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Puromycin: Action on Neuronal Mitochondria

Abstract. *Puromycin, in dosages that inhibit cerebral protein synthesis and expression of memory in mice, produces swelling of neuronal mitochondria. Acetoxycycloheximide, which inhibits cerebral protein synthesis to the same extent as puromycin, fails to produce swelling of neuronal mitochondria. Puromycin and heximide mixtures produce severe inhibition of protein synthesis, but result in a minimal swelling of neuronal mitochondria and in a decrease of peptidyl-puromycin complexes to a level of 30 percent of that following the injection of puromycin alone. It is concluded that swelling of neuronal mitochondria in the presence of puromycin is not due to inhibition of cerebral protein synthesis per se, but is related to a specific action of puromycin on ribosomal protein synthesis. The findings are consistent with the hypothesis that peptidyl-puromycin complexes are responsible for mitochondrial swelling.*

A previous ultrastructural study showed that intracerebral injections of puromycin, in dosages that suppress expression of the memory of maze-learning in mice, produce extensive swelling of mitochondria in neuronal perikarya and dendrites (*1*). Although no experiments were performed to investigate the mechanism of the action of puromycin on these mitochondria, it was suggested that peptidyl-puromycin complexes, by-products of the action of puromycin on ribosomal protein synthesis, could be responsible for the observed changes. Furthermore, it was pointed out that understanding of the mode of action of this antibiotic on mitochondrial membranes might contribute to an understanding of its assumed effect (*2*) on other cytomembranes, yielding further insight into its effect on memory.

The present ultrastructural and biochemical study was undertaken to investigate the mechanism of the mitochondrial swelling in the presence of puromycin. The following three possibilities have been tested: (i) The swelling is caused by some unknown cytotoxic action of puromycin unrelated to the inhibitory effect of this drug on protein synthesis; (ii) it is secondary to inhibition of protein synthesis per se; or (iii) it is specifically related to the particular mode of action of puromycin on ribosomal protein synthesis.

For ultrastructural study, three mice were injected bitemporally with a mixture containing 90 μg each of puromycin and acetoxycycloheximide; three others were injected with 90 μg of heximide alone, and four control animals were injected with saline. The heximide was used because, when administered alone, it inhibits cerebral protein synthesis at least as extensively as puromycin does (*3*), but by a different mechanism (*4–6*), while in combination with puromycin it inhibits the formation of peptidyl-puromycin complexes (*7*). Some of the mice from each group (those treated with puromycin, a puromycin-heximide mixture, and controls) were killed 7 to 10 hours after injection; the others were killed 18 to 19 hours after injection. Details of the various procedures have been reported previously (*1*, *8*).

Swelling of mitochondria was not observed in the mice treated with heximide. Swollen mitochondria were present after injection of the puromycin-heximide mixture (Fig.1A), but the incidence of swelling was definitely lower than it was after puromycin alone. There was disappearance of the matrix and diminution of the number and length of the cristae of the swollen mitochondria; these changes produced by the mixture were qualitatively similar to those observed after administration of puromycin alone. As with puromycin alone, the abnormal mitochondria were seen mainly in neuronal perikarya or, more rarely, in dendrites. Swollen mitochondria were not seen in axons, presynaptic endings, or glial cells; they were more numerous 18 to 19 hours after injection of the puromycin-heximide mixture than 7 hours after. No more than three abnormal mitochondria per section of neuronal perikaryon were ever observed, while 19 hours after administration of puromycin alone the great majority of the mitochondria of the perikarya and dendrites were swollen (Fig. 1, A and B). Furthermore, the number of neurons with abnormal mitochondria was very small compared with the number found after puromycin alone.

The ribosomes of neuronal perikarya measured about 250 Å in diameter and corresponded in size and number to those of the control animals. Unlike the results observed after the administration of puromycin alone (*1*), disaggregation or abnormal aggregation of polysomes was not observed in either group of mice treated with heximide or with the puromycin-heximide mixture. This finding agrees with the results of biochemical studies in vivo (*7*) and in vitro (*4*), showing that cycloheximide [which has a common mode of action with heximide (*5*)] does not disaggregate the polysomal complexes and partially protects them from the disaggregation caused by puromycin (*7*, *9*).

The amounts of peptidyl-puromycin were investigated in one series of 11 mice that received bitemporal injections of 90 μg of puromycin and in a second series of 5 mice injected bitemporally with a mixture of 120 μg each of puro-

mycin and heximide. Both solutions were labeled with radioactive puromycin (Methoxy-H^3; specific radioactivity, 2 mc/mg) (*10*). Each injection of 12 μl contained 0.9 μc of the tritiated compound. The mice were killed from 1 to 48 hours after the injections. Both temporal cortices, including the entorhinal cortices, were sampled from all of the animals. The tissue was homogenized with 3 ml of ice-cold, 6 percent trichloroacetic acid (TCA) in a glass hand-homogenizer. After the precipitate was allowed to stand in ice for 30 minutes, it was plated in a glass-fiber filter, washed with 20 ml each of cold water and acetone, and dried. The radioactivity of the TCA precipitate was measured in a Packard Tri-Carb liquid scintillation counter. The values obtained were converted into picomoles of peptidyl-puromycin per 100 mg of tissue, since it has been demonstrated that puromycin binds to peptide chains in a 1:1 ratio (*11*).

It was found that peptidyl-puromycin complexes were precipitable by TCA for at least 48 hours after administration of either puromycin alone or the puromycin-heximide mixture (Fig. 2). The peak concentration of the precipitated peptidyl-puromycin after injection of the mixture of puromycin and heximide was about 30 percent of that obtained with puromycin alone. The highest concentration of peptidyl-puromycin was found 8 to 13 hours after puromycin and 16 to 24 hours after the puromycin-heximide mixture.

Acetoxycycloheximide, which, like puromycin, causes a severe and sustained inhibition of cerebral protein synthesis (*3*), fails to produce the mitochondrial swelling observed in the presence of puromycin alone; therefore, inhibition of protein synthesis per se cannot be the cause of this lesion.

The limited extent of the swelling of neuronal mitochondria after administration of a puromycin-heximide mixture, compared to the extensive swelling of these organelles after puromycin alone, can be explained by the different mode of action of the two drugs. Puromycin, combining with the carboxyl terminals of growing peptide chains, causes a continuous release of peptidyl-puromycin complexes (*9*, *11*), while heximide inhibits the growth and the release of polypeptide chains (*5*, *6*). When both drugs are injected simultaneously, the effect of puromycin on the synthesis of ribosomal proteins is significantly inhibited, as indicated in our experiments by the reduction of puromycin-peptides to a level of 30 percent of that after injection of puromycin alone. The presence of puromycin peptides indicates that heximide does not block the entry of puromycin into the cell. Furthermore, after injection of the two drugs, the amounts of TCA-soluble puromycin were essentially the same as those obtained after puromycin alone. The minimum extent of swelling of the mitochondria that was found in the presence of both puromycin and heximide sug-

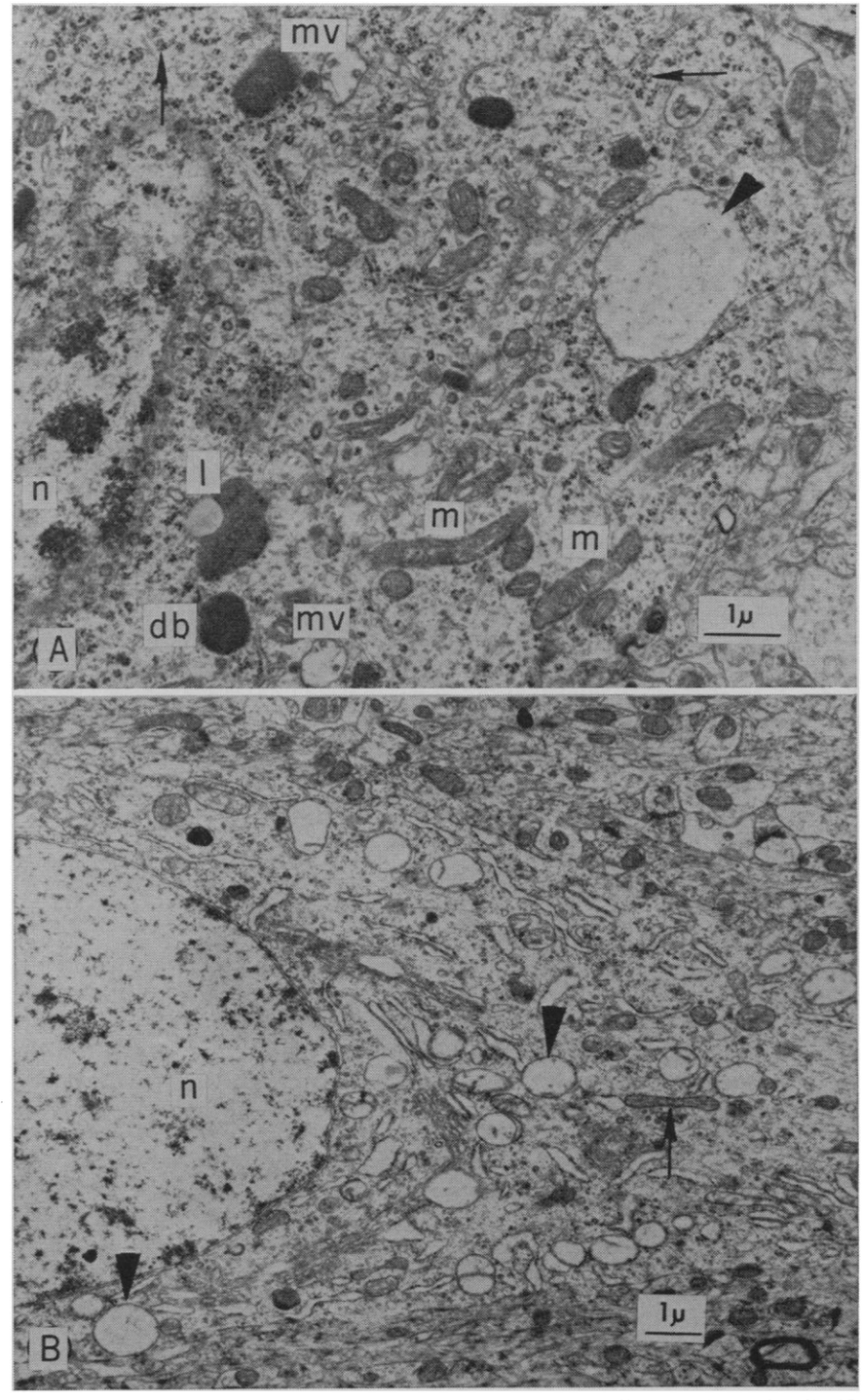

Fig. 1. (A) Neuron with a swollen mitochondrion in the perikaryon (arrowhead) 18 hours after injection of puromycin and heximide; all the other mitochondria are normal (*m*). Polyribosomes are normally aggregated (arrow); *n*, nucleus; *db*, dense bodies; *mv*, multivesicular bodies; *l*, lipofuscin (× 16,000). (B) Numerous swollen mitochondria (arrowhead) are present in the perikaryon of neurons 19 hours after injection of puromycin. The ribosomes are diminished in number and polysomes are in part disaggregated. The adjacent neuropil is normal. Arrow, normal mitochondrion; *n*, nucleus.

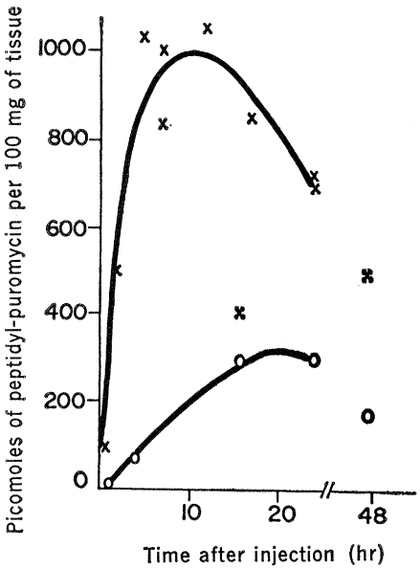

Fig. 2. Amounts of the peptidyl-puromycin mixture, precipitated by TCA, present in 100 mg of temporal cortex (wet weight) as a function of time after injections into each temporal lobe of 90 μg of puromycin alone (×) or of 120 μg each of puromycin and heximide (○).

gests that this lesion is not related to some cytotoxic effect of puromycin other than that on protein synthesis. Nor can the swelling be related solely to the effect of puromycin on the synthesis of mitochondrial protein, since there is evidence that heximide, unlike puromycin (*12–14*), has no effect on this mitochondrial activity (*14, 15*) and should not interfere with puromycin in this action.

At this time, it appears most likely that swelling of neuronal mitochondria in the presence of puromycin is due to the formation of peptidyl-puromycin. This interpretation is supported by the finding that the extent of mitochondrial swelling appears to be directly related to the amount of puromycin-peptides that are precipitated by TCA. It should be pointed out that puromycin blocks expression of memory indefinitely, whereas a mixture of puromycin and heximide has no such effect (*16*). The present findings are consistent with the possibility that the effect of puromycin on memory is due to a widespread action of peptidyl-puromycin on neuronal cytomembranes.

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Histamine Release in vitro: Inhibition by Catecholamines and Methylxanthines

Abstract. *Methylxanthines and catecholamines both inhibit antigenically induced histamine release from human leukocytes. They act synergistically to inhibit the reaction, but must be present when antigen is added; preincubation is not effective. Since both increase cellular levels of cyclic 3′,5′-adenosine monophosphate it is postulated that this compound plays a role in the regulation of allergic histamine release.*

We have previously described an in vitro system of antigen-induced histamine release from human leukocytes (*1*). The reaction follows the interaction of ragweed antigen E (a protein of molecular weight 38,000) and reaginic (IgE) antibody on the surface of leukocytes isolated from donors sensitive to ragweed (*2*). The system serves as a model of human allergy inasmuch as the sensitivity of the leukocytes to antigen E (based on the concentration of antigen required for 50 percent histamine release) reflects the clinical severity of the allergic diathesis (*3*).

The mechanism of this type of reaction, which we have called allergic histamine release, has not as yet been defined. We have shown that the cell is not injured as a result of the immune reaction, but rather that the antigen elicits an active multistep response which has some similarities to secretory phenomena. In order to release histamine the cell must be viable and the glycolytic system must be intact. Oxidative metabolism does not appear to be required (*4*).

In the course of studies on inhibitors of histamine release the action of commercial preparations of the methylxanthines (theophylline, theobromine, and caffeine) and the catecholamines (epinephrine and isoproterenol) was explored (*5*). Leukocytes were isolated from the peripheral blood of allergic donors as previously described, and suspended in a serum-free tris buffer (*1*). The inhibitory capacity of the drugs was determined by adding fractions of a cell suspension to a series of tubes containing a constant amount of antigen E and variable concentrations of the inhibitor. The reaction mixtures were then incubated at 37°C for 60 minutes, and the percentage of the total histamine released from the cells into the fluid phase was measured fluorometrically (*1, 5*). The percentage of inhibition caused by each drug was calculated from the formula $(C - E)/C \times 100$, where C and E stand for the percentage of histamine release in the control and experimental (inhibitor-containing) tubes, respectively. Inhibition studies were carried out with the leukocytes of six to eight different allergic donors.

All of the methylxanthines inhibited histamine release; theophylline was the most active (Fig. 1). Inhibition began at 1 to 3 × $10^{-5}M$, and 50 percent inhibition was reached with concentrations about tenfold higher. Two- or threefold higher concentrations of caffeine or theobromine were required to produce an equivalent inhibitory effect. Theophylline stopped histamine release immediately, even if the reaction had begun and an appreciable percentage of the histamine had already been released. In another type of experiment cells were exposed to theophylline for 15 minutes at 37°C in the absence of antigen. They were then centrifuged, resuspended in a theophylline-free buffer, and exposed to antigen. No inhibi-