

THE FINE STRUCTURE OF PUROMYCIN-INDUCED CHANGES IN MOUSE ENTORHINAL CORTEX

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ABSTRACT

Bitemporal intracerebral injections of puromycin in mice suppress indefinitely expression of memory of avoidance-discrimination learning. Ultrastructural studies of the entorhinal cortex of puromycin-treated mice revealed the following: (a) Abnormalities were not observed in presynaptic terminals and synaptic clefts; many postsynaptic dendrites or somas contained swollen mitochondria. (b) Dispersion of polyribosomes into single units or condensation of ribosomes into irregular aggregates with loss of "distinctiveness" was noted in a few neurons 7-27 hr after puromycin treatment. (c) Cytoplasmic aggregates of granular or amorphous material were frequently noted within otherwise normal neuronal perikarya. (d) Mitochondria in many neuronal perikarya and dendrites were swollen. Mitochondria in axons, presynaptic terminals, and glial cells were unaltered. The relationships between these lesions and the effect of puromycin on protein synthesis and memory are examined. It is suggested that the disaggregation of polysomes is too limited to explain the effect of puromycin on memory. Special emphasis is given to the swelling of mitochondria. The possible mechanisms and the significance of this lesion are discussed.

Memory of maze learning in mice disappears for at least 3 months after intracerebral injection of puromycin (1), a powerful inhibitor of protein synthesis (2-3). The mechanism of this effect of puromycin on memory is obscure (1, 4).

The present ultrastructural investigations on mice treated with intracerebral injections of puromycin were undertaken with the hope that they would contribute to an understanding of the mode of action of puromycin on memory. Cortical synapses were examined in detail because of the current belief that memory depends upon modification of their properties (5-6) and because of the recent finding of morphological abnormalities in the neocortical synapses of patients with mental

retardation or dementia (7-10). No significant changes were noted in synaptic endings. Neuronal ribosomes were also studied in detail. Disaggregation of ribosomes from polysomes and reduction in number of ribosomes were noted in only a few neurons. The most striking abnormality consisted of a series of changes in certain neuronal mitochondria. The possible relationship of these findings to the effect of puromycin on memory will be discussed.

MATERIALS AND METHODS

10 adult white mice weighing about 30 g were injected intracerebrally with puromycin. Just before use, puromycin dihydrochloride was dissolved in

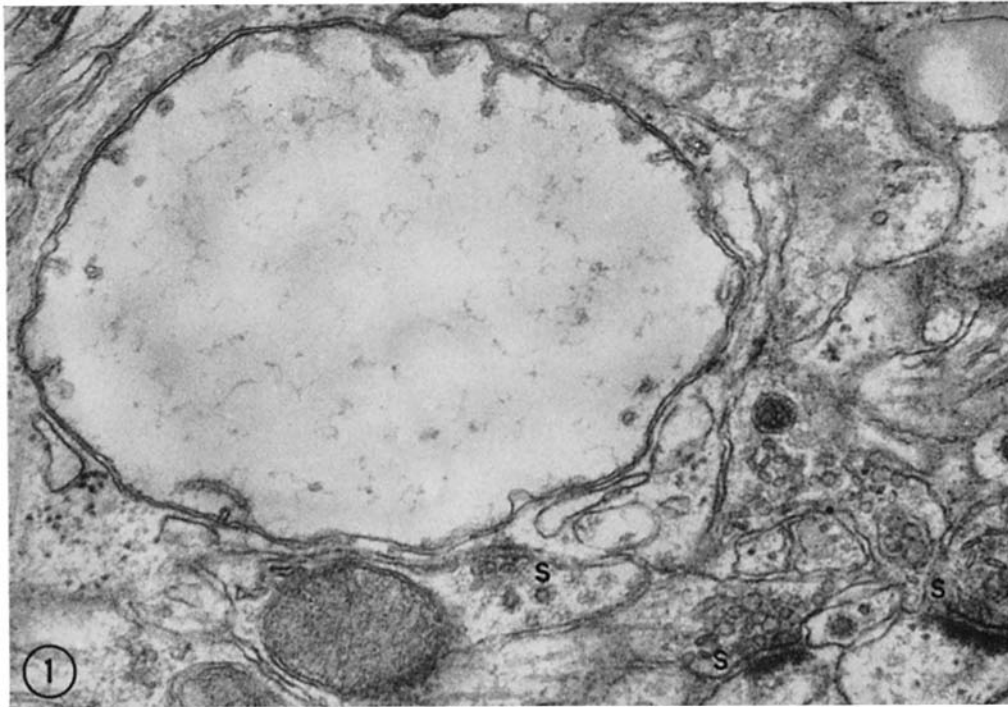


FIGURE 1 Severely swollen mitochondrion in postsynaptic ending. Only a few short cristae are present. The matrix has disappeared and the inner space shows filamentous material. *s*, presynaptic terminals. 7 hr after injection of puromycin. $\times 44,500$.

water and the solution brought to pH 6 with 0.1 N NaOH. 12 μ l of this solution, containing 90 μ g of puromycin, were injected bitemporally through small holes in the skull just above the angle between the caudal sutures of the parietal bones and the origin of the temporal muscles, as previously described (11). Following the same procedure, NaCl equal to puromycin in volume, pH, and osmolarity was injected in five control mice. The mice treated with puromycin were divided into three groups: two mice were sacrificed 7 hr after injection when inhibition of protein synthesis in the entorhinal cortex and hippocampus was at its height; six were sacrificed after 19–27 hr when protein synthesis was largely restored and memory had disappeared (1); and two were sacrificed after 36 hr. For each group there was one or more controls. The mice were perfused for 20 min with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and 2% 0.2 M CaCl_2 (12). Both entorhinal cortices with part of the adjacent ventral hippocampi (3–4 mm from the point of injection) were sampled from all animals. In addition, the dorsal hippocampus (about 1 mm from the point of injection) was sampled from one mouse sacrificed 26 hr after puromycin injection

and from its control. For electron microscopy, fixation was continued by immersion in glutaraldehyde, at 4°C, for an additional 2½ hr. Specimens were washed in 10% sucrose in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 2% osmium tetroxide in Millonig's buffer (13). The samples were embedded in English Araldite.¹ Sections were cut on an LKB Ultratome with a diamond knife. Contrast was enhanced by staining with uranyl acetate and lead citrate (14, 15). A Siemens Elmiskop 1 electron microscope was used with objective apertures 50 μ in diameter and at an accelerating current of 80 v. 57 blocks of tissue were sectioned and approximately 700 electron micrographs were studied. For light microscopy, samples were dehydrated with alcohol, embedded in paraffin, and stained with hematoxylin and eosin. Three mice (one at 7 hr and one at 17 hr after injection with puromycin, and one control) were perfused with Zenker's fixative (16); entorhinal cortex was sampled and stained with malachite-green-pyronin stain, for demonstration of RNA according to Baker and Williams (17).

¹ Araldite, Ciba, Duxford, Cambridge, England.

RESULTS

Light Microscopy

Sections from the entorhinal cortex were examined after staining with hematoxylin and eosin and with malachite-green-pyronin. No significant differences were seen in the neuronal cytoplasmic RNA of control and puromycin-treated mice; cytological or histological lesions were not observed.

Electron Microscopy

CONTROL MICE: No abnormalities were seen in the entorhinal cortex or ventral hippocampus of mice injected with saline. The neuronal synaptic complexes, glial cells, and blood vessels were unremarkable in their appearance, which corresponded to that described by a number of investigators (18-21). The following observations are noted because of their relevance in evaluating the effect of puromycin. The neuronal perinuclear cytoplasm had large numbers of ribosomes, about 250 Å in diameter, arranged in circles or small

rosettes, for the most part, containing four to six ribosomes (Fig. 3 *b*). Isolated ribosomes were rarely seen. The cisterns of the endoplasmic reticulum were flattened and contained amorphous material of low electron opacity. The mitochondria had a dense matrix, and the cristae generally were arranged in an irregular, transverse direction. The outer and inner mitochondrial membranes measured about 75 Å in thickness (the inner being generally slightly thicker); the center-to-center distance between the outer and the inner membranes was variable, but usually measured 140-170 Å. Localized vacuolization of the mitochondrial matrix with displacement of the cristae was occasionally observed in a random fashion in neuronal and glial cells. This change was recognized as an artefact of aldehyde fixation (22). The synaptic complexes were composed of presynaptic terminals (about 1 μ in diameter), which contained mitochondria and a highly variable number of round or flat synaptic vesicles, about 400-600 Å in diameter; the postsynaptic element (dendrite, axon, or soma) was unremarkable.

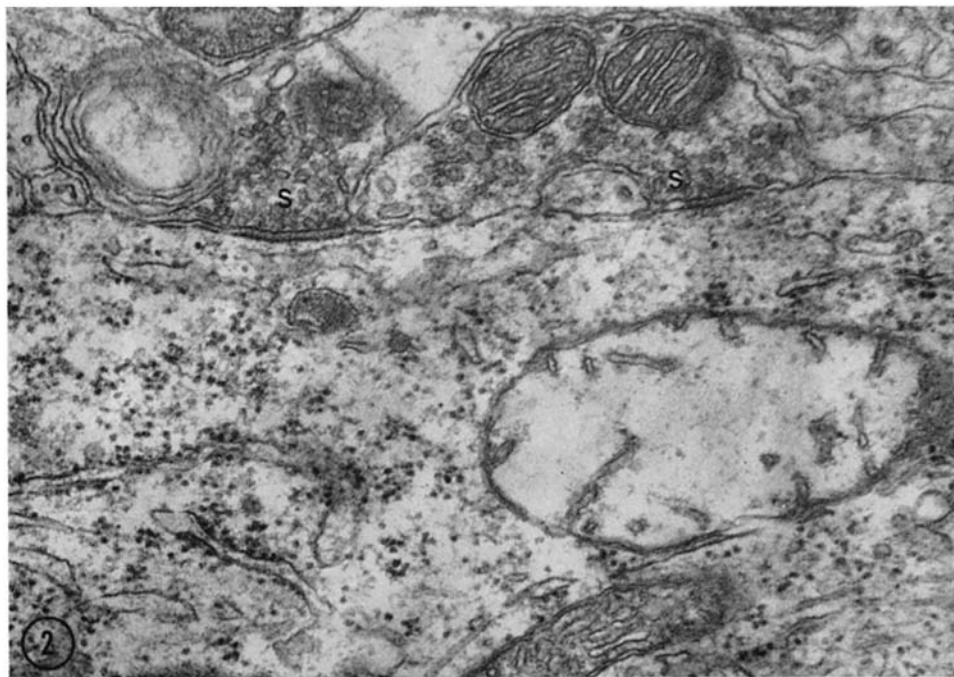


FIGURE 2 Presynaptic terminals (*s*) of axosomatic synapses. Most ribosomes in neuronal cytoplasm are dispersed. Note the normal appearance of mitochondria in one presynaptic ending; in contrast, a neuronal mitochondrion is swollen. 7 hr after injection of puromycin. $\times 47,000$.

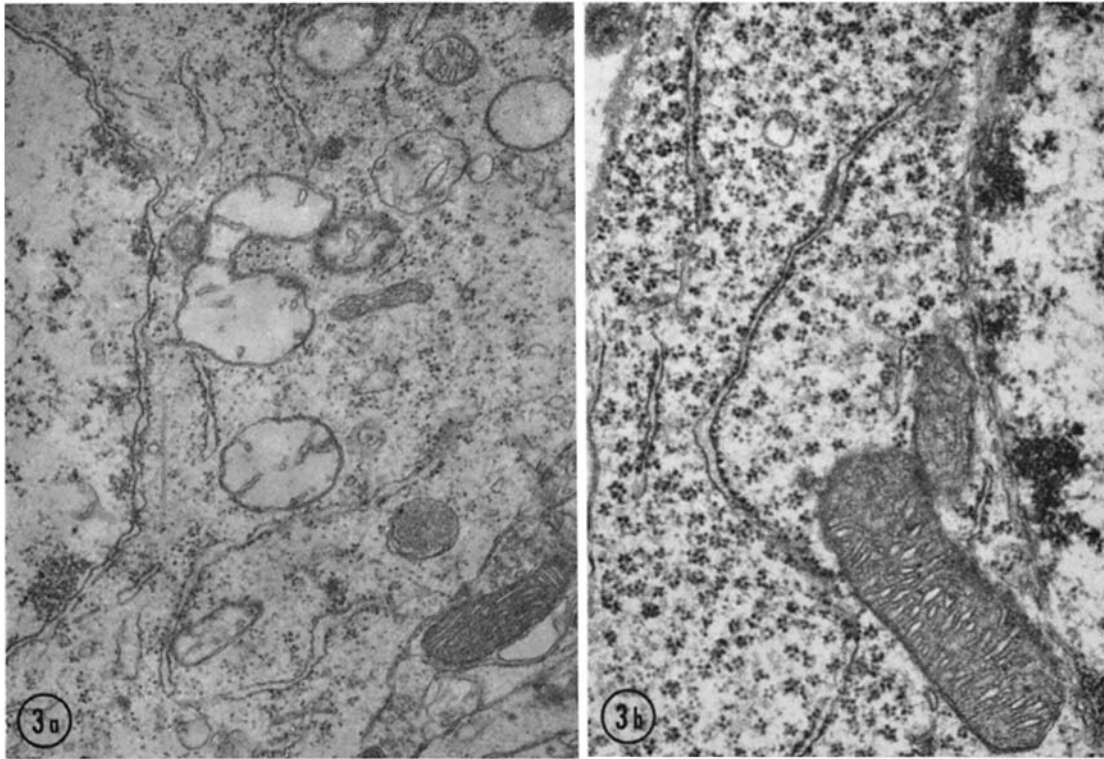


FIGURE 3 *a* Disaggregation of the polyribosomes into single units. The after injection of puromycin.

FIGURE 3 *b* The regular aggregation of ribosomes in polysomal "rosettes" in control animal injected with saline solution. $\times 24,000$.

PUROMYCIN-TREATED MICE: Because of limitations inherent to the technique, no attempt was made to identify, in the electron microscope, the various cell types and layers of the entorhinal cortex and ventral hippocampus. The changes to be described were noticed in numerous, randomly obtained sections from the above areas and, probably, represent diffuse lesions.

SYNAPTIC COMPLEXES: No abnormalities were found either in the presynaptic terminals or in the synaptic clefts. Except for swollen mitochondria, no abnormalities were seen in the post-synaptic dendrites of axodendritic synapses (Fig. 1). The subsynaptic web appeared normal. Axosomatic synapses at times involved abnormal neuronal perikarya (Fig. 2).

NEURONAL PERIKARYA AND PROCESSES: In a few neurons, the typical aggregates or "rosettes" of ribosomes were almost completely absent. In these instances, the polysomes were

dispersed in single units (Fig. 3 *a*) or were arranged in irregular aggregates with loss of individual distinctiveness (Fig. 4). The ribosomes were also clearly decreased in number in some areas of these perikarya, and especially there was depletion of polysomes in the entire perinuclear cytoplasm. Serial sections did not suggest that these changes were secondary to cellular swelling. Dispersed ribosomes appeared slightly smaller than in the control; they measured about 200 Å in diameter, as compared to 250 Å for normal ribosomes. Changes in polysomes, when present, were always associated with swelling of mitochondria. Occasionally, the cisterns of the endoplasmic reticulum were distended, and the usual amorphous material of low density was still noticeable within their lumen. These changes in polysomes and endoplasmic reticulum were present throughout the postinjection period from 7-27 hr, but the disaggregation and dispersion of polysomes in single

units appeared more evident 7 hr after treatment. None of these changes was observed in any cells 36 hr after puromycin injection.

Another abnormality, frequently noted within otherwise normal perikarya, and present in all mice treated with puromycin, consisted of rounded aggregates, about 0.8μ in diameter, of a granular or amorphous material containing a few, clear, vacuolar spaces and lacking a limiting membrane (Fig. 5). At high magnification, the apparent granular structure was found to consist of a mixture of loose, amorphous, granular, or fibrillar material (Fig. 6 *a*). Polysomes were in contact with these cytoplasmic bodies. Similar cytoplasmic aggregates were not seen in control mice. They have been found, however, by Shimizu and Ishii in the normal rat hypothalamus (23).

The most prominent abnormality consisted of swelling of mitochondria of neuronal perikarya and dendrites (Figs. 1, 7). Swollen mitochondria were not found in axons, presynaptic endings, or glial cells.

Swollen mitochondria were characterized by increase in size, disappearance of matrix, and diminution in number and length of cristae (Figs. 1, 2, 7, 8). Occasionally, mitochondria with a diameter as great as 2μ were seen (Fig. 1). The inner space, normally filled with matrix, was often empty except for dispersed filamentous or granular material. The remaining cristae were usually in continuity with the inner membrane at one or both of their ends. Often, fine filamentous material was attached to the surface of the cristae. The center-to-center distance between the outer and inner membranes and the thickness of these two membranes were unchanged or slightly reduced (100–170 Å and 60–70 Å, respectively) (Figs. 1, 8). In most damaged neurons, the only detectable abnormality was limited to mitochondria. Images suggesting complete breakdown of mitochondria were not observed.

The incidence of mitochondrial swelling varied considerably. In many neurons, practically all of the mitochondria of the perikarya and dendrites

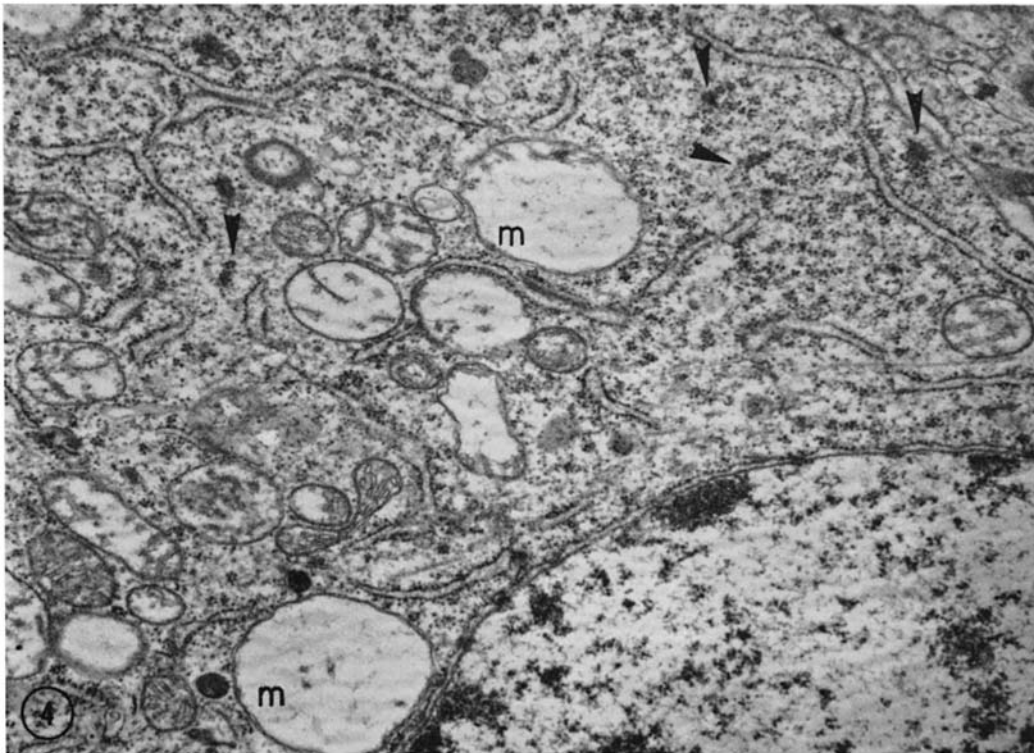


FIGURE 4 Irregular aggregates of ribosomes with loss of individual distinctiveness (arrowheads). *m*, swollen mitochondria. 26 hr after injection of puromycin. $\times 24,000$.

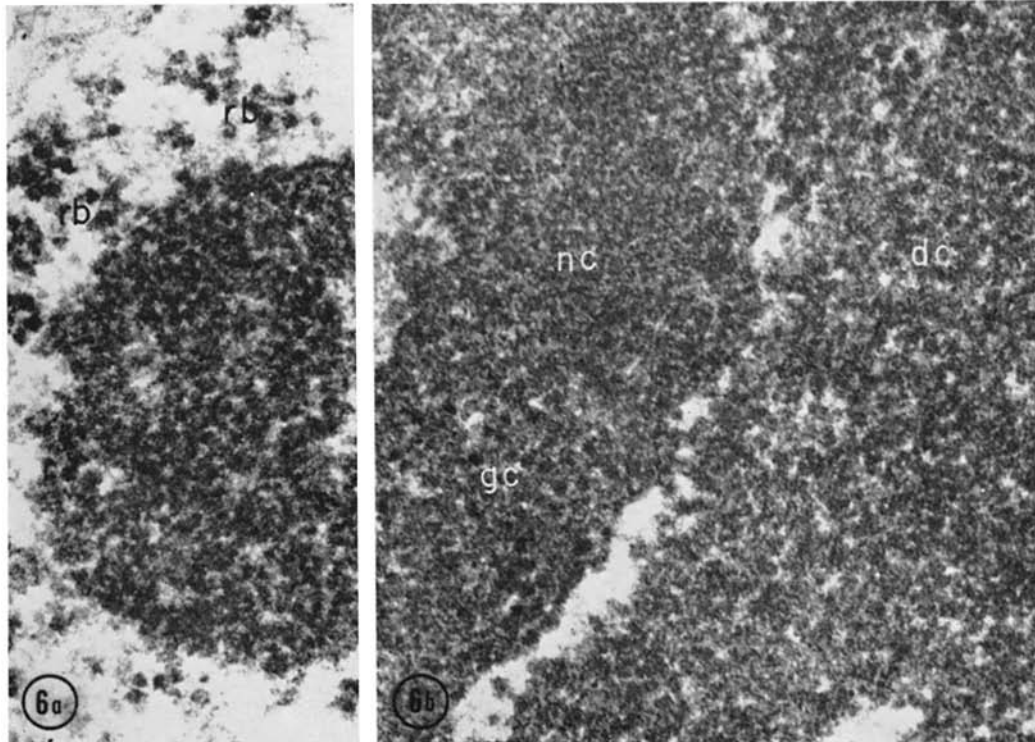
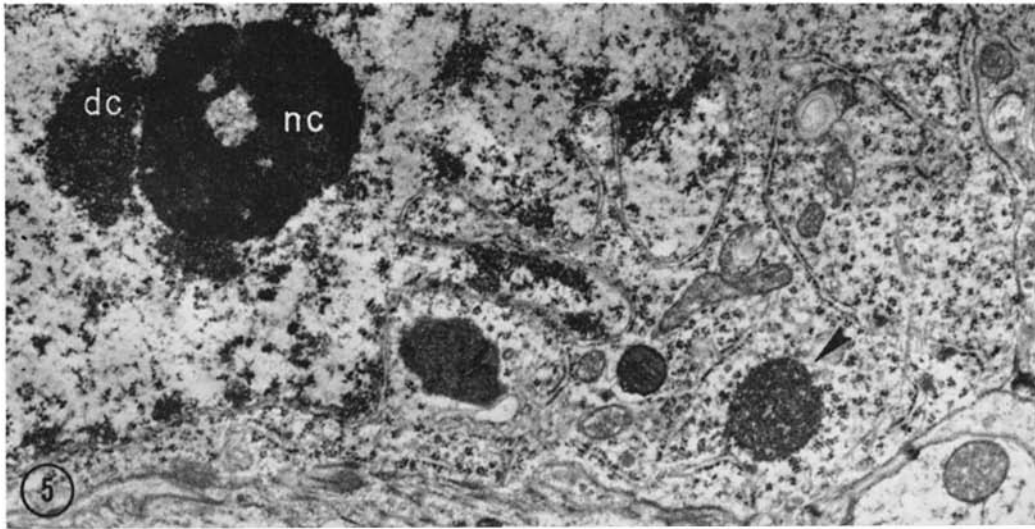


FIGURE 5 Neuronal cytoplasmic aggregate (arrowhead). *dc*, dense chromatin in the form of "nucleolar cap"; *nc*, nucleolus. 26 hr after injection of puromycin. $\times 16,500$.

FIGURE 6 *a* Neuronal cytoplasmic aggregate in contact with polyribosomes (*rb*).

FIGURE 6 *b* Part of a normal neuronal nucleolus (*nc*) with adjacent "nucleolar cap" of dense chromatin (*dc*). Note similarity between the neuronal cytoplasmic aggregate in Fig. 6 *a* and the granular component (*gc*) of the nucleolus. 26 hr after injection of puromycin. $\times 100,000$.

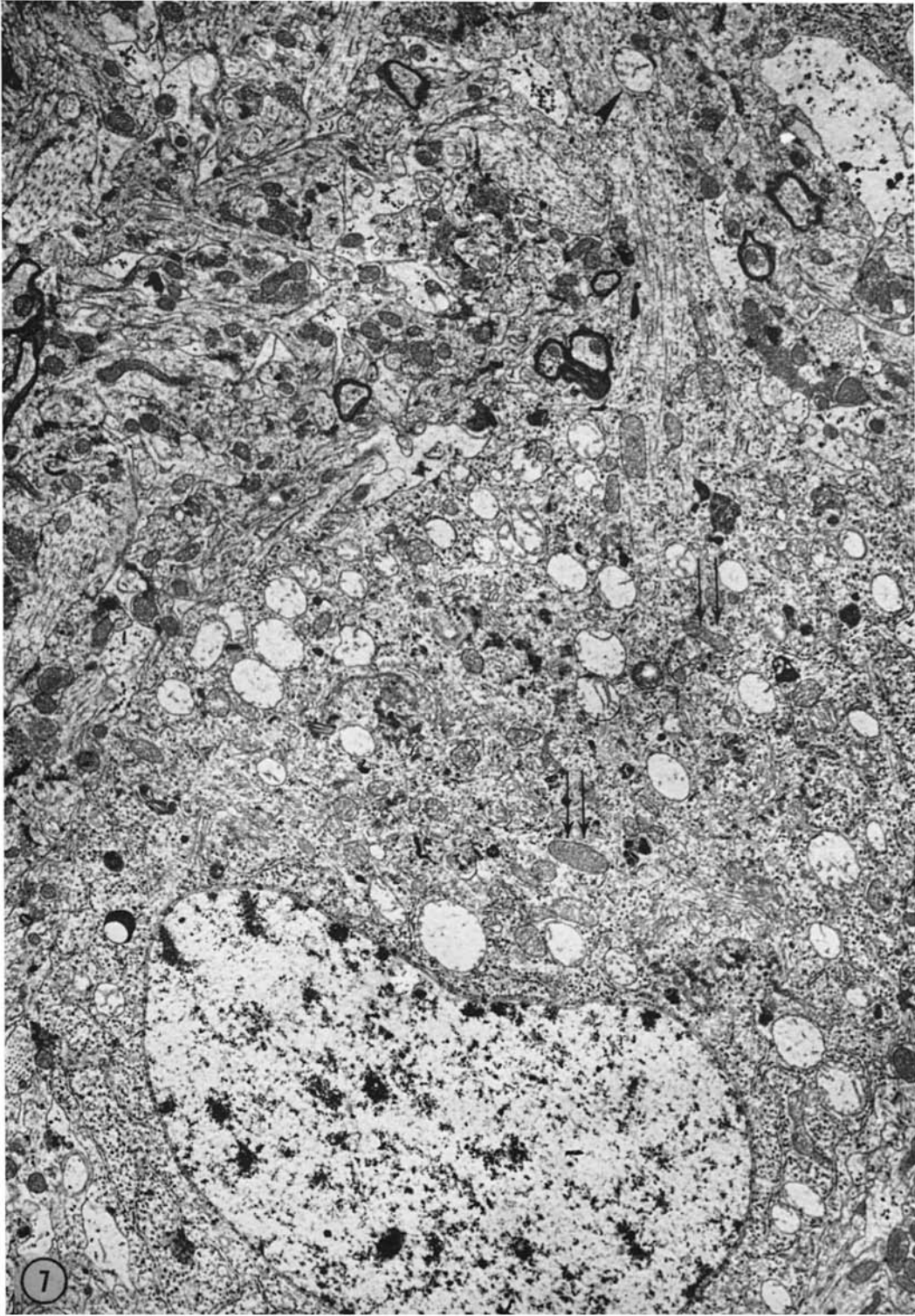


FIGURE 7 Neuron with numerous swollen mitochondria in perikaryon and apical dendrite (arrowhead); double arrows, normal neuronal mitochondria. Normal adjacent neuropil. 26 hr after injection of puromycin. $\times 9,000$.

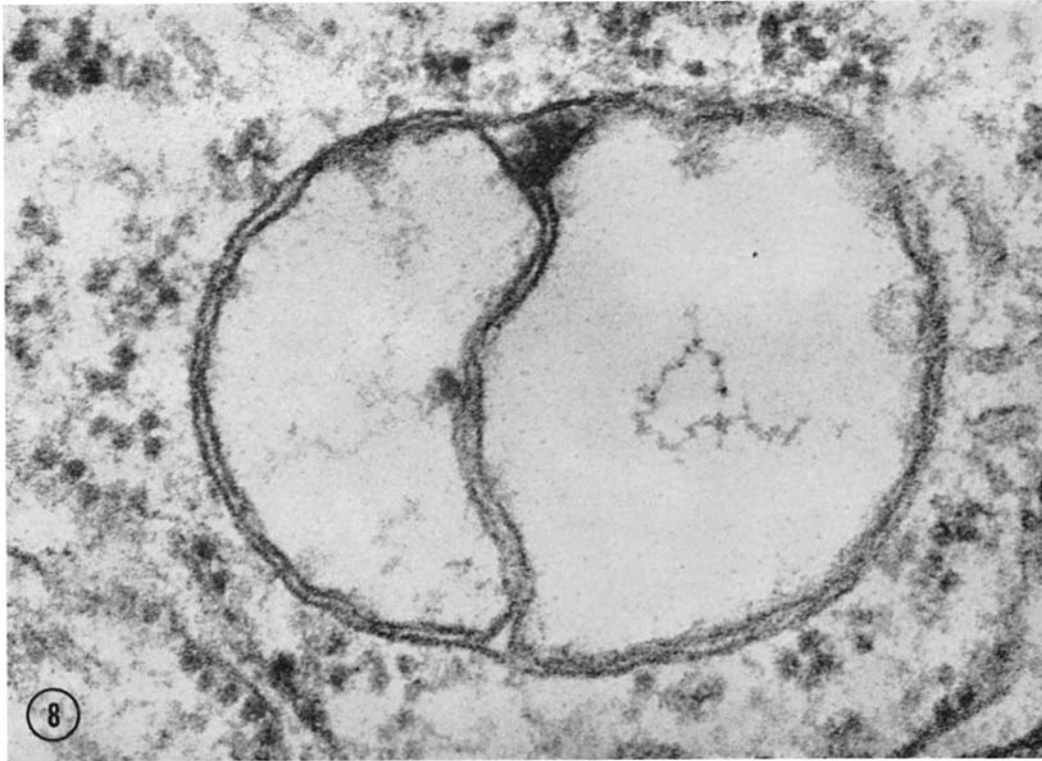


FIGURE 8 Swollen mitochondrion. Dispersed filamentous material is present within the inner space. 25 hr after injection of puromycin. $\times 139,000$.

were swollen; in others there was a predominance of normal mitochondria; in still others, no abnormalities were seen. Even in the most severely damaged areas, normal neurons were frequently seen next to abnormal ones (Fig. 9).

The degree of mitochondrial damage varied also with the time after injection of puromycin. Abnormal mitochondria were numerous from 7–27 hr after injection, and most numerous from 19–27 hr after injection. In mice sacrificed 36 hr after injection, only a few scattered abnormal mitochondria were found in a small number of neurons.

Neuronal nuclei and nucleoli were normal. There was no apparent difference in the size and distribution of the nucleolar caps between control and experimental mice. Cytoplasmic organelles such as Golgi complex and lysosomes, as well as lipofuscin, showed no changes.

In the dorsal hippocampi close to the sites of injection, similar but more pronounced lesions, particularly the disaggregation of polysomes, were found.

GLIAL CELLS AND BLOOD VESSELS: At times, astrocytes showed a slight increase of glycogen particles in the cytoplasm; in one cell, small amounts of glycogen were present in the nucleus. Oligodendroglia and blood vessels were normal.

DISCUSSION

No ultrastructural alterations of synapses were found which could be related to the persistent loss of expression of memory which follows treatment with puromycin. The abnormal mitochondria seen in postsynaptic dendrites and in neuronal perikarya have almost completely disappeared 36 hr after injection of puromycin. All other aspects of synaptic structure appeared normal.

The finding of disaggregated polysomes in neurons is of interest in the light of current thoughts that maintenance of the basic memory trace requires preservation of mRNA formed as a result of a learning experience and having the function of directing the synthesis of one or more proteins essential for the expression of memory

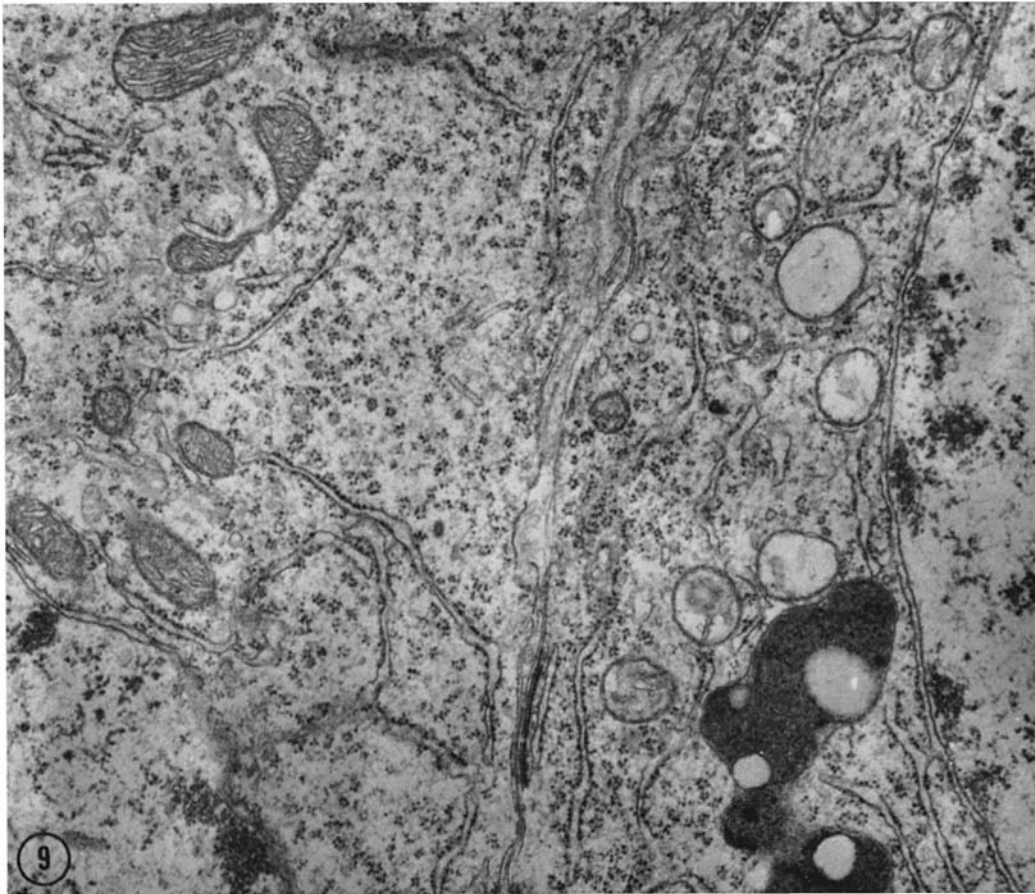


FIGURE 9 To the left of figure, neuronal cytoplasm with normal mitochondria. To the right of figure, another neuron shows swollen mitochondria. Polyribosomes are normal in both cells. 25 hr after injection of puromycin. $\times 22,000$.

(24). Our findings do not exclude this possibility. It might be supposed that disaggregation of neuronal polysomes in the present experiment is accompanied by degradation of mRNA. However, disaggregation of polysomes was observed in so few cells that, even if accompanied by degradation of mRNA, this loss would not explain the drastic effect of puromycin on memory. Furthermore, it has been found that, in a cell-free system, disaggregation of polysomes in the presence of puromycin can be satisfactorily explained without assuming the destruction of mRNA (25).

In addition, recent experiments have shown that the basic memory trace is maintained after puromycin treatment, since intracerebral injection of saline solution restores its expression in puromycin-treated mice (4).

Two observations require further comment. The first of these concerns the cytoplasmic aggregates of granular or amorphous material. Similar cytoplasmic inclusions, believed to be extruded nucleoli, have been observed in normal and diseased tissues (26). In a recent study, ultrastructural dissimilarities between the nucleolus and "nucleolus-like" cytoplasmic inclusions have been pointed out, and the suggestion has been made that the inclusions represent a reorganized nucleolar component extruded from the nucleus probably as a consequence of an extreme demand for protein synthesis (23). Studzinski has observed these inclusions in HeLa cells treated with puromycin; he has demonstrated that they contain RNA and that their appearance can be prevented by inhibiting RNA synthesis with actinomycin D

(27). In the present study, similar cytoplasmic aggregates, usually found in otherwise normal neurons, were frequently present after puromycin treatment. The structure of these cytoplasmic aggregates was similar to that of the areas of the normal nucleolonema containing the granular component (28, 29); however, these cytoplasmic aggregates did not resemble in structure the entire nucleolus. The cytoplasmic aggregates were generally situated near the nuclear membrane and were in contact with numerous polysomes. A direct proof of the origin of these aggregates from the nucleus or nucleolus is lacking; it is possible that their frequent occurrence after puromycin treatment represents an exaggeration of a normal finding.

The second observation concerns the presence, in many neurons, of swollen mitochondria with damaged cristae and disappearance of matrix. The swelling probably involves only the matrix of the mitochondria because the distance between the outer and the inner limiting membranes of the swollen mitochondria and the width of the space within the folds of the inner membrane forming the cristae are not increased. This mode of mitochondrial swelling, proposed by Lehninger (30), has been observed in the nervous tissue in various pathological states (31, 32). Although mitochondrial swelling has been extensively studied *in vitro* (33), observations on mitochondrial swelling *in vivo* are limited. According to Lehninger, the mitochondrial volume in the intact cell is the result of a balance of opposing forces promoting and inhibiting mitochondrial swelling (30). Puromycin may either alter this balance or have some other direct effect on mitochondrial volume. Several hypotheses can be given to explain the *in vivo* swelling of mitochondria which we have observed. We suggest three.

(a) Puromycin may have a general cytotoxic effect, particularly evident in mitochondria and unrelated to its inhibition of protein synthesis; mitochondrial swelling limited to the matrix, as mentioned above, has been found in various types of cellular injury.

(b) The mitochondrial lesions could be related to the inhibitory effect of puromycin on mitochondrial protein synthesis (34-36). Indirect support for this possibility comes from the demonstration in yeast that inhibition of mitochondrial protein synthesis by chloramphenicol is correlated with severe reduction in the number of cristae, while the outer membrane appears normal (37).

(c) Abnormal peptides, released from ribosomes in the presence of puromycin (38), could cause swelling of mitochondria since it is known that the most active of the mitochondrial "swelling agents" are polypeptides (33). This possibility is supported by the finding that mitochondrial swelling is absent in axons and presynaptic endings, in which polysomes and endoplasmic reticulum, sites of protein synthesis, are not present.

These hypotheses are presently being tested with other potent inhibitors of protein synthesis which have a mode of action different from that of puromycin. The degree of the functional impairment of swollen mitochondria and the possible effect of this impairment on the function of neurons cannot be assessed from morphological studies. However, it is hoped that an understanding of the action of puromycin on neuronal mitochondria will contribute to an understanding of its suppression of memory. This possibility becomes particularly attractive should puromycin peptides interact with mitochondrial membranes, for then insight would be gained into their possible effect on neuronal cytomembranes (4).

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