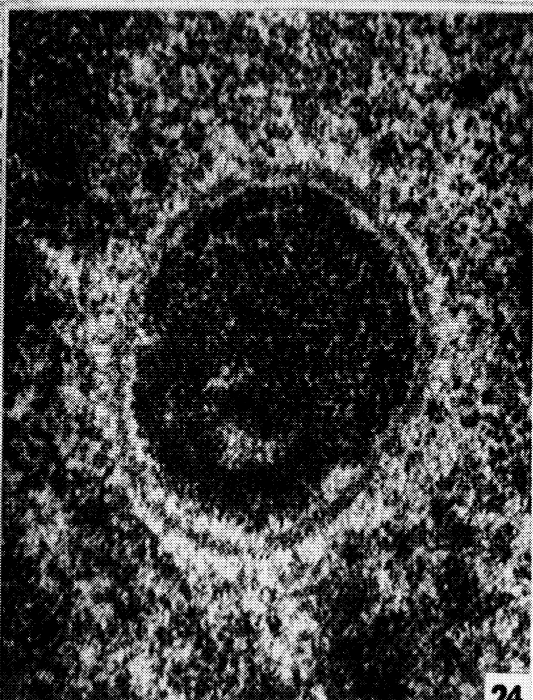
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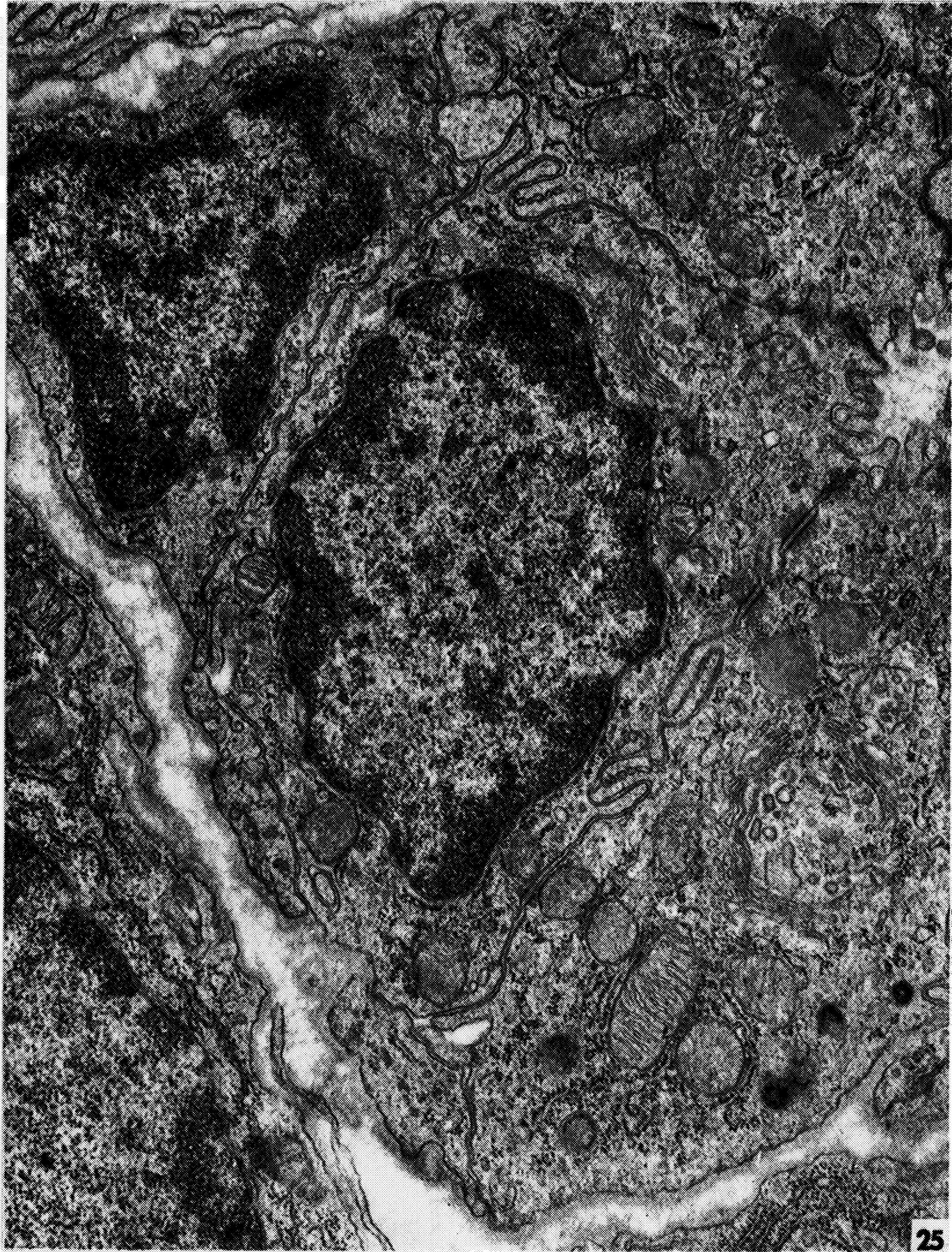
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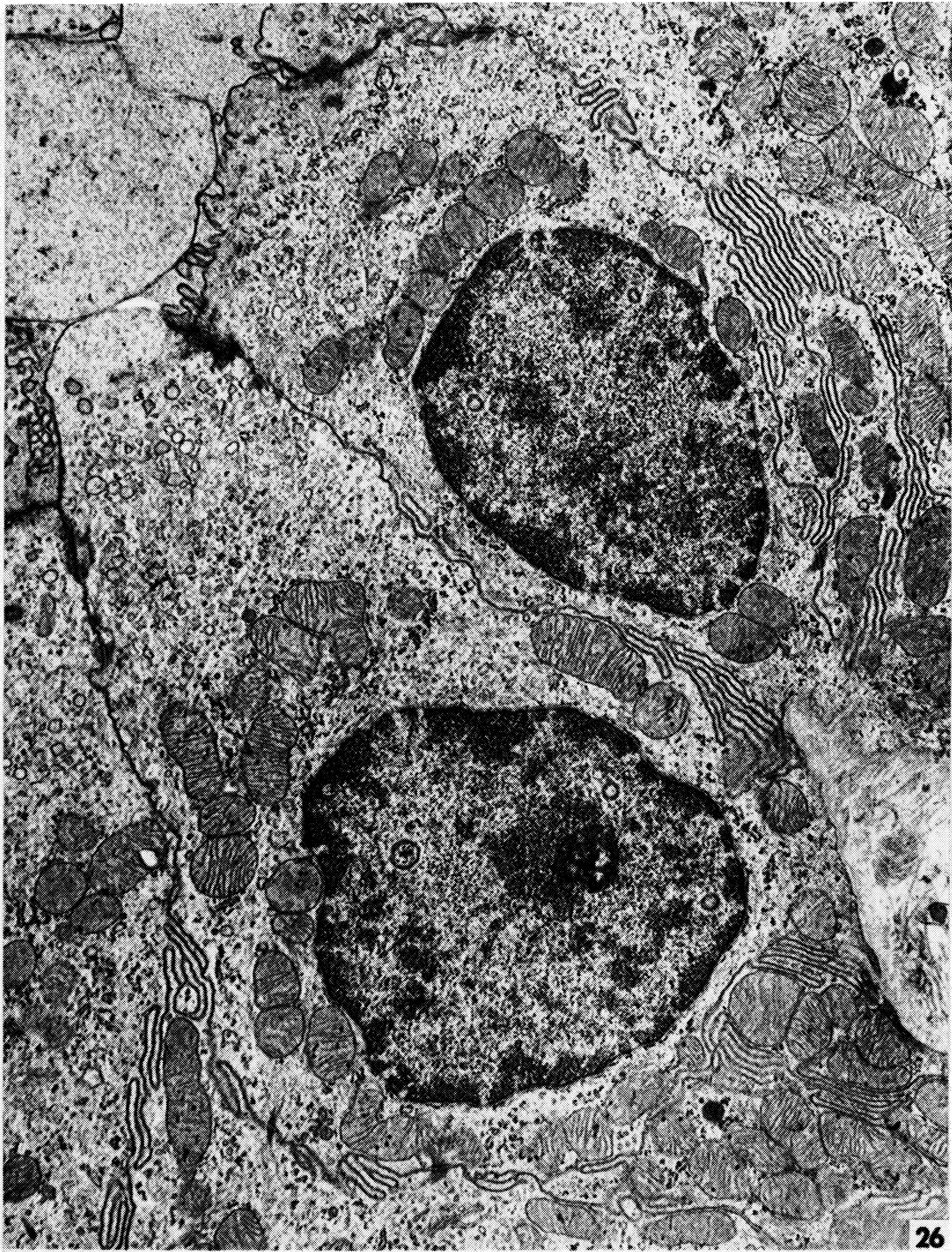
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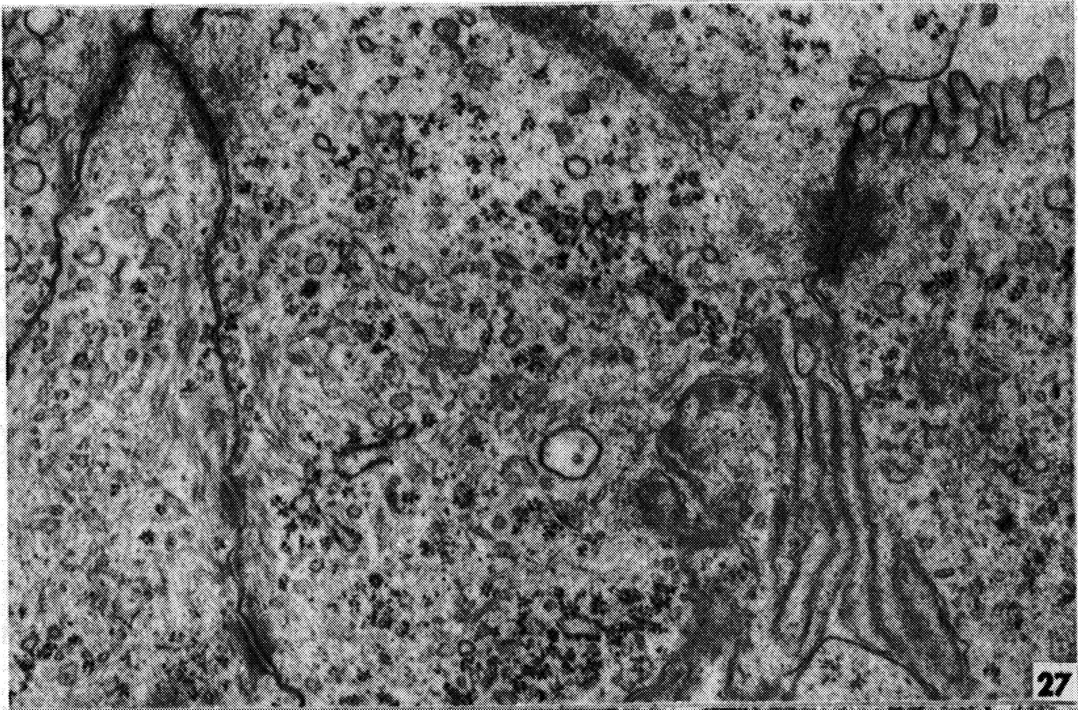
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the difficulty is compounded because of problems related to differentiating a real effect from an artifact and quantitating changes. Based on what has been known from biochemical studies, one might define the primary structural effect of actinomycin to be changes in the nucleolar structure and the number of ribosomes, whereas alterations of the membrane system of the RER, Golgi apparatus, and the number of zymogen granules, might be taken as necessary consequences, secondary to the reduced secretory function. The formation of cytolysomes, lipid droplets, and crystalloids, can be regarded as further degrading changes related to an abrupt imbalance of various intermediary metabolic processes, which might also come after other types of physiologic insults. For instance, the formation of lipid droplets in fibroblasts from scorbutic guinea pigs were related to a reduced synthesis of collagen in healing wounds (Ross and Benditt, 1964), and the increase in cytolysomes has been related to various aspects of reduced cellular functions (Norikoff, 1964; and others).

Nucleolar alterations observed in this study are somewhat different and unique from the acute effect of actinomycin D observed in pancreatic acinar cells and cultured kidney cells by Jezequel (1964) and Schoefle (1964). Rather than showing the segregation of nucleolar component, nucleoli of the experimental animals in our hands showed a drastic reduction in number of ribosome-like granules, and might be of significance in view of the role nucleoli play in RNA metabolism (Perry, 1963). The formation of incapsulated bodies within the nucleus has been occasionally observed in some of the normal lymphoid cells as well as in duct cells of salivary glands, although none of these previously noticed intranuclear bodies contained what could be identified as materials originating from the nucleolar structure, namely, the ribosome-like granules and the homogeneous dense substance of the nucleoli.

However, the real significance of the nucleolar incapsulation remains to be elucidated. It is tempting to speculate the following possibilities on purely logical grounds. It might represent a means by which the cell might preserve a vital intranuclear organelle concerned with RNA metabolism during a period of time when RNA synthesis is severly restricted, or, alternately, it might be an expression of the final stage of nucleolar deterioration, which result in the extrusion of the incapsulated matter into the cytoplasm. The latter possibility is particularly intriguing, since the nuclear extrusion has been observed in other types of cells (Bak, 1966). Furthermore, the extrusion might be a mandatory affair, if the content of the incapsulated bodies were degraded masses destined to be hydrolyzed by lysosomal enzymes which so far as is known are exclusively cytoplasmic.

The fact that the cells of the secretory duct showed accumulation of apical vacuoles with an increasing electron density of their content might be an expression of a possible retension of secretory products as the result of poor functioning of the duct cells. In fact, a similar situation was observed in following fasting and atropin administration, conditions which are known to suppress duct functions.

3. BIOCHEMICAL DETERMINATIONS OF URIDINE-H^3 AND LEUCINE-C^{14} IN ACTINOMYCIN-TREATED RATS

Since the short-term experiments of Jezequel and others in which lethal dose of actinomycin D were administered have indicated that in the rat pancreas, the nucleoli underwent irreversible structural damage, a series of biochemical experiments was made in order to determine the extent of effect on RNA and protein syntheses by the parotid and pancreas after different doses of actinomycin administration. The following represents the biochemical aspects of these experiments.

In each experiment of this series, 6 to 8 pairs of rats were used. An intraperitoneal injection of actinomycin in the dose of 200 or 1,000 μg/kgm of body weight was given to the experimental animal of the pair. The control rat was given an equivalent amount of isotonic saline. Following the administration of actinomycin D the animals were given either 15 μc/kg of leucine-C^{14} (U.L.) in 0.3 ml isotonic saline, 1 hour prior to sacrifice, or 50 μc/kg of uridine-H^3 in ml isotonic saline, 30 minutes prior to sacrifice.

The animals were sacrificed under ether anesthesia at intervals of 1, 3, 5, 8 or 24 hours after the actinomycin D administration. The parotid gland and pancreas were excised and immediately frozen on dry ice. The tissues were weighed just prior to the extraction and assay procedures. For determination of the amino acid incorporation, protein was extracted by the method of Siekevitz (1952) modified as follows: After cold and hot TCA extraction and washes with 95% ethanol and a 2:2:1 mixture of alcohol: ether :chloroform, the solvents were removed by suction filtration and the residue was air-dried. This residue was then weighed in a tared counting vial, and was then dissolved in 1 ml of hydroxide of Hyamine. The vial was heated for 30 minutes at 65°-70° to dissolve the residue. To this, 3 ml of absolute ethanol and 10 ml of flour composed of 100 mg dimethyl-POPOP and 3 gm PPO per liter of toluene. Following the preparation the radioactivity of the sample was determined by a Packard Tricarb liquid scintillation spectrometer.

The extraction of RNA was performed according to the method by Schmidt and Tannhauser. The radioactivity was measured in aliquots of the supernatant fraction containing RNA. Total RNA was spectrophotometrically measured by determining the absorption of the same supernatant fraction at 260 mμ.

The results are summarized in Tables I and II. It may be noted that the incorporation of uridine-H^3 into RNA in control animals might be about the same in both glands, and that the effect of the actinomycin D administration is clearly observed in both organs, especially following the administration of 1,000 μg/kg body weight. The effect appears to be most dramatic at 8 and 24 hours following the treatment. With the dose of 200 μg/kg body weight, only a slight suppression is noted in the parotid gland, whereas the suppression is more obvious and observed earlier in the pancreas.

TABLE I

EFFECTS OF ACTINOMYCIN D ON URIDINE-H^3 INCORPORATION

(DPM/gm protein)

Organ	Dose (μg)	Hours after Injection 1	3	5	8	24
Pancreas	0	923 (96)*	1025 (205)	1080 (125)	1268 (98)	1321 (180)
	200	258 (38)	730 (57)	1020 (89)	935 (25)	891 (123)
	1000	425 (21)	731 (118)	687 (63)	280 (51)	310 (17)
Parotid	0	754 (82)	1240 (103)	1104 (145)	1352 (72)	1405 (122)
	200	482 (30)	862 (120)	855 (67)	983 (128)	958 (81)
	1000	515 (12)	613 (43)	653 (39)	205 (27)	243 (22)

* ± Standard Deviation in Parentheses

TABLE II

EFFECTS OF ACTINOMYCIN D ON LEUCINE-C^{14} INCORPORATION

(DPM/gm protein)

Organ	Dose (μg)	Hours after Injection 1	3	5	8	24
Pancreas	0	2286 (302)*	2472 (144)	2533 (420)	2123 (267)	2181 (368)
	200	2402 (145)	2454 (168)	2245 (115)	2397 (301)	2256 (228)
	1000	2240 (168)	2672 (309)	2336 (253)	2262 (189)	2302 (98)
Parotid	0	225 (14)	242 (22)	240 (12)	265 (12)	252 (18)
	200	212 (10)	169 (19)	165 (20)	180 (9)	123 (30)
	1000	128 (8)	132 (12)	153 (17)	178 (21)	68 (14)

* ± Standard Deviation in Parentheses

Contrary to the effect on RNA synthesis the incorporation of leucine-C^{14} into protein fraction showed rather dramatic difference between the two organs. In the control animals one may notice that the pancreas is 8 to 10 times as fast in incorporating the amino acid. In animals receiving actinomycin D, it is apparent that the pancreas is not affected even with the higher dose throughout the experimental period, whereas the suppressive effect is obvious in the parotid gland even within an hour at 1,000 μg/kg dose. This combined with the pictures of the RNA synthesis, tempts one to arrive at the conclusion that the actinomycin D, in the dose used under these experimental conditions, seriously suppresses the synthesis of proteins in the parotid gland within a relatively short period of time, while little effect is noted in the pancreas taken from the same animals.

Since the nucleic acid synthesis is suppressed in both organs, and since the rate of uridine-H^3 incorporation in the control animals of both organs were more-or-less the same, this might be interpreted as indicative of the possibility that the life-span of mRNA in the pancreas might be very long, that is close to 24 hours, whereas that of the mRNA in the parotid gland might be a much shorter one. However, this would not negate the possibility that the pancreas has a reservoir of unused mRNA which might be used by the cells during the experimental period, even though the new mRNA synthesis is suppressed to an equal degree, and that the life span of mRNA, both in pancreas and parotid gland, might be comparable. A third possibility explaining the discrepancy might be related to the affinity of actinomycin D to different organs. It has been shown, for instance, that there are actinomycin D resistant and sensitive cell lines among cultured cells and that the binding of the actinomycin with DNA of sensitive cells is much firmer than that observed in resistant cells. It is possible, therefore, that the parotid gland cells might concentrate or combine more firmly with the actinomycin D molecules, although this does not completely explain the discrepancy, since the effect on the uridine-H^3 incorporation was about the same.

Whatever is the molecular basis for such difference, these results support our previous observations which showed a rather erratic behavior of the pancreas in terms of the organ weight changes under the influence of actinomycin D, whereas the parotid gland showed a uniform and drastic reduction. Furthermore, the electron microscopic studies to date suggest that the detrimental changes are more pronounced in the parotid gland as compared to the pancreas.

B. Radioautographic Studies on Protein and RNA Syntheses in the Parotid Gland and Pancreas of Normal and Hypophysectomized Mice

It has been established that the cytology of digestive glands in animals from which the hypophysis has been removed indicates a reduced amount of ribonucleic acid in these cells, as indicated by the reduction in the cytoplasmic basophilia as well as the reduction in size and number of nucleoli. It has further been shown that the removal of the master gland also results in the reduced capacity of the digestive glands to produce enzymes for secretion. As such biochemical and cytological results indicate that the glandular cells in the absence of hypophyseal hormones may show a suppression of nucleic acid synthesis correlated with the reduced protein biosynthesis, a radioautographic study on the subject aimed at clarifying the point has been carried out. Up to now we have completed studies on the protein biosynthesis, and the portion of the study concerning the nucleic acid synthesis is in progress. Therefore, in this report, details will be described only with regard to the effect of hypophysectomy on protein biosynthesis.

Mice were hypophysectomized via transauricular route, according to the method by Giovanni and others while under sodium pentobarbital aesthesia. The hypophysectomized mouse was paired with a normal mouse of comparable weight and pair-fed for a period of 40 days. Since the intake of food is known to produce immediate and transitory fine structural changes of digestive organs, the animals were starved for 8-12 hours prior to sacrifice, in order to minimize the irregularity in the appearance of secretory granules. Tritiated leucine, with the specific activity of 3.9 c/mM, was injected in the amount of 10 μc/gm body weight intraperitoneally, and the mice were sacrificed 15 minutes, 1 hour, 4 hours, and 24 hours after the injection of radioactive precursor. At the time of the sacrifice, a piece of pancreas and parotid gland was removed, fixed and embedded in the manner described elsewhere. The completeness of hypophysectomy was determined in each case by making micrascopic examinations of serial sections of the sphenoid bone containing the hypophysial fossa. The bone was decalcified in 5.5% Versene at pH 7.4 prior to paraffin embedding.

Sections of the pancreas and parotid gland, 6 μ in thickness, were made and placed on slides that had been previously coated with a subbing solution consisting of 0.5 g of gelatine and 0.05 g of chromium potassium sulphate in 100 ml of distilled water. The slides were coated with Kodak NTB-3 and exposed for various periods of time. Following the exposure, the slides were developed in 2, 4-diaminophenol dihydrochloride, and stained with Harris hemotoxyling and acidic eosin Y. In addition, a series of slides were made for histological observation of the glandular cells. For this purpose the slides were stained with Harris hemotoxilyn and acidic eosin Y, or PAS and azure II. The evaluation of the radioautographic materials was made by

counting the number of grains superimposing individual cells and by obtaining the average number of grains per cell type in both duct and acinar portions of the two glands. The results were analyzed with a program in IBM 7090.

As indicated in Table I, the mean body weight of the experimental animals before operation was 22.5 ± 2.1 g (standard deviation), which was reduced to 17.5 ± 1.18 g at the time of sacrifice, whereas the weight of the control mice was maintained at about the same level. Thus, the average weight loss in hypophysectomized mice is in the order of 22% of the preoperational value. Figures 1 through 4 illustrate the changes in the cytological appearance of both parotid and pancreatic acinar cells and confirm observations made by previous workers. In the experimental animal the size of the acinar cells is notably smaller, as compared to that of the pair-fed control animal, and the basophilic substance in the cytoplasm is markedly reduced. Both nuclei and nucleoli are also smaller than those of the control, and chromatin materials are more compactly arranged.

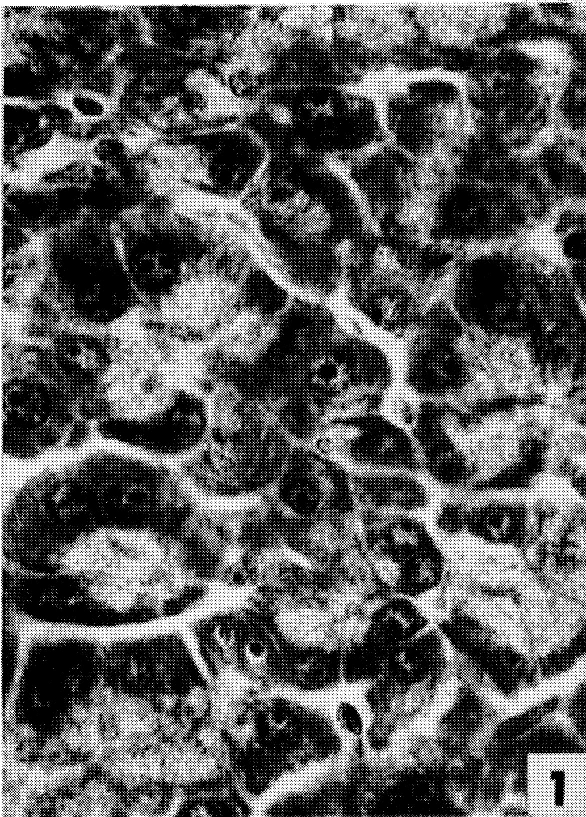
TABLE I

BODY WEIGHTS OF HYPOPHYSECTOMIZED AND PAIR-FED MICE

Number of Animals	Pre-experimental Weight in Grams (SD)	Weight at Sacrifice in Grams (SD)
Control (4)	21.9 (±2.0)	21.2 (±2.1)
Hypophysectomized (4)	22.5 (±2.1)	17.5 (±1.8)

The preparations processed for radioautography show similar cytologic features when the focusing is made at the level of the tissues, as indicated in Figs. 5 and 6, which compare the pancreatic acinar cells of the control animal with those of a hypophysectomized mouse both injected with leucine-H^3 15 minutes prior to sacrifice. As the level of focusing of the same fields is brought up to that of silver grains, as depicted in Figs. 7 and 8, numerous silver grains appear superimposed on these cells. In the subsequent photomicrographs only those photographs that are taken at the level of silver grains will be represented.

At 15 minutes it is already clear that the number of silver grains is far greater in the control gland (Fig. 7) as opposed to the experimental (Fig. 8). Although the overall grain number is lower in the parotid gland than that of the pancreas, a similar situation is observed in the parotid (Figs. 9 and 10).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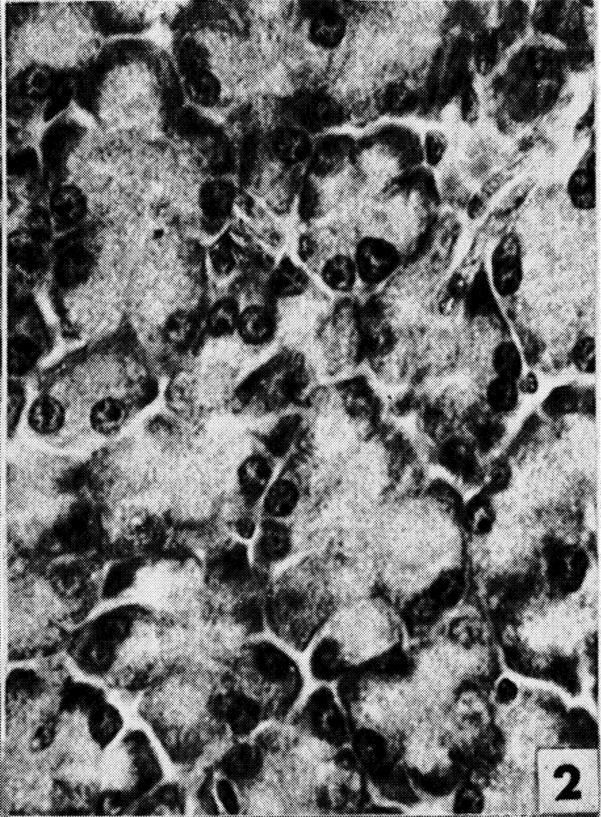
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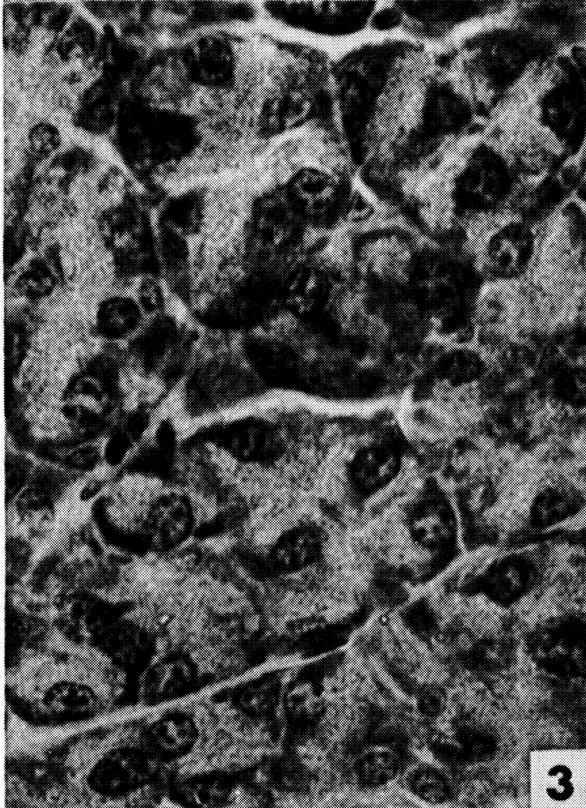
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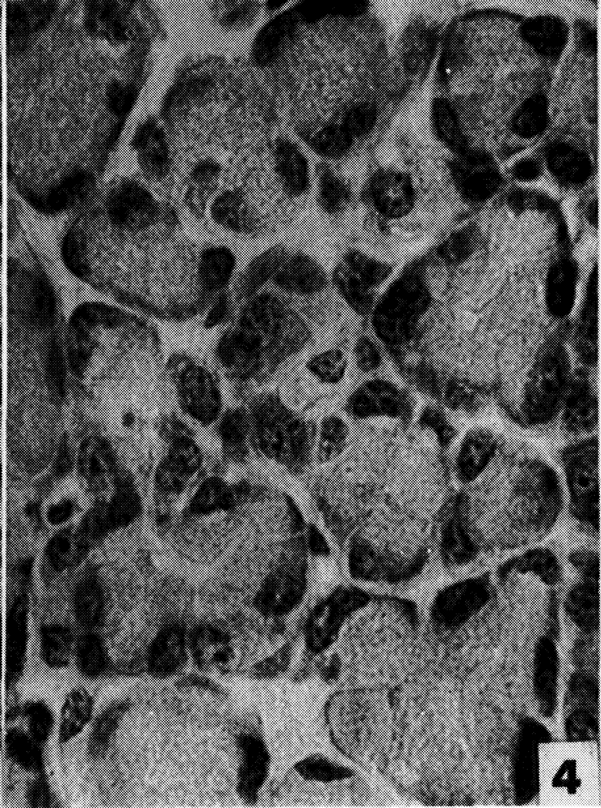
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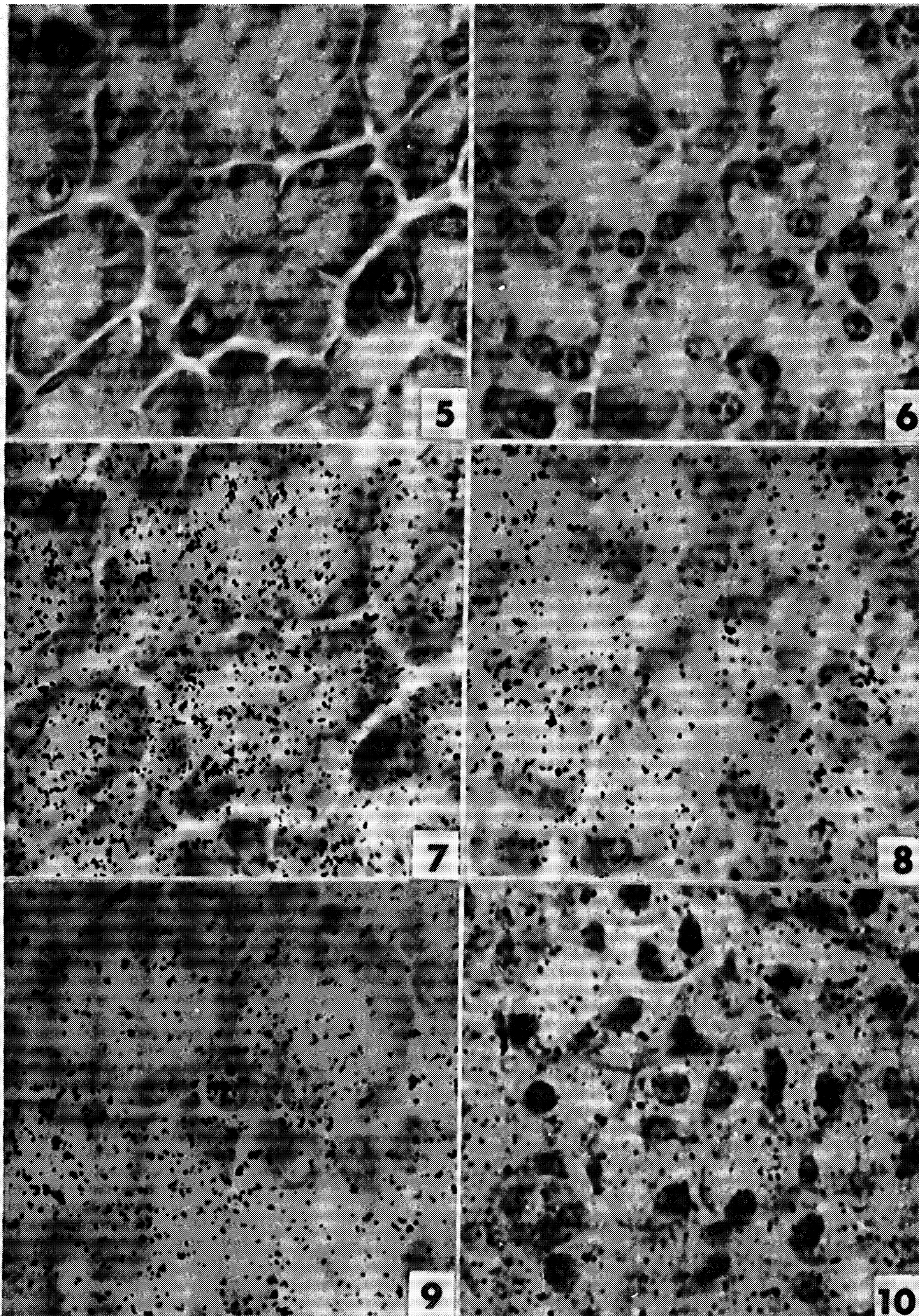
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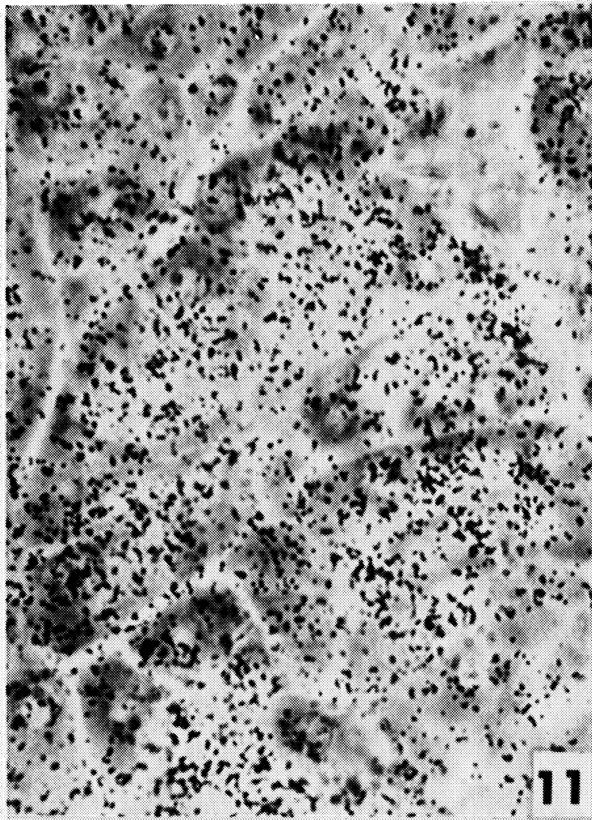
By 1 hour after the injection of the labeled amino acid, the number of grains in both glands are increased considerably (Figs. 11 through 14). However, when one compares the experimental to that of the control, the control gland shows a much greater number of grains which are primarily distributed in the supranuclear region of the acinar cells.

At 4 hours after the injection a similar tendency is observed, except that the number of grains in both glands is somewhat reduced from that of the 60-minute group (Figs. 15 through 18). After 24 hours of leucine-H^3 injection, the differences are insignificant, and the average grain number is much reduced (Figs. 19 through 22).

An observation of the radioactive grain in the lumen of ducts may indicate the secretory functions of these cells. After 15 minutes of leucine injection, ducts of both control and experimental animals show few silver grains (Fig. 23), whereas by 60 minutes the majority of ducts in the control animals becomes filled with radioactive grains, as indicated in Fig. 24. This is observed both in the pancreas and parotid gland. However, in experimental animals, only a very small amount of grains is observed in the duct lumen, even after 1 hour of injection (Fig. 25), and the amount does not seem to vary thereafter. On the other hand, the duct lumen in control animals showed a rapid reduction in the grain number by 4 hours following the injection (Fig. 26). These observations of the silver grains in the lumen of ducts indicate that in control animals a great deal of newly synthesized secretory product is excreted out of the gland in about 60 minutes. On the other hand, the experimental glands show a very small amount of secretory materials accumulating in the ducts at 60 minutes, indicating a slower rate of uptake and secretion of the final product.

For quantitative purpose the grain counts were made on 10 or more randomly selected acini, and, after obtaining the mean number per nucleus in each cell type, the results were statistically analyzed (Tables II and III). It may be seen that throughout the first 4 hours significant differences are present between the experimental and control glands, and that, during this period, the control glands show approximately twice as many grains in most cases. It may also be noted that while these figures represent glands taken from the same animals, the average number of grains is far greater in the pancreas than in the parotid gland. Thus, it is concluded that the parotid gland cells might synthesize proteins at a much less rate as compared to the pancreatic acinar cells, and that in both glands the removal of pituitary gland produces a significant suppression of the protein synthesis. The discrepancy between the parotid and pancreas in terms of leucine-H^3 incorporation was also observed in rats through biochemical means, and, therefore appears to be a universal one.

While significant difference is also noted in animals sacrificed 4 hours after the precursor injection, the difference was less marked than in animals sacrificed at 60 minutes. This, along with the observation on the appearance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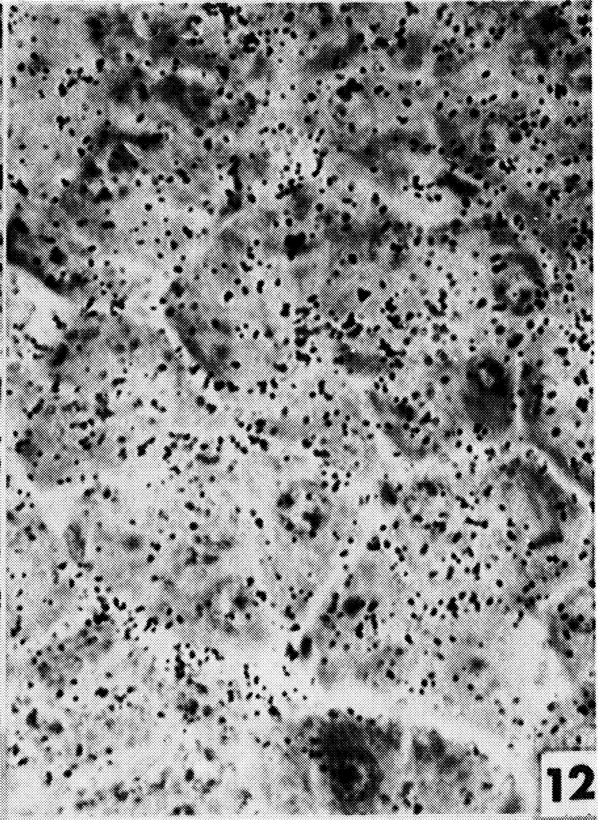
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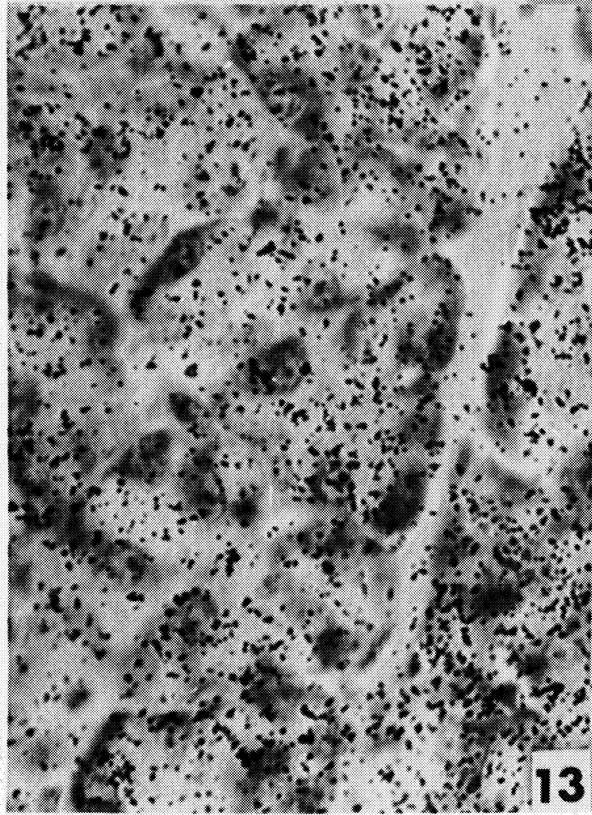
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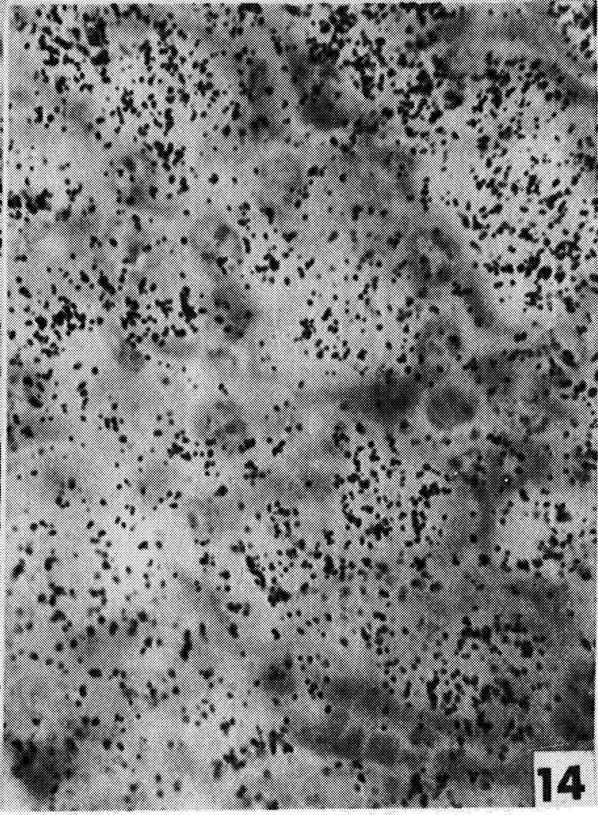
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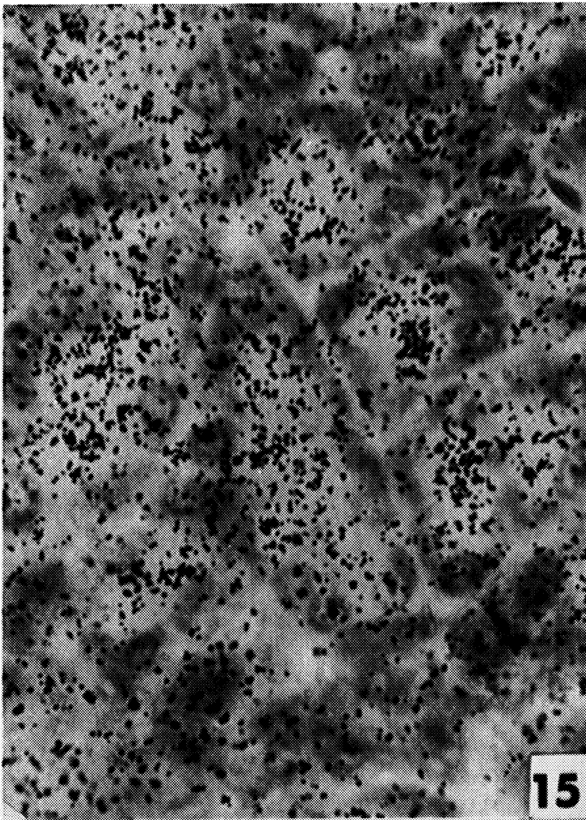
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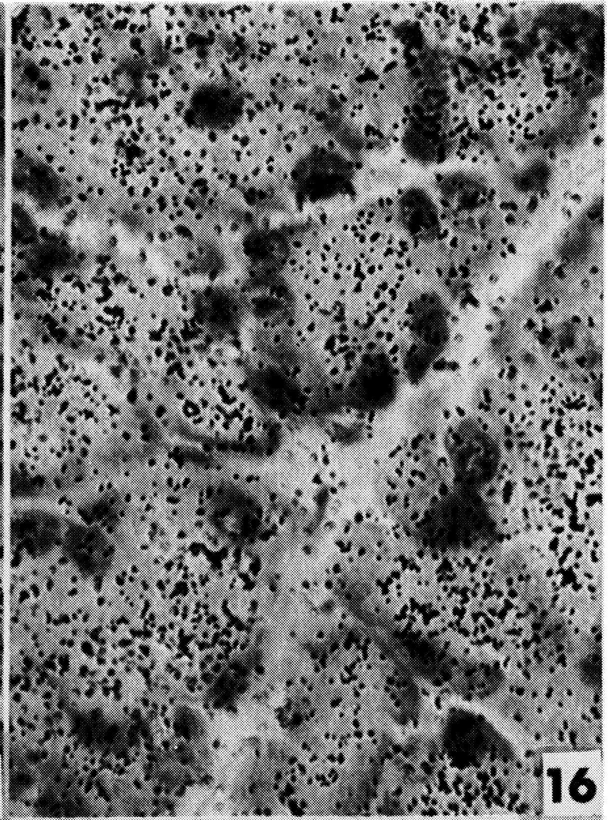
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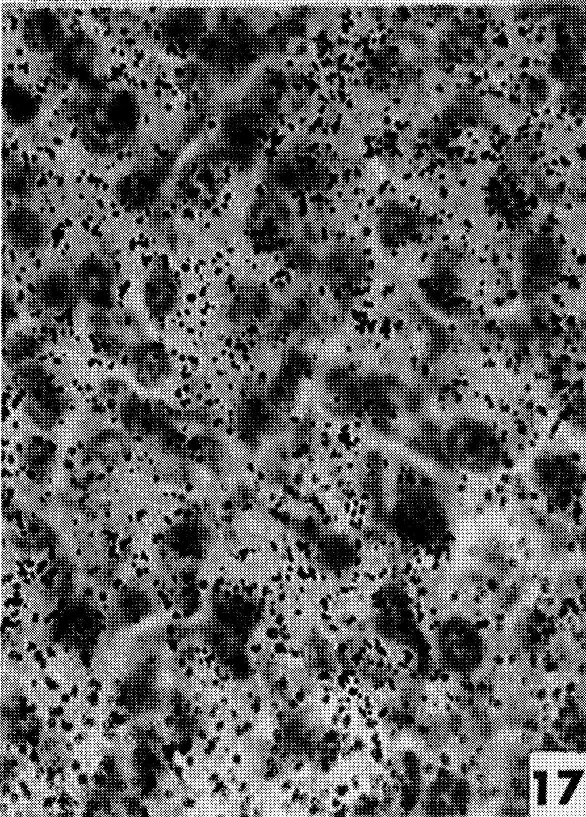
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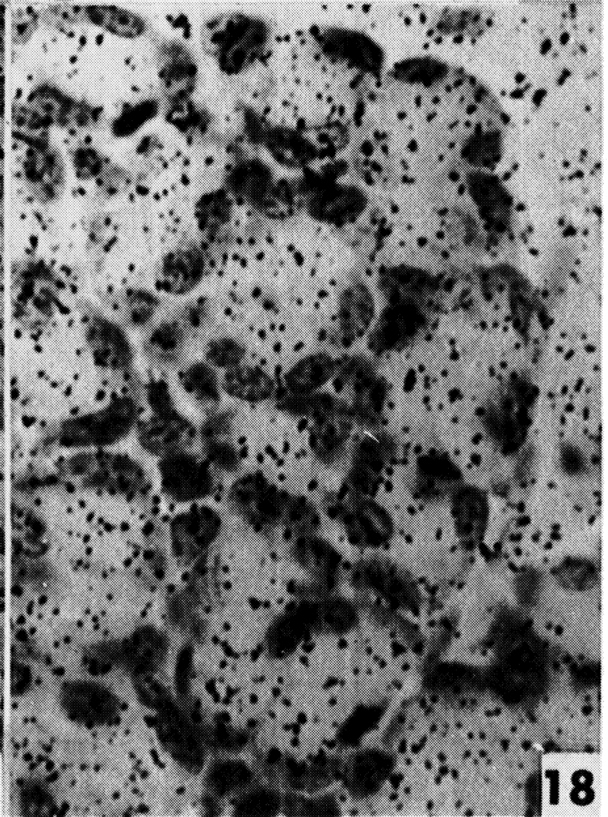
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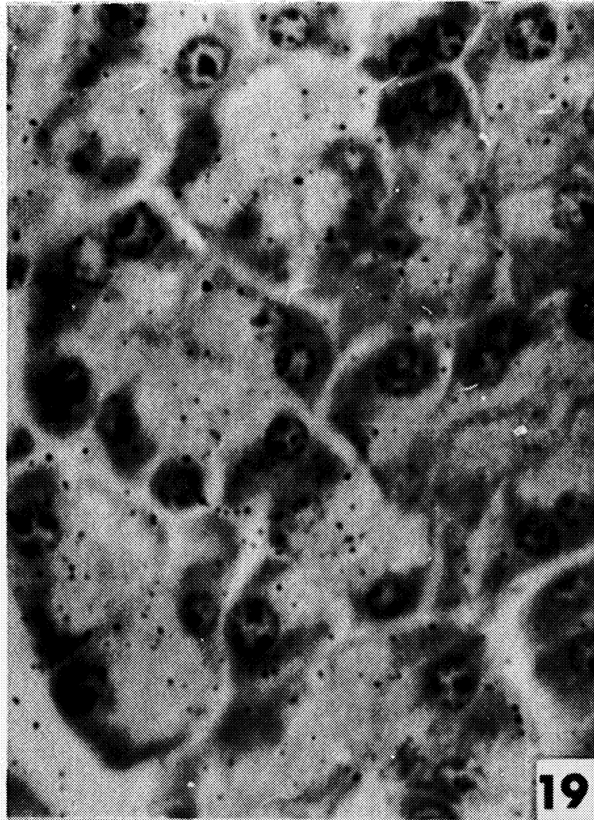
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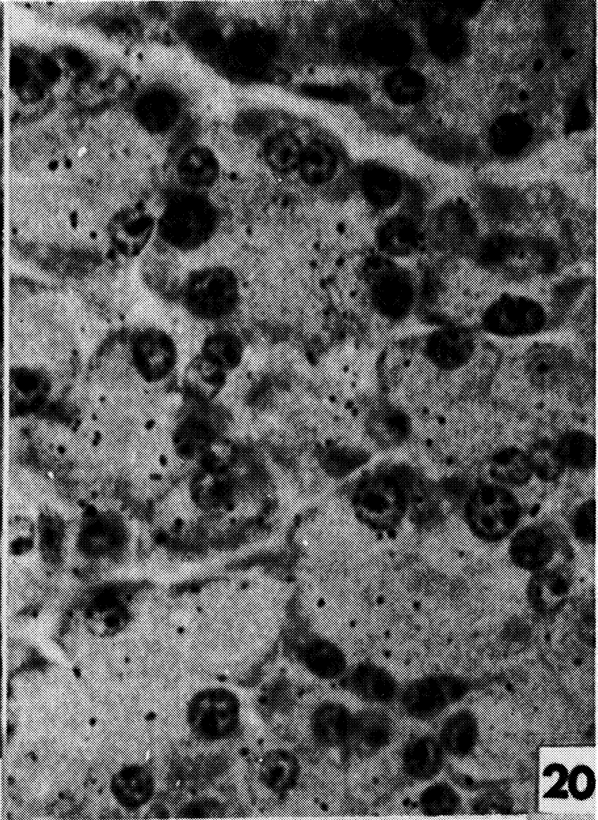
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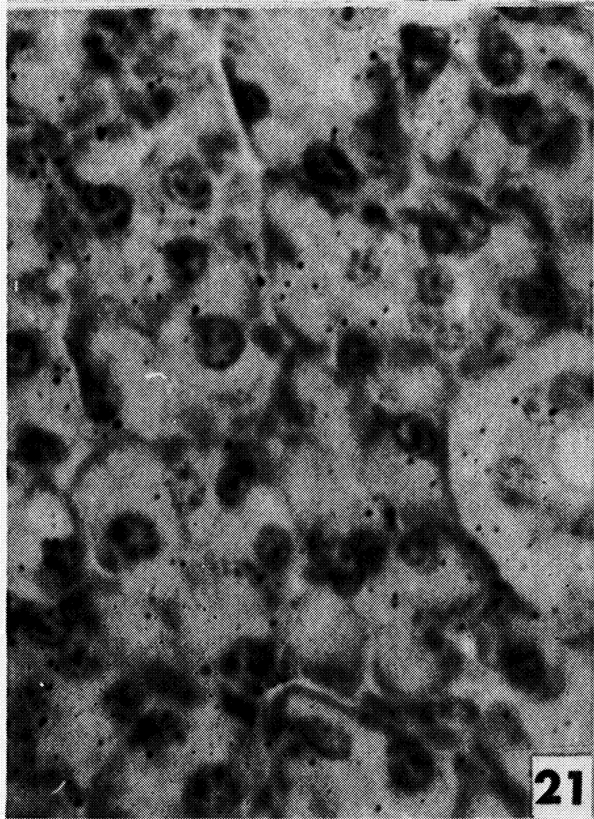
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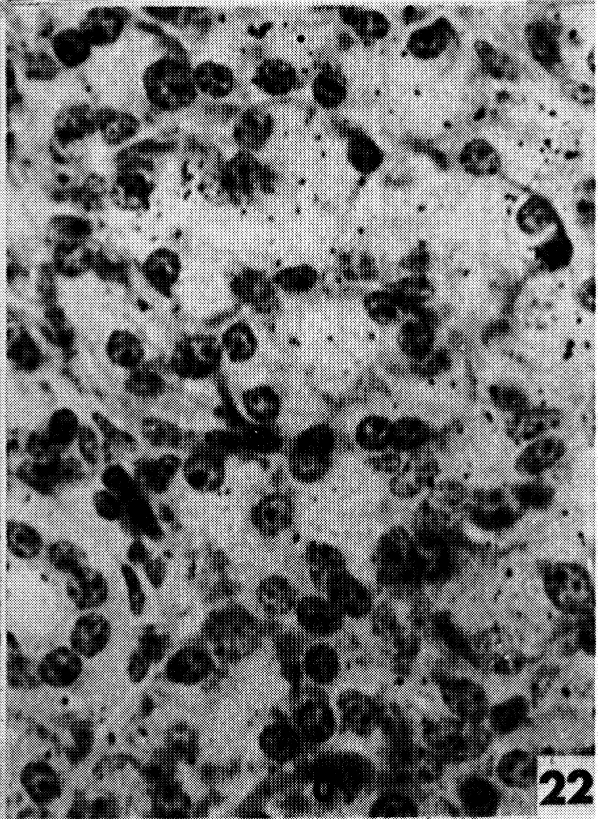
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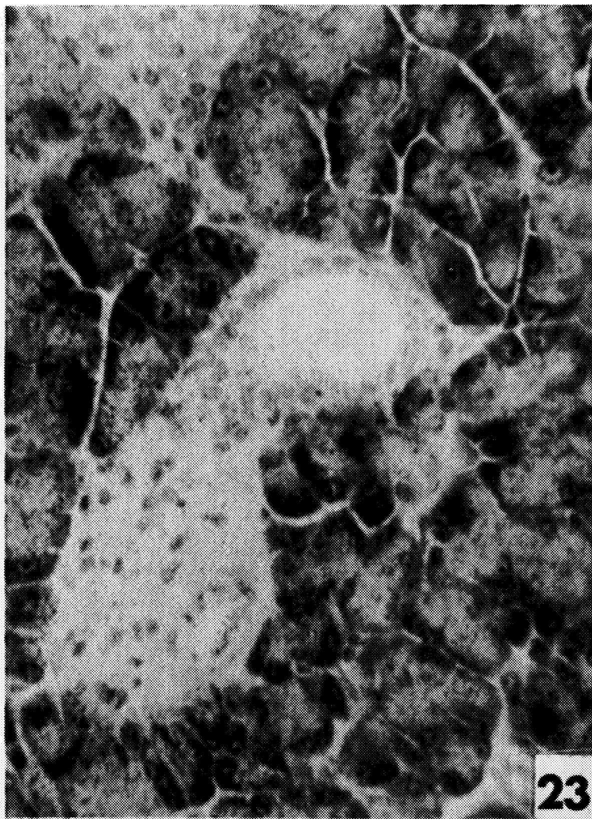
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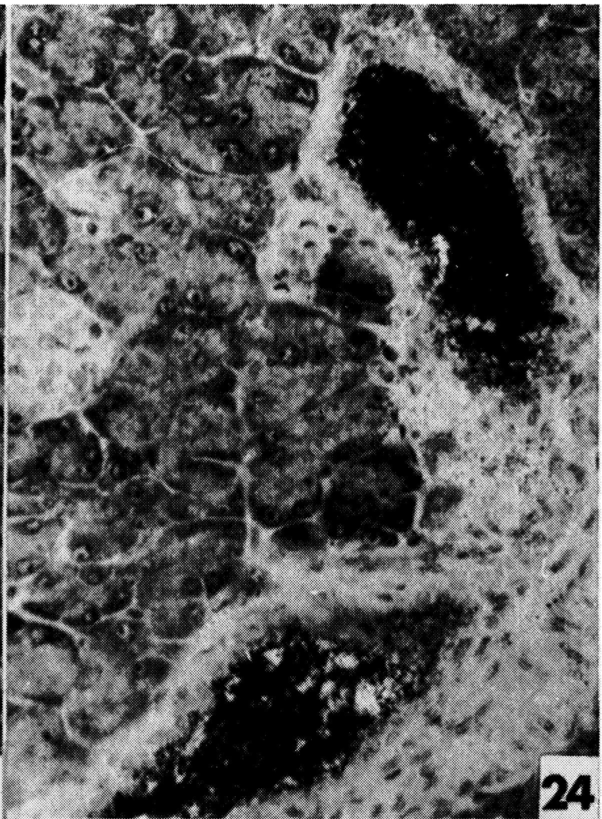
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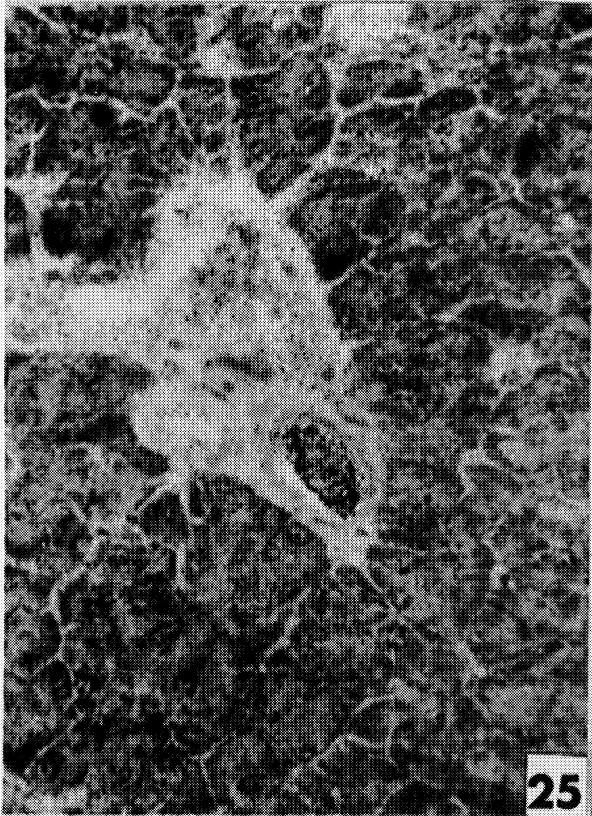
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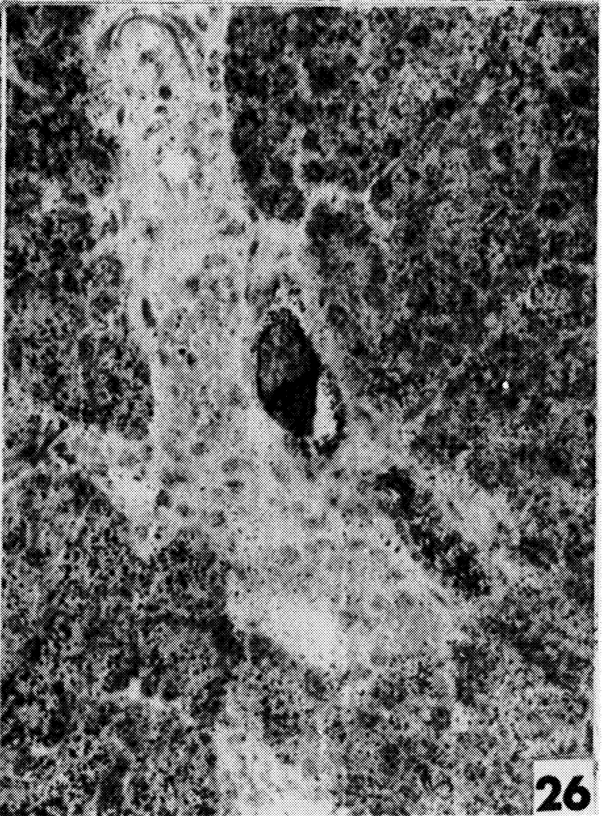
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TABLE II

QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF HYPOPHYSECTOMY IN MICE*
(Leucine-H^3 Incorporation by Pancreatic Acinar Cells)

Time After H^3 Injection	Treatment	No. Nuclei Counted	Mean Grain No. (±S.D.)	Probability
15 min	Control Hypophysectomy	114 151	94.46 (17.06) 40.17 (9.37)	$P < 0.001$
60 min	Control Hypophysectomy	112 159	99.70 (22.92) 46.10 (6.39)	$P < 0.001$
4 hr	Control Hypophysectomy	142 168	68.36 (13.84) 45.77 (6.92)	$P < 0.001$
24 hr	Control Hypophysectomy	121 184	5.02 (1.44) 4.92 (1.06)	$P < 0.8$

*Hypophysectomized and pair-fed control mice maintained for 55 days and sacrificed at 15 minutes, 1 hour, 4 hours or 24 hours after injection of leucine-H^3 (10 μc/gm B.W.; specific activity 3.9 C/mM).

Radioautographic exposure, 10 days.

TABLE III

QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF HYPOPHYSECTOMY IN MICE*
(Leucine-H^3 Incorporation by Parotid Acinar Cells)

Time After H^3 Injection	Treatment	No. Nuclei Counted	Mean Grain No. (±S.D.)	Probability
15 min	Control Hypophysectomy	202 177	24.79 (3.75) 14.84 (1.62)	$P < 0.001$
60 min	Control Hypophysectomy	178 190	51.79 (9.24) 41.51 (3.01)	$P < 0.001$
4 hr	Control Hypophysectomy	147 189	42.11 (7.46) 20.88 (2.91)	$P < 0.001$
24 hr	Control Hypophysectomy	148 153	1.56 (0.40) 3.87 (0.77)	$P < 0.001$

*Hypophysectomized and pair-fed control mice maintained for 55 days and sacrificed at 15 minutes, 1 hour, 4 hours or 24 hours after injection of leucine-H^3 (10 μc/gm B.W.; specific activity 3.9 C/mM).

Radioautographic exposure, 10 days.

of secretory product in the duct lumen, may be taken to suggest that the figures from the 4-hour group might be less meaningful than the figure from the 60-minute group.

Quantitative evaluations, made of cells of the striated ducts in both glands, show that, as suspected from the histological appearance of the duct cells, the number of grains in duct cells are insignificantly low, and therefore no valid comparison could be made. On the basis of this low rate of incorporation of leucine-H^3 it might be stated that the synthesis of proteins by striated ducts is minimal and no significant effect is exerted by hypophysectomy.

TABLE IV

QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF HYPOPHYSECTOMY IN MICE*

(Leucine-H^3 Incorporation by Striated Duct Cells of Pancreas)

Time After H^3 Injection	Treatment	No. Cells Counted	Mean Grain No. (±S.D.)
15 min	Control	40	3.60 (0.53)
	Hypophysectomy	40	2.47 (0.38)
60 min	Control	40	5.02 (0.80)
	Hypophysectomy	40	3.20 (0.41)
4 hr	Control	40	4.63 (0.39)
	Hypophysectomy	40	5.82 (0.37)
24 hr	Control	40	1.55 (0.28)
	Hypophysectomy	40	1.45 (0.28)

*Hypophysectomized and pair-fed control mice maintained for 55 days and sacrificed at 15 minutes, 1 hour, 4 hours or 24 hours after injection of leucine-H^3 (10 µc/gm B.W.; specific activity 3.9 C/mM).

Radioautographic exposure, 10 days.

TABLE V

QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF HYPOPHYSECTOMY IN MICE*

(Leucine-H^3 Incorporation by Striated Duct Cells of Parotid Gland)

Time After H^3 Injection	Treatment	No. Cells Counted	Mean Grain No. (±S.D.)
15 min	Control	40	2.22 (0.26)
	Hypophysectomy	40	3.77 (0.34)
60 min	Control	40	3.88 (0.39)
	Hypophysectomy	40	4.35 (0.59)
4 hr	Control	40	6.15 (0.69)
	Hypophysectomy	40	2.70 (0.30)
24 hr	Control	40	1.27 (0.26)
	Hypophysectomy	40	1.90 (0.31)

*Hypophysectomized and pair-fed control mice maintained for 55 days and sacrificed at 15 minutes, 1 hour, 4 hours or 24 hours after injection of leucine-H^3 (10 µc/gm B.W.; specific activity 3.9 C/mM).

Radioautographic exposure, 10 days.

C. Experiments on the Effect of Anoxia on Protein and Nucleic Acid Syntheses by Salivary Gland Cells

During the past few years we have found that an anoxic insult during the early postnatal period reduces the connective tissue matrix formation by the fibroblast. Since it was thought that such suppression of matrix formation by connective tissue cells might be a secondary reflection related to an overall suppression of protein synthesis, we have decided to study the effect of anoxia on protein and nucleic acid syntheses in developing salivary glands. Of the several studies that are in progress dealing with all of the major digestive glands, the following is the result of an experiment in which the effect of hypoxia on the leucine-H^3 incorporation by submandibular gland cells of the rat neonate was studied.

New-born Sprague-Dawley rats from an inbred colony were subjected to a total anoxia for a period of 20 minutes by placing them in a bell jar flushed with a continued flow of purified nitrogen. Oxygen content in the bell jar under the condition judged by a Westinghouse oxygen analyzer, was approximately 40 PPM. Immediately following the exposure to anoxia, the rats were injected with 3 μc/g body weight of leucine-H^3. The specific activity of this preparation was 5 C/mM. The control animals were handled in an identical manner, except that during the period within the bell jar an adequate supply of fresh air was made available for normal respiration. Animals were sacrificed in pairs at 15 minutes, and 4, 24, 48, and 72 hours after the injection.

At the time of sacrifice the rats were decapitated, and, following the removal of brain tissues from the calvarium, the head was dissected through a mid-sagittal plane and fixed in Bouin's solution for 48 hours. The fixed tissues were double-embedded in parlodion and paraplast. The blocks were so oriented as to allow the sections to go through the developing glands in parasagittal planes. Serial sections were made at 6 μ and mounted on microscope slides that had previously been cleaned and treated with a subbing solution consisting of 0.5% pure gelatine and 0.05% of chromium potassium sulphate in distilled water.

Slides having comparable histological areas were selected microscopically and coated with Kodak NTB-3 liquid emulsion in complete darkness. After drying, the slides were placed in a small plastic box that contained packets of desiccant. The boxes were wrapped with two layers of lead sheet and an additional layer of light tight paper. They were sealed in a plastic bag in which desiccant packets were placed and were left in a refrigerator for a period of 5 or 8 weeks for exposure. At the end of the exposure period the slides were developed in complete darkness in 2, 4-diaminophenol dihydrochloride for 1 minute. After fixation, slides were stained in Harris hemotoxylin and alcoholic eosin Y. From each slide grain counts were made over an average of 70

or more cells, and expressed in terms of the number of grains per cell for comparison. The results were analyzed with a computer program in IBM 7090.

Table I records the data from quantitation of the radioautographic slides that were exposed for 5 weeks. It may be seen that in control animals there is a gradual increase in the average number of grains per acinar cell in the first 4 hours, whereas the average grain number is gradually reduced thereafter. Thus, the average grain number reached a peak of 11.62 at 4 hours from 2.66 and 10.46 at 15 minutes and 1 hour, respectively. In contrast, the average grain number of anoxia-treated animals in the early period was much less than that of the controls, showing only 1.35 per cell at 15 minutes, 3.99 at 1 hour, and 8.26 at 4 hours. The level of significance at these 3 periods was $P < 0.001$. The grain number in anoxia-treated animals increased to a level similar to that of the controls by 24 hours and thereafter. The slight difference observed during the late periods was insignificant.

TABLE I

QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF NEONATAL ANOXIA IN RATS

(Leucine-H^3 Incorporation by Submandibular Gland*)

Time After H^3 Injection	Treatment	No. Cells Counted	Mean Grain No. (S.D.)	Probability
15 min	Anoxia Control	44 102	1.35 (±0.24) 2.66 (±0.69)	$P < 0.001$
60 min	Anoxia Control	104 40	3.99 (±0.87) 10.46 (±1.47)	$P < 0.001$
4 hr	Anoxia Control	41 73	8.26 (±0.60) 11.62 (±2.15)	$P < 0.001$
24 hr	Anoxia Control	40 102	11.25 (±1.03) 10.38 (±1.67)	$P < 0.2$
48 hr	Anoxia Control	56 51	6.30 (±0.73) 7.08 (±1.50)	$P < 0.3$

*Neonates subjected to total anoxia for 20 minutes and immediately injected with 3 μc/gm B.W. of leucine-H^3 (specific activity: 5 C/mM).

Radioautographic exposure, 5 weeks.

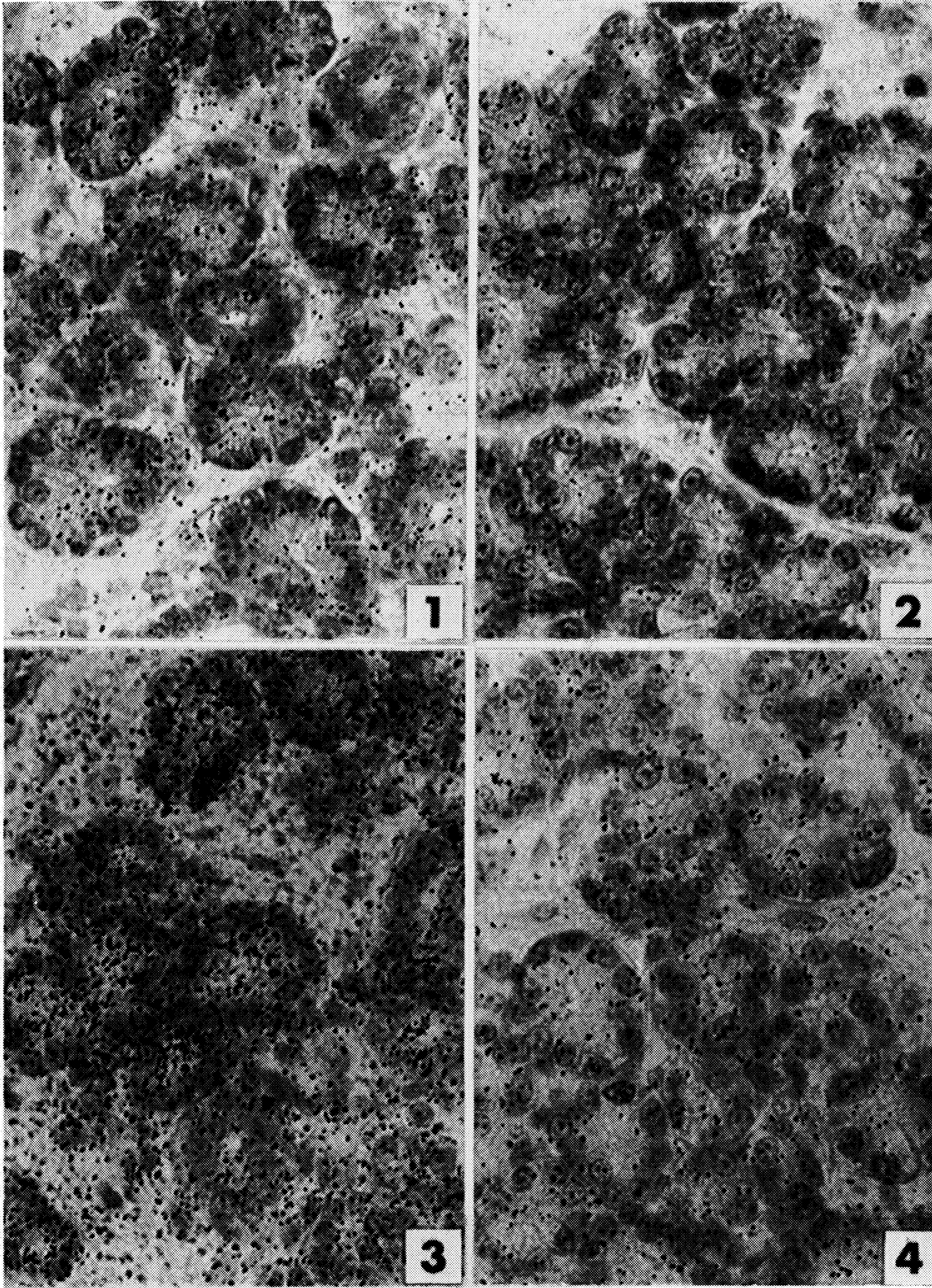
While the geographic problems inherent in the light microscopic radioautography of paraffin sections do not allow an accurate histologic representation of the quantitative data gained through grain counts, photomicrographs in Figs. 1 through 4 illustrate clearly the differences observed between control and anoxia-treated animals during the first hour. It may be noted that at 15 minutes after the injection there are several grains per cell in the control

(Fig. 1) as opposed to the experimental gland in which none or only a few grains were localized in each cell (Fig. 2). By 1 hour the amount of grains superimposing upon the cells in the control became so great that details of the cell structure were hard to resolve on photomicrographs (Fig. 3). However, it might be noted that the grains were more concentrated in the apical portion of the cytoplasm. On the other hand, in anoxia-treated animals only a few to several grains were localized in a cell (Fig. 4). A similar tendency was observed in sections from animals sacrificed 4 hours after the injection of the radioactive amino acid. Since there were no meaningful differences after 24 hours, as indicated in Text-Fig. 1, no actual radioautographs are presented. Histologically, occasional mitotic figures were observed throughout the series, both in experimental and control animals.

The incorporation of a universal amino acid, such as leucine-H^3, into the salivary glands of neonatal animals may reflect either or both of the following phenomena, namely, the synthesis of secretory proteins, and the elaboration of cytoplasmic proteins needed for growth and division. Thus, the changes in the average grain number of control animals during the first several hours might be regarded as an expression of the accumulation of newly synthesized cytoplasmic proteins for growth, and/or an accumulation of secretory proteins, since these neonatal animals have attained a minimum level of functioning of digestive glands. The gradual reduction of the grain number at later periods could reflect the reduction in the level of available precursors, the rapid attainment of secretory capabilities by the acinar cells, and/or the dilution of cytoplasmic grains by progressive divisions.

On the other hand, the definite and rapidly occurring discrepancy of the average grain number between anoxia-treated and control rats during the early hours after the insult should indicate (1) the actual reduction of the synthesis of secretory or cytoplasmic proteins, (2) the increase in the rate of secretion, and/or (3) the increase in the rate of division leading to a speedy dilution of the radioautographic grains. Although there is no direct evidence to negate the last two possibilities, it is tempting to conclude tentatively that the early discrepancy in grain numbers might be the direct result of suppression of protein synthesis, either for secretory or cytoplasmic growth purposes, as there is no evidence that hypoxia serves to stimulate the secretory functions or divisions of cells.

In this connection we have observed that the synthesis of secretory proteins as expressed by the incorporation of proline-H^3 into various types of connective tissue cells in neonatal animals is not suppressed during the first 6 hours after the insult, but was significantly suppressed by 24 hours after the insult (Smith and Han, 1968). This late effect was thought to reflect the damage done to such cytoplasmic machinery needed for protein secretion as the endoplasmic reticulum, Golgi apparatus, etc., the lack of which would reduce the formation of secretory proteins even after the recovery from an immediate suppression of overall protein synthesis. If this is the case, the results from this study showing an earlier suppression of leucine-H^3 incorporation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might be related not so much to the secretory function of these developing glands, but rather to the suppression of overall protein syntheses. The lack of difference at 24 hours and thereafter suggests that the synthesis of proteins might have recovered within 24 hours.

Other studies of the effects of the hypoxic stress on protein synthesis have shown varying results. Based on radioautographic evidence Lubiere and others (1964) reported that different organs have varying sensitivity toward the hypoxic insult, while Sanders et al. (1965), and Turner and Turner (1965) both reported hypoxia induced suppression of protein synthesis in the liver and pancreatic cells respectively. Morphological studies of the immediate effect of hypoxia by using electron microscopes have also revealed various types of detrimental changes, such as a swelling of mitochondria (Hager et al. 1960; Oudea, 1963; and Webster and Ames, 1965) or an increase in lysosomes (Oudea, 1963; Confer and Stenger, 1964; and Glinsmann and Ericsson, 1966). All of these authors have dealt with adult animals.

Whether the suppression of leucine-H^3 incorporation observed in this study is due to a direct effect of anoxia on the cytoplasmic protein synthesis, and/or due to an indirect effect mediated through the suppression of ATP production, remains to be seen. The fact that the tissues in perinatal periods are in the process of changing from a relatively anaerobic state to a more complete aerobiosis tempts one to speculate on the possibility of a direct and relatively immediate effect on the protein assembly machines, i.e., polyribosomes, etc., although this might be superimposed by the effect on energy metabolism. Attempts are being made in our laboratory to answer this question through correlated biochemical and electron microscopic studies.

PAPERS IN PRESS OR IN PREPARATION

1. Effects of hypoxia on leucine-H^3 incorporation by submandibular gland cells of rat neonates, Proc. Soc. Exp. Biol. Med., J. H. Kim and S. S. Han.

2. Biochemical and electron microscopic studies of differential effects of actinomycin D on pancreas and parotid glands, P. Stern and S. S. Han.

3. Radioautographic studies of uridine-H^3 incorporation by pancreas and parotid glands of normal and hypophysectomized mice, Y. G. Kim and S. S. Han.

4. Effects of hypophysectomy on protein synthesis by pancreas, parotid and submandibular glands in mice, Y. G. Kim and S. S. Han.

5. Electron microscopic studies of digestive glands following hypophysectomy, S. S. Han and Y. G. Kim.

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