ELECTRON MICROSCOPE STUDIES ON PUROMYCIN-INDUCED CHANGES IN NERVE CELLS OF THE MEDULLA OBLONGATA

KIYOSHI KISHI. From the Department of Anatomy, Faculty of Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-Ku, Tokyo, Japan

It has been reported that puromycin, commonly known as an inhibitor of protein synthesis (35), induces the following ultrastructural changes: mitochondrial swelling, cisternal dilatation of rough endoplasmic reticulum, formation of intracisternal granules, disaggregation of polysomes, and an increase in number of autophagic vacuoles (11, 18, 19). In addition, two kinds of membrane-unbound bodies have been observed after puromycin injection: the small, amorphous, electron-dense granular mass in pancreatic acinar cells (18) and gastric chief cells (19), and the rounded aggregate of granular or amorphous material similar to the cytoplasmic nucleolus-like bodies in the nerve cells of the mouse entorhinal cortex (11).

During the course of cytochemical studies on the cytoplasmic nucleolus-like bodies (manuscript in preparation), our attention was called to the fact that puromycin induces aggregates of tubular material which are ultrastructurally different from the nucleolus-like body (14–17). The present report describes such aggregates in the dorsal sensory nucleus of the vagus of the medulla oblongata of the mouse and rat.

MATERIALS AND METHODS

Eighteen mice of both sexes weighing 30–35 g and six male and female rats weighing 200–250 g were used in this study. Puromycin dihydrochloride was purchased from Makor Chemicals Ltd., Jerusalem, Israel and Sigma Chemical Co., St. Louis, Mo. Puromycin in 0.4 ml of distilled water, adjusted to pH 6 with NaOH, was injected subcutaneously in the middle of the back of the mouse. The dose of puromycin used was 0.42 mg/g body weight, which is reported to cause 80% inhibition of protein synthesis in the mouse cerebellar cortex (9). 2, 5, and 30 h after the single injection, the animals were fixed by perfusion with an aldehyde mixture according to Palay et al. (22). Double injections, i.e., the same dose injected 9 h after the first one, were performed 2–8 h before the fixation. Further, 0.5 mg puromycin in 0.03 ml distilled water, adjusted to pH 6, was injected into the lateral ventricle of the rats with the use of a stereotaxic instrument according to Fenstermacher (8). The rats were fixed by perfusion 6 h after the injection. Small blocks of brain stem were postfixed for 3 h with cold, buffered osmium tetroxide (22). Before dehydration, they were stained with 0.5% uranyl acetate in acetate-Veronal buffer (7). They were then dehydrated and embedded in Epon. Ultrathin sections were stained with saturated aqueous uranyl acetate followed by lead tartrate (20) and examined in a Hitachi HS-7 or a JEOL JEM-100B electron microscope. In addition, 1-μm thick sections were cut from the same blocks, stained with toluidine blue, and examined with a light microscope.

RESULTS

Untreated Animals

The morphology of the cytoplasmic nucleolus-like body in the dorsal sensory nucleus of the vagus of the rat has been reported previously (14, 15). The nucleolus-like bodies are also found in the perikarya of the small nerve cells in the same nucleus of the mouse medulla oblongata. They are electron-dense, round or oval masses, and their maximum diameter is 2 μm. Each body consists of an entanglement of 70–100-Å filaments and granules of 80–120 Å in diameter (Figs. 1, 3). The body frequently has excavations, and there are satellite bodies at its periphery (Fig. 3).

Puromycin-Treated Animals

The majority of puromycin-injected animals showed a typical behavioral effect, a drowsiness or

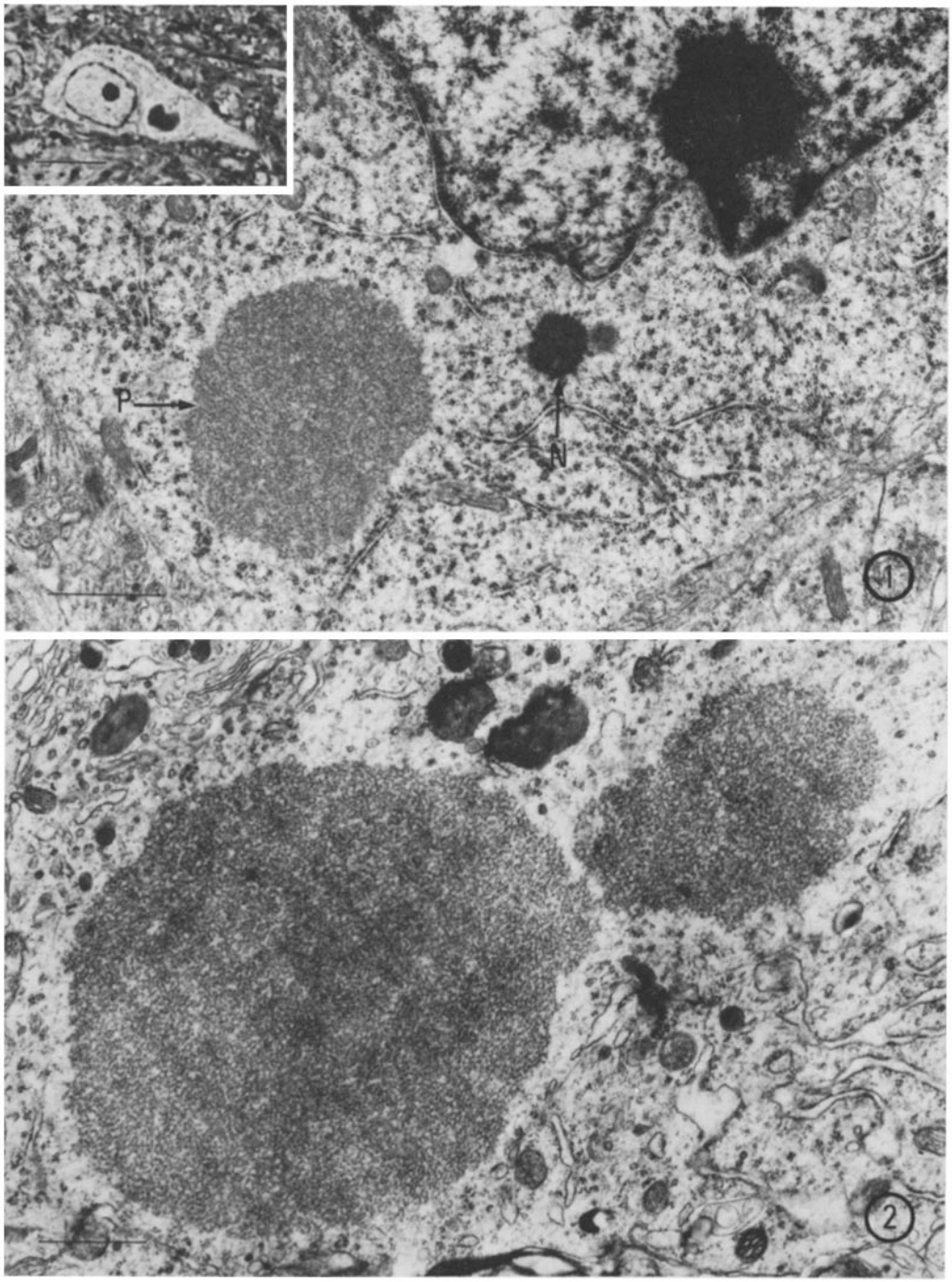

FIGURE 1 Nerve cell of dorsal sensory nucleus of the vagus of the mouse, 5 h after subcutaneous injection of puromycin. Note that the puromycin-induced body (*P*) is less dense than the cytoplasmic nucleolus-like body (*N*). The *inset* shows a light micrograph of a nerve cell in a 1-μm Epon section stained with toluidine blue. The polygonal inclusion in the cytoplasm is surrounded by a pale halo. × 16,500. Scale marker 1 μm. *Inset*, × 1,000. Scale marker, 10 μm.

FIGURE 2 Two first-type bodies in the same perikaryon of the mouse nerve cell, 6 h after double injection of puromycin. The dilatations of cisternae of the rough endoplasmic reticulum are observed at lower right. × 15,600. Scale marker, 1 μm.

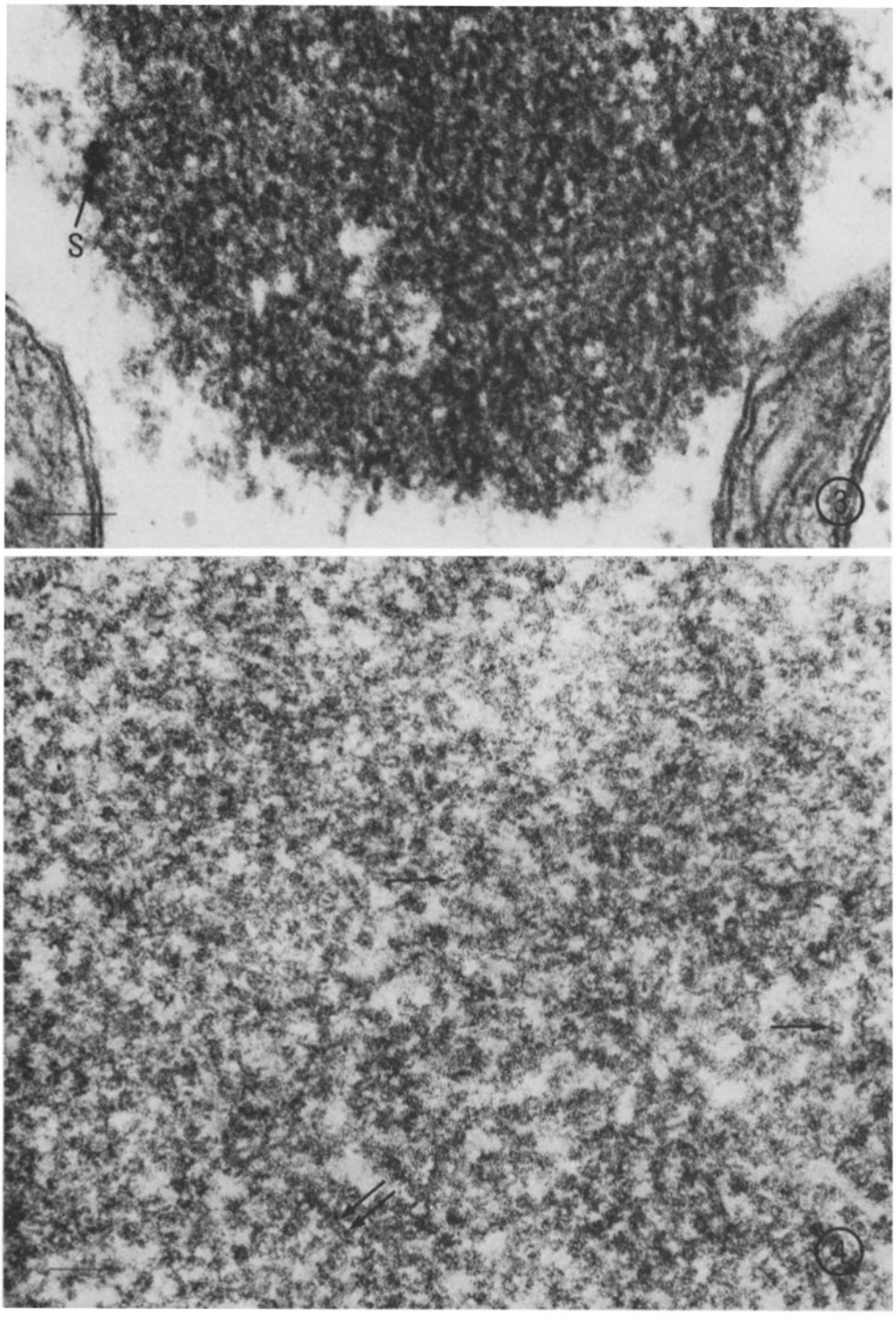

FIGURE 3 Cytoplasmic nucleolus-like body containing 70–100-Å filaments and granules 80–120 Å in diameter. Satellite body (*S*). × 105,000. Scale marker, 0.1 μm.

FIGURE 4 Higher magnification of the first type of body containing randomly arranged tubular elements and fine fibrils. The oval or circular profiles (arrows), 170–190 Å in diameter, are cross sections of tubules. Parallel double lines (double arrows) appear to be longitudinal sections of tubules. × 105,000. Scale marker, 0.1 μm.

a sleep which has been described as an indicator of the 80% inhibition of protein synthesis in the brain (9). Only such animals showed the puromycin-induced bodies in the nerve cells as described here. The inclusion is identified under a light microscope by its shape, size, and location in a 1-μm thick Epon section. It is round, oval, gourd-shaped, or polygonal, and stains purple with toluidine blue. Its size ranges from 2 to 8 μm. Gourd-shaped and polygonal inclusions are frequently surrounded by a pale halo (Fig. 1, *inset*). About 4% of the neuronal population in the dorsal sensory nucleus of the vagus have these inclusions 6 h after the double injection. In the animals receiving a single injection, the inclusions are found in about 2% (2 h and 5 h after the injection) and 1% (30 h after the injection) of the neuronal population.

In electron micrographs, puromycin-induced bodies are found not only in the perikaryon of nerve cells, but also in the nerve processes. The bodies are not membrane-bound and are far less electron dense than the nucleolus-like bodies (Fig. 1). A single body is usually found in one nerve cell, but two are not infrequent (Fig. 2). These bodies can be classified into two types.

The first type had a round, oval, or gourd-shaped profile, 2–8 μm in diameter (Figs. 1, 2, 4). This structure occurred after all the injection procedures, including the subcutaneous and the intraventricular injections. Its size reached up to 5 μm 2 h after, and 8 μm 5 h after, the single subcutaneous injection. There was no apparent reduction in size even 30 h after the injection. The inclusion was a compact mass of short tubules, 170–190 Å in diameter × 300–600 Å long, and fine fibrils of 40–50 Å. The tubules had an electron-transparent center about 60–80 Å in diameter, and their walls were poorly preserved as compared with the normal 240–260-Å neurotubules. They often showed oval profiles which were considered to represent oblique sections of the short tubules (Fig. 4).

The second type of body, paracrystalline in appearance, had a polygonal profile, 3–7 μm in diameter (Figs. 5, 6). Its electron density was higher than that of the first type. This type appeared considerably less frequently than the first type, and it could not be found after the intraventricular injection, probably because of the smaller dose of puromycin. This body consisted of intersecting, parallel, alternately electron-dense and electron-transparent lines. The dense lines were 170–190 Å thick, and the transparent lines varied in their thickness, 80–340 Å, with a mean of 240 Å. The acute angles of the intersections varied from 40° to 90° (Fig. 5). Under a higher magnification, the dense lines were often seen as parallel double lines 60–80 Å apart which were considered to represent longitudinal sections of the tubules. Further, periodic dot patterns were observed in some sections. Each dot consisted of a small circle, 170–190 Å in diameter, with a lumen of about 60–80 Å (Figs. 5, 6). Therefore, this type was considered to be made up of a lattice of 170–190-Å tubules and not to be a true crystal. Both types of bodies were occasionally found in the same perikaryon.

The body appeared to have no apparent relationship to other organelles, including the neurotubules and neurofilaments. Normal neurotubules, 240–260 Å thick, were sometimes found around the body.

The number and the sizes of the cytoplasmic nucleolus-like bodies did not change remarkably after the injection of puromycin. Swelling of the mitochondria and dilatation of the cisternae of the rough endoplasmic reticulum were frequently observed (Fig. 2). Double membrane-bound bodies containing clearly recognizable cytoplasmic organelles, which appeared to be autophagic vacuoles, were rarely found. No change was observed in the nucleus or polyribosomes.

DISCUSSION

The present findings indicate that the puromycin-induced body is different from the cytoplasmic nucleolus-like body in many details, ranging from its elements to its gross morphology.

Considering the tubular element of puromycin-induced body, it would be of interest to compare it with other tubular structures in the cell cytoplasm.

Segmental degradation of microtubules has been reported in heliozoan axopodium treated at a low temperature (31). However, the diameter (about 340 Å) of such segmental tubule is larger than that of short tubules of the present first type. The intersecting pattern of the second type of body is different from the pattern of the paracrystals induced by Vinca alkaloids (2, 25). Further, it has been reported that puromycin itself cannot induce microtubular paracrystals (2, 29). It is unlikely that tubulin constitutes the present body.

The short tubules of the present body bear some resemblance to the negatively stained 22S protein

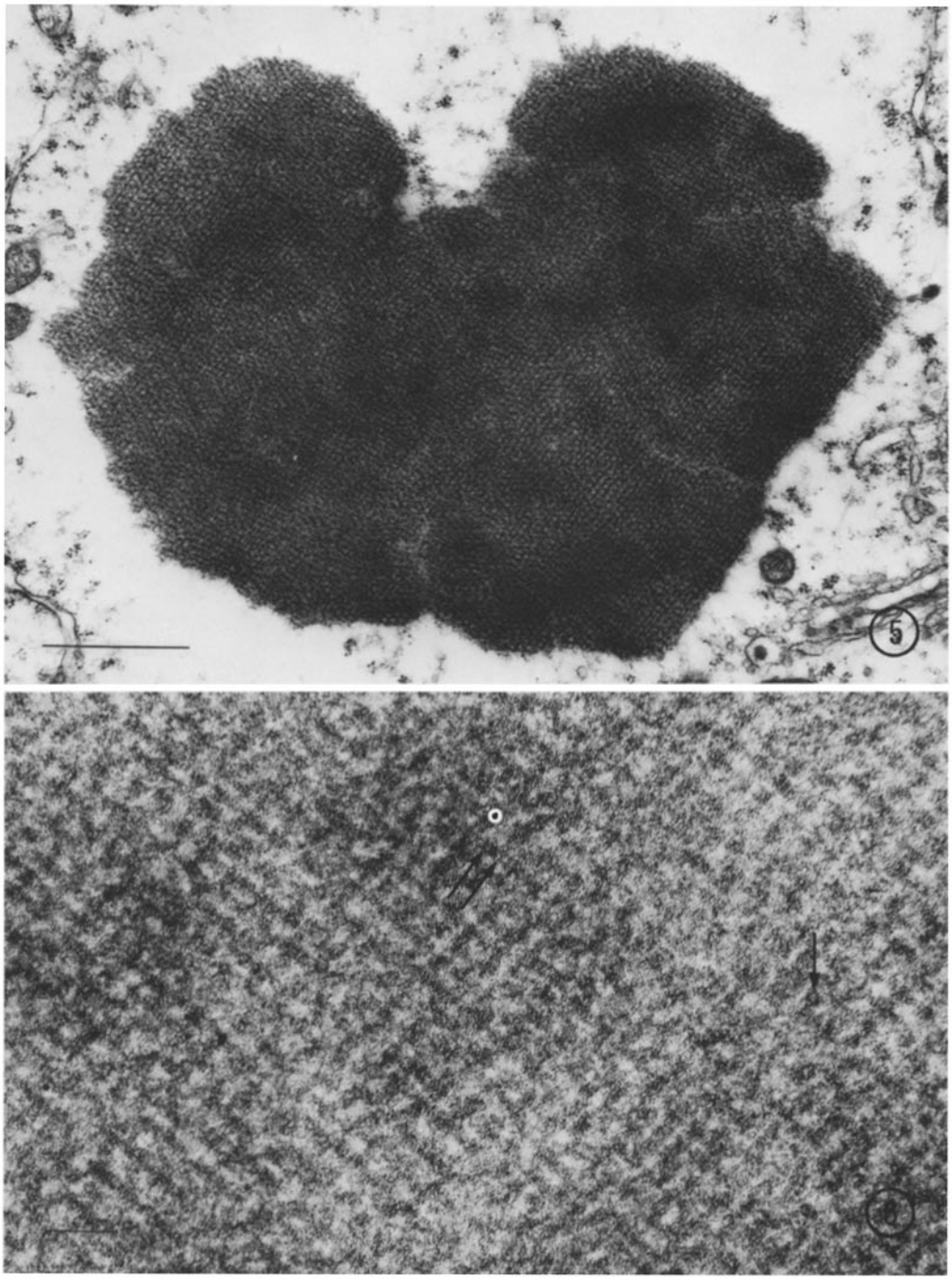

FIGURE 5 Second type of body in the perikaryon of mouse nerve cell, 6 h after double injection of puromycin. It consists mainly of intersecting, parallel, alternately electron-dense and electron-transparent lines. × 21,000. Scale marker, 1 μm.

FIGURE 6 Higher magnification of the second type of body consisting mainly of intersecting, parallel, alternately electron-dense and electron-transparent lines. The acute angle of the intersection is 84°. The width of the dense line is 170–190 Å. The parallel double lines (double arrows), 70 Å apart, appear to be a longitudinal section of a tubule. The circular profile (arrow), 180 Å in diameter, may be a cross section of a tubule. × 105,000. Scale marker, 0.1 μm.

of sea urchin, which has a cylindrical shape with approximate dimensions of 150 × 200–250 Å (4, 28). However, in the ultrathin section, 22S protein shows an amorphous appearance, and is located in the isolated mitotic apparatus of sea urchin eggs (3).

The tubular elements of the present body are morphologically distinguished from the twisted tubules in the affected neurons of Alzheimer's disease (30, 34).

In its ultrastructure, the puromycin-induced body is similar to the aggregates of helical nucleocapsids (180–200 Å in diameter) which have been observed in various cells infected with paramyxoviruses (5, 6, 13, 23, 24, 32). However, in the electron micrographs, the nucleocapsids of these viruses appear longer than the element of the first type of the present body. The length of nucleocapsid of Sendai virus is 1 μm or more (12). The paracrystalline array of nucleocapsids of measles virus is different from the array of the second type of the present inclusion. No viral particle or viron was observed in this study. The present inclusion was found consistently after puromycin injection, and was never found in an untreated animal. In addition, the inclusions are found in nerve cells of the medulla oblongata even 2 h after subcutaneous injection of puromycin. On the other hand, the nucleocapsids have been observed in cultured cells 8 h (parainfluenza virus) to 6 days (measles virus) after the virus inoculation (5, 13, 23). In addition, it seems unlikely that every sample of puromycin purchased from two different sources for this study is contaminated by a virus. It is hardly probable that puromycin produces activation of latent virus in the brain, because puromycin itself is known to inhibit the multiplication of a virus (33). Furthermore, membrane-unbound unique structures have been reported in other cells after the injection of puromycin (11, 18, 19). Therefore, it seems reasonable to conclude that the present inclusion is of nonviral origin, and that puromycin per se induces these inclusion.

It has been reported that puromycyl peptides are released from ribosomes when puromycin arrests protein synthesis (21, 27). Puromycyl peptides accumulate in the brain after treatment with puromycin (10). It is possible that such puromycyl peptides would in some way be related to the occurrence of the present body. Another possible explanation is sought in the inhibition of 3′,5′-cyclic AMP phosphodiesterase by puromycin (1). The elevated level of cyclic AMP in the brain through the inhibition of the phosphodiesterase could be related to the assembly of the present body in such a manner as the model suggested for microtubule assembly (26).

Received for publication 11 February 1974, and in revised form 8 August 1974.

REFERENCES

1. APPLEMAN, M. M., and R. G. KEMP. 1966. Puromycin: a potent metabolic effect independent of protein synthesis. *Biochem. Biophys. Res. Commun.* **24**:564.
2. BENSCH, K. G., and S. E. MALAWISTA. 1969. Microtubular crystals in mammalian cells. *J. Cell Biol.* **40**:95.
3. BIBRING, T., and J. BAXANDALL. 1969. Immunological studies of 22S protein from isolated mitotic apparatus. *J. Cell Biol.* **41**:577.
4. BORISY, G. G., and E. W. TAYLOR. 1967. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. *J. Cell Biol.* **34**:535.
5. COMPANS, R. W., K. V. HOLMES, S. DALES, and P. W. CHOPPIN. 1964. An electron microscopic study of moderate and virulent virus-cell interactions of the parainfluenza virus SV5. *Virology.* **30**:411.
6. DUC-NGUYEN, H., and E. N. ROSENBLUM. 1967. Immuno-electron microscopy of the mumps virus. *J. Virol.* **1**:415.
7. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
8. FENSTERMACHER, J. D. 1972. Ventriculocisternal perfusion as a technique for studying transport and metabolism within the brain. *In* Research Methods in Neurochemistry. N. Marks and R. Rodnight, editors. Plenum Press, New York-London. **1**:165.
9. FLEXNER, J. B., L. B. FLEXNER, E. STELLAR, G. DE LA HABA, and R. B. ROBERTS. 1962. Inhibition of protein synthesis in brain and learning and memory following puromycin. *J. Neurochem.* **9**:595.
10. GAMBETTI, P., and N. K. GONATAS. 1968. Puromycin: action on neuronal mitochondria. *Science (Wash. D. C.).* **161**:900.
11. GAMBETTI, P., N. K. GONATAS, and L. B. FLEXNER. 1968. The fine structure of puromycin-induced changes in mouse entorhinal cortex. *J. Cell Biol.* **36**:379.
12. HOSAKA, Y., and H. KITANO. 1966. Studies on the Pleomorphism of HVJ virions. *Virology.* **29**:205.
13. HOWE, C., C. MORGAN, C. DE VAUX ST. CYR, K. C. HSU, and H. M. ROSE. 1967. Morphogenesis of type 2 parainfluenza virus examined by light and electron microscopy. *J. Virol.* **1**:215.

14. KISHI, K. 1972. Fine structural and cytochemical observations on cytoplasmic nucleolus-like bodies in nerve cells of rat medulla oblongata. *Z. Zellforsch. Mikrosk. Anat.* **132**:523.
15. KISHI, K. 1972. Two types of cytoplasmic nucleolus-like bodies. Observation of the serial sections of rat neurons. *Arch. Histol. Jap.* **35**:83.
16. LE BEUX, Y. J. 1971. An ultrastructural study of the neurosecretory cells of the medial vascular prechiasmatic gland, the preoptic recess and the anterior part of the suprachiasmatic area. *Z. Zellforsch. Mikrosk. Anat.* **114**:404.
17. LE BEUX, Y. J. 1972. An ultrastructural study of a cytoplasmic filamentous body, termed nematosome, in the neurons of the rat and cat substantia nigra. *Z. Zellforsch. Mikrosk. Anat.* **133**:289.
18. LONGNECKER, D. S., H. SHINOZUKA, and E. FARBER. 1968. Molecular pathology of in vivo inhibition of protein synthesis. Electron microscopy of rat pancreatic acinar cells in puromycin-induced necrosis. *Am. J. Pathol.* **52**:891.
19. LONGNECKER, D. S., and T. T. EDMONDS. 1969. Electron microscopy of gastric mucosa from puromycin-treated rats. *Am. J. Pathol.* **57**:65.
20. MILLONIG, G. 1961. A modified procedure for lead staining of thin sections. *J. Biophys. Biochem. Cytol.* **11**:736.
21. NATHANS, D. 1964. Puromycin inhibition of protein synthesis: incorporation of puromycin into peptide chains. *Proc. Natl. Acad. Sci. U. S. A.* **51**:585.
22. PALAY, S. L., C. SOTELO, A. PETERS, and P. M. ORKAND. 1968. The axon hillock and the initial segment. *J. Cell Biol.* **38**:193.
23. RAINE, C. S., L. A. FELDMAN, R. D. SHEPPARD, and M. B. BORNSTEIN. 1969. Ultrastructure of measles virus in cultures of hamster cerebellum. *J. Virol.* **4**:169.
24. RAINE, C. S., L. A. FELDMAN, R. D. SHEPPARD, and M. B. BORNSTEIN. 1971. Ultrastructural study of long-term measles infection in cultures of hamster dorsal-root ganglion. *J. Virol.* **8**:318.
25. SCHOCHET, S. S., JR., P. W. LAMPERT, and K. M. EARLE. 1968. Neuronal changes induced by intrathecal vincristine sulfate. *J. Neuropathol. Exp. Neurol.* **27**:645.
26. SHELANSKI, M. L. 1973. Chemistry of the filaments and tubules of brain. *J. Histochem. Cytochem.* **21**:529.
27. SMITH, J. D., R. TRAUT, G. M. BLACKBURN, and R. E. MONRO. 1965. Action of puromycin in polyadenylic acid-directed polylysine synthesis. *J. Mol. Biol.* **13**:617.
28. STEPHENS, R. E. 1967. The mitotic apparatus. Physical chemical characterization of the 22S protein component and its subunits. *J. Cell Biol.* **32**:255.
29. STRAHS, K. R., and H. SATO. 1973. Potentiation of vinblastine crystal formation in vivo by puromycin and colcemid. *Exp. Cell Res.* **80**:10.
30. TERRY, R. D. 1963. The fine structure of neurofibrillary tangles in Alzheimer's disease. *J. Neuropathol. Exp. Neurol.* **22**:629.
31. TILNEY, L. G., and K. R. PORTER. 1967. Studies on the microtubules in heliozoa. II. The effect of low temperature on these structures in the formation and maintenance of the axopodia. *J. Cell Biol.* **34**:327.
32. WATERSON, A. P. 1962. Two kinds of myxovirus. *Nature. (Lond.).* **193**:1163.
33. WHEELOCK, E. F. 1962. The role of protein synthesis in the eclipse period of Newcastle disease virus multiplication in HeLa cells as studied with puromycin. *Proc. Natl. Acad. Sci. U. S. A.* **48**:1358.
34. WISNIEWSKI, H., R. D. TERRY, and A. HIRANO. 1970. Neurofibrillary pathology. *J. Neuropathol. Exp. Neurol.* **29**:163.
35. YARMOLINSKY, M. B., and G. L. DE LA HABA. 1959. Inhibition by puromycin of amino acid incorporation into protein. *Proc. Natl. Acad. Sci. U. S. A.* **45**:1721.