

## Studies on Hypoxia

### VIII. Ultrastructural and Biochemical Effects of Prolonged Exposure on Rat Parotid Glands

A. P. MORAWA AND S. S. HAN

*Departments of Anatomy, Oral Biology and Pedodontics,  
The University of Michigan, School of Medicine and Dentistry*

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The effect of chronic hypoxia upon the rat parotid gland is studied by electron microscopy and biochemistry. Male Sprague-Dawley rats are subjected to 88% N<sub>2</sub> and 12% O<sub>2</sub> at less than 2 psi pressure for 7 days. For electron microscopy, the tissues are perfusion-fixed, embedded and sectioned in a routine manner. In the biochemical studies, rats are injected with <sup>3</sup>H-phenylalanine (2 μCi/g; s.i. ~5 Ci/mM) 60 min before sacrifice. Fresh glands are then prepared for analysis of amylase (Cibachrome-amylase substrate method), DNA (diphenylamine reaction), and total protein (Lowry method). The radioactivity in the acid precipitable and soluble fractions is determined by liquid scintillation spectrometry. The control animals are pair-fed and handled identically except that they are maintained in an ambient atmosphere. The ultrastructure of the hypoxic cells is altered in several areas. The Golgi apparatus demonstrates a decrease in organization and contains fewer transport vesicles. The rough-surfaced endoplasmic reticulum is broken and presents many vesicular and concentric profiles. The mitochondria have undergone several changes including swelling, clumping of intramitochondrial matrix, and fragmentation of cristae. The nucleus demonstrates a fibrillar pattern and contains a reduced amount of heterochromatin. The biochemical results indicate that, compared with controls, the hypoxic cells contain only 55% of amylase and 84% of the DNA, and thus suggest a drastic reduction of exportable protein production and an increase in cellular size, respectively. On the contrary, the incorporation of <sup>3</sup>H-phenylalanine demonstrates an increase in the amount of radioactivity in the acid precipitable fraction of the hypoxic cells. These results lead to the conclusion that hypoxic stress causes a suppression of exportable protein synthesis, but may induce other cytoplasmic protein production, reflecting the biochemical and morphologic adjustment of the cell to a recovery phase.

## INTRODUCTION

Numerous publications have characterized the ultrastructure and biochemistry of the exocrine glands. The essential features of the secretory cycle of exocrine glands have been defined by many investigators, including Schramm and Dannon (1961), Palade, Siekevitz and Caro (1962), Warshawsky, Leblond, and Droz (1963), Caro and Palade (1964), Schramm and Bdolah (1964), Schramm, Ben-Zvi, and Bdolah (1965), Redman, Siekevitz, and Palade (1966), Sreebny and Johnson (1969), Jamieson and Palade (1967 and 1971), and Lillie and Han (1973).

Robinovitch, Sreebny, and Smuckler (1966) developed a fixative utilizing

buffered glutaraldehyde and osmium tetroxide which resulted in excellent preservation of acinar cell granules of the rat parotid and various other intracellular organelles. Redman and Sreebny (1971) examined the cellular differentiation and activation of enzymes in the developing rat parotid gland and suggested that substrates in the food provided secretory stimulation, and had an inductive significance in the differentiation of the parotid gland.

The chemical nature of the secretory process in the rat parotid gland was studied, among others, by Badad, Ben-Zvi, Bdolah, and Schramm (1967). It was demonstrated that, as in the pancreas (Jamieson and Palade, 1967 and 1968),  $\alpha$ -amylase synthesis is critically dependent on the availability of energy and cannot occur in the absence of oxygen or in the presence of inhibitors of oxidative phosphorylation. Farber and Sidransky (1956) investigated the changes in amino acid pool size which were related to stimulation of secretory enzyme synthesis.

Studies of the alterations subsequent to hypoxia are numerous in areas of teratology, perinatal biology, and physiology, biochemistry and morphology of the heart, lung and liver. Work by Ingalls and Curley (1957), Murakami, Kameyama, and Kato (1956), Morawa and Han (1968), and Degenhardt (1960) have established the teratogenic effect of hypoxia. Studies by Morawa and Han (1968) indicated that hypoxia caused a retardation of cellular differentiation, reduction of cell size, and presumably a suppressed protein biosynthesis. This has been substantiated by subsequent work from our laboratory in which effects of anoxia on tooth bud (Smith and Han 1968), salivary glands (Kim and Han, 1969a; Han, Kim, and Burdi, 1971) and connective tissue (Kim and Han, 1969b) in rat neonates were studied.

With respect to ultrastructural effects of hypoxia, Confer and Stenger (1964) studied the hepatic parenchyma after hypoxic stress and found an increase in the number of lysosomes and cytolysosomes. Oudea (1963), and Glinsmann and Ericsson (1966) reported that these changes were reversible. They also noted the presence of vacuoles with colloidal particles and lipid, and an increase in mitochondrial size containing fewer and smaller cristae than the controls. Orth and Morgan (1962) investigated changes in myocardial tissue subsequent to hypoxic stress, and reported similar ultrastructural alterations, as well as a notable increase in the number of cytoplasmic lipid inclusions.

Biochemically, marked polysomal disaggregation with a subsequent decrease in incorporation of L-leucine- $\mu$ - $^{14}$ C was reported by Surks and Berkowitz (1971). The total protein and RNA contents of hepatic cells were also reduced. These investigators felt that hypoxia might interfere with the production of aminoacyl-tRNA via a decreased concentration of aminoacyl-tRNA synthetase or reduced availability of ATP which supplied energy for the synthetic reactions.

Shertzer and Cascarano (1972) and Aithal and Ramasarma (1969) examined the changes in mitochondrial activity after a prolonged exposure to hypoxic environment. They reported increases in cytochromes *b* and *c* with a concurrent increase in succinic dehydrogenase activity. These were interpreted as suggestive of oxidation of NADH, and hence, a shift which favored anaerobic energy production. However, Feinstein and Schramm (1970), and Ueha, Catanzaro, Hanson, and Lindsay (1971), reported that this modification of cellular respiration did not occur in the rat parotid.

Since the level of oxidative metabolism in myocardial tissue was very high and was dependent on the availability of ADP as a phosphate acceptor, Aschenbrenner, Zak, Cutilletta, and Robinowitz (1971) studied the respiratory enzymes and incorporation of labeled amino acids into the mitochondrial and acid soluble proteins of cardiac muscle under conditions of reduced partial pressure of oxygen and carbon dioxide. On the basis of the reduction in specific radioactivity of cytochrome *c*, it was hypothesized that up to 25% of the mitochondria were damaged or destroyed by hypoxia.

In light of the investigations cited above, it was considered that the parotid gland would be an excellent model to evaluate changes in exportable protein synthesis resulting from hypoxic stress. The objectives of this study were to evaluate the effects of chronic hypoxia on the fine structure and biochemical functioning of the rat parotid gland cells.

### MATERIALS AND METHODS

*Animals.* A total of 96 male Sprague-Dawley rats (*Rattus norvegicus*) weighing  $190 \pm 15$  g were used throughout this experiment. They were maintained on Purina rat chow and tap water *ad libitum* and provided with 14 hr of light and 10 hr of dark (8:00 PM–6:00 AM). The control animals were pair-fed with the experimental animals. Rats of normal groups were provided with food and water *ad libitum*. At 5:00 PM on the day preceding sacrifice, food was withdrawn from all rats. All experimental procedures were initiated between 8:00–9:00 AM and the dosage of radioactive amino acid was calculated from the animal weight at this time.

*Environment.* Hypoxic conditions were produced in an environmental chamber custom-fabricated for these experiments. The hypoxic chamber was provided with a continuous flow of 12% O<sub>2</sub> and 88% N<sub>2</sub> (Cryogenics Corporation) under a pressure of less than 2 psi and at a constant temperature of  $20 \pm 1^\circ\text{C}$ . Carbon dioxide was removed with a soda-lime trap. The control groups of animals were maintained in a similar chamber except that atmospheric air was circulated under a pressure of less than 2 psi. All experimental rats were subjected to a hypoxic environment for a period of 7 days.

*Preparation of tissues for microscopy.* Five minutes before sacrifice, the animals were sedated by an intraperitoneal injection of 3.5% saline solution of chloral hydrate (1 ml/100 g body wt). Using care not to disturb the vascular supply to the gland, the parotid was exposed by removing the overlying skin, fasciae and adipose tissue, and then was covered with a saline-soaked gauze. The rats were perfused with a prewarmed half-strength Karnovsky fixative (1965) through the left ventricle. One hundred fifty to 200 ml of the fixative was perfused over a period of 15–18 min. The gland was then removed, placed in fresh fixative, and diced with a razor blade. Fixation was continued at room temperature for 60 min. The tissues were washed in 0.1 M cacodylate buffer with 4.5% sucrose and postfixed for 60 min in 1% osmium tetroxide buffered with 0.1 M cacodylate containing 4.5% sucrose. Following fixation, the tissue blocks were dehydrated through serially graded alcohols and propylene oxide prior to infiltration with a monomeric mixture of resins and curing according to Luft (1961).

One  $\mu$  thick sections were made on an LKB Ultratome I for light microscopy. They were stained with 1% toluidine blue veronal acetate buffer (pH 9.0) and mounted in Permount (Fisher Scientific Co.). Micrographs were taken with a Zeiss photomicroscope using Normaski interference optics.

Ultrathin sections for electron microscopy were made with glass knives ( $65^\circ$ ) on an LKB Ultratome III. Sections were collected on bare 200 mesh grids, stained with saturated uranyl acetate and lead citrate (Reynolds, 1963). All observations were made in a Hitachi HS-8 electron microscope.

*Nucleolar Index.* Previous work from our laboratory indicated that a nucleolar index is a convenient means of demonstrating changes in synthetic abilities in secretory cells at light microscopic level (Jhee, Han, and Avery, 1965). The nucleolar index was derived by counting the number of nucleoli present in 3000 nuclei of parotid acinar cells for each group, and computed using the following formula.

$$\text{Nucleolar Index} = \frac{\text{Number of Nucleoli}}{\text{Number of Nuclei}} \times 100$$

The significance of differences between groups were evaluated by using the student *t* test.

*Biochemical techniques.* The radioactive precursor, L- $^3\text{H}$ -phenylalanine with a specific activity of 5 Ci/mM ( $^3\text{H}$ -phe) was used to evaluate the protein synthesis. All rats were injected with 2  $\mu\text{Ci/g}$  body wt of the precursor 60 min before sacrifice. All injections were made at the same time of day in order to minimize the diurnal rhythmic effect.

The ability of the gland to incorporate  $^3\text{H}$ -phe into total protein was studied *in vivo*, employing a procedure modified from Hinrichs, Petersen, and Baserga (1964). Sixty minutes after injection of  $^3\text{H}$ -phe, the animals were killed by a blow to the base of the skull and the parotid gland was exposed, excised, and placed on chilled glass ( $0^\circ\text{C}$ ). All visible adipose and fibrous tissues were removed rapidly, and the gland was weighed and homogenized in a known volume of cold distilled water. Parotid tissues from 3 animals of each group were pooled and assayed in triplicate runs. Cold perchloric acid (0.6 N PCA) was added to aliquots of the homogenate suspended in distilled water to bring the final concentration of PCA to 0.3 N. The precipitate was centrifuged at 9000 rpm for 10 min, and subsequently solubilized in Soluene (Packard Instrument Co.) prior to adding 10 ml of scintillation fluid. The acid soluble fraction derived from the previous procedure was added to Bray's solution. All fractions were counted in model 3320 Packard Tri-Carb liquid scintillation spectrometer. The quench correction was made using the external standard method.

By estimating DNA and total protein content from a single tissue homogenate, it is possible to compare the rate of incorporation of precursors into proteins on the basis of DNA. This is particularly pertinent in studies of exocrine cells which contain varying amounts of exportable proteins under different conditions. The diphenylamine reaction was employed to determine DNA content using calf thymus DNA as standard (Burton, 1968). Total proteins in the parotid tissues were measured by the folin phenol reagent and plotted against

TABLE I  
EFFECTS OF HYPOXIA UPON BODY WT<sup>a</sup>

| Time/Group | Hypoxic <sup>b</sup> | %    | Control <sup>c</sup> | %    | Normal <sup>d</sup> | %     |
|------------|----------------------|------|----------------------|------|---------------------|-------|
| Beginning  | 187.9<br>(172-219)   | 100  | 194.4<br>(175-220)   | 100  | 205.0<br>(186-217)  | 100   |
| Sacrifice  | 172.3<br>(148-193)   | 91.7 | 186.1<br>(162-214)   | 95.7 | 262.2<br>(246-281)  | 128.1 |

<sup>a</sup> Mean weight changes in g from 34 rats in each group.

<sup>b</sup> Hypoxic rats subjected to 12% O<sub>2</sub> and 88% N<sub>2</sub> for 7 days.

<sup>c</sup> Pair-fed control.

<sup>d</sup> Fed *ad libitum*.

known dilutions of bovine serum albumen (Lowry, Rosenbrough, Farr, and Randall (1951)).

For determination of  $\alpha$ -amylase activities, a colorimetric procedure with the Cibachrome Blue F<sub>3</sub> GA-Amylose substrate (Roche Co.) was utilized. This technique allows a direct quantitation of the enzyme content, using a single substrate of dyc-sugar complex which renders a blue color upon cleavage of the 1-4 bonds.

Upon sacrifice, blood samples of approximately 8  $\mu$ l per animal were collected in heparinized capillary tubes [Clay Adams Heparinized Capillary Tubes, (75 mm  $\times$  1.2 mm)] and processed according to the method of Frankel, Whalley, Knorpp, and Korst (1962). The blood level of <sup>3</sup>H-phe was comparable and uniform in all control and experimental rats.

## OBSERVATIONS AND RESULTS

Changes in body weights during the experimental period are recorded in Table I. It may be noted that, following hypoxic stress, the rats lost approximately 15 g or 8% of their original weight. That this was largely due to reduced food intake as a consequence of the experimental conditions is indicated by similar weight loss (4%) of the pair-fed control animals. During the same period, the normal rats gained an average of 57 g or 28% of their original weight. Since the primary focus of these experiments was to determine the changes related to the synthesis of secretory proteins, the microscopic observations reported below will be limited to the acinar portions of the parotid gland.

### Light Microscopy

The cells from the pair-fed controls were generally pyramidal in shape and had apices filled with discrete and darkly stained zymogen granules (Fig. 1). The basal portion of the cells was intensely basophilic. The nuclei were rounded in contour. Occasionally, binucleate cells were observed. Supranuclear clear regions representing the negative images of the Golgi complex were visible in many cells.

In contrast to acinar cells of the control gland, the cells from hypoxic animals were larger and had a less basophilic cytoplasm (Fig. 2). Zymogen granules were more numerous and less dense than those of the control. The peri-

nuclear Golgi complex was small but evident in some cells, while in others it was obscure. Lipid droplets which could be identified in sections stained with *p*-phenylenediamine were more numerous in the basal region of the experimental acinar cells (Ledingham and Simpson, 1970).

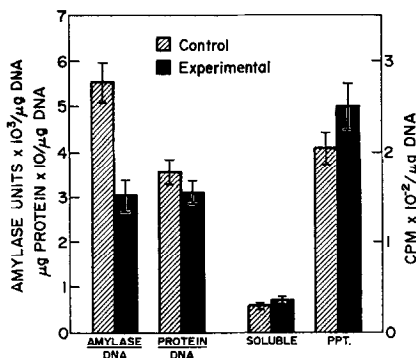
**Nucleolar index.** The nucleolus is known to be the site of ribosomal RNA synthesis and has been shown to change as a function of variations in cellular protein synthesis (Caspersson and Schultz, 1940; Busch and Smetana, 1970). Alterations in the size and/or number of nucleoli were determined by using 1  $\mu$ m thick sections of epon-embedded tissue. Table II records the nucleolar indices which indicate that, while the pair-fed control rats have a nucleolar index of 61, that of the hypoxic groups is depressed to 54. This is a reduction of over 12% in the nucleolar index. Although the *ad libitum* fed normal rats had a slightly greater index than the pair-fed control, the difference was insignificant ( $P < 0.1$ ).

### Electron Microscopy

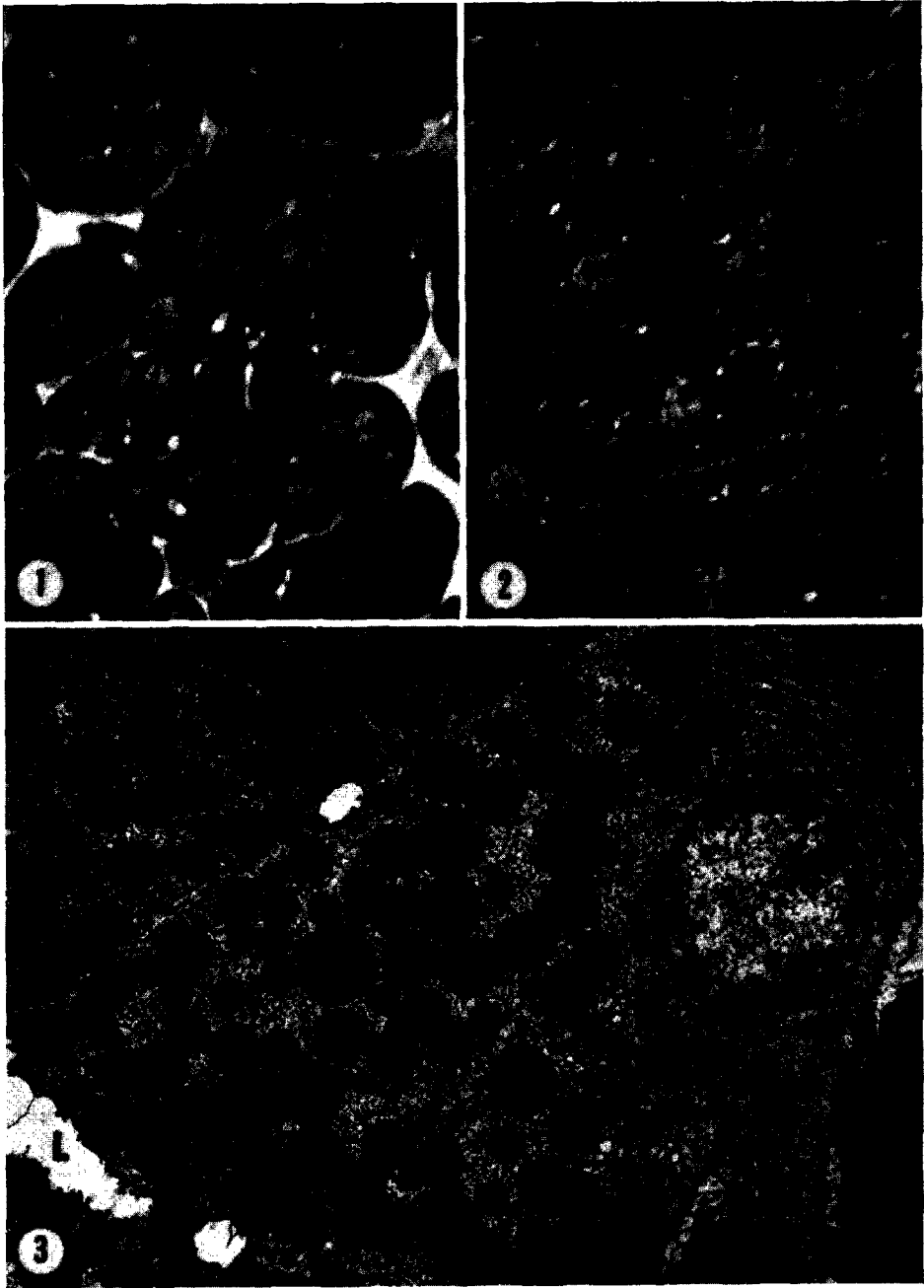
**General features.** Low magnification electron micrographs of the control and the hypoxic acinar cells confirmed the results from light microscopy. The control cells were pyramidal in shape, having a basally positioned nucleus (Fig. 3). The RER was well-developed at the base. The secretory granules and condensing vacuoles were rounded and uniform in electron density. The mitochondria contained well-developed and numerous cristae.

The hypoxic cells also demonstrated the typical pyramidal shape with a basally situated nucleus (Fig. 10). Despite the clear reduction in cytoplasmic basophilia, the RER was also fairly well-developed, except for certain unusual features to be elaborated later. A number of cytolysosomes were visible in the experimental cells.

**Transitional cell.** A number of cells that existed between the acini and the intercalated ducts were moderately developed in terms of cytoplasmic structures concerned with secretory protein synthesis, hence, called transitional cells in this article (Figs. 4 and 11). The electron micrographs of this cell type from the hypoxic and control groups were generally similar. They were both low pyramidal in shape with a basally positioned nucleus. The density of ground cytoplasm was less than that of the mature acinar cells. The RER was rather



TEXT FIG. 1. Effects of Hypoxia on Rat Parotid glands: amylase, protein, and  $^3\text{H}$ -phenylalanine incorporation.



FIGS. 1 and 2. Normarski interference micrographs of parotid glands.

FIG. 1. Control cells displaying the typical appearance of acinar cells. Supranuclear, clear regions representing the negative images of the Golgi complex are visible in several cells.  $\times 900$ .

FIG. 2. Hypoxic cells showing enlarged granules which are more numerous than the control.  $\times 900$ .

FIG. 3. An electron micrograph of an acinar cell from a control parotid gland. The nucleus is basally positioned, the granules are uniform in electron density, and the mitochondria located throughout the cytoplasm. The acinar lumen is seen in the upper left hand corner (L).  $\times 4725$ .

TABLE II  
EFFECTS OF HYPOXIA UPON RAT PAROTID ACINAR CELLS:<sup>a</sup> NUCLEOLAR INDEX

| Group                | $\frac{\text{No. of nucleoli}}{\text{No. of nuclei}} \times 100$ | % of Control | % of Normal | Significance |
|----------------------|--|--------------|-------------|--------------|
| Hypoxic <sup>b</sup> | 53.59<br>( $\pm 3.22$ )  | 87.6         | 85.5        | <0.01        |
| Control <sup>c</sup> | 61.21<br>( $\pm 3.31$ )  | 100          | 97.7        | <0.1         |
| Normal <sup>d</sup>  | 62.67<br>( $\pm 3.84$ )  | 102.1        | 100         |              |

<sup>a</sup> Results are derived from counting 3000 cells from 5 animals.

<sup>b</sup> Hypoxic rats subjected to 12% O<sub>2</sub> and 88% N<sub>2</sub> for 7 days.

<sup>c</sup> Pair-fed control.

<sup>d</sup> Fed *ad libitum*.

diffuse and somewhat serpentine in appearance, and the granules of the transitional cell in control rats were frequently bizonal in character, although many cells contained the typical granules of uniform density (Fig. 4). The number of granules in the experimental cell was considerably less than that of the control cell (Fig. 11). However, the small number of transitional cells observed precludes a generalized conclusion regarding this point.

**Golgi apparatus.** The observations of this organelle tended to support the previous reports regarding suppressive effects on protein biosynthesis under hypoxic conditions (Han, Kim, and Burdi, 1971; Meerson, 1971; Cohen, 1972). The ultrastructure of the Golgi apparatus from the control cells demonstrated well-organized stacks of smooth-surfaced lamellae with numerous transport vesicles (Fig. 5). The condensing vacuoles were discrete with distinct membranes. The cisternae of the more proximal elements of the Golgi apparatus contained small amounts of flocculent material, while the distal portions demonstrated accumulations of products which had an electron density and texture more typical of the condensing vacuoles and the prozymogen granules. Many well-developed mitochondria were visible in the adjacent cytoplasm.

The Golgi complex from the hypoxic cell had several distinct modifications observed consistently. The entire Golgi apparatus was not as well organized as that of the control (Fig. 12). The lamellar elements were reduced in size and number. Smooth vesicular profiles which were different from the transport vesicles and appeared to be fragmented lamellae were present. The number of Golgi vesicles was reduced. The condensing vacuoles and prozymogen granules were also reduced in number. They were bordered by interrupted and jagged membranes of less uniform electron density when compared with the control.

**Granules.** In the control cells, two types of zymogen granules were observed. The more numerous were the dense mature granules and prozymogen granules mentioned above. They would correspond respectively to the types G<sub>1</sub> and G<sub>2</sub> described by Robinovitch *et al.* (1966). The G<sub>1</sub> granules were generally smaller and more dense than the G<sub>2</sub> granules; the G<sub>2</sub> granules were usually found in close association with Golgi stacks (Fig. 6).



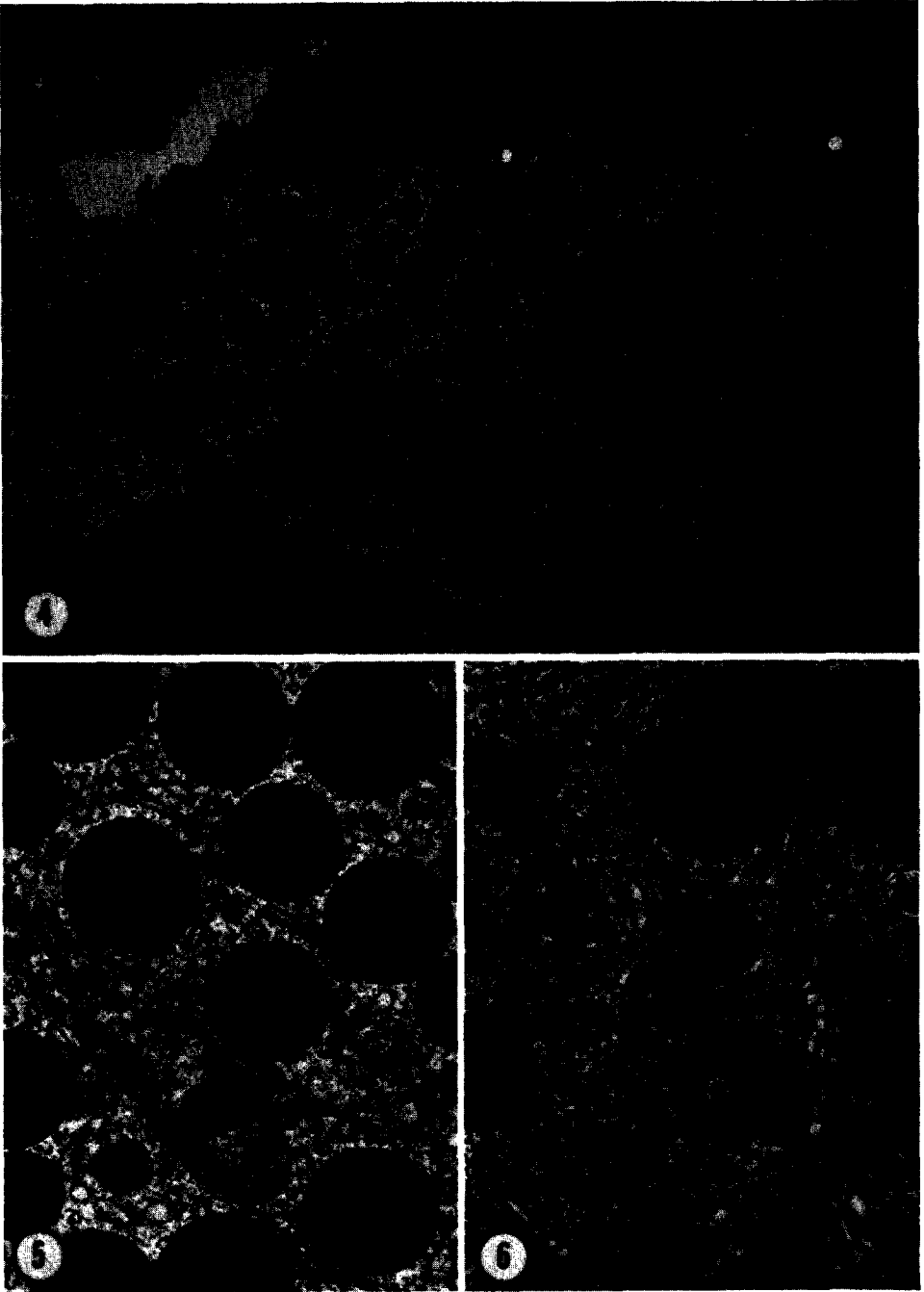


FIG. 4. An electron micrograph of a transitional cell from the control gland. The cell has a polarized appearance and a small number of zymogen granules. Compared to the regular acinar cells the cytoplasm is more electron-lucent, and the RER is somewhat serpentine in configuration, and the granules have a bizonal appearance. The Golgi complex is small  $\times 5725$ .

FIGS. 5 and 6. Electron micrographs of portions of parotid acinar cells from control rats.

FIG. 5. Distinct and uniformly dense granules such as shown in this electron micrograph are typical of the control acinar cells.  $\times 12,275$ .

FIG. 6. This typical Golgi complex exhibits well-developed and extensive lamellae, numerous transport vesicles, and newly formed ( $G_2$ ) and mature zymogen ( $G_1$ ) granules.  $\times 14,625$ .

In contrast to the control cells, the granules from the hypoxic cells were of less electron density and often contained secretory material of an irregularly mottled character (Fig. 10). The granules appeared to be more variable in size and density than those of the control.

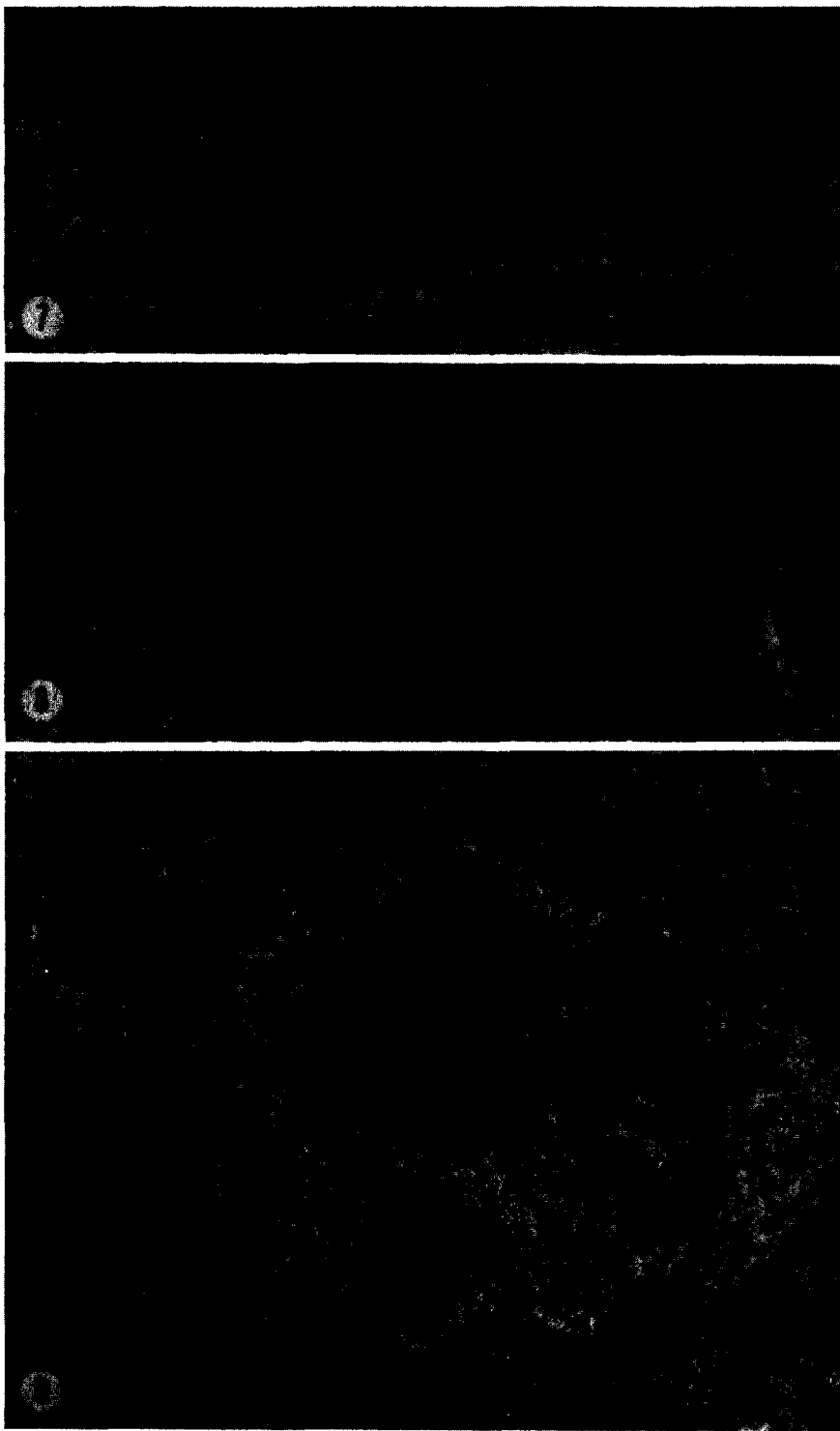
*Endoplasmic reticulum.* The RER in the control cells consisted of parallel and anastomosing rows of membranes covered with numerous attached ribosomes. The RER of the hypoxic gland was present in two primary patterns; the first was similar to that of the control cells with many elongated profiles while the second, and more prevalent pattern, consisted of vesicular and concentric profiles that were diffusely interspersed with many free ribosomes (Fig. 13).

*Mitochondria.* The fine structure of mitochondria from the control gland demonstrated two typical patterns which might be related to differences in energy states. The first type (Fig. 7) was of the classical elongated shape with moderately electron dense matrix, well-developed inner and outer membranes, and many long cristae that traversed the entire diameter of the organelle. The second type (Fig. 8) was pleomorphic in shape and contained a matrix of greater electron density than the previous example. Although they demonstrated distinct inner and outer membranes, their cristae appeared distended and shorter than those previously described. A few intramitochondrial granules were visible in both types of mitochondria.

Following hypoxic stress, the mitochondria had undergone several gradations of change (Figs. 14 and 15). The shape varied from the usual elongated form to more pleomorphic configurations. The density of the matrix also varied over a considerable range (cf. Figures. 14 and 15). Many of them appeared to have a more rounded outer contour suggesting swelling. The cristae were often small in size, reduced in number and appeared fragmented. Areas of disruption were present between the inner and outer membranes. One of more consistent findings was the presence of numerous aggregates of the matrix which had a flocculent appearance.

*Nucleus and nucleolus.* The nucleus from both control and hypoxic cells was spherical in shape, basally positioned and of comparable size. The nucleoplasm of the control cell was of slightly greater density and contained a greater number of irregularly shaped heterochromatin clumps distributed at the margin and subjacent to the nuclear envelope (Fig. 9). Compared to this, the chromatin pattern of the hypoxic cells appeared more diffuse (Fig. 18); an observation which had puzzled us until biochemical findings corroborated this. The nucleolus of the control cells was generally larger and of greater electron density (Fig. 9). It contained a well-developed spongelike nucleolonema; a less dense pars amorpha filled the interstices between the twisted components of the nucleolonema. Patches of chromatin associated with the rim of the nucleolus were somewhat smaller in hypoxic gland (Fig. 18). In these cells, the nucleolonema and pars amorpha were also reduced in size. The chromatin associated with the nucleolus was reduced in electron density.

*Lysosomes, cytolysosomes, and lipids.* Lysosomes and cytolysosomes were present in both the control and hypoxic cells (Figs. 16 and 17). The number of cytolysosomes appeared greatly increased in the experimental cells, corresponding with the findings reported by Glinsmann and Ericsson (1966) and Oudea (1963).



FIGS. 7-9. Electron micrographs of portions of parotid acinar cells from control rats.  
FIG. 7. An example of the most prevalent type of mitochondria observed in the control acinar cells. It has distinct inner and outer membranes and well-developed long cristae. Note the regular lamellar pattern of the adjacent RER.  $\times 41,950$ .

Increased amounts of lipid droplets were evident in the hypoxic cells (Fig. 16). Most of the lipids were present along the basal surface of acinar cells, supporting similar observations by Orth and Morgan (1962), Confer and Stenger, (1964), and Oulea (1963).

### *Biochemical Results*

The results from amylase and total protein determinations, and  $^3\text{H}$ -phe incorporation into the acid-precipitable fraction per mg of tissue are recorded in Table III. That the hypoxic gland had least DNA and proteins indicate the possible swelling and enlargement of cells observed in microscopy. It may be noted that the DNA content of the hypoxic group was approximately 16% less than that of the pair-fed control, indicating that cells from the experimental glands were larger than those of pair-fed control. The DNA content in *ad libitum* fed rats was higher than both the pair-fed control and the hypoxic groups. In light of this, the translation of the biochemical data into units per  $\mu\text{g}$  DNA was considered essential to evaluating the hypoxic effect on a cellular basis.

The amount of total proteins and amylase in glands from the hypoxic group was significantly lower than that of the pair-fed control. As indicated in Table III, the hypoxic groups contained only 72% and 47% of proteins and amylase, respectively. No significant differences were found between the pair-fed control and the normal groups.

The same data, expressed as units of amylase and proteins per  $\mu\text{g}$  DNA (Text-Fig. 1), show that the experimental gland cells contained only 55% of the amylase when compared to the control ( $P < 0.002$ ). To our surprise, however, the total protein content was reduced by less than 10% of the control ( $P < 0.533$ ), suggesting the possibility that a greater amount of nonexportable protein may be present in the experimental cells.

The results of the  $^3\text{H}$ -phe incorporation into proteins also shown in Text Fig. 1, help clarify the discrepancy between amylase and total protein content described above. The radioactivity of the acid soluble fraction in both the experimental and control glands is approximately the same, and therefore indicate the lack of any significant differences in amino acid pool size between the two ( $P < 0.667$ ). On the other hand, the incorporation of  $^3\text{H}$ -phe into the acid precipitable fraction is significantly higher in the experimental group (21% at  $P < 0.053$ ). This is contrary to what had been anticipated, and suggests that the cells may be undertaking an enhanced synthesis of nonexportable proteins.

## DISCUSSION

### *Structural Evidence for Suppression of Exportable Proteins*

One of the salient points of the present study is that despite an increase in overall protein synthesis, the cytoplasmic structures engaged in secretory protein

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FIG. 8. The mitochondria depicted here exhibit a dense matrix, short cristae, and distinct inner and outer membranes. This type of mitochondria were thought to represent an energy-rich state.  $\times 45,900$ .

FIG. 9. A nucleus, showing the margination of the heterochromatin subjacent to the nuclear envelope. The heterochromatin is dense and the nucleolus is large and well-developed.  $\times 10,800$ .

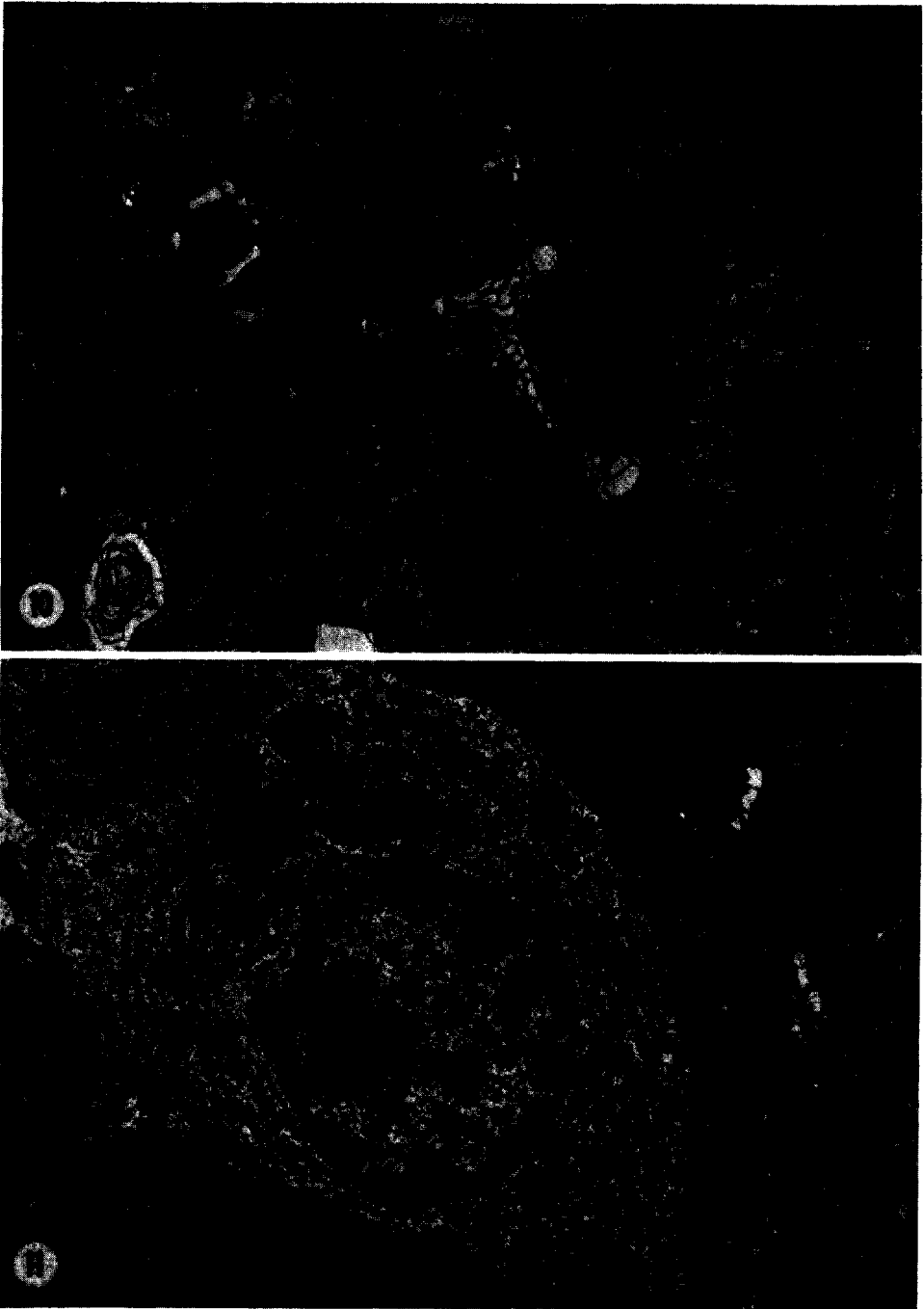


FIG. 10. An electron micrograph of an acinar cell of the parotid gland from an experimental rat. Mottling of certain zymogen granules and an increased number of cytolysosomes are evident in the cytoplasm of this secretory cell.  $\times 4500$ .

FIG. 11. An electron micrograph of a transitional cell from the parotid gland of an experimental rat. Although this cell contains mature and "newlyformed" zymogen granules, they are fewer than in the control. The cytoplasmic matrix is electron-lucent and RER appears more serpentine.  $\times 4725$ .

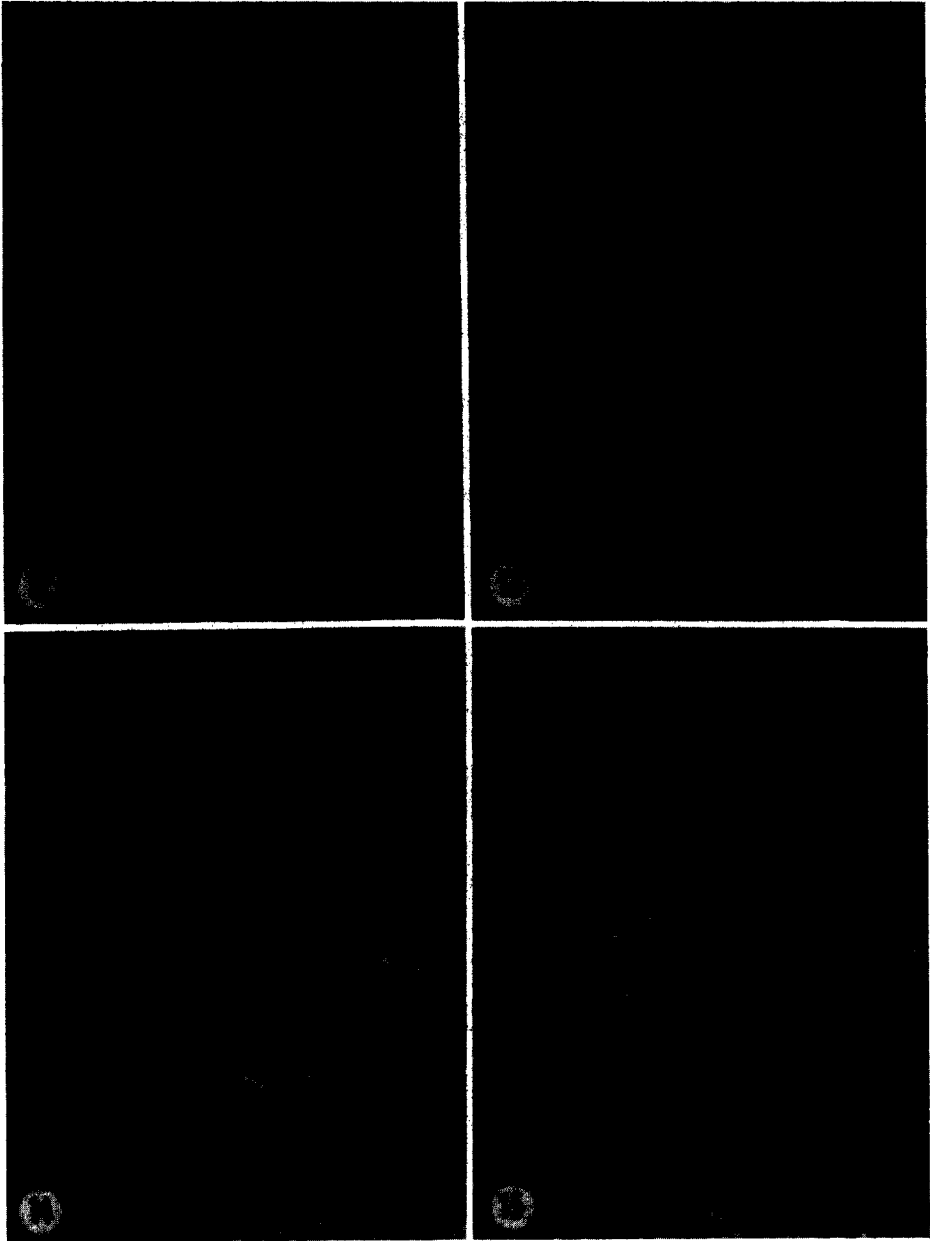


FIG. 12 and 13. Electron micrographs of portions of parotid acinar cells from experimental rats.

FIG. 12. A Golgi apparatus demonstrating a reduction in the number and organization of Golgi membranes and a jagged contour of the prozymogen ( $G_2$ ) granules. The Golgi lamellae show budding off of vesicles containing secretory product (arrows).  $\times 15,937$ .

FIG. 13. The RER has assumed a number of concentric patterns. Note the free ribosomes scattered among the profiles.  $\times 14,662$ .

FIG. 14. Mitochondria show signs of swelling, as they appear large and contain a lucent matrix. Cristae are fragmented and the flocculent dense masses (arrows) are evident in the matrix.  $\times 14,500$ .

FIG. 15. The mitochondria in this electron micrograph have distinct inner and outer membranes, some fragmented cristae, and several flocculent electron dense clumps in the matrix. Note the vesicular profiles of the RER.  $\times 13,175$ .

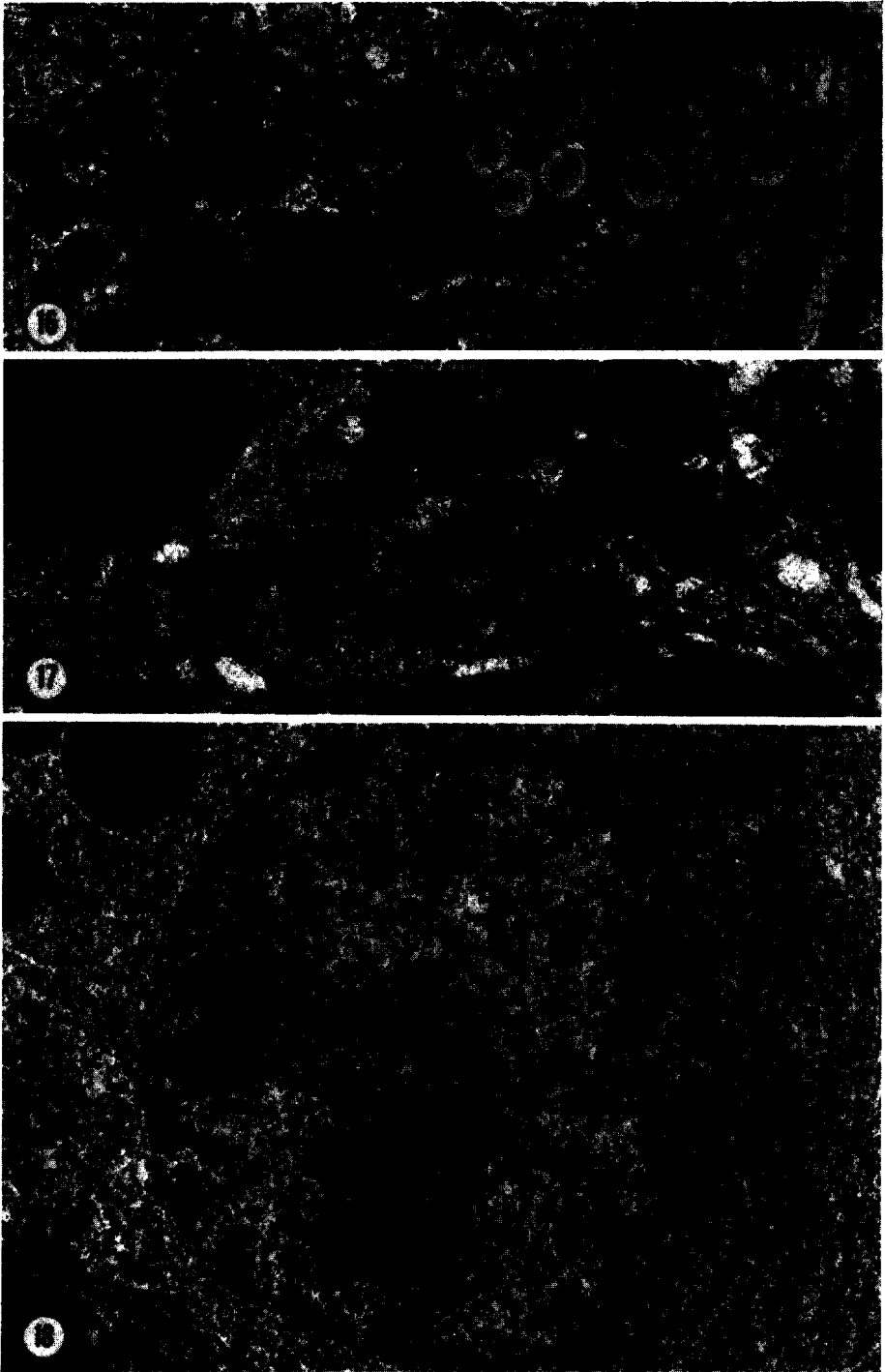


FIG. 16-18. Electron micrographs of portions of acinar cells from experimental parotid glands.

FIG. 16. Lipid droplets (Li) of this character were found in increased numbers after hypoxia.  $\times 13,600$ .

FIG. 17. Cytolysosomes as the ones depicted here as well as in Figure 16 are increased in number in the cytoplasm of the experimental cells.  $\times 57,200$ .

TABLE III  
EFFECTS OF HYPOXIA UPON CELLULAR CONTENTS OF DNA, PROTEIN, AND  
AMYLASE IN RAT PAROTID GLANDS

| Amount<br>mg<br>Tissue    | Control <sup>a</sup> | Hypoxic <sup>b</sup> | Normal <sup>c</sup>  | X/C  |
|---------------------------|----------------------|----------------------|----------------------|------|
| DNA ( $\mu\text{g}$ )     | 4.9 $\pm$ 0.15       | 4.1 $\pm$ 0.0        | 5.9 $\pm$ 0.10       | 0.84 |
| Protein ( $\mu\text{g}$ ) | 178.3 $\pm$ 9.8      | 128.3 $\pm$ 9.8      | 171.7 $\pm$ 4.1      | 0.72 |
| Amylase (units)           | 26,889<br>$\pm$ 1706 | 12,533<br>$\pm$ 1442 | 27,111<br>$\pm$ 2171 | 0.47 |

<sup>a</sup> Pair-fed with hypoxic group.

<sup>b</sup> Hypoxic rats subjected to 12% O<sub>2</sub> and 88% N<sub>2</sub> for 7 days.

<sup>c</sup> Fed *ad libitum*.

synthesis are reduced in size and extent of their organization following hypoxia. This is evidenced by the frequent breakdown of the profiles of the RER, the reduction in size of the Golgi apparatus, and the low nucleolar index observed in the parotid acinar cells of the experimental group. The reduction in amylase content corroborate these structural changes.

The secretory protein synthesis in exocrine cells such as pancreas and parotid is an energy dependent phenomena (Jamieson and Palade, 1971). Therefore, the modifications of mitochondrial structure present in the parotid acinar cells from the hypoxic group suggest that the primary site of the effects of oxygen deficiency might be the mitochondria which have shown swelling, increased pleomorphism, aggregation of matrix materials, and fragmentation of cristae. Other cytoplasmic changes such as the disorganization of the RER, or the reduction in size of the Golgi apparatus could then be regarded as secondary modifications resulting from a partial depletion of ATP supply, necessary for the maintenance of the synthetic activities, as a consequence of mitochondrial degeneration (Aschenbrenner *et al.*, 1971). Thus, our results support an earlier conclusion by Cohen (1972) who studied the biochemical effects of hypoxia on heart, lung and liver. Feinstein and Schramm (1970) and Ueha *et al.* (1971) have demonstrated that amylase synthesis by rat parotid glands could not be shifted to depend upon anerobic glycolysis. In the absence of adequate oxygen supply, it might be expected that secretory protein synthesis in this particular gland of this species, could therefore be turned off.

Since approximately 85% of the protein synthesis in the rat parotid gland represents exportable proteins, the reduction in the nucleolar morphology, which reflect the state of a cell's synthesis of ribosomal RNA (Busch and Smetana, 1970), can also be taken as indicative of a depression of the overall synthesis of exportable proteins. In contrast, the more loose appearance of the nuclear chromatin pattern in hypoxic group suggests that the heterochromatin in experimental animals is reduced when compared to that of the control. The chromatin pattern in the experimental glands shows a fine, fibrillar organization, resembling

FIG. 18. This nucleus has a fine, fibrillar pattern to the chromatin. The nucleolus is well-developed, but small. Note the adjacent pleomorphic mitochondria and the vesicular profiles of the RER.  $\times 9600$ .



the appearance of the active euchromatin as has been characterized in various types of cells (Hay and Revel, 1963; and others). Such a nuclear appearance could be a reflection of activated chromatin in terms of DNA replication. However, in the absence of DNA synthesis as in mature parotid acinar cells, the same structural characteristics probably reflect transcriptional activities of the nucleus. In light of this it is significant that, although the present study did not look into the changes in DNA synthesis under hypoxic conditions, the amount of total DNA per mg of wet parotid tissue was significantly lower in the stressed animals.

#### *Discrepancy Between Amylase and Total Protein Content*

While the amount of total protein contained in the experimental glands was moderately reduced (approximately 12% at  $P < 0.05$ ), the amount of amylase in the experimental animals was only 55% of the pair-fed control (Text Fig. 1). Since this is the result of a comparison of the quantity of proteins and amylase units per  $\mu\text{g}$  of DNA, it represents a clear discrepancy between a precipitous reduction of amylase content and a limited decrease in the total protein content. In the rat parotid, 50% of the total glandular proteins of the control represent secretory proteins (Schramm, 1968). Therefore, the relatively small difference in total protein content between the experimental and control glands, in the presence of a much greater difference in amylase units, suggests the cells from the experimental animals contain a larger amount of sedentary proteins. In fact, our calculations indicate there could be as much as 15% more sedentary proteins in the experimental glands at this time. In other words, the experimental animals, while having one-half as much exportable proteins (Table III), contain about 15% more sedentary proteins which must represent the new proteins synthesized since the beginning of this experiment. Considered together, the changes in the appearance of nuclear chromatin and the increased amount of sedentary proteins suggests an enhancement of sedentary protein synthesis in the parotid acinar cells of hypoxic animals.

This conclusion is supported by the results from  $^3\text{H}$ -phe incorporation studies. The levels of radioactivity present in the acid-precipitable fractions clearly indicate that the amino acid incorporation in the experimental gland is increased by 21% over that of the control. The 60 min time period between the radioactive amino acid injection and sacrifice is considered adequate in terms of allowing the amino acid incorporation into protein fractions, and yet not long enough to cause a spillage of the synthesized proteins through the discharge of secretory products by parotid cells (Castle, Jamieson, and Palade, 1972; and Han, 1973). In contrast to the findings by Farber and Sidransky (1956) which showed an amino acid pool change related to changes in secretory protein synthesis, the suppression of amylase synthesis by hypoxia has not resulted in a modification of radioactivity in acid soluble fractions.

*Overall significance of this study.* The observations from this study indicate that, while secretory protein synthesis has been drastically suppressed by a reduced partial pressure of  $\text{O}_2$ , the acinar cells may be undergoing a series of possible adaptive changes which are reflected in the nuclear morphology and the amount of sedentary proteins present, as well as in the increased incorporation of amino acids into the acid precipitable fractions. It should be emphasized

that, if there were a genuine enhancement of sedentary protein synthesis for adaptation to an O<sub>2</sub> deficient environment, this is happening under the same reduced O<sub>2</sub> pressure which depressed secretory protein synthesis. Therefore, the increased protein synthesis during this adaptive phase may well represent those synthetic activities that are capable of capitalizing upon anerobic mechanisms of energy supply.

A support for this conclusion has been recently obtained in our laboratory in which hypoxic animals have shown a total recovery of gland function after two weeks of hypoxic exposure under the same conditions that have been used in the present study (Kim, Morawa, and Han, 1973). In this study, experimental gland cells exposed to hypoxia have been found to synthesize  $\alpha$ -amylase at the same level of efficiency as controls, a change that would require either a greater number of ATP generating units (mitochondria) or a greater amount of cytoplasmic machinery for synthesis. In view of this, the enhanced sedentary protein synthesis, observed at the end of one week exposure to hypoxic environment, could be regarded as reflecting adaptive changes which may eventually lead to the supplementation of the cytoplasmic organelles necessary to attain the functional level of the glands observed in the two week study.

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