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Cytochrome oxidase activity of mitochondria in sensory nerve endings of mouse palatal rugae

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INTRODUCTION

The existence of an abundance of tightly packed mitochondria in a number of specialized sensory nerve terminals is well established (Pease & Quilliam, 1957; Cauna & Ross, 1960; Merrillees, 1960) and they have been considered important in the supply of energy required for the transduction of physical stimuli into sensory nerve impulses at these sites (Bleichmar & De Robertis, 1962; Vinnikov, 1966). Recent electron microscopic studies (Weiss & Pillai, 1965; Weiss & Mayr, 1972) have suggested that mitochondria that have 'stalled' at neuronal terminations, such as the presynaptic endings of motor end-plates and the endings on intrafusal fibres of muscle spindles, lose their viability by undergoing a massive and rapid disintegration. This hypothesis may have gained support from the work of Hajós & Kerpel-Fronius (1969, 1971), who reported a block of the citric acid cycle in presynaptic mitochondria in the central nervous system.

Cytochemical studies of the central nervous sytem have demonstrated high cytochrome oxidase activity associated with presynaptic mitochondria (Kerpel-Fronius & Hajós, 1967) and studies of muscle spindles have revealed a high level of citric acid cycle enzyme activity (Ovalle, 1971). Whether the histochemical reaction products were merely in the vicinity of disrupted mitochondria or localized within them was not determined. The precise localization of the reaction products could not be inferred, either because of inadequate tissue fixation or because of lack of positive identification of the histochemical reaction product.

The purpose of the present study was to determine if mitochondria in sensory endings in palatal rugae were morphologically and physiologically competent, through the use of a precise ultrastructural cytochemical method (Seligman, Karnovsky, Wasserkrug & Hanker, 1968) for the demonstration of their cytochrome oxidase activity.

Their activity was compared with that of mitochondria of epithelial cells of the kidney and the choroid plexus, since in certain of these cells with high metabolic activity similarities in the arrangement of the mitochondria could be associated with specific physiologic function (Pease, 1956). Moreover, previous studies (Karnovsky & Himmelhoch, 1961) had suggested that a decrease in cytochrome oxidase activity of kidney tubular epithelium was not due to a disappearance of mitochondria but rather to a decrease in their metabolism.

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MATERIALS AND METHODS

Studies were performed on C57BL/10 and C57BL/6J-*dt*^{J} mice (The Jackson Laboratory, Bar Harbor, Maine), 1 and 16–25 days old. Animals were anaesthetized with ether and, depending on the size of the animal, 3–5 ml of physiological saline were administered through the left ventricle to flush most of the blood out of the organs prior to perfusion by the same route with 2 % formaldehyde (freshly prepared by depolymerization of paraformaldehyde) 0·15 M in pH 6·7–7·4 phosphate buffer. The tissues were excised rapidly after evidence of a satisfactory perfusion (judged by leakage of fixative through a small lip incision), and fixation was continued by immersion in the same fixative for a total period of 60–90 minutes. The tissues were then rinsed for 24 hours in several changes of 0·22 M sucrose which was 0·1 M in pH 5·6 acetate buffer.

For light microscopic histochemistry, cryostat sections (4–10 μm) of the fixed tissues were prepared and air-dried on coverslips. The sections were incubated in a medium for cytochrome oxidase (Seligman *et al.* 1968) for 30 minutes at 37 °C. Frequently, 1–2 μm sections of tissues processed for electron microscopy and embedded in Durcupan were also examined. For electron microscopy, 200 μm parasagittal sections of hard palate, or 50 μm sections of the other tissues, were prepared with a Sorvall TC-2 sectioner (Smith & Farquhar, 1965). The sections were briefly rinsed in pH 7·2 phosphate buffer (0·05 M) prior to incubation for cytochrome oxidase activity by the method used for light microscopy. Subsequent treatment of the tissues, as well as the use of inhibitors and intensification of the mitochondrial stain on the ultrathin sections by bridging through thiocarbohydrazide to osmium, was performed according to the directions of Seligman *et al.* (1968). As an alternative to intensification by the bridging procedure, ultrathin sections were counterstained with methanolic uranyl acetate (Stempak & Ward, 1964) and lead citrate (Reynolds, 1963).

Since the enzyme staining procedure gave an end product which was visible as well as electron-opaque, areas for ultrathin sectioning were selected by examining 2 μm plastic sections under the light microscope.

OBSERVATIONS

Light microscopy

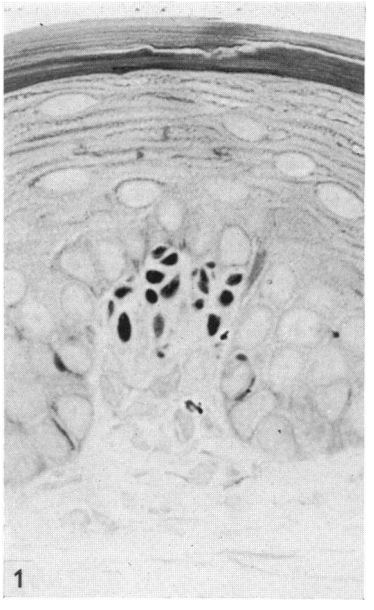
The staining for cytochrome oxidase observed in all tissues was limited to mitochondria. In parasagittal sections of hard palate, intensely stained areas were seen subjacent to the epithelium (Fig. 1), especially at the summits of the rugae, in

Fig. 1. Sensory nerve terminations in ruga of mouse palate. Stained for cytochrome oxidase activity. Parsagittal section. 1 μm. × 1200.

Fig. 2. Cytochrome oxidase in mitochondria of the epithelium of convoluted tubules of mouse kidney. Glomerulus (G) and nuclei (↑) are unstained. 1 μm section. × 550.

Fig. 3. Cytochrome oxidase in mitochondria of cuboidal epithelium of choroid plexus of fourth ventricle of mouse brain. Mitochondria of capillary endothelium and nuclei are unstained. 10 μm cryostat section. × 550.

Fig. 4. Sensory receptor in palate showing mitochondria of neurite (N), laminar cells (L) and epithelium (E), stained with equal intensity. Dalton's chrome-osmium fixative (Dalton, 1955), uranyl acetate and lead citrate stain. × 34500.



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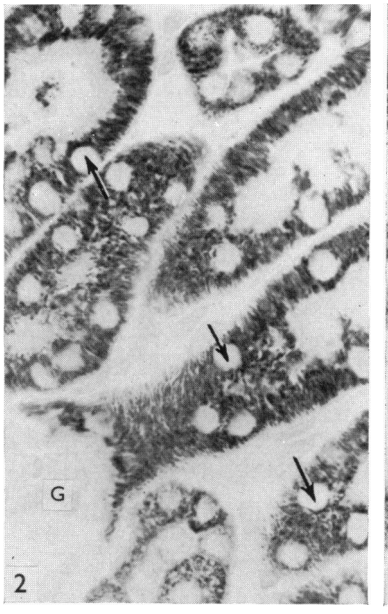

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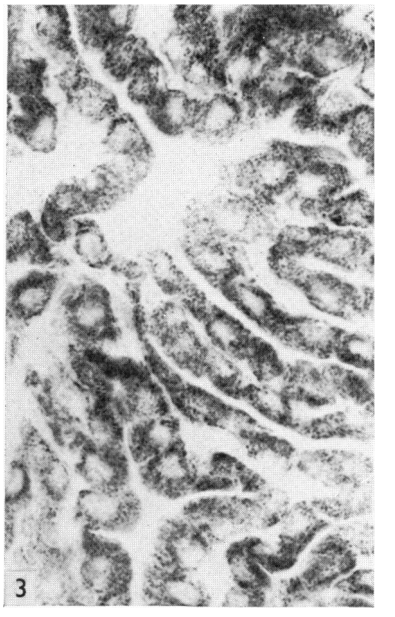

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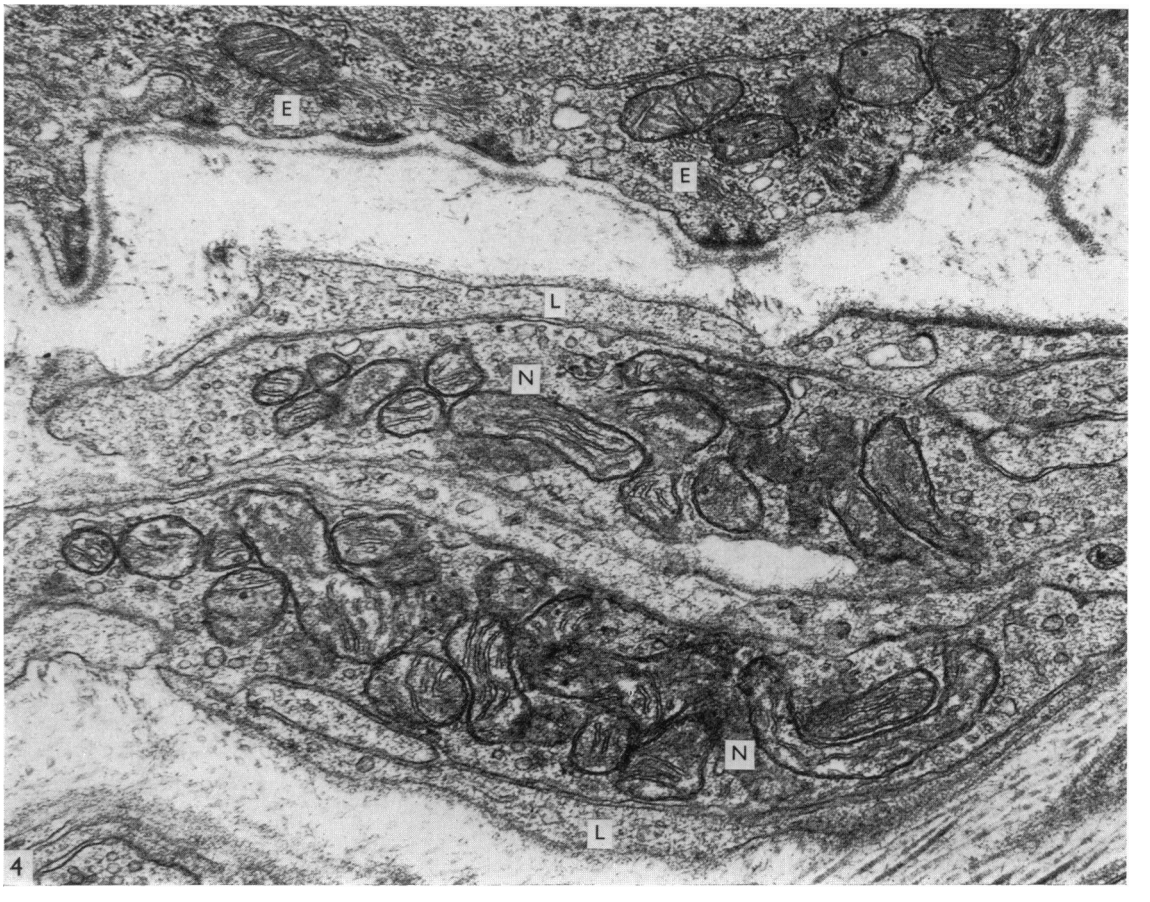

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situations known by other neurohistological methods to contain sensory receptors. In contrast, the cells of the oral epithelium and the connective tissue cells of the supporting tissues were scarcely stained. However, the mitochondria of the basal layer of the epithelium appeared to be stained more deeply than those of the other layers. We confirmed that the intense staining was due to the cytochemical activity of the neurite mitochondria by our electron microscope studies. Light microscopy of the other tissues confirmed the limitation of staining to mitochondria. Rod-like mitochondria of the epithelial cells in the proximal and distal convoluted kidney tubules and the cuboidal epithelium of the choroid plexus stained very intensely (Figs. 2, 3). Mitochondria in other areas of the kidney tubules, in the intervening connective tissues, or in the endothelium of capillaries of the choroid plexus, showed much less activity. Staining was absent in the kidney glomerulus.

Electron microscopy

Oral mucosal sensory receptors were recognized readily in electron micrographs by their central nerve cell process, or neurite, which contained tightly packed mitochondria (Fig. 4). The neurites were surrounded by flattened laminar cells containing hollow-cored vesicles (Figs. 4, 5). The receptors took various forms and were frequently found a short distance beneath the oral epithelium (Fig. 4).

The pattern of staining of mitochondria observed in palatal receptors fixed routinely in chrome-osmium and counterstained with uranyl acetate and lead citrate was quite different from that seen in tissues incubated with diaminobenzidine and cytochrome *c* for cytochrome oxidase demonstration. In routinely stained preparations (Fig. 4) mitochondria in epithelial cells, in laminar cells, and within the neuroplasm of the endings stained with equal intensities. In cytochrome oxidase preparations (Fig. 5) staining of mitochondria was much more intense in the sensory endings than in the mitochondria of either the laminar cells or the neighbouring oral epithelial cells.

Disrupted mitochondria were more frequently observed in neuronal endings (Fig. 6) than in the epithelia of kidney tubules, choroid plexus, or non-nervous elements in the hard palate; they were also seen in fibroblasts. Mitochondria of laminar cells showed much less disruption than those of neuronal endings. Degenerating mitochondria were seen less frequently than disrupted mitochondria in endings (Fig. 6); they were usually much smaller than the other mitochondria in the neurite. Occasionally, however, endings containing no disrupted mitochondria were seen on the same section adjacent to endings in which most of the mitochondria were disrupted. Swollen mitochondria were frequently seen in these preparations. The criteria for classifying mitochondria as 'normal', 'degenerate', 'disrupted', or 'swollen' were those described by Rouiller (1960). The most usual type of degenerating mitochondria observed had ribbon-like lamellae which gave them a whorl-like appearance (Fig. 6). They were rare, and their frequency was not increased by changing the pH or osmolality of the fixative; they never displayed broken or 'disrupted' membranes. On the other hand, the frequency of disrupted or swollen mitochondria was very dependent on the pH of the fixative, and to a lesser degree on the osmolality. Disrupted mitochondria were more common in tissues fixed at pH 6·7 than in tissues fixed at pH 7·2 or 7·4, although enzyme activity was better preserved at the lower pH.

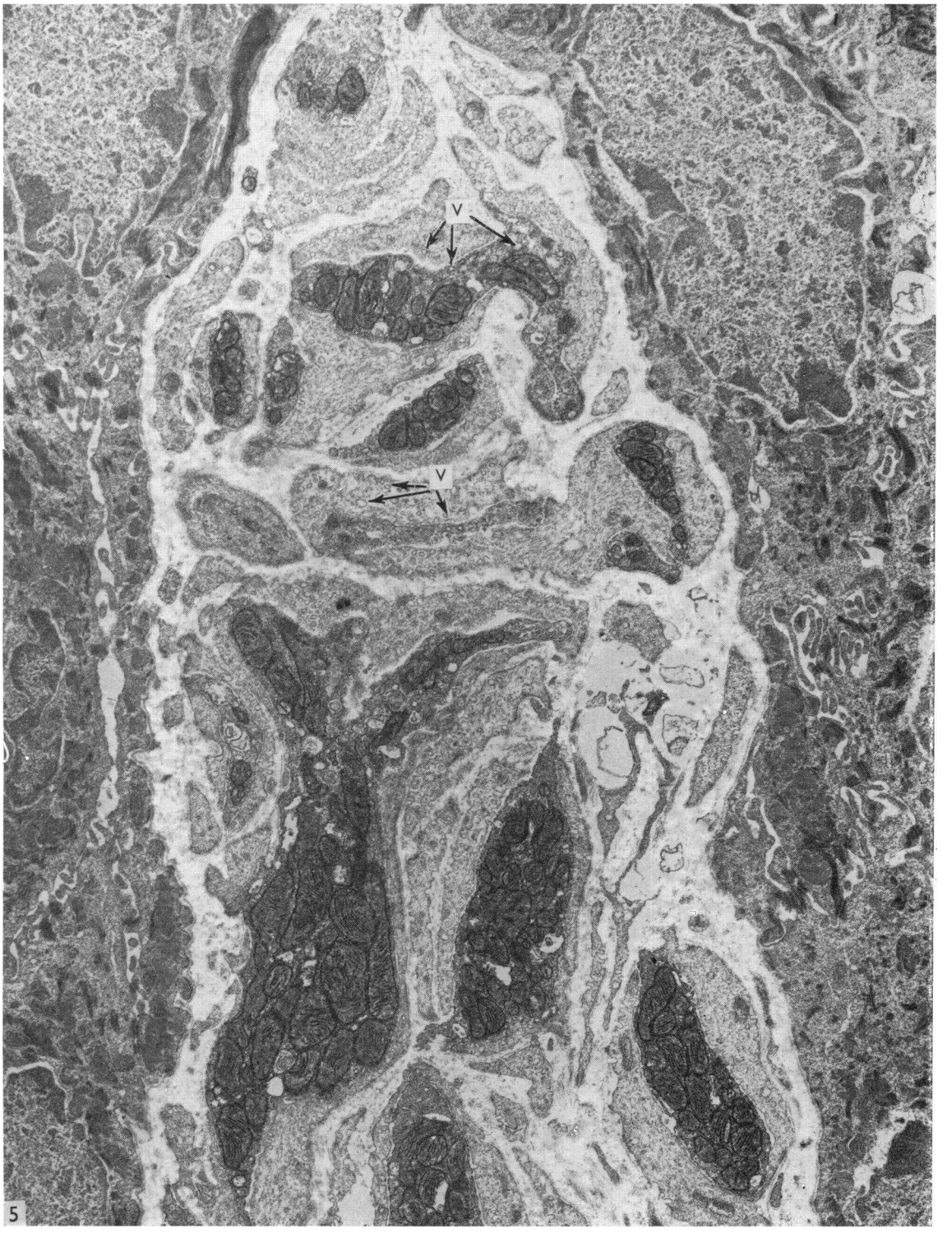

Fig. 5. Cytochrome oxidase activity in mitochondria of neurites of palatal sensory receptor. Note hollow-cored vesicles (V). No counterstain. × 14900.

Intense staining for cytochrome oxidase activity was observed on cristal remnants of the disrupted mitochondria. Vacuolization of mitochondria, as reported by Weiss & Pillae (1965), was also rare. Vacuoles were more conspicuous in the neuroplasm of endings. Moreover, swollen mitochondria were the rule in sections where vacuolization was observed.

Staining of the neurite mitochondria was limited to the outer and inner (cristal) membranes. The cristae, which were frequently longitudinally arranged (Figs. 4–7), usually stained more intensely than the outer mitochondrial membrane (Fig. 7). Mitochondria in other cell types showed a similar pattern of cytochemical staining. The only extramitochondrial sites stained were desmosomes and tonofilaments of palatal epithelial cells (Fig. 5).

The distinction between the cytochrome oxidase activity of mitochondria of neuronal endings and those of palatal epithelium was less if unfixed tissues were incubated for enzyme activity. Staining was again limited almost exclusively to mitochondria, but mitochondria of endings and epithelium stained equally well. Fixation of the tissues usually made it possible to distinguish mitochondria having different levels of metabolic activity. Thus, in fixed tissues, mitochondria in intraepithelial sensory endings stained more intensely than mitochondria in epithelial cells.

Although activity was observed in tissues fixed for more than 90 minutes, especially in cells containing the more enzymically active mitochondrial types, it was very sporadic. Fixation mixtures containing glutaraldehyde were also more inhibitory toward cytochrome oxidase. Within a given cell mitochondria sometimes varied in enzyme activity. Although the outer mitochondrial membranes were quite uniformly stained for cytochrome oxidase within a particular neuronal ending, large variations in staining intensity of the cristae of mitochondria within the same ending or of cristae within the same mitochondrion were frequently observed (Fig. 7).

Hollow-cored vesicles (Nishi, Oura & Pallie, 1969) were occasionally observed in the neuroplasm in the palatal endings (Fig. 4); they were very numerous in the laminar or lamellar cells adjacent to the endings at both the proximal and distal borders of the laminar cells and at the borders of the neuronal terminations (Fig. 7).

DISCUSSION

Marked differences in the cytochrome oxidase activity of mitochondria in different cell types have been observed in the present study. Since the method for cytochrome oxidase permits the use of fixed tissues rather than the fresh frozen tissues which were employed in previous studies (Seligman *et al.* 1967; Hajós & Kerpel-Fronius, 1969, 1971) with succinic dehydrogenase, the discrepancies met with in those studies did

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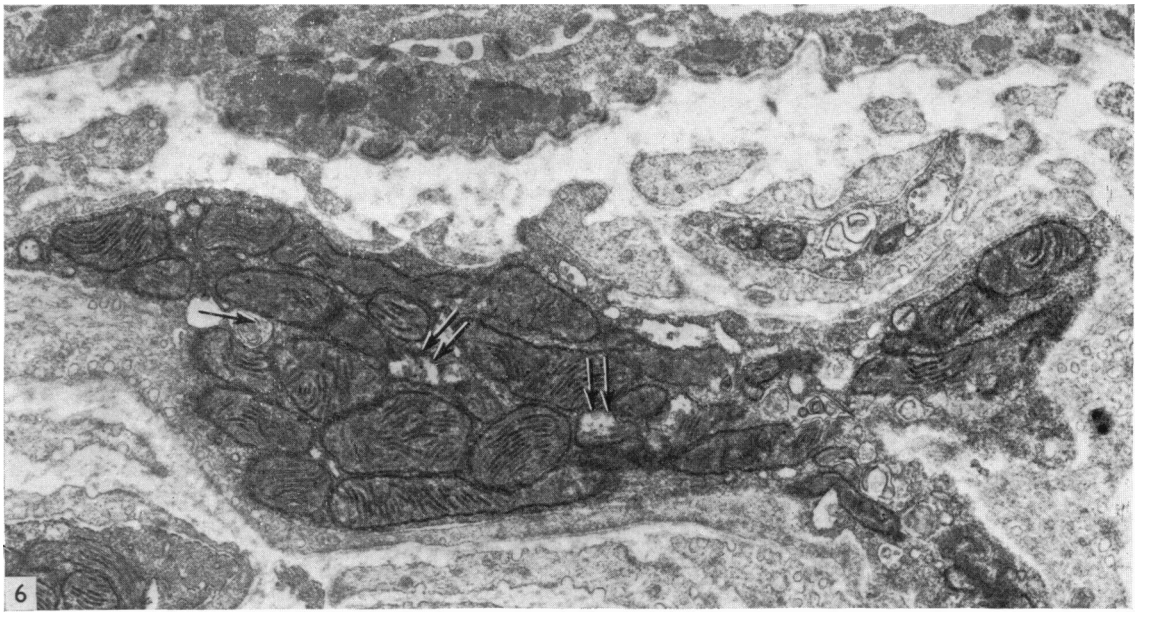
Fig. 6. Area of Fig. 5 at higher magnification showing degenerated (↑) and disrupted (↑↑) mitochondria in neurites. Cytochrome oxidase. No counterstain. × 25000.

Fig. 7. Area of Fig. 5 enlarged to show detail of staining of mitochondrial membranes for cytochrome oxidase. Note relatively unstained mitochondria (↑) in laminar cells and longitudinal cristae of neurite mitochondria. × 32000.

Fig. 8. Cytochrome oxidase in neurite and fibrocyte (F) mitochondria in palatal ruga. The neurite mitochondria stain more intensely. No counterstain. × 14500.



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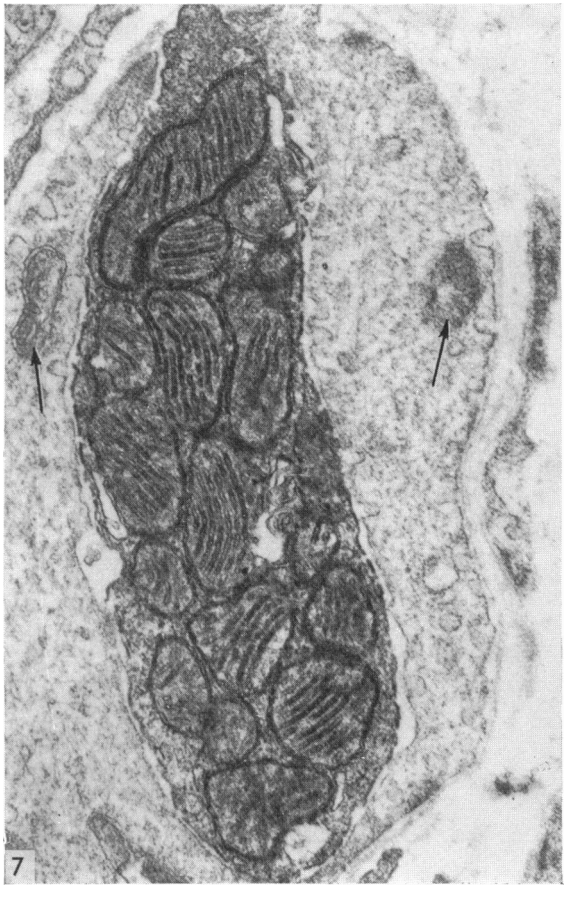

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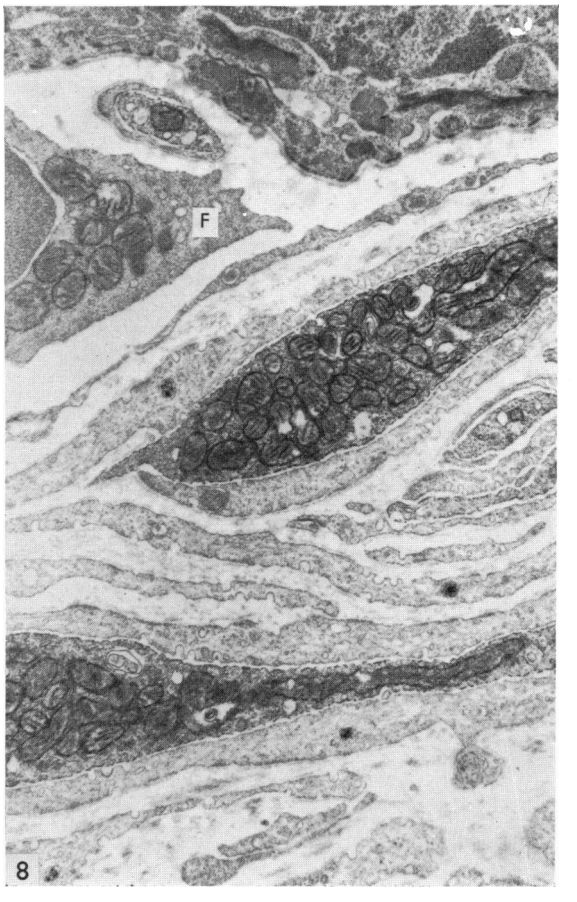

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not occur. In particular, artefacts due to the ready dissociability from the cristae of dehydrogenases such as succinic dehydrogenase were not observed.

The fact that mitochondria in the basal portion of kidney tubular epithelial cells had intense staining for cytochrome oxidase activity, whereas glomerular mitochondria were unstained, is consistent with the need for adenosine triphosphate in these epithelia to supply the energy for active transport processes (Liisberg, 1969). The staining of mitochondria in the cuboidal epithelium of the choroid plexus of the brain also corresponds to expectations of metabolic demands on these mitochondria indicated by ultrastructural studies (Pease, 1956). These results suggest that the intense staining for cytochrome oxidase of mitochondria in the nerve endings of palatal rugae is likewise due to a high metabolic activity.

The relatively high activity of mitochondria in neurons when compared with those in supporting cells has been observed previously (Hamberger, Blomstrand & Lehninger, 1970). In addition to supplying energy for the transduction of physical stimuli into nerve impulses, it has been proposed that they may play a part in the synthesis of enzymes concerned in the secretion and metabolism of neurotransmitters (Lehninger, 1970).

Another observation in the current study suggests an active metabolic role for mitochondria in the terminations in palatal rugae. The presence of vesicles along with tightly packed mitochondria in the endings (Fig. 4) suggests the probability of special metabolic processes in the nerve terminal (Quilliam, 1958).

Mitochondrial preservation is a common problem in cytochemical studies. That the frequent disruption of mitochondria observed in the palatal endings was an artifact of preparation is supported by several observations, including the greatly increased tendency to disruption noted when tissue was fixed at a pH lower than 7·2, and the swelling of mitochondria noted in areas of disruption. The cristae that could be observed in partially disrupted mitochondria stained intensely by the enzyme cytochemical procedures. 'Swollen' or 'disrupted' mitochondria could be due to physiological or pathological changes, or simply to an artifact of preparation. 'Degenerating' mitochondria could also result from the same events, but more probably indicate the turnover of the mitochondrial population. In certain instances, mitochondria described as 'swollen' could actually be normal mitochondria cut tangentially rather than transversely or longitudinally. If disrupted mitochondria were moribund or dead, their cristal remnants would be expected to stain less intensely. The low frequency with which degenerating mitochondria were observed in the endings could be explained by the turnover expected in the mitochondrial population, and appeared to be independent of the way the tissue was handled. It is also important that, whereas the cristae of 'disrupted' mitochondria had intense cytochrome oxidase activity, the whorls of degenerating mitochondria had no activity. Although the frequency with which abnormal mitochondrial profiles occurred was not measured, it appeared to depend (to some extent at least) on the incidence per unit area of cytoplasm of all types of mitochondria.

SUMMARY

The cytochrome oxidase activity of mitochondria in terminations of afferent nerve fibres adjacent to the rugae of hard palate of the mouse has been studied with a cytochemical method, with results which indicate that they are metabolically competent. The mitochondria in the neuroplasm of endings stained more intensely than those in laminar cells, epithelial cells or fibroblasts. Although disrupted mitochondria were frequently seen in terminations, especially in tissues fixed at low pH, their cristae showed no diminution in enzyme activity. This is consistent with the disruption being due to inadequate cellular preservation.

The high metabolic activity of mitochondria in the sensory terminations suggests that these mitochondria may supply the energy required for sensory transduction or for the synthesis of enzymes concerned in the elaboration or transport of vesicles in the ending. This is contrary to previous suggestions that these are moribund mitochondria that are dammed up as a result of neuroplasmic flow.

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