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Progress Report

HYPOXIC EFFECT ON TEETH AND SALIVARY GLANDS

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SUMMARY PAGE

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(g) Summary statement of the report which follows:

The earliest efforts of this project, during the -01 grant year, have been concerned with the biochemical, fine structural, and functional alterations in the normal development of teeth and salivary glands in neonatal rats whose mothers had been insulted by hypoxic atmospheric conditions during pregnancy. Published articles based on this research are listed elsewhere in this report.

More recent studies have indicated that (1) as indicated by radioautographic quantitation following leucine-H^3 injection of hypoxia insulted pregnant rats, the overall synthesis of proteins in normal rat neonates is less in the submandibular gland than in comparable pancreatic acinar cells; (2) it appears that the submandibular gland is affected more profoundly than the pancreas even though it demonstrates a greater rate of leucine incorporation; and (3) protein synthesis in both the pancreas and submandibular gland was suppressed significantly in all the hypoxia insulted animals. As related to the possible types of secretory granules found in the developing submandibular gland, electron microscopic evidence in these studies of rat neonates show marked differences between acinar and ductule granules. Moreover, the differences between the ductule-type granules are great. As for the question concerning the sites of hypoxia effect, electron microscopic evidence shows a marked alteration in submandibular mitochondria which were initially characterized by selling of select mitochondria, vacuolization of their matrices, the appearance of whirl-like figures, and the reduction in organization of the mitochondrial cristae.

DETAILED PROGRESS REPORT

The following report describes some of the major efforts and findings, during the -02 and -03 years of grant HD-03147, with respect to the hypoxic effect on developing teeth and salivary glands.

A. Effects of Prenatal Anoxic Exposure on Leucine-H^3 Incorporation By the Pancreas and Submandibular Gland in Rat Neonates

In a previous communication from this laboratory, it was reported that exposure of pregnant Sprague-Dawley rats to an atmosphere of purified nitrogen at different times of gestation period produced a rather serious lag in the differentiation of epidermal and connective tissue cells in offsprings from such animals. This was in addition to the skeletal anomalies and retardation of dentition which were most pronounced in animals subjected to hypoxia at days 10 and 20 of fetal life, respectively.

Inasmuch as the growth and differentiation depend directly upon the normal production of enzymes, and proteins in general, it was suspected that the lack of differentiation of epithelial and connective tissue cells might be due to the sustained effect of hypoxia on the capacity for protein biosynthesis by these cells. The present experiment was designed to evaluate whether or not a single hypoxic insult during the fetal life would show a sustained suppression of protein biosyntheses in the developing submandibular gland and pancreas during the neonatal period.

MATERIALS AND METHODS

Animals and Anoxic Conditions

In each experiment 4 pregnant rats were subjected to total anoxia for a period of 20 minutes in a manner described previously (Morawa and Han, 1968). Following the exposure to anoxia, the rats were allowed to give natural birth until day 22 of gestation. If the delivery was not made by 22 days, however, the pregnant rats were anesthetized with ether and the fetuses were removed. These subjects were regarded as being equal to those neonates which were delivered naturally. The neonatal rats were used within hours after delivery so that none of them would have experienced suckling. For each experiment, a total of 12 selected neonates were used for radioautographic preparation. These consist of 3 litter mates from the control animal, 3 litter mates each from the 3 experimental groups representing animals insulted on days 12, 15, and 18 of fetal life. Experiments were repeated three times.

Radioautographic Preparation

Each of the neonatal rats was intraperitoneally injected with leucine-H^3 in the amount of 10 μc/gm body weight. The specific activity of the labelled amino acid was 3.90 C/mM. One animal from each of the 4 groups was sacrificed at 15, 60 or 120 minutes following the injection of leucine-H^3. The submandibular gland and the pancreas were rapidly excised, fixed for 24 hours in 10% neutral formalin and embedded in parlodion and paraplast in the standard manner. Sections 6 micra thick were made and collected on slides, coated with a subbing solution consisting of 0.5% pure gelatin and 0.05% chromium postassium sulphate in distilled water.

Five sets of the slides were coated with Kodak-NTB 3 nuclear track emulsion as described elsewhere and were exposed for varying periods. An exposure period of 8 days turned out to be optimal for grain counts and therefore used in the quantitation of average grain numbers. Because of the poor differentiation of duct elements during the neonatal period, the grain counts were made only in definite acinar cells. The results of the grain count were processed for student t test by using an IBM 7090 computer program.

RESULTS

Since the quantitative data are recorded in Tables I and II, the illustrative figures of radioautographs are chosen only from the tissues of the neonates that had been insulted on the 12 day of gestation and were sacrificed 120 minutes after the injection of leucine-H^3.

Figures 1 and 2 compare the pancreatic tissues of the neonates from the control and experimental animals. It is clear that the number of grains in the experimental animals is notably fewer than that of the control pancreas. Submandibular gland, depicted in Figs. 3 and 4, shows a similar tendency.

The results of grain counts from all of the experimental and control animals, given radioactive precursor at different times prior to sacrifice, are summarized in Tables I and II. Individual data are based on pooled counts obtained from all 3 experiments. Although a significant difference between the experimental and control animals is observed in rats injected with radioactive precursor 15 minutes before the sacrifice, the difference is most pronounced in animals sacrificed at 120 minutes. In animals of this group, the average grain number of the pancreas of experimental animals varies between 50 and 75% of the control value (Table I). In all cases the difference between the experimental and control pairs is significant at the level of $p < 0.001$.

Table II represents the quantitative data obtained by counting cells of the submandibular glands. It may be seen that the mean grain number in control animals is only about 70% of what is found for pancreatic acinar cells at 120 minutes when the mean grain number of the controls was the greatest.

The results from the experimental animals are somewhat irregular in animals sacrificed at 15 minutes, although it is generally lower than that of the control. However, in animals sacrificed at 60 and 120 minutes after the injection of the radioactive amino acid, the number of grains per cell is the lowest in animals insulted on day 12 of gestation, reaching down to 30% or less of the control value. On the other hand, the experimental animals insulted on days 15 and 18 of gestation have a mean grain number which is twice as high as the ones exposed to hypoxia on day 12 of gestation, although they were significantly low when compared to the control. Excluding the ones that were sacrificed at 15 minutes the level of significance between the control and the experimental animals is $p < 0.001$.

DISCUSSIONS

The results described throw light upon the following three points: (1) As indicated by radioautographic quantitation following leucine-H^3 injection, the overall synthesis of proteins in normal rat neonates goes on at a greater rate in the pancreatic acinar cells than those of the submandibular gland of neonatal rats. (2) Of the two glandular tissues, the submandibular gland is affected more profoundly despite the greater rate of leucine incorporation observed in the pancreas. (3) In both organs the protain synthesis was suppressed significantly in all animals insulted at different gestation periods.

Several previous workers have reported that, following exposure to anoxia, the synthesis of proteins was variously suppressed in adult organisms (Sanders, Hale and Miller, 1965; Turner and Turner, 1965). Earlier work from our own laboratory has demonstrated that similar suppressive effects were found in neonatal animals following acute anoxic exposure during the first day of life (Smith and Han, 196 ; Kim and Han, 1968 and 1969). Such effects were evident in glandular cells as well as among various types of connective tissue cells.

Insofar as the prenatal effects are concerned, past studies have dealt primarily with teratological aspects observable in neonates born of hypoxia-treated pregnant mother (Degenhardt, 1960; Murakami, Kameyama, and Kato, 1956; Ingals and Curley, 1957; Morawa and Han, 1968) or in chickens rendered hypoxic to hatching. These studies have clearly shown that a single prenatal residence (Grabowski, 1964) in hypoxic environment could produce profound malformations in skeletal morphology of the offspring. This was suggested to be due to the accumulation of lactic acid (Grabowski, 1964) and possible derangement of sequential evolution of enzymes and other proteins at crucial points in differentiation (Smith and Han, 1968). These statements support earlier observations of Murakami, et al., and Ingals and Curley who pointed out that the malformations were most serious in offsprings of the mothers, insulted during the time when somites were in the process of differentiation.

Except for the histological observation by Morawa and Han (1968) who suggested the possibility of a sustained impairment of protein synthesis in epidermis, few have reported any continued effects of prenatal exposure to anoxia on protein synthesis. Whether this is due to prolonged effects on rate of transcription and translation of secretory products, or due to simple retardation in degree of differentiation cannot be determined as yet, and awaits for future experimental data.

TABLE I. QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS IN RATS OF ANOXIA GIVEN AT DIFFERENT TIMES OF GESTATION LEUCINE-H^3 INCORPORATION BY ACINAR CELLS OF PANCREAS*

Time After H^3 Injected (min)	Treatment	Mean Grain No. (S.D.)	% of Control	Significance
	Control	9.38 (±1.99)	100	-
	12 D. Anoxia	6.23 (±1.30)	66.4	$P < 0.001$
	15 D. Anoxia	4.93 (±1.01)	52.5	$P < 0.001$
	18 D. Anoxia	7.19 (±1.98)	76.8	$P < 0.001$
60	Control	11.64 (±1.80)	100	-
	12 D. Anoxia	7.64 (±1.60)	65.7	$P < 0.001$
	15 D. Anoxia	9.26 (±1.88)	79.6	$P < 0.001$
	18 D. Anoxia	6.24 (±1.13)	53.9	$P < 0.001$
120	Control	12.01 (±1.32)	100	-
	12 D. Anoxia	6.09 (±1.45)	50.7	$P < 0.001$
	15 D. Anoxia	8.01 (±1.68)	66.6	$P < 0.001$
	18 D. Anoxia	7.31 (±1.32)	60.9	$P < 0.001$

*Pregnant rats subjected to total anoxia for 20 minutes and immediately injected with 10 μc/gm b.w. of leucine H^3 (specific activity: 3.90 c/mM), radioautographic exposure, 8 days.

TABLE II. QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS IN RATS OF ANOXIA GIVEN AT DIFFERENT TIMES OF GESTATION
LEUCINE-H^3 INCORPORATION BY ACINAR CELLS OF SUBMANDIBULAR GLAND*

Time After H^3 Injected (min)	Treatment	Mean Grain No. (S.D.)	% of Control	Significance
15	Control	4.29 (±0.57)	100	-
	12 D. Anoxia	3.47 (±0.79)	80.9	$P < 0.02$
	15 D. Anoxia	3.41 (±0.72)	79.5	$P < 0.01$
	18 D. Anoxia	2.67 (±0.50)	62.2	$P < 0.001$
60	Control	7.48 (±0.84)	100	-
	12 D. Anoxia	2.47 (±0.44)	33.0	$P < 0.001$
	15 D. Anoxia	4.59 (±0.69)	61.4	$P < 0.001$
	18 D. Anoxia	6.36 (±0.84)	71.7	$P < 0.001$
120	Control	8.94 (±1.30)	100	-
	12 D. Anoxia	2.31 (±0.50)	25.8	$P < 0.001$
	15 D. Anoxia	5.63 (±0.55)	63.0	$P < 0.001$
	18 D. Anoxia	4.92 (±0.96)	55.0	$P < 0.001$

*Pregnant rats subjected to total anoxia for 20 minutes and immediately injected with 10 μc/gm b.w. of leucine-H^3 (specific activity: 3.90 c/mM), radioautographic exposure, 8 days.

EXPLANATION OF FIGURES

Plate I

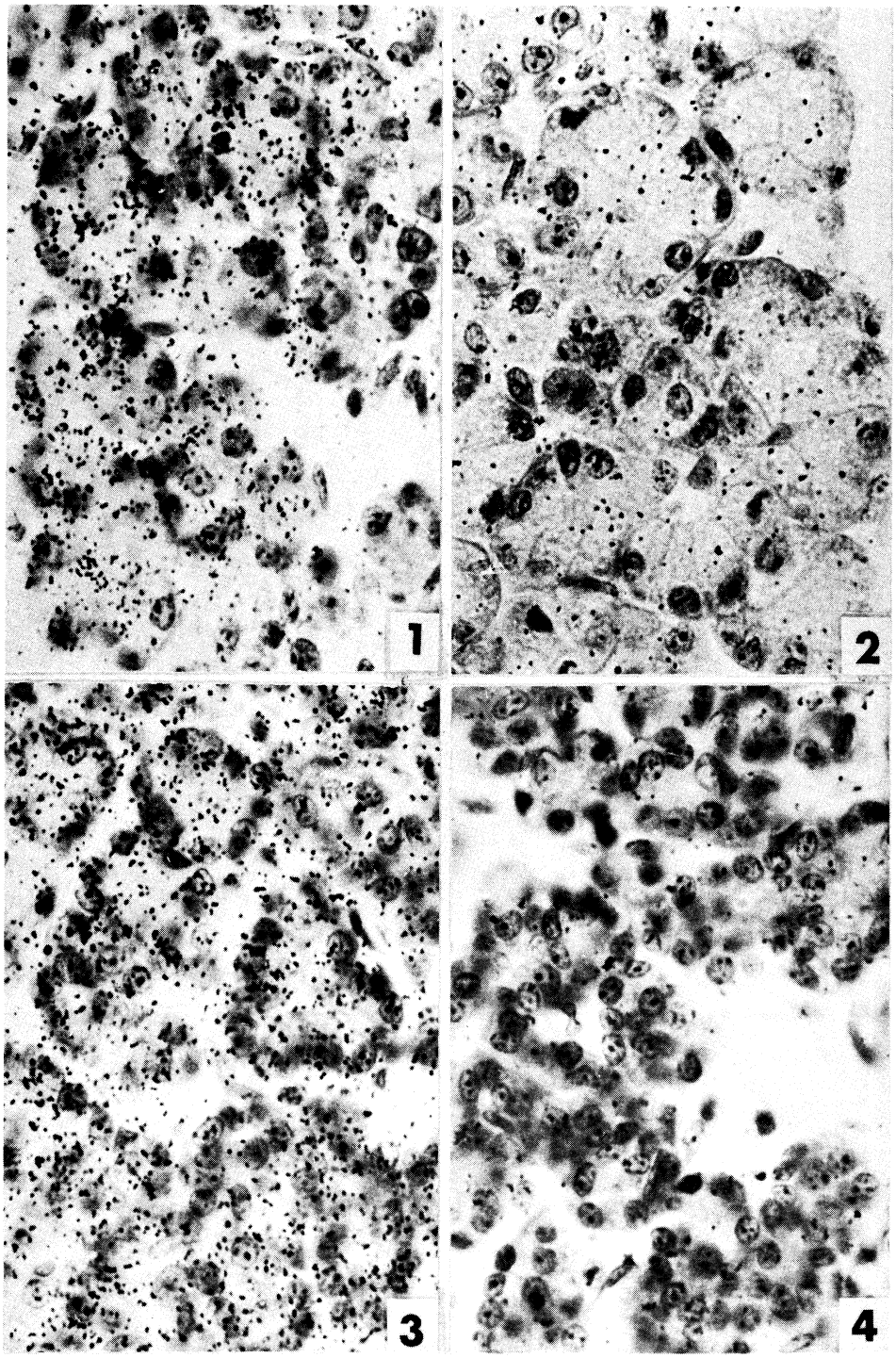
Radioautographs of pancreas (Figs. 1 and 2) and submandibular gland (Figs. 3 and 4) of rat neonates. Rats were scarificed at 2 hours after the injections of leucine-H^3. Pictures were focused at the levels of silver grains, and therefore the cells appear somewhat out of focus. Magnified at 630 X.

Fig. 1. Pancreas of a control rat.

Fig. 2. Pancreas of a rat exposed to hypoxia on day 12 of gestation period. The number of grains over individual cells is less than that of Fig. 1.

Fig. 3. Submandibular gland of a control rat.

Fig. 4. Submandibular gland of rat exposed to hypoxia on day 12 of gestation period.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B. The Fine Structure of Secretory Granules in Submandibular Glands of The Rat During Early Postnatal Development

Two types of secretory granules occur in the submandibular gland of the adult rat; one is found in acinar cells and the other in the granulated portion of secretory ducts. The granules of the acinar cells are of a mucous type and those of the ducts are of a serous type (Shackleford and Klapper, 1962). In the electron microscope, the granules of acinar cells are characterized by the ill-preserved appearance of their membrane and electron translucent interior (Scott and Pease, 1959; Kurtz, 1964; Tamarin and Sreebny, 1965; and Tamarin, 1967). The granules of ducts, on the other hand, are bound by a distinct membrane and appear dense with no obvious internal structure. These granules occur in the convoluted portion of secretory ducts and are of varying density (Scott and Pease, 1959; Leeson and Jacoby, 1969; and Tamarin and Sreebny, 1965).

Submandibular glands of the rat are immature during the early postnatal period in terms of their structure (Jacoby and Leeson, 1959; and Leeson and Jacoby, 1959), enzyme content (Fukuda, 1967) and secretory activity (Sreebny et al., 1955). Microscopic observations of submandibular glands of the newborn rat have shown that neither typical acini nor granular ducts are present in them. In the neonatal rat, secretory granules are located in the terminal endpiece intralobular ducts, which has been described as the "terminal tubule" (Jacoby and Leeson, 1959).

The differentiation of mucous acini and granulated ducts from the terminal tubule proceeds to completion during the first six weeks of life.

The chemical composition of the secretory granules of the terminal tubules appears to be different from that of the granules of the mature gland, as indicated by the difference in various staining reactions (Jacoby and Leeson, 1959). Furthermore, the content of various enzymes in the granules of terminal tubules is different from the granules of the granular duct (Fukuda, 1967). Such differences in chemical composition are expected to be reflected in the structure of the secretory granules. With the exception of Leeson and Jacoby (1959), few have studied the differentiating submandibular glands of young rats with the electron microscope.

The present study describes the fine structure of "transitory" serous granules of the terminal tubule in the submandibular gland of the neonatal rat during the first week of life.

MATERIALS AND METHODS

Small pieces of submandibular glands were dissected from 1-, 3- and 7-day-old Sprague-Dawley rats and fixed in 2% paraformaldehyde buffered to pH 7.4 with 0.1 M cacodylate, followed by a post-fixation with 1% O_sO_4 in the same buffer. The tissues were dehydrated in graded concentrations of ethanol and embedded in a mixture of epoxy resin in a routine manner (Luft, 1961). Ultra-thin sections were made on a Porter-Blum MT II microtome with a diamond knife, and stained with 1% aquaeous uranyl acetate and lead citrate (Reynolds, 1963). Final preparations were studied in a Hitachi 11c electron microscope at the accelerating voltage of 50 KV.

OBSERVATIONS

Observations of the submandibular gland in this article will be limited to the terminal tubule of intralobular ducts where transitory secretory granules occur. Two types of cells constitute the terminal tubule (Fig. 1). One type contains granules of ill-preserved appearance, having a content of low electron density and resembles a mucous secreting cell. The other type contains spherical granules of an increased density and is delineated better than the former type. For convenience in description these granules of the terminal tubule may be named on the basis of their appearance. The granules of lower density and ill-preserved appearance may be called the "acinar-type," as they resemble in appearance the mucous granules of acinar cells of the adult gland. The granules of higher density and of distinct contour are similar to the granules of the granulated duct in adult glands and hence may be referred to as the "duct-type."

Both types of granules are localized in the apical portion of the cytoplasm in respective cells. In cells which contain acinar-type granules, the cytoplasm is more abundant and parallel profiles of the endoplasmic reticulum are present throughout the cytoplasm. In the cells containing the duct-type granules, segments of endoplasmic reticulum are scattered mostly at the basal portion of the cytoplasm.

The acinar-type granules are of one type and their structure remains the same throughout the first week of the gland development. Although the acinar-type granules are membrane bound, the membrane is not distinct in many granules (Fig. 1). The ill-preserved interior of these granules is difficult to describe and has dense fibrillar masses of various thicknesses. Irrespective of their size, however, the acinar type granules are of similar structure.

The duct-type granules, on the other hand, show widely different structures during the first week of postnatal development. Granules of different internal structure occur in cells of the terminal tubule at different days and sometimes within the same cell (Figs. 2 and 6). In cells with these granules, Golgi complexes are often found among the granules (Fig. 2). However, the Golgi

complexes are not as elaborate as those which are associated with the acinar-type granules. The granules of small size near the Golgi are homogeneously dense. Small bits of endoplasmic reticulum are present among the granules, as well as numerous membrane free ribosomes.

Since the duct-type granules of a single gland may vary widely in their structure, it is difficult to generalize that the granules of one structure represent a given stage of the gland development. However, there are certain structural variations which occur more frequently in the gland at one stage than in other stages during the week.

The granules which are most frequently encountered in the gland of 1-day-old rats are membrane-bound and show an intermediate density (Fig. 3). Within each granule, circular or slightly elongated structures of about 660Å in diameter occur, which resemble a shape of short cylinders or shells.

Granules containing long extended tubules are also found in the gland of 1-day-old rats. However, the tubule containing granules appear to be more abundant in the gland of 3-day-old rats (Figs. 4 and 5). In these granules the diameter of tubules are the same as the diameter of shells or short cylinders shown in Fig. 3. The number of tubules per granule is variable among different cells within a single gland.

Granules of still a different appearance occur in the terminal tubules of 3-day-old and 7-day-old rats. These granules are shown in Fig. 2. Spheres or rods of low internal density are present in the dense matrix of these granules. The diameter of these spheres or rods is the same as the interior diameter of shells or tubules in the granules described earlier. However, the outline of these spheres or rods is not clear, and patches of dense material occur at the periphery of these granules.

More granules of widely different internal structures occur in the terminal tubules of 7-day-old rats than in younger rats. The granules of 7-day-old rats have in general, a more complicated internal structure than that of younger rats (Fig. 7).

While the granules of different internal structures occur in different cells the morphology of granules may also vary within a single cell (Fig. 6). In many instances the matrix of the granule is denser than that of the ground cytoplasm (Figs. 7 and 8). Occasionally a fingerprint pattern which appears to be due to a regular alignment of electronlucent rods, is observed (Fig. 7).

DISCUSSION

The terminal portions of the developing intralobular ducts in submandibular glands of rats consist of acinar and duct type cells, at least during the first week of postnatal period. The cell with ill-preserved appearing granules

is similar in appearance to the acinar cells of the adult gland which has previously been described (Scott and Pease, 1959; Kurtz, 1964; Sreebny and Tamarin, 1965; Tamarin, 1967) and contains stacks of parallel endoplasmic reticulum and large Golgi regions. The cell which contains dense and distinct granules resembles the serous cells of granular ducts of the adult gland; Golgi zone is somewhat small (Scott and Pease, 1959) and the endoplasmic reticulum is not oriented parallel to each other in most part (Tamarin, 1967).

The acinar cells of the rat submandibular gland, has been described to "bud off" from the terminal tubules (Jacoby and Leeson, 1959; Leeson and Jacoby, 1959). It is possible that the acinar type cells which were found in the terminal tubule during the first week of the gland development are those cells which subsequently are forming acini. The presence of two cells containing different types of secretory granules has recently been observed in the submandibular gland of neonatal mice (Park and Han, 1969); one cell contains mucous-like granules and the other serous type granules. It appears that the occurrence of acinar- and duct-type cells in the terminal tubules of submandibular glands during neonatal period is not unique for rats.

The chemical composition of the granules in the terminal tubules has not been determined in this study. However, the granules of the acinar-type cells resemble morphologically the granules of adult acinar cells which have previously been described (Scott and Pease, 1959; Leeson and Jacoby, 1959; Kurtz, 1964; Sreebny and Tamarin, 1965; Kanda, et al., 1968). In short, the granules are bound by a disrupted membrane and show aggregates of fibrillar materials in an amorphous interior. The granules of the duct-type cells are, to a certain extent, similar in appearance to the serous-type granules of parotid acinar cells (Parks, 1961; Kurtz, 1964) and granular-duct cells (Scott and Pease, 1964; Sreebny and Tamarin, 1965) in adult rats. The granules are distinctly membrane bound and have dense interior. However, the elaborate internal structure seen in the duct-type cells of the terminal tubules has not been observed or does not occur in the serous-type cells of the adult submandibular gland.

The duct-type granules of various structures in the terminal tubules during the early postnatal period are a transitory type, before an adult type granule appears. Granules containing portions of the secretory duct, granular ducts, are not present in the gland until rats reach about 5 to 6 weeks of age (Jacoby and Leeson, 1959; Fukuda, 1967). Simultaneous with the appearance of secretory granules in the granular duct, the granules of the terminal tubule disappear and the tubule transforms into a intercalated duct (Jacoby and Leeson, 1959; Leeson and Jacoby, 1959). Granules of an unusual structure, which is different from the structure of the granules in adult glands, have been observed in the submandibular gland of newborn mice, but not in 5-day-old mice (Kumegawa, Cattoni and Rose, 1967).

Wide variations which occur in the structure of the duct-type granules in the terminal tubule can be explained in several ways. The first possible explanation is that all granules are of one type with one function, and the

granules of different structures represent various stages of the synthetic cycle. The second possibility is that the granules are of various type, each with an independent different function, and the granules of different structure represent the mature form of these granules. The third possibility is that there are more than one type of granules, some granules of different structures are of one type and the granules of various structures represent mature and immature stages of different granules.

Obviously, a morphological study, such as this, alone cannot provide a convincing evidence for or against any of these possible explanations.

In the cells of the terminal tubule, the granules of small size, which occur near the Golgi complex are of a homogeneous interior with no obvious structure. The larger granules of various structures might be those which are at different stages of the granule formation. The occurrence of more than one type of granule within a cell also indicates that the granules of various structures may be those which are forming. Furthermore, some granules of the terminal tubule appear to be related in their structure. It can be imagined that the tubular structures are derived by the elongation of the shells or short cylinders. Unusual forms of secretion granules have been observed in the acinar cells of the adult rat submandibular gland during the synthetic period following the administration of a secretion stimulating drug (Kanda, Mayfield, and Coggeshall, 1968). These granules of unusual forms were thought to be an early form of secretion product.

However, wide variations in the structure of secretory granules in the duct cells of the adult gland have not been observed. Granules of different density and size occurred in the duct cells of adult glands, as a result of fasting and feeding, which increased the synthetic activity (Scoot and Pease, 1964). Similarly, in the granular duct portion of the adult submandibular gland, three types of cells occur; and agranular cell, a light granular cell and a dark granular cell. These cells have been interpreted as representing different secretory stages of the same cell type.

In the terminal tubules, some granules with more elaborate internal structures, such as those with thick and thin fingerprints like structures or those with dense patches of material, are not readily related to the granules of simpler interior, such as those with shells or cylinders. This obviously indicates that either there are more than one type of granules, or many intermediate type granules between those present in differentiating glands have not been observed in this study.

Regardless of these uncertainties, however, it is possible that the difference in structure shown by the secretory granules of young rats from the granules of the adult rat gland is a reflection of the differences in the chemical composition of these two granules. Histochemical studies have demonstrated that the granules of the terminal tubule show staining reactions different from the granules of the adult rat gland (Jacoby and Leeson, 1959). The enzyme con-

tent appears to be also different in the gland of young and adult rats; various enzymes do not reach the adult level until about 5 weeks after birth (Fukuda, 1967). Similarly, biochemical analysis of the submandibular gland have shown that the proteolytic activity of the gland increase with the increasing age of rats from about 15 days after birth (Sreebny, et al., 1955). It is not unlikely that qualitative and/or quantitative differences in the chemical components of the granules would be reflected in the structure.

SUMMARY

The secretory endpieces in the submandibular gland of rats during the first week of postnatal development have been studied by electron microscopy. The terminal portion of the ducts during the acinar differentiation consists of two types of cells which contain secretory granules. These cells are different in the organization of their cytoplasm and in the appearance of the granules they contain. One cell is an acinar-type and the other is duct-type of the adult gland. In the acinar-type cells, granules of electron-lucent interior are present and their structure remains the same throughout the week. In the duct-type cells, granules of widely different structures are present even within a single cell, as well as in different cells of the same gland at different stages of the development. The granules of the duct-type cells are of an unusual type, which is not found in the adult gland. Granules from younger glands contain circular or short cylindrical profiles of about 660Å. In older animals, the tubular substructures elongate, take irregular courses and occasionally form a regularly arranged bundle. The matrix of the granules is frequently much denser than that of the tubular substructure.

EXPLANATION OF FIGURES

Plate I

Fig. 1. A terminal tubule of the submandibular gland from a 7-day-old rat, illustrating two types of cells which constitute the tubule during the first week of the gland development. Acinar type cells (AC) contain ill-preserved appearing granules of lower density. Duct-type cells (DC) contain distinct granules of somewhat higher density. Note the difference in the pattern of endoplasmic reticulum organization in these two types of cells. X 5,000

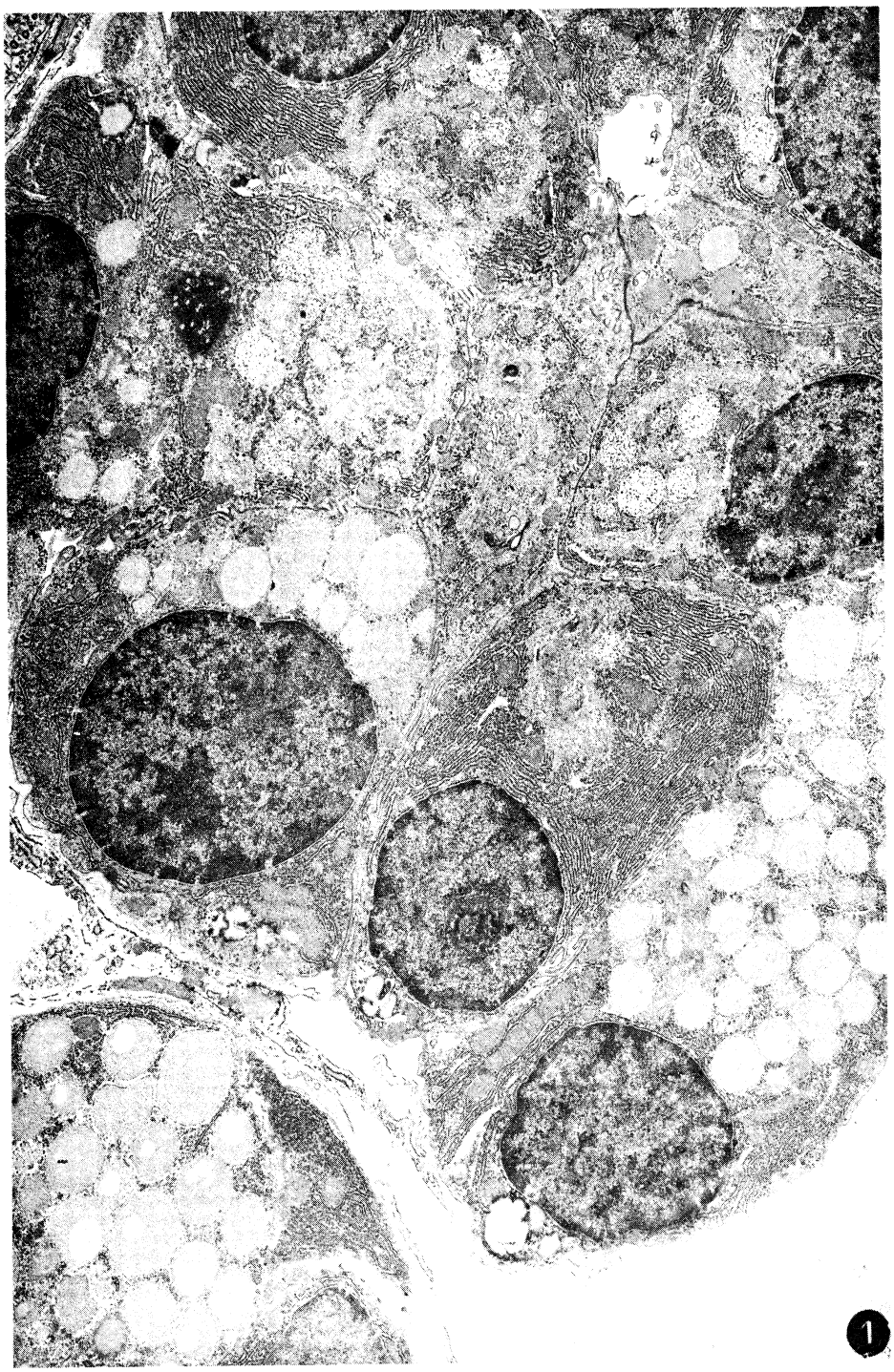

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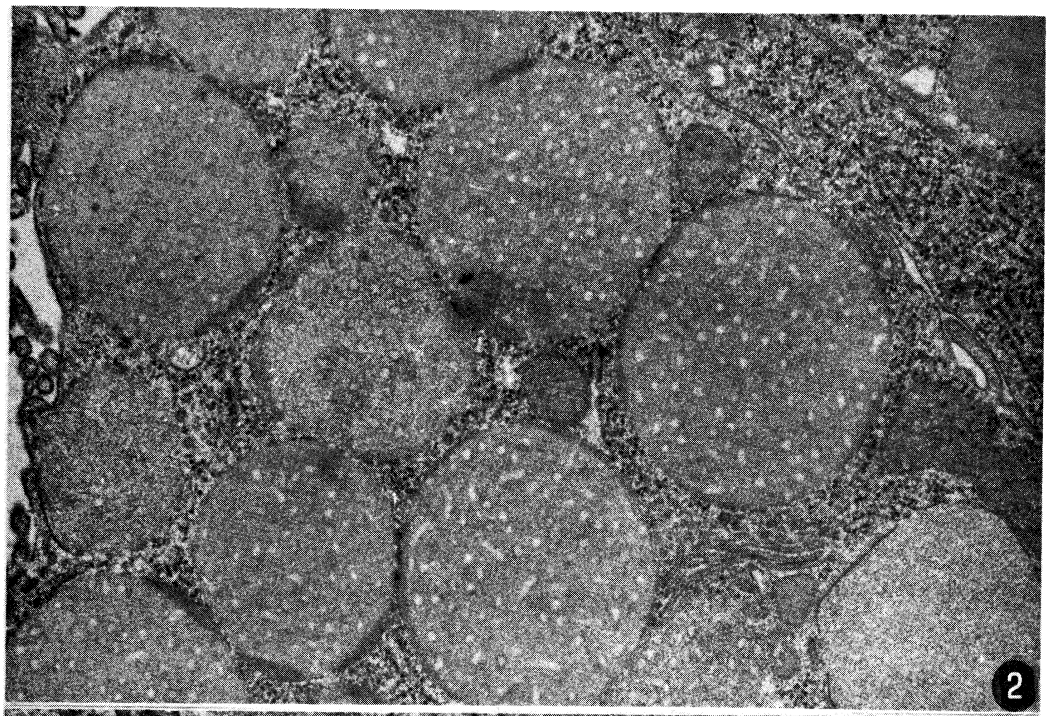
Plate II

Fig. 2. A portion of an acinar type cell from the terminal tubule of a 3-day-old rat. Golgi complexes occur in close association with the granules (AG). X 30,000

Fig. 3. A portion of a duct type cell from the terminal tubule of a 1-day-old rat. Granules of various kinds occur. Small granules of homogeneously dense interi or are near the Golgi (G). Numerous free ribosomes are among the granules. X 25,000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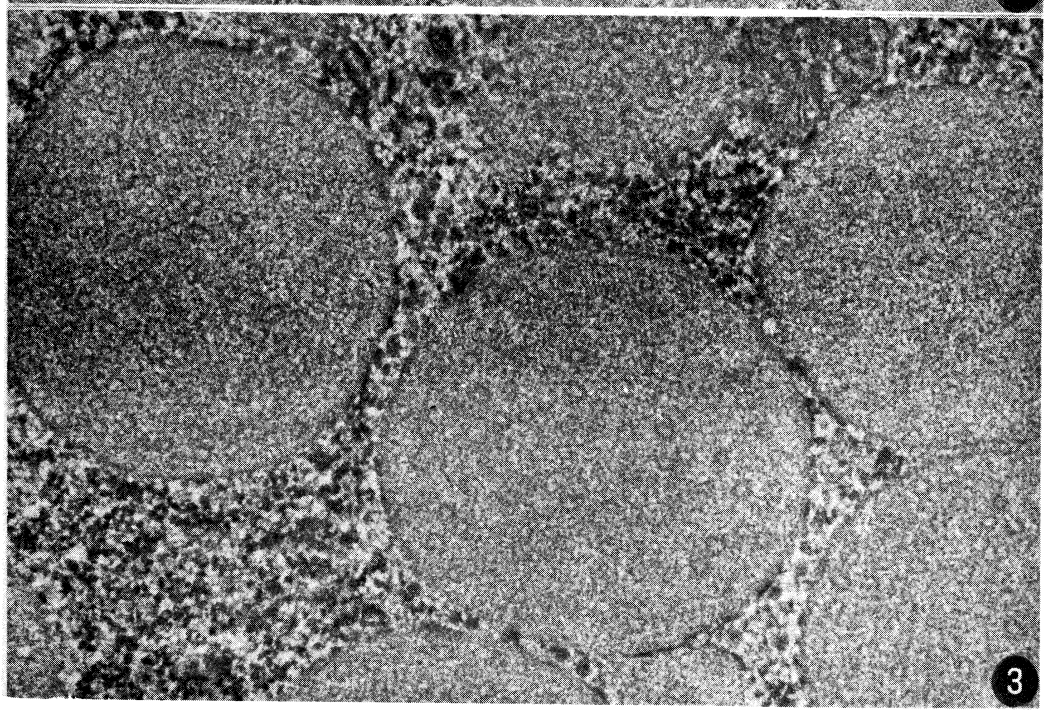
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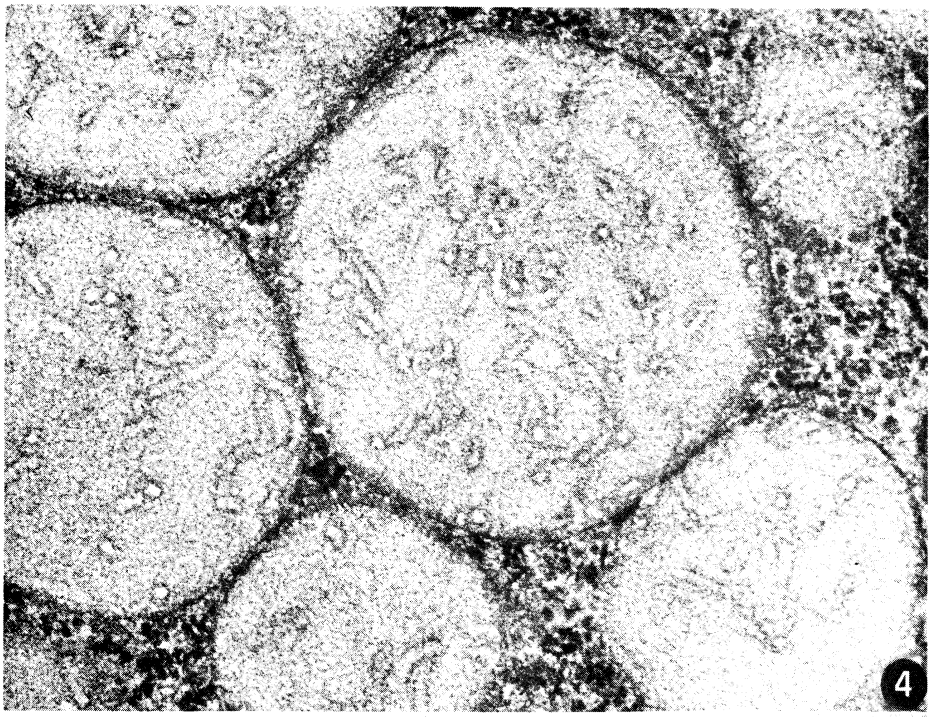
Plate III

Fig. 4. A type of granule which is most frequently encountered in the duct-type cells of 3-day-old rats.

Fig. 5. Granules with tubular substructure occur frequently in the duct-type cells of the terminal tubule on day 3. X 50,000



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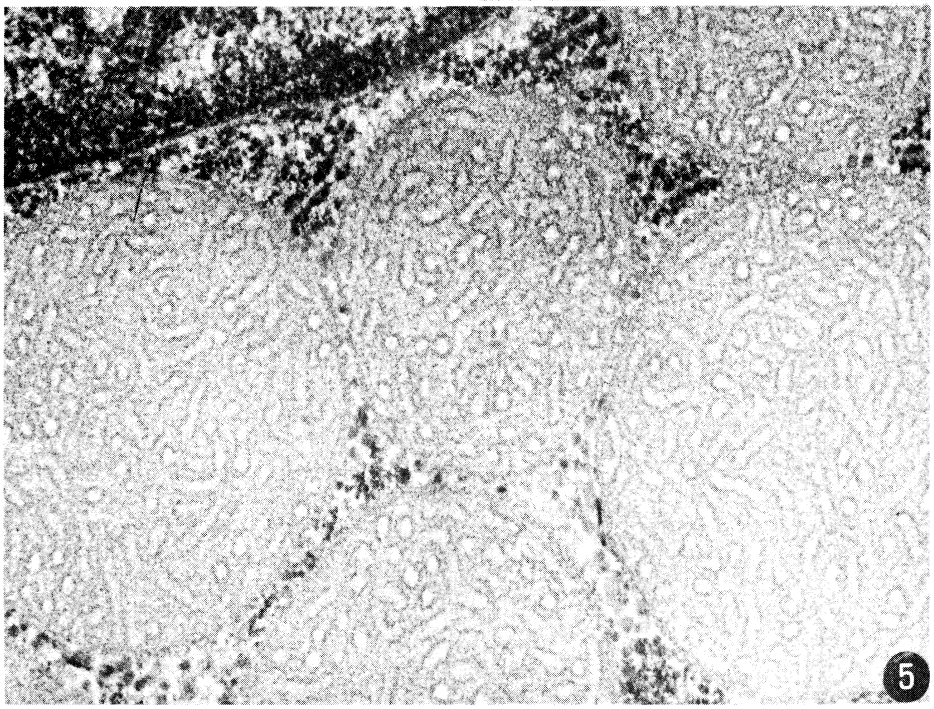

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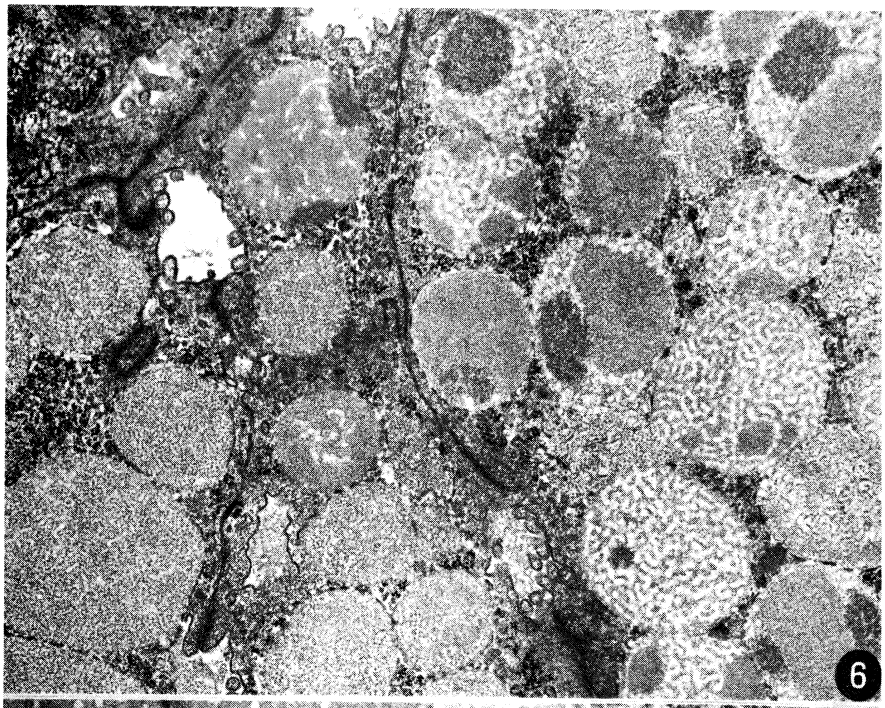
Plate IV

Fig. 6. Apical portions of the terminal tubule cells in the gland of a 7-day-old rat. Granules of various structures occur in different duct-type cells as well as within a cell. X 12,000

Fig. 7. Granules which contain tubules and are found in the duct-type cells of 7-day-old rats. The tubules of these granules are clumped together. X 50,000

Fig. 8. Granules of the duct type cells in the terminal tubules of 7-day-old rats. X 50,000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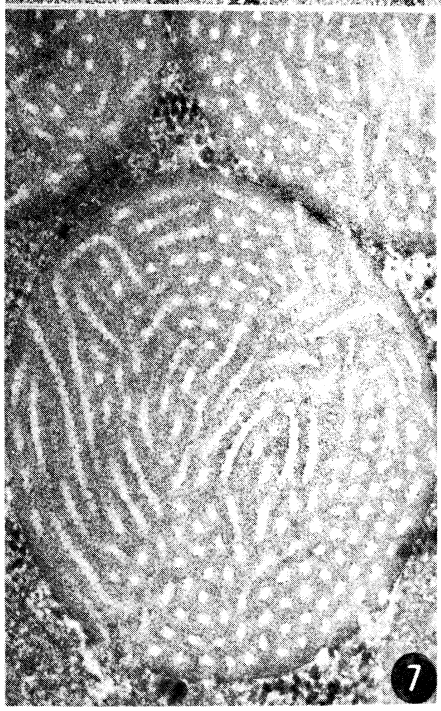
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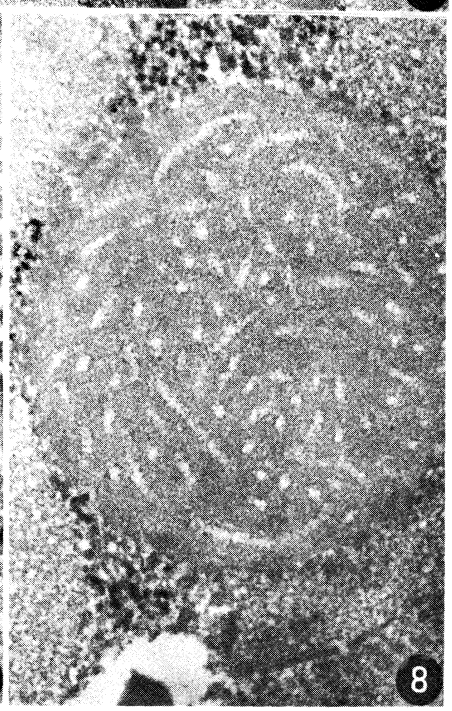
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C. Hypoxia Induced Modifications of Mitochondrial Ultrastructure in Submandibular Glands of Neonatal Rats

During the past decade a number of investigators have observed the fine structure of rodent submandibular glands (Scott and Pease, 1959; Kurtz, 1964; Tamarin and Sreebny, 1965; Tamarin, 1967) including the structure of developing glands during the early postnatal period of life (Leeson and Jacoby, 1959; Kim and Han, 1969). Much of these studies were concentrated on the appearance of evolving granules and other dytoplasmic organelles that are related to the production of secretory granules, and only casual comments have been made with respect to the fine structure of mitochondria.

On the other hand, electron microscopic observations of acute hypoxic effect in various tissues have centered primarily around the structural modification of mitochondria. Such observations include the blistering of the mitochondrial membrane and overall swelling of the mitochondria volume.

In the course of studying the immediate cytologic effect of a brief exposure to anoxia in digestive glands of neonatal mice, we have found a frequent reorganization of mitochondrial substructure which involves the formation of numerous small tubules in the mitochondria of terminal duct cells of the submandibular gland. The purpose of this article is to document the above mentioned observation.

MATERIALS AND METHODS

Forty-eight neonatal mice were paired according to the weight and one of each pair was subjected to a totally anoxic condition for 20 minutes as described elsewhere (Kim and Han, 1969). After the exposure the experimental animals were appropriately marked with a laboratory marker and returned to the mother until the time of sacrifice. Three pairs each of these animals were sacrificed at 30 minutes, 1, 3, 6, 12, 24, 48, and 72 hours after the completion of the anioxic exposure.

Small pieces of submandibular gland were dissected and fixed in 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.4 followed by a post-fixation with 1% osmium tetroxide in the same buffer. The tissues were dehydrated in graded ethanol and embedded in a mixture of epoxy resin in a routine manner (Luft, 1961). Thin sections were made on a LKB Ultratome III with a diamond knife and stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963). Final preparations were studied in a Philips 300 electron microscope at the accelerating voltage of 60 KV.

OBSERVATIONS

The mitochondria of cells of the terminal tubules of submandibular glands from control animals is large and usually rod shaped, measuring up to 10 μ in length with an average diameter of 0.7 μ. They are frequently folded over, presenting a somewhat pleomorphic appearance (Fig. 1). Occasionally a fusion of such folded portion is observed presenting a rather complex structure. Within the mitochondria, are well developed cristae which are spaced between 60 to 80 mμ apart from each other. These cristae originate from the internal limiting membrane and traverse the entire diameter of the mitochondrial body giving a rather regular shelf-like appearance to the longitudinally sectioned mitochondria. Along the periphery of the individual cristae, one finds many circular profiles (long arrows, Fig. 1), some of which appear to be continuous with the crista. Thus a suggestion is made that mitochondria might have small finger like projections along the free ends of its cristae which might project in random directions.

Elsewhere in the matrix are found a number of intramitochondrial granules (short arrows, Fig. 1) presumed to be inorganic salts composed of divalent ions (Peachy, 1962) and numerous smaller and less electron dense granules. The size of these moderately electron dense granules vary from that of ribosomes (LG, Fig. 1) to much smaller granules (SG, Fig. 1), measuring only 50 to 60 Å units in diameter. All of the granular materials are invested in a finely fibrillar and moderately electron dense matrix.

As early as 30 minutes after the anoxic exposure, mitochondria of these cells show certain notable changes. These are the swelling and the formation of small clusters of tubular profiles that are approximately 230 Å units in diameter (Fig. 2). The mitochondrial swelling was evident as indicated by the loosening of dense fibrous texture of the matrix, as well as the decrease in its electron density and the greater distance between cristae. Furthermore, there were frequent separations of space within individual cristae.

The formation of small clusters of tubule cell structures is best observed in glands taken after 2 to 6 hours of exposure. Comparative examinations of many static micrographs indicate that these tubular structures are characteristically of uniform diameter and may begin as small patches of irregularly arranged tubules. Larger patches show more regular arrangement of these tubules as indicated in Fig. 3. It may be noted that each of the two clusters depicted in this figure is composed of at least 30 or more of such tubular profiles that appear to be in the form of clusters.

Observations of longitudinal profiles indicate that such clusters develop between mitochondrial cristae (Figs. 4 and 5) and may present a straight and almost crystalline appearance. The longest registration of such profiles has been observed to measure up to 0.4 μ. In mitochondria where more than one such structure was found in a single plane of section, individual patches were located in random directions (Fig. 6). In between such straight profiles were

irregularly crossing tubules that appeared to be in the process of further organization. Elsewhere one could find occasional vacuolization (Fig. 6) and whirl-like deposits of membrane materials.

DISCUSSION

The preponderant size of mitochondria and abundance of glycogen granules in the developing terminal tubule cells of rodent submandibular gland give an impression that the level of metabolic activity and hence consumption of oxygen by these cells are high. This is further amplified by the fact that mitochondrial cristae are regular and extensively differentiated; a feature that has been linked to the differentiation of oxidative phosphorylating functions (Green, 1960; and Lehninger, 1964).

The sensitivity of mitochondrial structure to oxygen tension has been experimentally demonstrated under in vitro conditions in which almost instantaneous changes in the structure of isolated mitochondria from liver cells was characterized by electron microscopy.

Past studies of the ultrastructural modification of anoxia-induced changes in heart muscle cells and certain neural elements have shown notable changes in the mitochondrial structure that was characterized by blistering of the limiting membranes and swelling. The results of the present study suggests that the earliest modifications of subcellular structures might be the swelling of certain mitochondria, vacuolization of mitochondrial matrix, deposition of whirl-like figures, and the reduction in the degree of organization of mitochondrial cristae. The latter observation was true only in those mitochondria where the array of tubular paracrystalline structures were noted, suggesting a possible causal relationship between the two phenomena.

Considering the probable functional impairment of phosphorlating capacity of these mitochondria due to lack of oxygen, it is tempting to speculate that such dissociation of enzyme assemblies might isolate the electron transport systems which might aggregate together until a reutilization of such enzyme assemblies become possible. Although such a hypothesis will have to be proven by clarification of the chemical identity of the paracrystalline structures and a possible replication of such damage under in vitro conditions, it might be pointed out that many enzymes could be recovered in crystalline form, and that certain cytoplasmic structures that are known to contain peroxides and hydrolytic enzymes show paracrystalline structure. These include the microbodies of the hepatic parenchymal cells and the crystalloid structures of the eosinophilic leucocytes.


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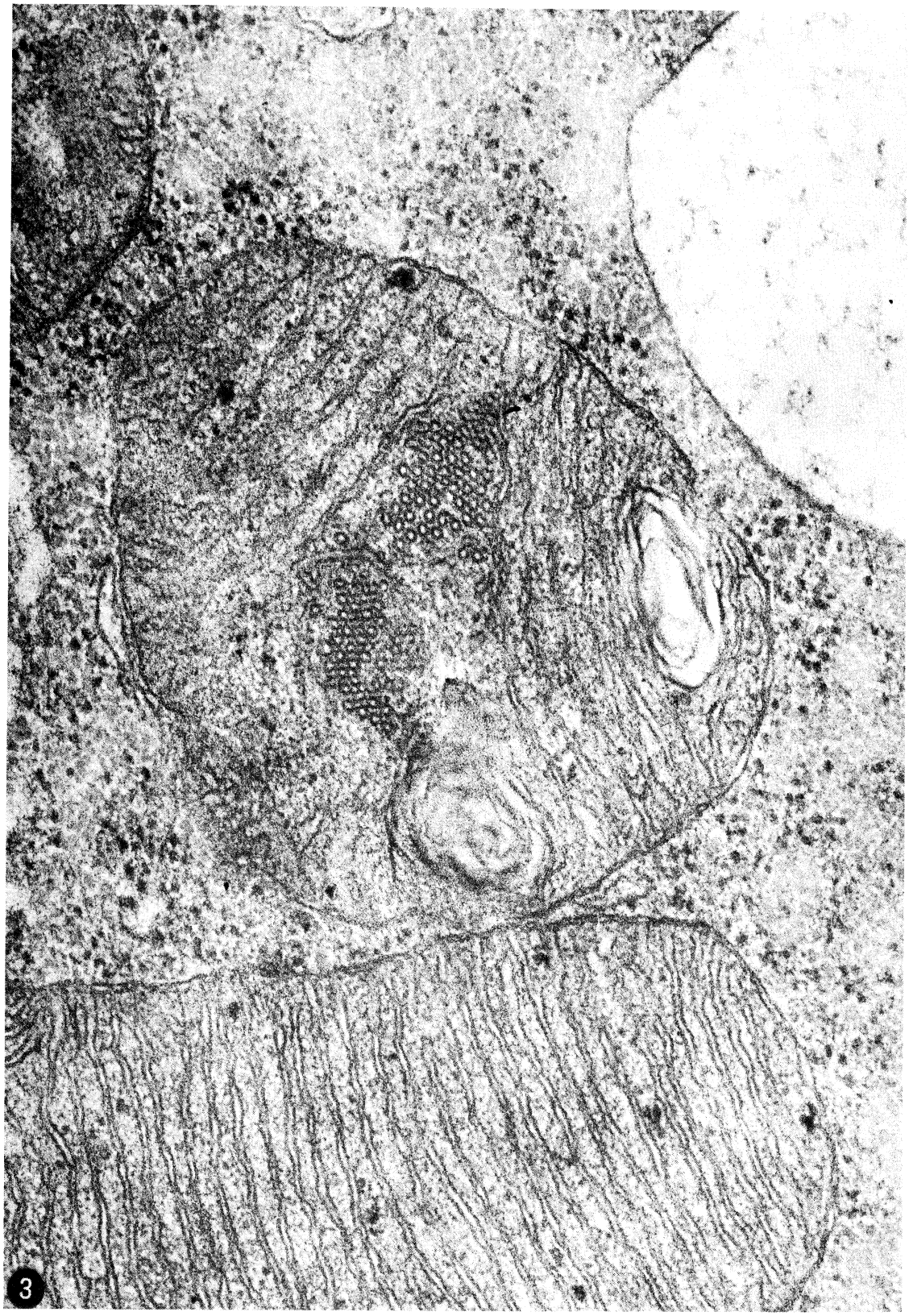
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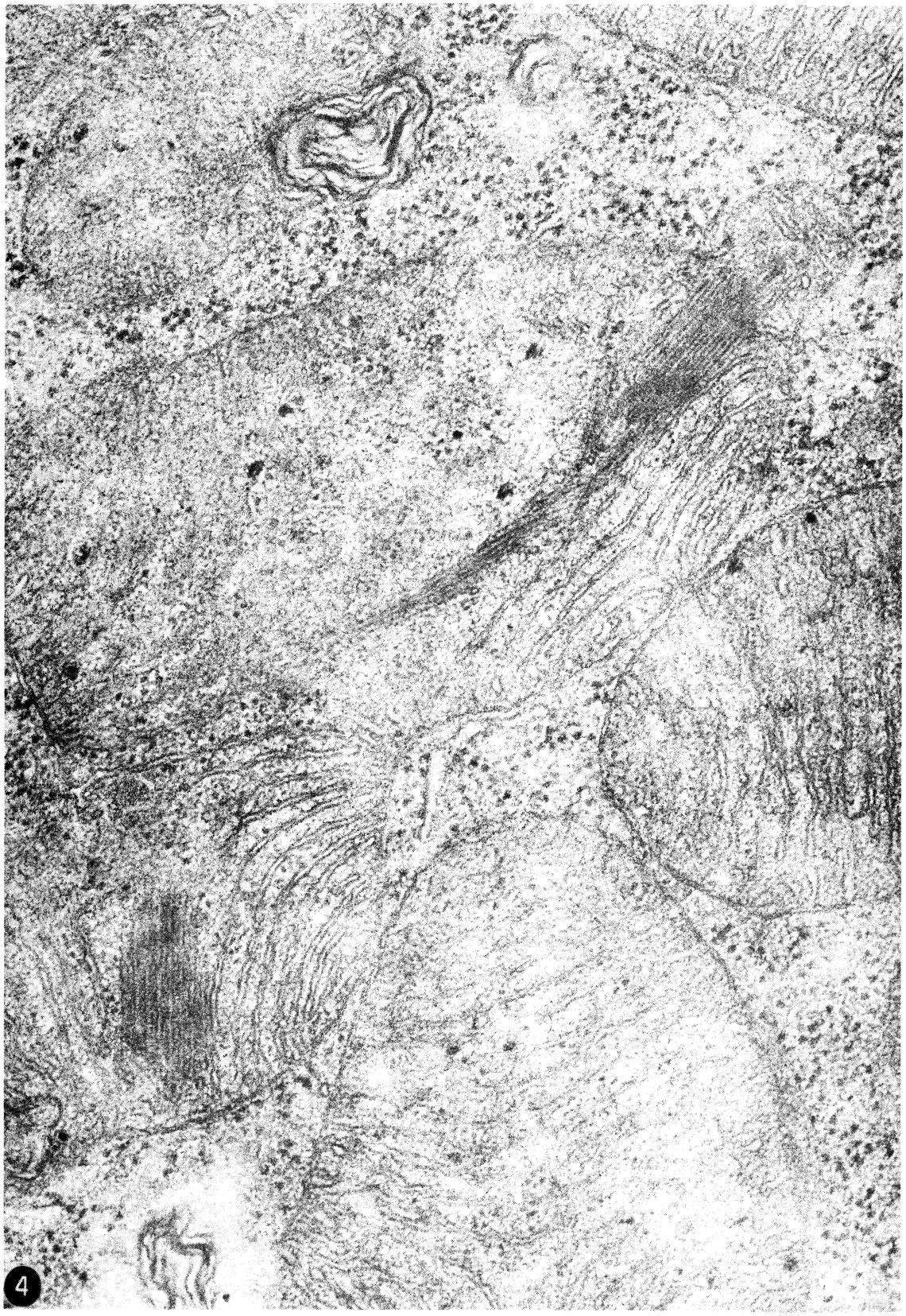
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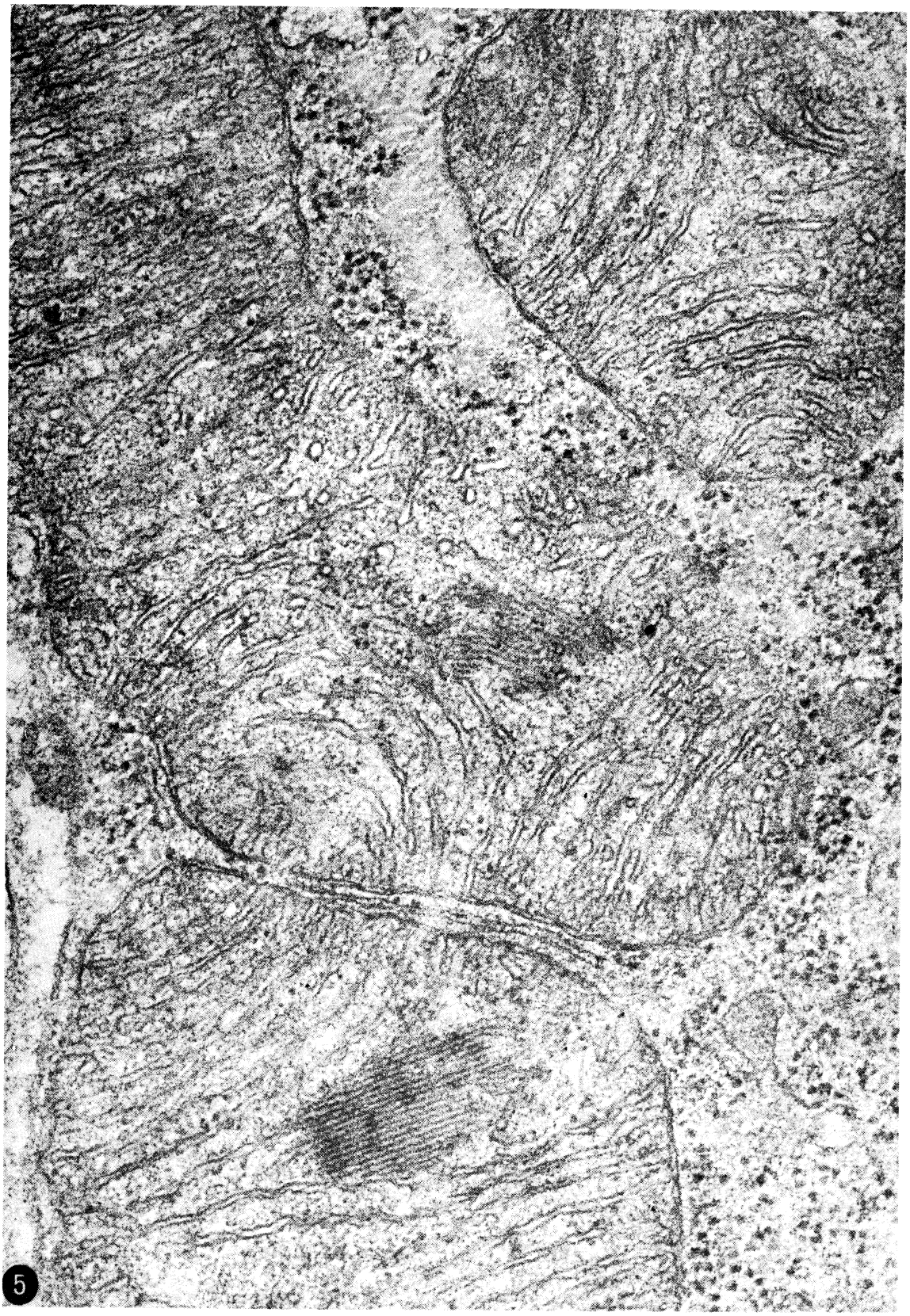
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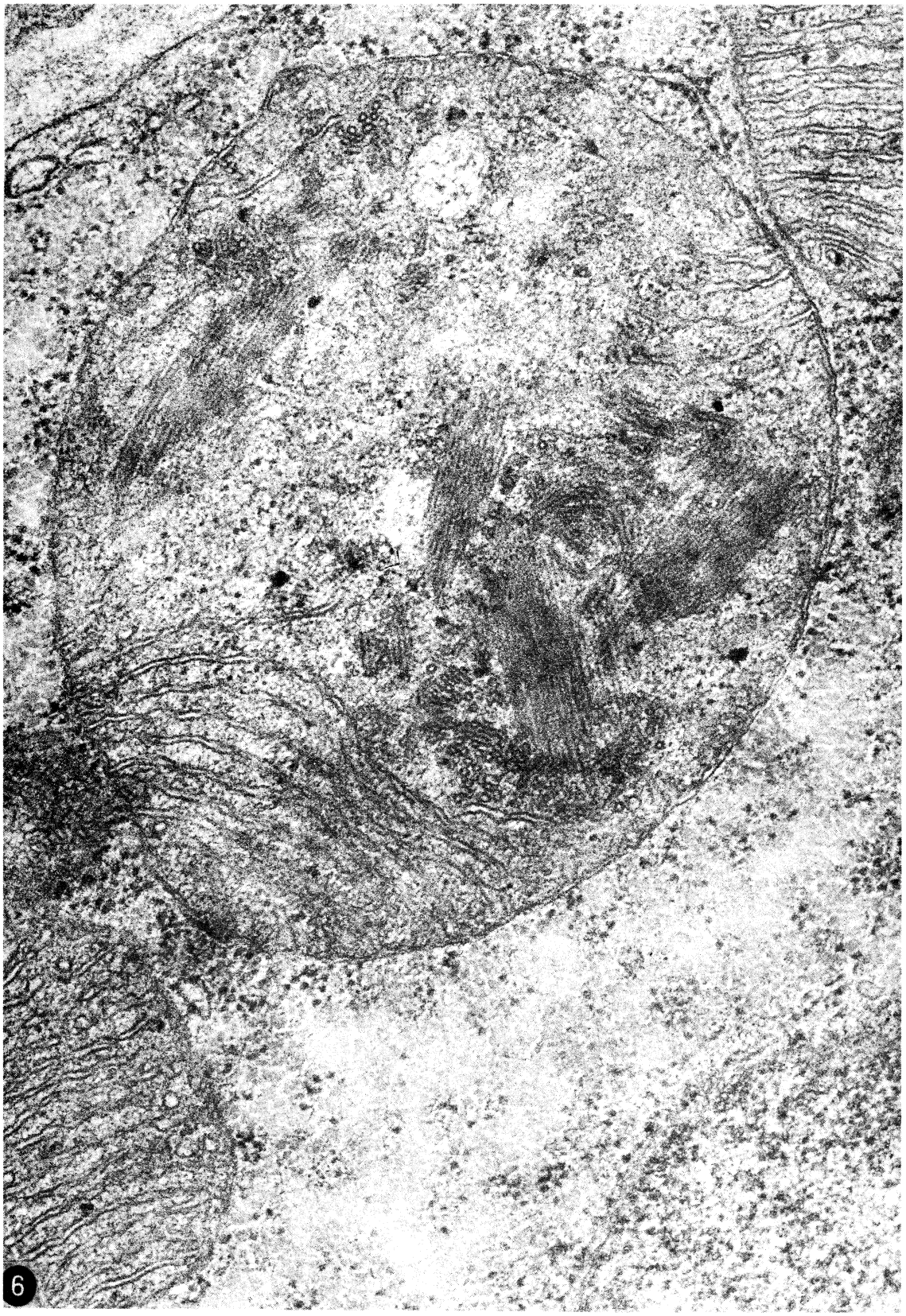
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D. Effects of Total Anoxia on Developing Connective Tissue Cells in Rats

Traditionally the neurophysiologic aspects of the hypoxic effect have been extensively studied from the utilitarian point of view relative to anesthesia. The literature in this regard is rather voluminous. More recently, a number of studies on the development of foetuses exposed to hypoxia have demonstrated serious birth defects (Murakami, Kameyama, and Kato, 1956; Ingalls and Curley, 1957; Degenhardt, 1960; Morawa and Han, 1968). The teratological results so produced have led to the idea that the malformation was due to the excessive accumulation of lactic acid and thereby to a chemical upset of the milieu in which the foetal tissues were developing (Grabowski, 1964).

In a previous study we noted, in addition to the skeletal deformities previously reported, a sharp reduction of basophilia and delayed differentiation of developing epithelial and connective tissue cells (Morawa and Han, 1968). This was thought to be due to a sustained suppression of protein synthesis caused by a single insult given during the prenatal period. Consequently, we performed experiments aimed at illuminating the effects of prenatal exposure to anoxia on protein biosynthesis in rapidly growing cells during the immediate postnatal period (Han and Kim, 1969). The results from these experiments indicated that there was a significant drop in the rate of labelled amino acid incorporation into a variety of cells, including connective tissue cells.

On the other hand, studies dealing with the neonates subjected to anoxia within hours of birth did not reveal any notable changes in cytology of protein-producing cells namely fibroblasts, osteoblasts and odontoblasts (Smith and Han, 1968). However, the protein synthesis (leucine-H^3 incorporation) in salivary glands from similarly treated neonates was dramatically inhibited as early as 60 minutes after the exposure to anoxia (Kim and Han, 1969). Whether a similar suppressive effect was exerted on connective tissue cells was not determined. Nevertheless, this is in contrast to the delayed effect on the production of secretory proteins (proline-H^3 incorporation) by connective tissue cells of neonates that showed the greatest reduction at 24 hours (Smith and Han, 1968).

The purpose of this experiment was to study the nature and extent of the immediate effect of anoxia on overall protein synthesis in fibroblasts, osteoblasts, and chondrocytes of rat neonates.

MATERIALS AND METHODS

Experimental Design

New-born Sprague-Dawley rats from an inbred colony were used throughout the experiment. They were subjected to anoxia for a period of 20 minutes by residence in a bell jar which was flushed with a continued flow of purified nitrogen. The oxygen content in the bell jar fell rapidly, within 2 minutes, to 30 to 40 ppm, as indicated by a Westinghouse oxygen analyzer, and was maintained at this low level for the rest of the time. Immediately after the anoxic period of time the rats were given intraperitoneally 3 μc of DL-leucine-4, 5-H^3 per gm of body weight. The specific activity of the leucine was 5 curies per mM. The same number of control animals were handled in an identical manner, except that during their residence in the bell jar the latter was continuously flushed with fresh air. Animals were sacrificed in pairs at 15 minutes and at 1, 4, 24, 48, and 72 hours after the injection of the labelled amino acid. Three identical experiments were carried out.

Preparation for Radioautography

Animals were sacrificed by decapitation. The brain was removed after the calvarium was excised. The head was then sectioned through the mid-sagittal plane and fixed in Bouin's solution for 48 hours. During double-embedding in parlodion and paraplast in a routine manner the tissue was so oriented as to allow the sections to be made through the molar region of the developing maxilliary arch. Serial sections, 6μ in thickness, were mounted on 1 x 3 inch microscope slides which had been pretreated with an adhesive solution consisting of 0.5% pure gelatin, 0.05% of chromium potassium sulphate in distilled water.

Five sets of slides, having comparable histological areas, were coated by dipping into Kodak NTB-3 emulsion at 45°C in total darkness. The slides were then placed on end on a wooden rack and dried for 15 minutes in an oven set at 45°C. After drying, the slides were placed in an air-tight box containing small packets of Drierite, shielded by wrapping with two layers of lead sheet, and wrapped with an additional layer of light-tight paper. They were sealed in a plastic bag in which Drierite packets were placed, and were kept in a refrigerator at 4°C for a period of 5 weeks.

At the end of the exposure period the slides were developed in 2, 4-diaminophenol dihydrochloride for 1 minute in complete darkness. After fixation in Kodak Rapid Fixer for 2 minutes and washing, the slides were stained in Harris hemotoxylin and alcoholic eosin Y. The average number of grains over each cell type was obtained by counting approximately 500 cells per animal. In all, over 15,000 cells were examined. These data were subjected to the student t test by using a computer program provided by The University of Michigan Terminal System IBM 7090.

RESULTS AND OBSERVATIONS

No difference in the cytological appearance between anoxic and control rats could be discerned throughout the experimental period. However, the histologic appearance of radioautographs elucidated well the cumulative effects of secretory processes by connective tissue cells, as there was a proportional increase in intercellular grain number relative to the rate of synthesis and secretion. On the other hand, the grain counts over cells showed even slight differences in intracellular grain numbers which might easily escape a histologic scrutiny. Therefore, both quantitative data from grain counts and histology of radioautographs are presented in this report.

The Fibroblast

Depending upon the region the fibroblasts showed a wide range of average grain numbers. For instance, the cells in the dental pulp and developing periodontal ligaments had fewer grains per cell than those located in the subcutaneous connective tissue. Differences were also noted among the fibroblasts present on the subcutaneous tissues of various regions. For this reason, only those fibroblasts that were located in the subcutaneous region covering the lower border of the mandible were selected for this presentation.

A summary of individual counts of silver grains over the fibroblasts is given in Table I. It can be seen that in control animals there was a progressive increase in the grain number from 4.9 at 15 minutes to 10.7 and 15.3 at 60 minutes and 4 hours respectively. By 24 hours, the average number was 9.8 per cell, and remained at this value essentially for the following 24 hours. On the other hand, the average number of grains per cell in the anoxic group was only 3.7 at 15 minutes, increased to 4.9 at 60 minutes, and then further increased to 13.9 by 4 hours after the injection. Thereafter, the average grain number dropped to 9.0 per cell, and no significant changes were noted in the following days.

Statistically, the differences at 15 and 60 minutes were significant at the level of $p < 0.001$, whereas at 4 hours the difference between the average grain count was significant at $p < 0.01$. The difference at 24 hours was only significant at the level of $p < 0.1$, and the same tendency was observed throughout the rest of the experimental period.

The photomicrographs appearing in Figs. 1 through 8 illustrate and supplement the results gained by the grain counting. Figures 1 and 2, which were from the experimental and control animals sacrificed at 15 minutes after the injection of labelled leucine, showed little difference on visual inspection. At 60 minutes, however, the number of grains superimposed upon these cells was definitely fewer in experimental as compared to control animals (Figs. 3 and 4). In addition, it was already evident by 60 minutes that there was an increasing number of grains localized in intercellular space, some distance away from the cell body. This point was even clearer in the pairs sacrificed at 4 hours (Figs. 5

TABLE I. QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF NEONATAL ANOXIA IN RATS

Leucine-H^3 Incorporation by Fibroblast*

Time After H^3 Injection	Treatment	Mean Grain No. (S.D.)	Probability
15 min	Anoxia	3.72 (±0.77)	$P < 0.001$
	Control	4.90 (±0.94)	
60 min	Anoxia	4.86 (±1.39)	$P < 0.001$
	Control	10.68 (±1.73)	
4 hr	Anoxia	13.86 (±1.81)	$P < 0.01$
	Control	15.32 (±1.99)	
24 hr	Anoxia	9.00 (±1.50)	$P < 0.1$
	Control	9.82 (±1.51)	
48 hr	Anoxia	9.70 (±0.98)	$P < 0.001$
	Control	11.17 (±1.38)	

*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.

and 6), and at 24 hours (Figs. 7 and 8), following the injection. Thus, the grain number over the intercellular space is definitely greater in the control tissue of both 4- and 24-hour animals, despite the relatively large number of grains over the cells in experimental animals at this time.

The Osteoblast

In Table II the counts of silver grains over osteoblasts are summarized. Recognizing the large regional variation in osteogenic activities of developing bones, we have limited our observations to the lower border of mandible directly under the forming molar teeth. It is pertinent that the pattern of changes of average grains per cell in the control animals was similar to that seen in the fibroblasts.

There was a difference between the experimental and control value throughout the first 4 hours, and the level of significance in all of these periods was $p < 0.001$. As was true in the case of fibroblasts, the greatest difference was observed at 60 minutes after the injection, namely 10.7 grains per cell in the control and 6.0 grains per cell in the experimental. The slight differences observed in the average value at 24 hours and at 48 hours were statistically insignificant in this case.

Figures 9 through 16 provide pictorial supports for the quantitative data. At 15 minutes (Figs. 9 and 10) the small difference was not readily noticeable in actual photomicrographic fields. By 60 minutes, however, the cells of the controls (Fig. 12) showed far more grains than those of the experimental animals (Fig. 11). In Figs. 13 and 14, which were from the experimental and control animals at 4 hours, a clear difference was discerned in the extracellular grain numbers as in the case of the fibroblasts. Although the average grains per cell did not indicate a meaningful difference, the animals sacrificed 24 hours after the injection of the radioactive amino acid demonstrated a real difference in the number of extracellularly located grains, which were greater in the control animals (Fig. 16) when compared to that of the experimentals (Fig. 15).

The Chondrocyte

In the new-born rat a fairly large amount of chondrogenic tissues are notable in spheno-occipital region of the cranial base. By selecting histologically comparable areas of such chondroid masses, meaningful quantitative efforts could be made. In Table III are recorded the results of grain counts made in these chondroid tissues. Paralleling the change in the fibroblast and osteoblast, the average number of grains per chondrocyte of the spheno-occipital region in the control showed a gradual increase during the early hours after anoxic insult and was followed by a gradual reduction thereafter. There were significant differences between experimental and control animals except in those that were sacrificed at 48 and 72 hours. The level of significance in these periods was $p < 0.001$, with the exception of the 4-hour group, which was significant at $p < 0.01$.

TABLE II. QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF NEONATAL ANOXIA IN RATS

Leucine-H^3 Incorporation by Osteoblast*

Time After H^3 Injection	Treatment	Mean Grain No. (S.D.)	Probability
15 min	Anoxia	2.5 (±0.81)	P < 0.001
	Control	4.62 (±0.98)	
60 min	Anoxia	5.96 (±1.04)	P < 0.001
	Control	10.67 (±2.01)	
4 hr	Anoxia	9.33 (±1.96)	P < 0.001
	Control	11.29 (±2.42)	
24 hr	Anoxia	12.13 (±1.82)	P < 0.6
	Control	12.37 (±1.67)	
48 hr	Anoxia	9.06 (±1.61)	P < 0.7
	Control	8.86 (±1.93)	

*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.

TABLE III. QUANTITATIVE RADIOAUTOGRAPHY ON EFFECTS OF NEONATAL ANOXIA IN RATS

Leucine-H^3 Incorporation by Chondrocytes of Nasosphenoidal Region*

Time After H^3 Injection	Treatment	Mean Grain No. (S.D.)	Probability
15 min	Anoxia	2.89 (±0.94)	P < 0.001
	Control	4.84 (±1.26)	
60 min	Anoxia	4.08 (±0.88)	P < 0.001
	Control	10.37 (±2.24)	
4 hr	Anoxia	11.79 (±1.93)	P < 0.01
	Control	13.25 (±2.07)	
24 hr	Anoxia	9.33 (±2.19)	P < 0.001
	Control	12.00 (±1.80)	
48 hr	Anoxia	8.63 (±1.77)	P < 0.3
	Control	9.21 (±1.42)	

*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.

As shown in photomicrographs it was evident that an increased number of extracellular grains was localized in the control animals sacrificed at 1 hour (Fig. 20) and 4 hours as compared to the 15 minute-control (Fig. 18). Throughout, the number of grains seen extracellularly was lower in the experimental animals than that of the control. By 24 hours the intercellular grains in the experimental animal (Fig. 23) had increased considerably and the net difference between the experimental and control (Fig. 24) appeared to be reduced.

DISCUSSION

Despite the problems relative to the fixation of free precursors or conversely leeching out of synthesized materials during the course of preparation, quantitative radioautography has been successfully applied in many problems that require the localization of radioactive precursors being incorporated into proteins at histological and cytological levels (Warshawsky, Leblond, and Droz, 1963; Young and Greulich, 1963, Ross and Benditt, 1962; Han, 1967; and many others). Although we have used, as others did, 1 micron-thick sections of epoxide-embedded materials for radioautographic quantitation of soft tissues, the limitation of the block size creates a serious problem in attempting to extend the same technique to studies of the hard tissues of craniofacial regions, which are necessarily large and have a marked functional and structural heterogeneity among cells present in different regions. Under these circumstances, the relatively thick sections of paraffin-embedded materials used in this study represent one of the best, if not the only method of studying the modification of the hard tissue metabolism at the level of individual cells.

It should be noted that, although statistically significant differences were present in all animals sacrificed at 15 minutes, 1 and 4 hours after anoxic exposure, the difference was the greatest at 60 minutes following the insult. The fact that such differences were largely insignificant after 24 hours of the insult suggests the transient nature of the suppression of leucine-H^3 incorporation. In this connection it is of interest to compare the data from the present study to those of our previous work in which the incorporation of proline-H^3 into the secretory proteins was studied in similar types of cells (Smith and Han, 1968). Of the cells studied, which included osteoblasts and fibroblasts, the incorporation of proline-H^3 was suppressed to the greatest degree at 24 hours following the anoxia, and only a small suppression was noted at 1 and 6 hours after the insult. This delay in maximum suppression of proline-H^3 incorporation is in sharp contrast with the early reduction of leucine-H^3 incorporation observed in this study.

Since proline-H^3 is incorporated into collagen and other proteins of the connective tissue matrix the delayed effect might be taken as indicative of an earlier effect upon the developing intracellular machinery necessary for secretory protein synthesis, such as the endoplasmic reticulum, Golgi apparatus, ribosomes, and so on. Furthermore, it is possible that the direct effect of anoxia on the overall protein synthesis by these cells is recovered to a near-normal

level by 24 hours after the insult, despite a markedly reduced incorporation of amino acids into secretory proteins. However, this particular point should remain as a question to be supplemented by future experiments, inasmuch as these two experiments were done under similar but not identical conditions.

Notwithstanding these, the fact that the synthesis of proteins is rapidly growing neonates is drastically reduced following a brief exposure to total anoxia provides us with another point that should be considered in conjunction with the etiology of malformation in developing animals exposed to hypoxic insult. As mentioned earlier, previous studies dealing with prenatal animals have suggested that the hypoxia related deformities malformation might be due to the accumulation of lactic acid and interference of tissue growth by such an accumulation of the lactic acid (Ingalls and Curley, 1957; Grabowski, 1964). While this probably is true, the serious, though transient, impairment of protein biosynthesis in various connective tissue cells at a crucial time during cellular differentiation might be responsible for the derangement of the pattern of sequential enzyme differentiation of various body regions. The importance of maintaining such a pattern of differentiation for the normal growth of an individual organism cannot be over-emphasized, inasmuch as the normal differentiation and growth of cells are implemented by the coordinated evolution of enzymes which are proteinaceous. In this sense the present results augment the previously expressed concepts, and offer a complementary alternative to the mechanistic view.

EXPLANATION OF FIGURES

Plate I

Figures 1 through 8. Radioautographs of the subcutaneous connective tissue from animals injected with 3 μc/gm body weight of leucine-H^3 in normal and anoxic animals. The photographs on the left column represent the experimental animals, while the ones on the right column are from the control animals sacrificed at the same intervals corresponding to those on the left.

Fig. 1. Experimental sacrificed at 15 minutes.

Fig. 2. Control sacrificed at 15 minutes. Only a few grains are localized over individual fibroblasts.

Fig. 3. Experimental animal sacrificed at 60 minutes after the injection. There is no increase in the number of grains as compared to that representing 15 minutes.

Fig. 4. Control animal sacrificed at 60 minutes. There is a notable increase in the intracellular grains as well as a greater number of extracellular grains as compared to Fig. 2.

Fig. 5. Experimental animal sacrificed at 4 hours. Note that a fairly large number of grains are found over individual cells.

Fig. 6. Control rat sacrificed at 4 hours. The number of grains superimposed upon individual cells is about the same as that of the experimental. However, a greater number of intercellular grains is observed.

Figs. 7 and 8. Experimental and control animals sacrificed at 24 hours. The appearance of grains of the cells is about the same between the two photomicrographs except that in the control animals there is a greater number of extracellular grains.

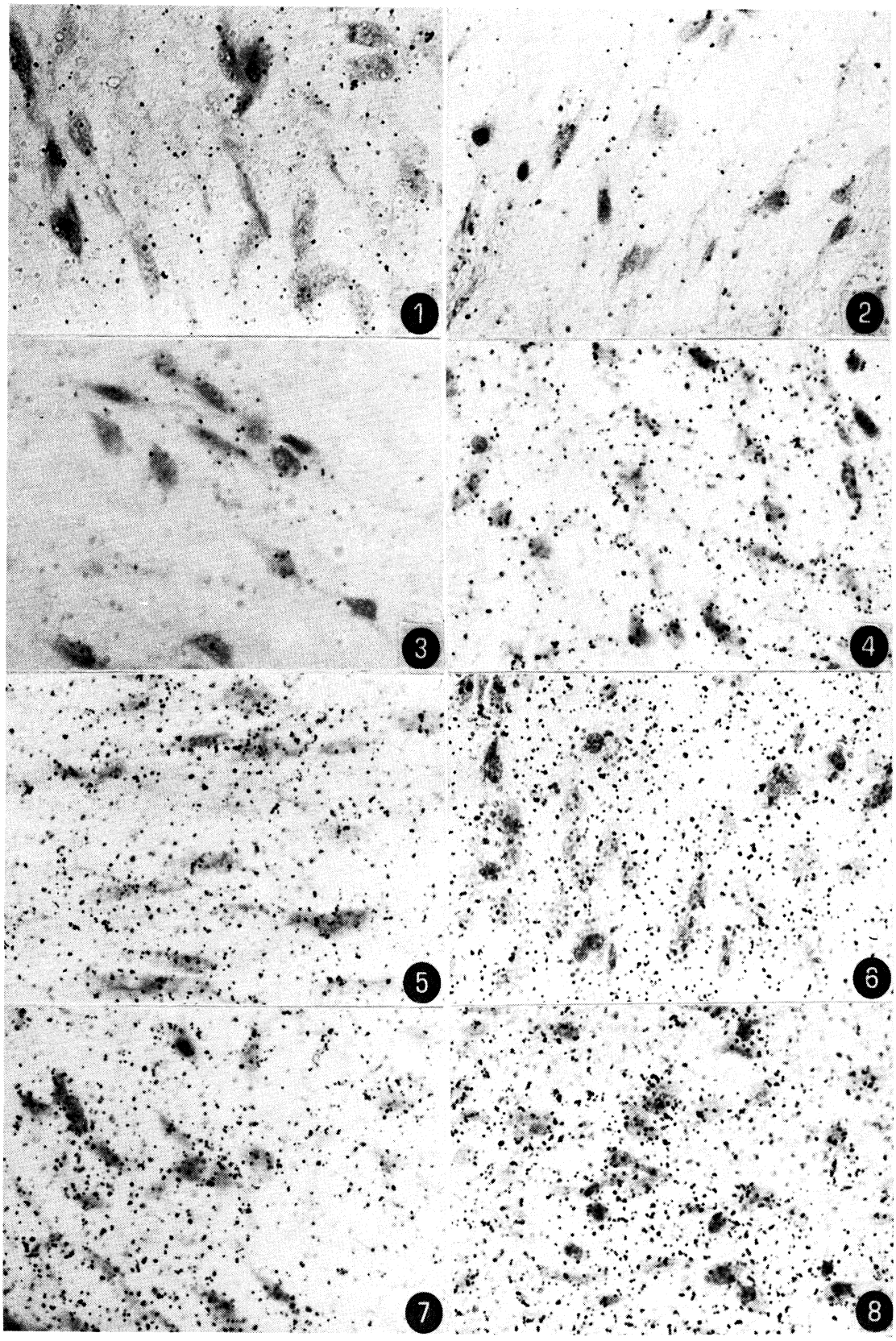
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Plate II

Figures 9 through 16. The appearance of radioautographs of the osteogenic region along the lower border of the mandible in rats with or without exposure to total anoxia. The photographs on the left column represent the ones from anoxic animals, whereas the ones on the right depict the comparable control rats.

Fig. 9. Experimental animal sacrificed at 15 minutes.

Fig. 10. Control animal sacrificed at 15 minutes. Note the relative paucity of grains in both the experimental and control rats.

Fig. 11. Experimental animal sacrificed at 60 minutes.

Fig. 12. Control animal sacrificed at 60 minutes. A much larger number of grains over osteoblasts as well as somewhat greater number of extracellular grains is noted.

Fig. 13. Experimental animal sacrificed at 4 hours. The number of grains over osteoblasts is increased over that shown in the experimental rat of 60 minutes.

Fig. 14. Control animal sacrificed at 4 hours. The number of extracellular grains is notably increased over that seen in the control at 60 minutes and is greater than that of the experimental.

Figs. 15 and 16. Experimental and control animals sacrificed at 24 hours. As is true in the case of fibroblasts, the number of extracellular grains is greater in the control animal.

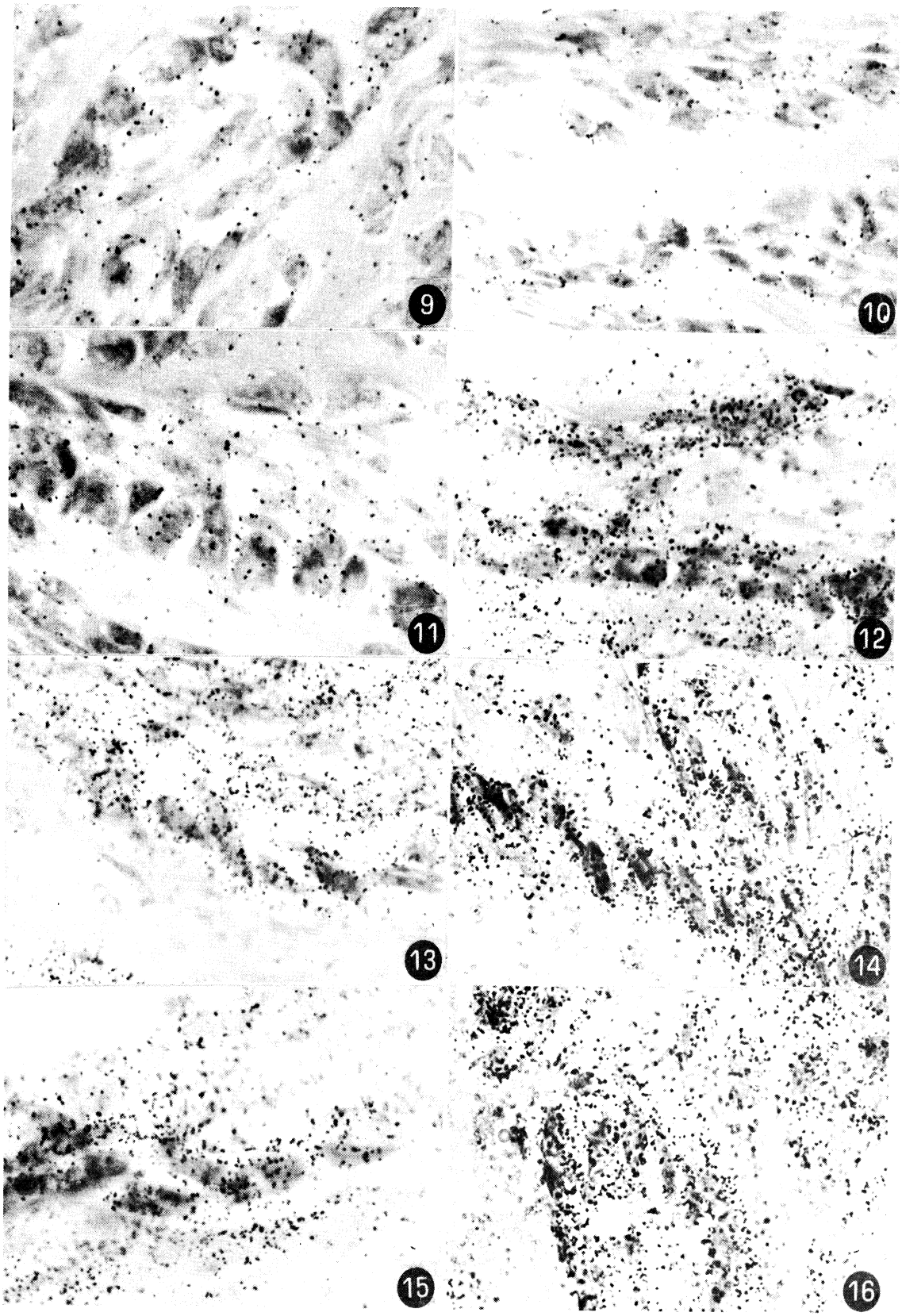
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Plate III

Figures 17 through 24. Radioautographs of chondroid mass of the spheno-occipital region of normal and anoxia-subjected neonatal rats. The photographs on the left column represent fields from anoxic animals, whereas the ones on the right depict comparable controls.

Fig. 17. Experimental animal sacrificed at 15 minutes. Only a few grains are seen over individual lacunae.

Fig. 18. Control animal sacrificed at 15 minutes after the injection. A slightly greater number of grains is observed.

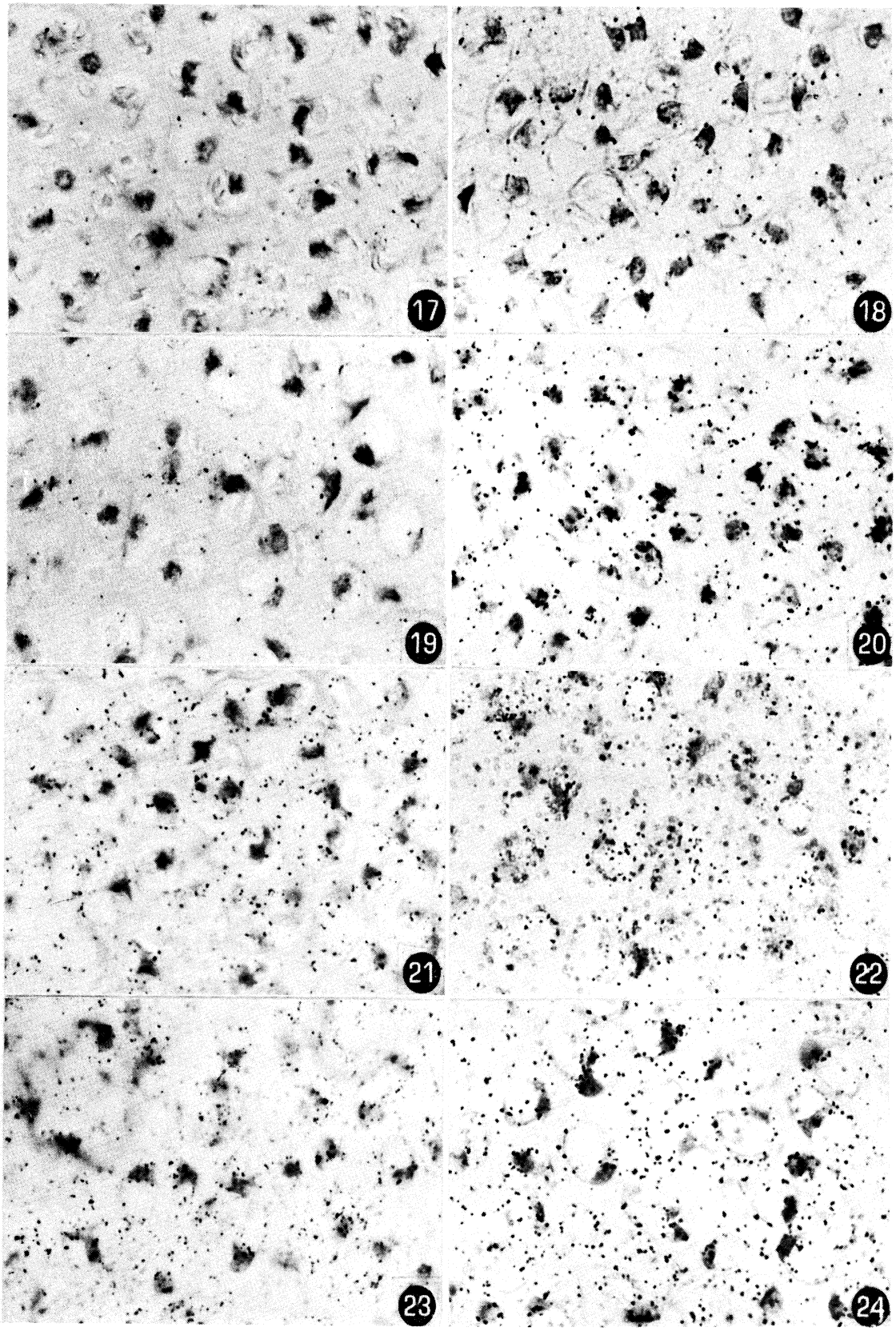
Fig. 19. Experimental animal sacrificed at 60 minutes. Again only a small number of intracellular granules are visible.

Fig. 20. Control animal sacrificed at 60 minutes. Notice a relatively large number of intracellular grains as well as a somewhat greater number of extracellular grains.

Fig. 21. Experimental animal sacrificed at 4 hours. The number of intracellular grains is notably increased over that seen at earlier periods and occasional extracellular grains are observed.

Fig. 22. Control animal sacrificed at 4 hours. In addition to a considerable number of intracellular grains, a large number of extracellular grains is visible, although many of the extracellular grains are somewhat out of focus.

Figs. 23 and 24. Radioautographs of experimental and control animals sacrificed at 24 hours. Note that the intracellular grains are concentrated in both cases, while extracellular grains are somewhat greater in the control.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PUBLISHED RESEARCH FINDINGS

The following publications are based on research conducted during the -01 year of grant HD 03147:

Articles:

(1) Studies on Hypoxia. I. Gross and histologic influences of maternal anoxia upon developing rat fetuses. Arch. oral Biol., 13:745-754, 1968.

(2) Studies on Hypoxia. II. Autoradiographic quantitation of proline-H^3 incorporation by connective tissue cells of the neonatal hamster. J. dent. Res., 47:244-251, 1968.

(3) Studies on Hypoxia. III. Effects on leucine-H^3 incorporation by submandibular gland cells or rat neonates. Proc. Soc. Exp. Biol. Med., 130:470-473, 1969.

(4) Studies on Hypoxia. IV. Differential response of respiratory enzymes in various organs of adult rats. Proc. Soc. Exp. Biol. Med., 130: 1042-1045, 1969.

Abstracts:

(1) Proline-H^3 incorporation in connective tissue cells of hamster neonates subjected to anoxia. J. dent. Res., IADR abstracts, p. 168, 1968.

(2) Leucine-H^3 incorporation by connective tissue cells of rat neonates subjected to anoxia. J. dent. Res., IADR abstracts, p. 168, 1968.

(3) Light and electron microscopic observations of the early postnatal development of the rat submandibular gland. J. dent. Res., IADR abstracts, p. 179, 1969.

(4) Respiratory enzyme activities in various organs of adrenalectomized rats exposed to anoxia. J. dent. Res., IADR abstracts, p. 70, 1969.

(5) A radiographic study of leucine-H^3 incorporation by the submandibular gland in rat neonates exposed to anoxia. J. dent. Res., IADR abstracts, p. 167, 1969.

Copies of each of the four published articles listed above are being submitted as supplementary material to this detailed report.