THE EFFECTS OF RESERPINE AND HYPOXIA ON THE AMINE-STORING GRANULES OF THE HAMSTER CAROTID BODY

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ABSTRACT

The carotid bodies from control, reserpine-treated, and hypoxia-treated hamsters were fixed with phosphate-buffered glutaraldehyde and osmium tetroxide, s-Collidine-buffered osmium tetroxide, or phosphate-buffered glutaraldehyde followed by potassium dichromate incubation. Following glutaraldehyde-osmium tetroxide fixation no differences in density or population of the electron-opaque granules in the glomus cells of either control or experimental animals were observed. With s-Collidine-buffered osmium tetroxide and the glutaraldehyde-dichromate technique a marked decrease in density without an appreciable reduction in number of granules was noted after reserpine treatment, while in hypoxia-treated hamsters the density and population of the granules were not different from those of the controls. The results indicate that reserpine depletes the amines without granule disappearance and that hypoxia does not affect the amine content of the granules. It is suggested that following glutaraldehyde-osmium tetroxide double fixation, persistence of the density of the granules in reserpine-treated animals is due primarily to the nonamine content, and that the amines in the glomus cells are probably not directly involved in the respiratory reflex.

INTRODUCTION

The carotid body is located in dense connective tissue at the bifurcation of the common carotid artery and consists of groups of epithelioid cells and intervening blood channels. The body is believed to be a chemoreceptor involved in respiratory reflexes. Electron microscopic studies (Duncan and Garner, 1957; Lever and Boyd, 1957; Ross, 1957) have shown that the glomus cells (Type I of the carotid body contain numerous electron-opaque granules (granulated vesicles) which are similar in appearance to those in cells of the adrenal medulla. Lever and Lewis (1959) stated that the granules might contain catecholamines which could act as transmitter substances. With microspectrophotometric techniques, Hamberger et al. (1966) demonstrated that some of the cells of the human carotid body contained serotonin while others stored catecholamines. Using electron microscopic radioautographic techniques, Chen et al. (1967) have localized labeled precursors of both catechol- and indolamines over the granules in the glomus cells.

The effects of the catecholamine-depleting agent, reserpine, and of hypoxia on the appearance of the electron-opaque granules have been interpreted differently. Lever et al. (1959) noted a decrease in the number of granules in the cells of animals treated with reserpine and fixed in osmium

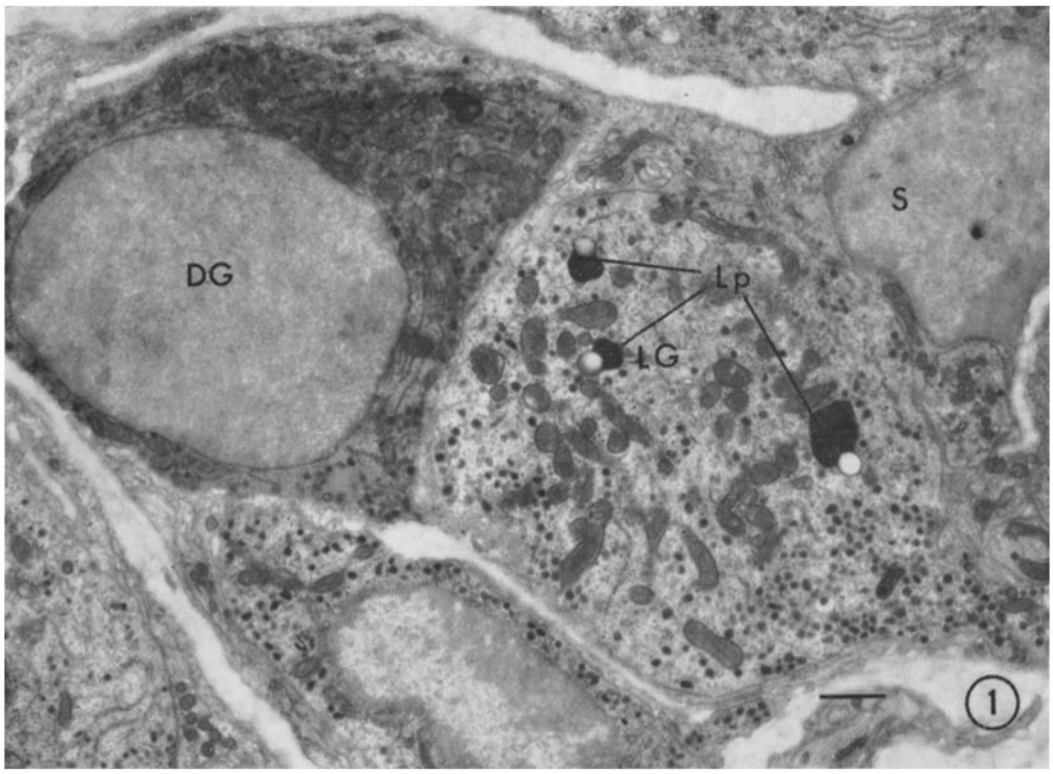


FIGURE 1 The carotid body from an untreated animal, showing three types of parenchymal cells. The supporting cell (*S*) sends thin, flattened processes around two types of glomus cells (*LG* and *DG*). One of the processes is also seen extending between two glomus cells. Mitochondria are more numerous and the cytoplasm is more opaque in the dark (*DG*) than in light (*DG*) cells. Numerous granules are seen in both light and dark glomus cells. In one of the light glomus cells three pigment granules (*Lp*) are illustrated. Phosphate-buffered glutaraldehyde and OsO_4 perfusion fixation. × 8700.

tetroxide while Duncan and Yates (1967) reported no such changes after glutaraldehyde-osmium tetroxide fixation. Hoffman and Birrell (1958) noted a marked decrease in the numbers of "vesicles" in the glomus cells following hypoxia, and Blümcke et al. (1967a) reported extracellular discharge with a consequent depletion of the granules. Al-Lami and Murray (1968) stated that conditions of hypoxia resulted in an increase rather than a decrease in the number of cytoplasmic granules. The varying results obtained by these investigators may be due to the fixatives employed. Callas and Wood (1965), Wood and Callas (1966), and Duncan and Yates (1967) noted that there were marked differences in the appearance of amine-storing granules in osmium tetroxide-fixed and glutaraldehyde-osmium tetroxide-fixed tissues.

The present research was undertaken to determine the effects of reserpine and hypoxia on the appearance and population of granules in the glomus cells by using various fixation techniques and a sensitive cytochemical method for the detection of unsubstituted amines.

MATERIALS AND METHODS

67 adult male Syrian hamsters weighing from 70–100 g were used in these studies. The animals were divided into three groups. Group I served as untreated controls. Some of the animals of Group II received subcutaneous injections of reserpine (0.75–1.25 mg/kg) daily for 2 to 20 days and were sacrificed 24 hr after the final injection; others received a single injection of reserpine (2 mg/kg) and were sacrificed 1, 2, 4, 8, 14, and 24 hr after treatment. The animals of Group III were placed in a 3 liter container in which the atmosphere consisted of one of the following gas mix-

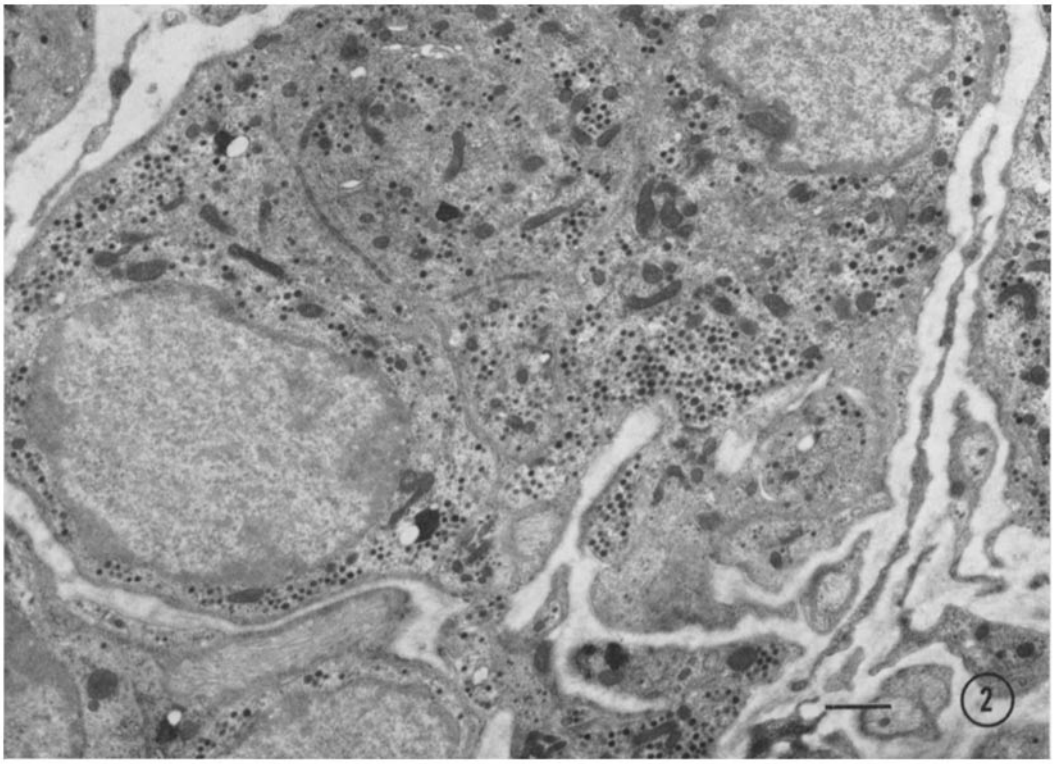


FIGURE 2 The carotid body from an animal injected daily with reserpine (1 mg/kg body weight) for 20 days. Essentially no structural changes are discernible except for the mitochondria which appear to be smaller and more dense as compared with those of control cells. A series of desmosomes occurs where the two adjacent glomus cells are in direct contact. Phosphate-buffered glutaraldehyde and OsO_4 perfusion fixation. × 8700.

tures: 3% oxygen and 97% nitrogen 4% oxygen and 96% nitrogen, or 5% oxygen and 95% nitrogen. The animals were subjected to this atmosphere for 20 min to 6 hr. During this period artificial respiration was frequently necessary to keep the 3% oxygen-treated animals alive until the end of the experiment. Carotid bodies were removed and processed through one of the following techniques.

(1) Glutaraldehyde-osmium tetroxide double fixation: The animals were anesthetized with an intraperitoneal injection of sodium nembutal. The trachea was exposed, a tracheal tube was inserted and connected to an artificial ventilator which, in turn, was connected to either room air (Groups I and II), or 3, 4, or 5% oxygen in a 97, 96, or 95% nitrogen gas mixture (Group III). About 200 ml of cold 3% glutaraldehyde in 0.1 M Sörensen's phosphate buffer (Sabatini et al., 1963) (pH 7.3–7.4) was perfused through the left ventricle. The right atrium was cut to allow egress of the blood and fixative, and the perfusion process lasted for 15-20 min. Following perfusion, carotid bodies from both sides were removed and placed in fixative with the same constituents as the perfusion fluid for an additional 2 hr at 4°C. After completion of fixation with glutaraldehyde the tissues were washed for 2 hr in 4–5 changes of phosphate buffer with 10% sucrose. The material was then postfixed in 1% osmium tetroxide in 0.1 M Sörensen's phosphate buffer for 1 to 1½ hr at 4°C. After osmification the carotid bodies were rinsed briefly with a 0.85% sodium chloride solution, rapidly dehydrated in ethanol and embedded in Epon 812 (Luft, 1961). Gold sections were cut perpendicular to the long axis of the internal carotid artery on a Porter-Blum ultramicrotome. The sections were picked up on copper grids, stained for 2 min with lead citrate (Reynolds, 1963) and examined in the electron microscope.

(2) s-Collidine-buffered osmium tetroxide fixation: The animals were anesthetized with sodium nembutal. For immersion fixation the left external, internal, and common carotid arteries were exposed and ligated. The left carotid bifurcation was removed

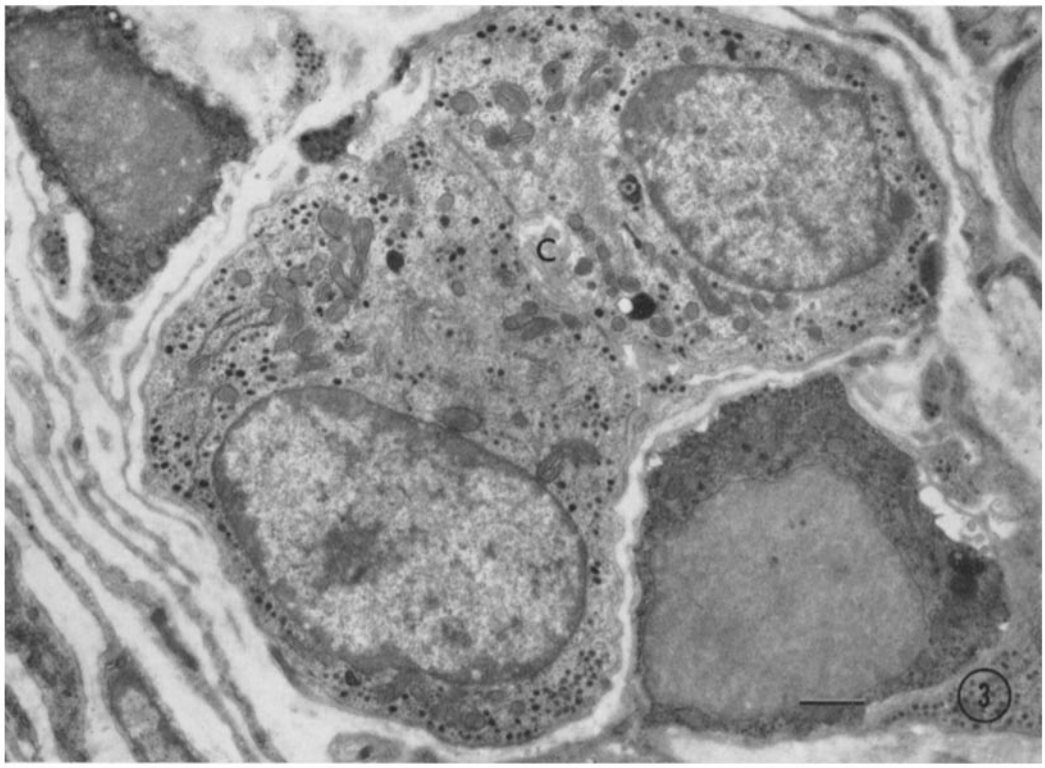
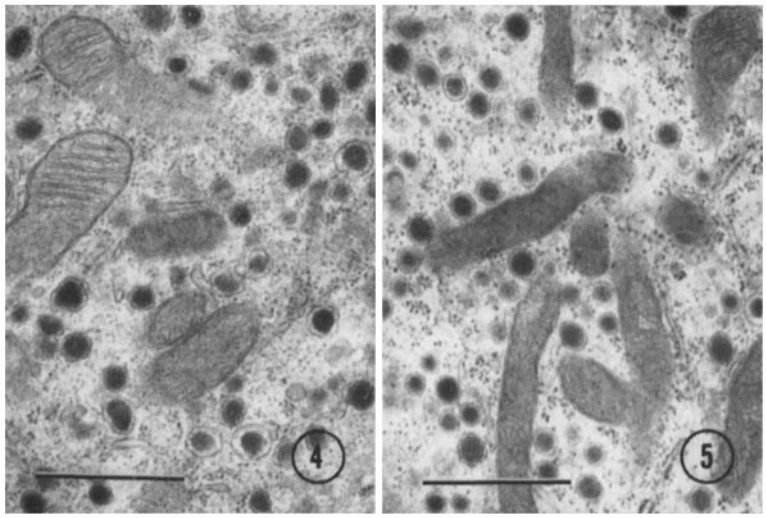


FIGURE 3 The carotid body of the animal subjected to a 4% oxygen, 96% nitrogen gas mixture for 3 hr. Two light and two dark glomus cells are shown. The appearance and population of the granules are within the normal range. Note that a canaliculus (*C*) is present between the two light glomus cells. Both sides of the structure are occluded by a series of desmosomes. Phosphate-buffered glutaraldehyde and OsO_4 perfusion fixation. × 8700.

and the carotid body was dissected free in the fixative (1.33% osmium tetroxide in 0.067 M s-Collidine buffer; pH, 7.35). Osmium tetroxide fixation by immersion frequently resulted in poor preservation of tissues, and consequently in later experiments a brief initial perfusion with fixative was performed. The left common carotid artery and the heart were exposed, the right atrium was cut and about 10 ml of Hanks' solution was rapidly perfused through the left ventricle. 10 to 20 ml of cold s-Collidine-buffered osmium tetroxide was then injected into the left common carotid artery over a period of approximately 5 min and the carotid body was excised and placed in fixative for an additional hour at 4°C. The tissue was rapidly dehydrated, embedded in Epon 812, thin sectioned, and examined in the electron microscope.

(3) Potassium dichromate incubation: A slight modification of Wood and Barrnett's (1964) potassium dichromate technique was used in this study. The carotid body and the adrenal medulla were removed from animals after perfusion with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and fixed for 2–4 hr in phosphate buffer containing 10% sucrose. After washing with buffer, each carotid body was cut into two parts and incubated together with pieces of the adrenal medulla in a solution of 2.5% potassium dichromate and 1% sodium sulphate in 0.2 M acetate buffer (pH, 4.1) for 18 to 24 hr at 4°C. One-half of the carotid body and adrenal medulla were subsequently postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer. The tissues were rapidly dehydrated and embedded in Epon 812. Unstained gold sections were examined in the electron microscope.

(4) Pronase digestion: Glutaraldehyde-osmium tetroxide-fixed Epon sections were oxidized with 10% hydrogen peroxide for 5 to 10 min at room temperature and subsequently subjected to pronase digestion (0.5% in 0.03 M phosphate buffer; pH, 7.4) (Monneron, 1966) for 5 to 40 min at 40°C. Sections treated with hydrogen peroxide alone were



FIGURES 4 and 5 Higher power electron micrographs of parts of carotid body cells from untreated (Fig. 4) and reserpine-treated (Fig. 5) hamsters. The mitochondria in the cell from the reserpine-treated hamster are smaller and exhibit a denser matrix than in the control (Fig. 4) cell. Note that most of the granules in both cells show an intact limiting membrane. Phosphate-buffered glutaraldehyde and OsO_4 perfusion fixation. Both figures × 19,600.

used as controls. Following digestion with pronase the sections were stained with uranyl acetate and lead citrate and examined in the electron microscope. The electron microscope used for all these studies was either an RCA EMU 3D or an RCA EMU 3G.

RESULTS

Untreated Animals

The parenchymal cells of the hamster carotid body are of two types, glomus or Type I and supporting or Type II (Fig. 1). The glomus cells exhibit numerous electron-opaque, membrane-bounded granules (granulated vesicles) in the cytoplasm which vary in number and tend to accumulate near the periphery and in the processes of the cells. Aside from the dense granules the cells are characterized by the presence of granular endoplasmic reticulum reminiscent of Nissl bodies, pigment granules, and aggregations of filaments (Figs. 1 and 6).

In tissues fixed in glutaraldehyde-osmium tetroxide either by immersion or by perfusion, the diameter of the granules ranged from 500 to 2000 A. A narrow electron-lucent rim separated the dense core of the granule from the surrounding smooth surfaced membrane, and occasionally granules with a ring-shaped interior were encountered. At the periphery of the cells the granules frequently were close to the plasma membrane, but profiles suggesting exteriorization of the dense core into the extracellular space as described by Blümcke et al. (1967a) were observed in only one instance. Two types of glomus cells, light and dark, could be distinguished on the basis of cytoplasmic density (Fig. 1). The dark cells were smaller and less numerous than the light ones and also exhibited more free ribosomes and mitochondria. No appreciable difference in the number of granules was noted in the two types of cells.

The dense cores of the granules in specimens fixed either by immersion or by perfusion in s-Collidine-buffered osmium tetroxide showed distinctly less opacity than did the cores of granules in material fixed with phosphate-buffered glutaraldehyde followed by osmium tetroxide (Figs. 1, 4, 6 and 9). With buffered osmium tetroxide alone considerable swelling of the granules occurred (the diameter ranging from 800 to 2500 A, with an average diameter of 1400 A) and rupture of the limiting membranes was observed (Fig. 9). After immersion fixation in collidine-buffered osmium tetroxide the mitochondria were more swollen and

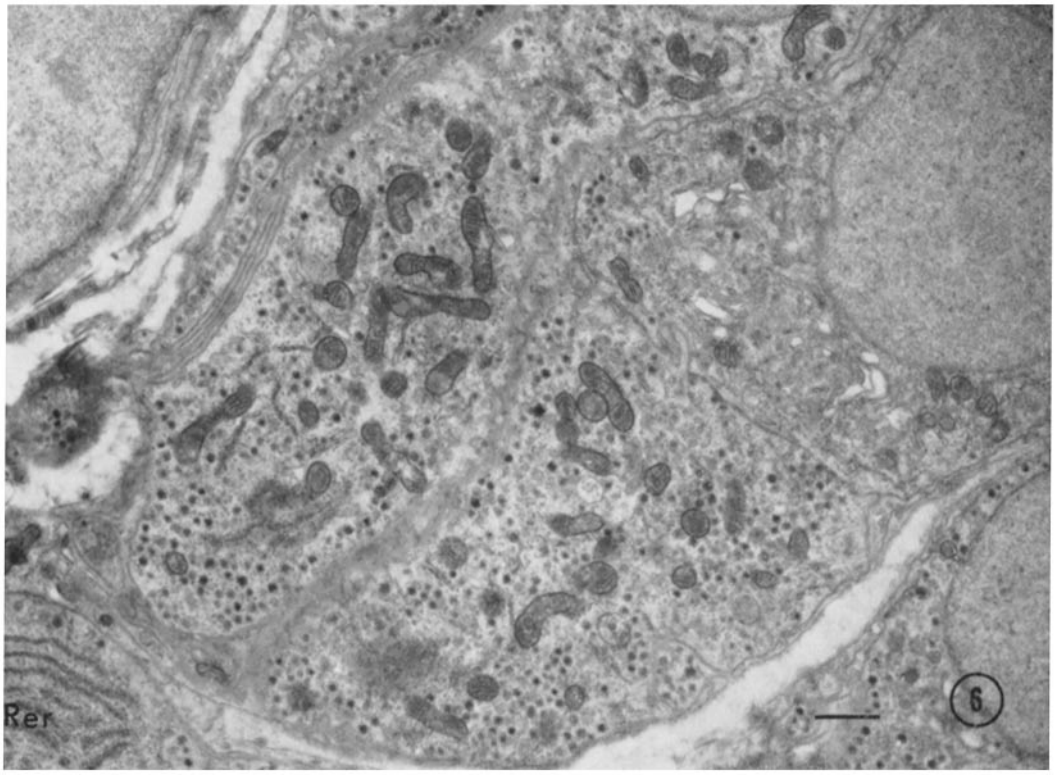


FIGURE 6 Portion of the carotid body from an untreated animal fixed by perfusion with s-Collidine–buffered OsO_4. Note that the density of the core of many of the granules is decreased in comparison with that of the cores of granules fixed with phosphate-buffered glutaraldehyde-OsO_4 (Figs. 1–3). Parallel-arranged membranes of the granular endoplasmic reticulum (*Rer*) are seen in the cell at the left lower corner of the field. × 8700.

vacuolated compared with those in cells from perfused animals.

Following the dichromate incubation technique, the granules in the glomus cells were more electron opaque (Fig. 12) than were those in the cells of unstained tissues which had been fixed sequentially with glutaraldehyde and osmium tetroxide. However, the granules appeared to be less dense than the norepinephrine-storing ones in adrenal medullary cells. The limiting membrane surrounding the granule was not seen with the dichromate technique. Most of the granules in the glomus cells gave a positive reaction after dichromate incubation. Those that did not were fewer in number and were generally smaller in size; such granules might represent either an early stage in amine synthesis, a component which remains after amine release from the granule, or they might correspond to the epinephrine-storing granules of the cat carotid body (Chiocchio et al., 1967). Postosmication following dichromate incubation defined the limiting membrane of the granulated vesicles in the glomus and adreno-medullary cells but it did not improve the contrast between the granules and the general cytoplasm.

With pronase digestion for 5 min the dense cores of the granules disappeared, leaving many clear spaces in the sections (Fig. 17). The mitochondria, ribosomes, and other organelles as well as the limiting membrane of the granules were still detectable though the contrast was greatly reduced.

Reserpine-Treated Animals

Following chronic reserpine administration the size of the glomus cells appeared to have decreased. No other changes in the histological features of the tissue were noted.

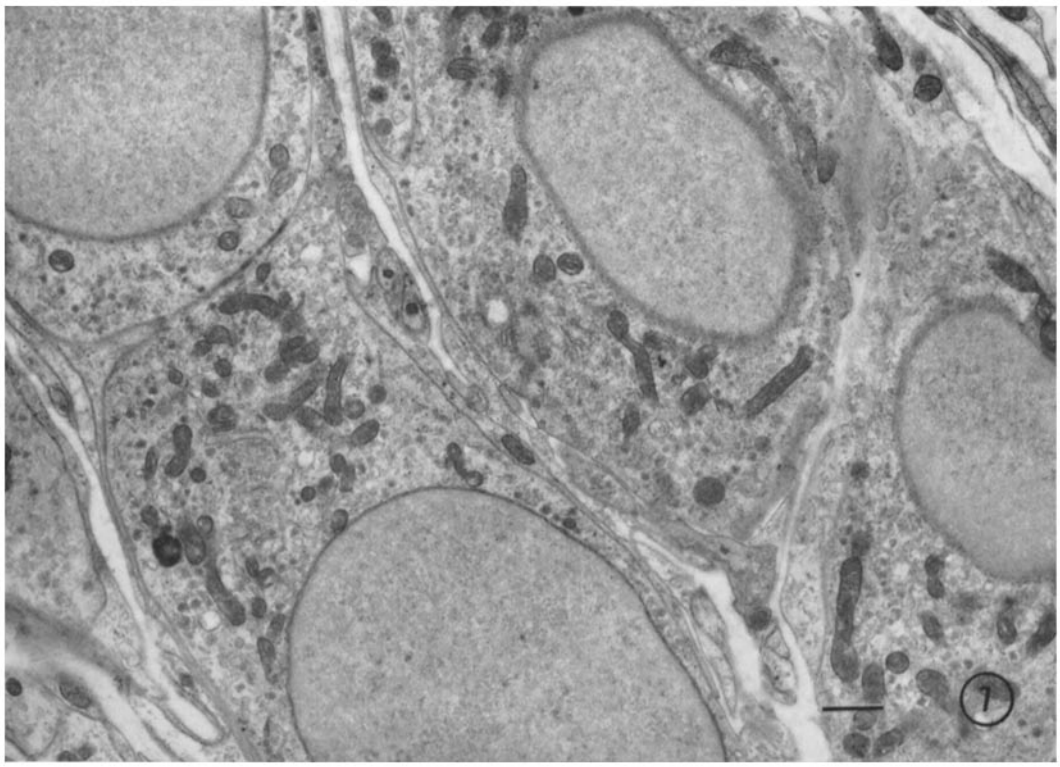


FIGURE 7 Portion of a carotid body from an animal treated daily with reserpine (1.25 mg/kg) for 9 days. The tissue was fixed by perfusion with s-Collidine-buffered OsO_4. Most of the dense cores of the granules in the glomus cells are markedly reduced in opacity. The mitochondria appear smaller and exhibit a denser matrix than do those in the control tissues. × 8700.

With sequential fixation either by immersion or perfusion in phosphate-buffered glutaraldehyde and osmium tetroxide the number and density of the granules appeared to be within the control range (Figs. 2 and 5). The mitochondria were smaller than normal and exhibited an opaque matrix (Fig. 2).

The carotid body of reserpine-treated hamsters seemed particularly vulnerable to handling before and during s-Collidine-buffered osmium tetroxide fixation, and better results were obtained with the perfusion technique. In most perfused tissues the mitochondria of the glomus cells were smaller than normal and exhibited an opaque matrix (Fig. 7). The density of most of the granules was markedly reduced and rupture of the surrounding smooth membranes was frequently seen (Figs. 7 and 10). No change in the distribution of the granules was observed. With immersion fixation the granules were comparable to those seen in perfused tissue but the mitochondria were usually swollen, vacuolated and distorted, or ruptured.

With the dichromate incubation technique no appreciable change in density of the granules was noted after treatment for one hour. However, a decrease in the density of the granules in some of the glomus cells (Fig. 13) and the cells of the adrenal medulla was seen as early as 2 hr after treatment. 24 hr following a single injection of reserpine the density of practically all the granules in the carotid body had decreased in comparison with those of control animals (Figs. 15 and 16).

Animals Subjected to Hypoxia

In tissues fixed with phosphate-buffered glutaraldehyde either by perfusion or by immersion followed by osmium tetroxide the only notable alteration in the ultrastructure of the glomus cells was an occasional mitochondrial swelling. These results are in agreement with the findings of Al-

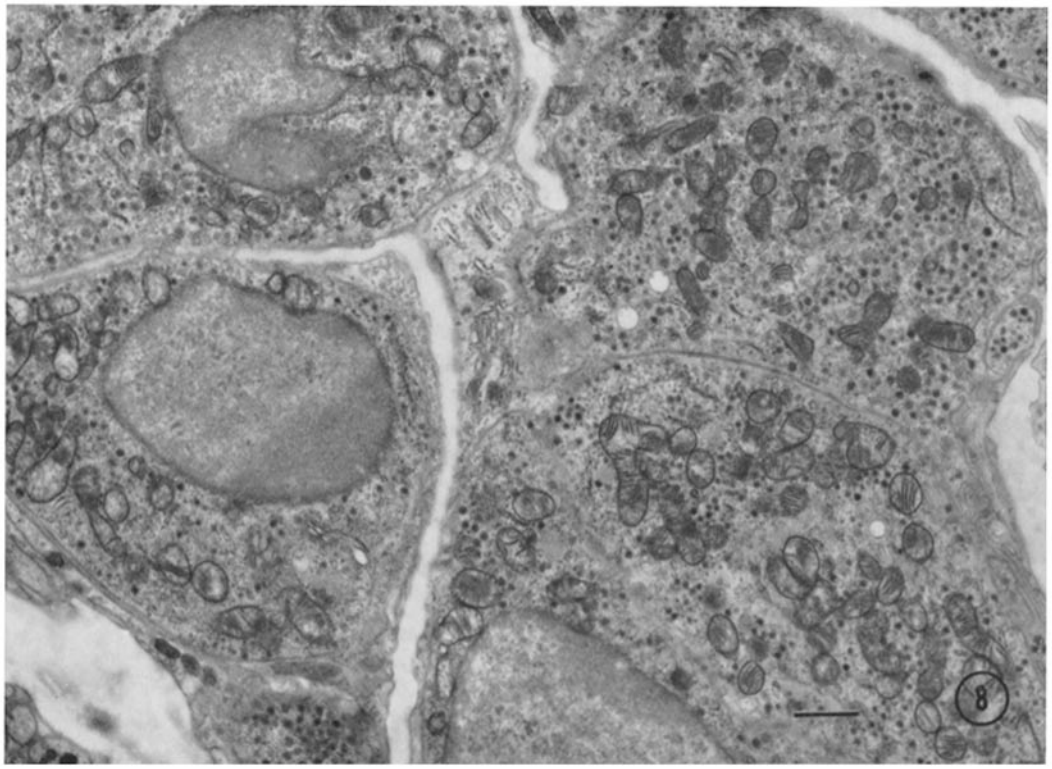


FIGURE 8 Portion of the carotid body of the animal treated with a 5% oxygen and 95% nitrogen gas mixture for 20 min. The tissue was fixed by perfusion with s-Collidine–buffered OsO_4. The mitochondria in most glomus cells appear swollen except in the cell which is located at the upper right part of the picture. However, the appearance of the granules is not different from that of granules of the controls. × 8700.

Lami and Murray (1968). The granules were normal in appearance (Fig. 3) and population, and there was no increase in cytoplasmic density in either the glomus (number of dark glomus cells) or supporting cells. No movement of granules toward the cell membrane or subsequent exteriorization of their content as described by Blümcke et al. (1967a) was observed.

In tissues fixed by immersion in s-Collidine-buffered osmic tetroxide rupture and swelling of the granules and mitochondria were seen frequently. Although there was a tendency of mitochondria to swell (Figs. 8, and 11) in perfused specimens, the appearance of the granules was not different from that of the controls. Profiles suggesting discharge of the granular contents into extracellular spaces were rarely observed.

With the glutaraldehyde-dichromate technique, no decrease in the density of the granules in the glomus cells was seen (Fig. 14).

DISCUSSION

The effects of reserpine on the granules of the glomus cells at the electron microscope level were first described by Lever et al. (1959). They noted that reserpine administration resulted in a general depletion of the electron-opaque granules and, therefore, they suggested that the granules probably contained catecholamines. Using microspectrophotometric techniques, Hamberger et al. (1966) reported that 5-hydroxytryptamine and catecholamines were present in human carotid body glomus cells. In the present study the sensitive cytochemical technique employed on the adrenal medulla and the carotid body (Wood and Barr-

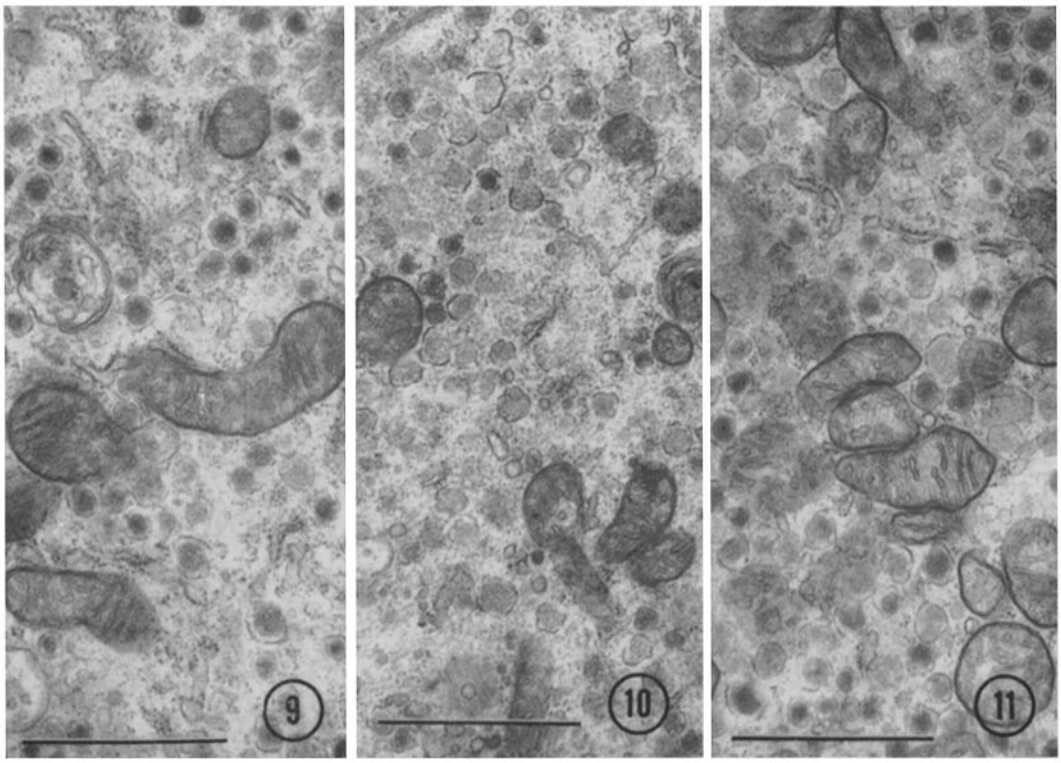
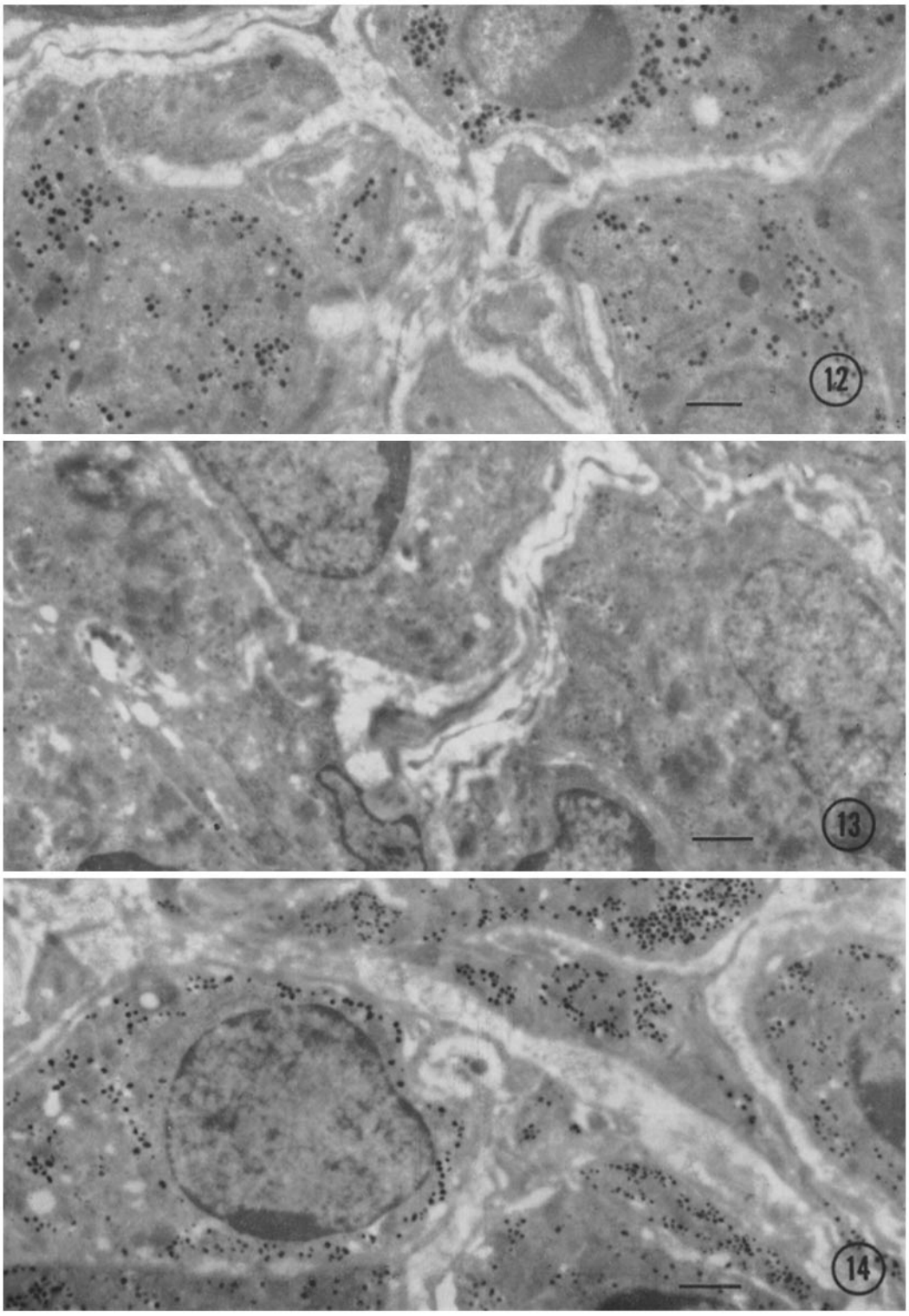


FIGURE 9 A part of a glomus cell from the carotid body of a control animal fixed with s-Collidine–buffered OsO_4. Although some of the granules have ruptured limiting membranes, most of them exhibit intact membranes. × 27,000.

FIGURE 10 In contrast to Fig. 9, a marked decrease in density of the cores of the granules and rupture of their limiting membranes are seen frequently in the glomus cells of reserpine treated animals fixed with s-Collidine–buffered OsO_4. × 27,000.

FIGURE 11 A micrograph from a hypoxia-treated animal fixed with s-Collidine–buffered OsO_4. The appearance of the granules is normal (compare with Fig. 9). × 27,000.

nett, 1964) is specific for unsubstituted amines which are apparently present in the granules of both cell types. Even though unsubstituted amines are present, the assumption should not be made that both glands function in a similar manner. According to Duncan and Yates (1967), reserpine treatment resulted in a depletion of granules and a concomitant increase in the number of empty vesicles of the same size in carotid body and adrenal medullary cells fixed with s-Collidine-buffered osmium tetroxide. On the other hand, when carotid bodies and the adrenal medulla were fixed first with glutaraldehyde and then with osmium tetroxide no appreciable change in the number or density of the granules in the carotid body was observed, but the medullary cell granules disappeared. Dichromate incubation following glutaraldehyde fixation in this study revealed a consistent decrease in opacity of the dense cores of the granules in the carotid body and adreno-medullary cells after reserpine injections. These findings suggest that reserpine treatment results in a release of the amine content from the granules. Microspectrophotometric and radiochromatographic techniques would further substantiate these observations. One explanation for the persistence of the dense granules in the glomus cells after reserpine administration and subsequent fixation in glutaraldehyde may be that the binding between catecholamines and the other components of the stor-



FIGURES 12–14 Portions of the carotid bodies of control (Fig. 12), reserpine-treated (Fig. 13), and hypoxia-treated (Fig. 14) hamsters. The tissues were fixed with glutaraldehyde and subsequently subjected to dichromate incubation. The sections are unstained. Note that the granules in the glomus cells of the control and hypoxia-treated animals (Figs. 12 and 14) appear very dense, while the opacity of the granules in the glomus cells of the reserpine-treated animal (Fig. 13) has essentially disappeared. All figures. × 9600.

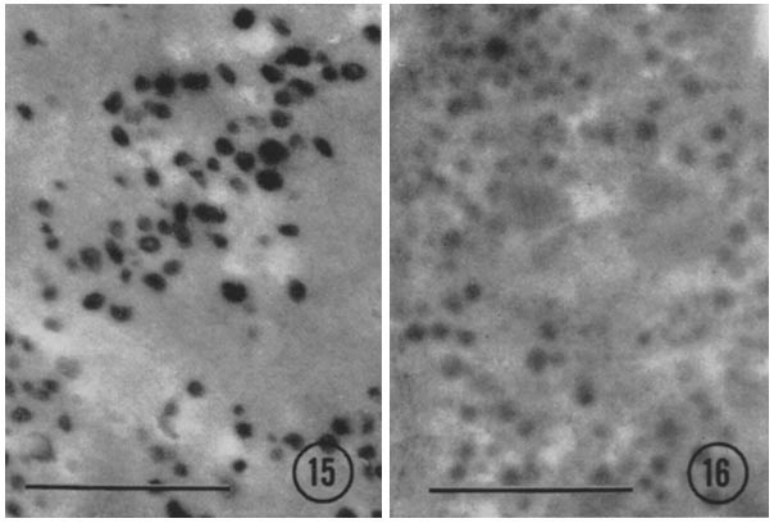


FIGURE 15 A higher power micrograph of a part of a glomus cell from a control animal. The tissue was prepared as described for Figs. 12–14. Most granules appear to be intensely stained. The ring-like structures of many granules are accentuated with this technique. Unstained. × 27,000.

FIGURE 16 A part of the glomus cell of a reserpine-treated animal. The tissue was prepared as stated for Figs. 12–14. Note the decrease in granule density. Unstained. × 27,000.

age granules is more resistant to the action of reserpine than are similar, but different, complexes in the adrenal medullary cells (Duncan and Yates, 1967). Another possible explanation could be that the amine content of the granules in the glomus cells is less than that of the granules in the adrenal medullary cells, and that the nonamine components are relatively resistant to reserpine treatment. Varying effects of reserpine on the release of the amine and nonamine content from different tissues have been described (von Euler et al., 1964). The density of the granules in the glomus cells of animals treated chronically with reserpine and fixed with glutaraldehyde and osmium tetroxide could be due primarily to the nonamine content (Lever et al., 1959; Yates, 1964). The pronase digestion experiments in these studies suggest that there is a sizeable amount of protein in the dense cores of the granules. It should be noted that after the dichromate incubation technique the granules in the glomus cells of nontreated animals appeared less dense than the norepinephrine-storing granules in adrenal medullary cells. Using light microscopic histochemical methods, Lever et al. (1959) showed that the staining of the glomus cells appeared to be approximately one tenth that seen in adrenal medullary cells.

The mechanism by which the amine content is released from the storage granules has not been fully established. de Robertis and Vaz Ferreria (1957) and Diner (1967) observed discharge of the content of the granules into the subendothelial spaces of the venous sinuses in the adrenal medulla. Pharmacological studies (Blaschko et al., 1967; Douglas, 1966) have suggested that all the soluble constituents of the granules are secreted. In electron microscopic studies, Yates (1963, 1964) postulated that the catecholamines could be released from the medullary cells without complete disappearance of granules. Histochemical data (Falck and Hellman, 1963, 1964) indicate that the granules in certain pancreatic islet cells contain biogenic amines and that reserpine treatment results in a decrease in the density of the granules. In the present study, although profiles of some granules appeared to be in contact with the plasma membrane, the ones which suggested a release of the contents into the extracellular spaces were rarely observed in either the control or treated animals. The consistent results showing a decrease in the opacity of the cores without an appreciable reduction in the number of granules after reserpine treatment suggests an intracellular release phenomenon at least in reserpinized animals.

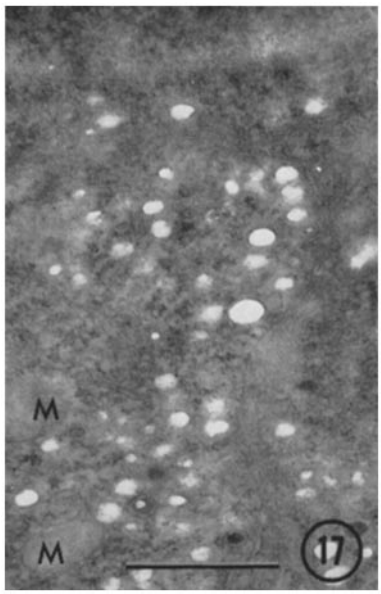


FIGURE 17 A part of the cytoplasm of a carotid body cell from tissue which had been fixed with glutaraldehyde and OsO_4, embedded in Epon, and subjected to pronase digestion. Note that most of the granules are absent, leaving clear spaces in the cytoplasm. Mitochondria (*M*) are shown. × 19,600.

Most electron microscopists who have attempted to correlate the fine structure of the carotid body with its function as a chemoreceptor have been confronted with such findings as: (1) there is no intimate relationship between the glomus cells and the blood stream; (2) the glomus cells are almost completely surrounded by supporting cells; and (3) the nerve terminals which contact the glomus cells appear to be morphologically efferent rather than afferent terminals. These facts cast serious doubt on the concept that the cells act as chemical receptors in the circulating blood stream (de Kock and Dunn, 1964). From the oncological viewpoint and from certain contradictory results of both animal experiments and clinical observations concerning the function of the carotid body, Karnauchow (1965) refuted the concept of the structure as a chemoreceptor.

The only observation suggesting that the glomus cells may be involved in impulse transmission is the presence of granules which are similar morphologically to those found in cells of the adrenal medulla which are known to contain catecholamines (Hagen and Barrnett, 1960). Lever and Lewis (1959) and Lever et al. (1959) first proposed the concept that the dense granules in the glomus cells contained catecholamines and that this substance might be responsible for chemical transmission. Hoffman and Birrell (1958) observed that the cells of the rat carotid body were filled with small "vesicles" which were depleted during anoxia. Blümcke and his co-workers obtained similar results after hypoxia (1967a) but somewhat different ones after hypercapnia (1967b). With hypoxia, they noted the mobilization of a few granules to the vicinity of the cytoplasmic membranes and a subsequent exteriorization into the extracellular space. Following hypercapnia, they observed disintegration of catecholamine granules, rupture of the limiting membrane surrounding the granules, and extrusion of the osmiophilic cores into the cytoplasm. The results obtained by these workers were based primarily on tissues fixed with buffered osmium tetroxide alone. In the present study with dichromate incubation, hypoxia caused no appreciable change in the density or number of the granules in the glomus cells. However, immersion fixation in osmium tetroxide resulted in rupture of granule limiting membranes and plasma membranes which occasionally appeared to fuse together to form profiles suggestive of the extrusion of the granular content into the extracellular space or extrusion of the dense core of the granule into the cytoplasm of the glomus cells.

The sensitive cytochemical technique for the detection of monoamines failed to reveal a depletion in the amine content of the granules of animals subjected to hypoxia. These results suggest that the biogenic amines in the glomus cells are not the active substances which directly initiate the transmission of nerve impulses preceding the respiratory reflex or, more conservatively, that the glomus cells do not release cytochemically detectable amounts of amines in response to hypoxia.

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