*PROTEIN SYNTHESIS IN THE ISOLATED GIANT AXON OF THE SQUID**

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Communicated by David Nachmansohn, February 2, 1968

The work of Weiss and his associates,[1–3] and more recently of a number of other investigators,[4–6] has established the occurrence of a flux of materials from the soma of neurons toward the peripheral regions of the axon. It has been postulated that this mechanism would account for the origin of most of the axonal protein, although the time required to cover the distance which separates some axonal tips from their cell bodies would impose severe delays.[4] On the other hand, a number of observations[7–9] have indicated the occurrence of local mechanisms of synthesis in peripheral axons, as suggested by the kinetics of appearance of individual proteins after axonal transection.

In this paper we report the incorporation of radioactive amino acids into the protein fraction of the axoplasm and of the axonal envelope obtained from giant axons of the squid. These axons are isolated essentially free from small fibers and connective tissue, and pure samples of axoplasm may be obtained by extrusion of the axon. Incorporation of amino acids into axonal protein has recently been reported using systems from mammals[10] and fish.[11]

Materials and Methods.—Giant axons of *Loligo pealii* were dissected and freed from small fibers; they were tied at both ends. Incubations were carried out at 18–20° in sea water previously filtered through Millipore which contained 5 mM Tris pH 7.8 and 10 μc/ml of a mixture of 15 C^{14}-labeled amino acids (New England Nuclear Co., Boston, Mass.). At the end of the incubation, the axons were washed with water, blotted on tissue paper, and the axoplasm was extruded after cutting open one end. Homogenization was carried out with 0.5 ml H_2O in a glass homogenizer and was repeated after addition of an equal volume of 10% trichloroacetic acid (TCA). After standing in the cold for at least 10 min, the precipitate was collected by centrifugation and washed with 1 ml of 5% TCA. Supernatants were combined, diluted five times with H_2O, and plated on copper planchets provided with concentric rings. Under these conditions the quenching of the radioactivity was 43%. The precipitate was treated with 1–2 ml of *N* NaOH at 50° for 5 min, any undissolved material being discarded by centrifugation. An aliquot was taken for protein determination with the method of Lowry,[12] and the remaining solution was reprecipitated with 0.6–1.2 ml of 50% TCA, heated at 90° for 15 min, left in the cold for at least 60 min, and filtered through Millipore with an excess of 5% TCA containing 1 mg/ml of a mixture of amino acids (Casamino acids, Difco). Radioactivity was measured in a gas-flow counter with a background of 5 cpm.

Results and Discussion.—Incubation of isolated giant axons of squid in buffered sea water containing a mixture of C^{14}-labeled amino acids resulted in uptake of radioactivity in the TCA-soluble and insoluble fractions of the axoplasm and of the axonal envelope.

Entrance of radioactive amino acids in the soluble pool of the axoplasm continued for several hours (Fig. 1). The rate of the process was linear during the first period of incubation, but appeared to decrease later. A saturation level was not reached, however, even after six to seven hours. A significant incorporation of radioactivity occurred also in the protein fraction of the axoplasm with a rate which remained approximately linear for several hours.

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A much higher rate of labeling occurred in the protein fraction of the envelope which progressed in an essentially linear fashion for the same length of time (Fig. 2). In this experiment the axons were incubated with 1 ml of medium per axon and transferred to fresh medium every two hours. It was noted that when the incubation was continued for a longer period, or was carried out in less than 1 ml of medium per axon, the rate of entrance of the radioactivity in the TCA-

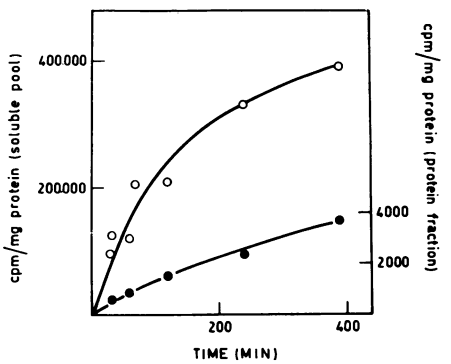

FIG. 1.—Kinetics of entrance of radioactive amino acids in the soluble pool and in the protein fraction of the axoplasm. Each giant axon was incubated with 1 ml of buffered sea water containing the mixture of C^{14}-labeled amino acids and transferred to fresh medium every 2 hr. O, Soluble pool; ●, protein fraction.

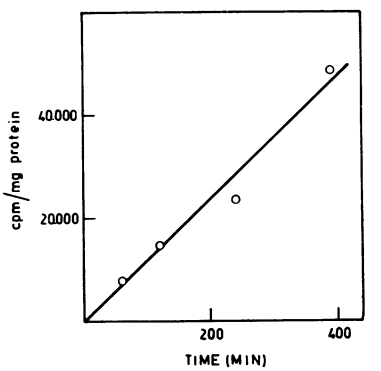

FIG. 2.—Kinetics of incorporation of radioactive amino acids into the protein fraction of the axonal envelope. Data obtained in the same experiment illustrated in Fig. 1.

soluble fraction of the envelope decreased considerably after 30 minutes, whereas the uptake of amino acids in the axoplasm remained essentially linear for a longer time.

In order to determine whether the incorporation of amino acids into the protein fraction of the axoplasm was due to the activity of a protein-synthesizing system, several known inhibitors of this process were tested for their effect on the rate of incorporation exhibited by isolated giant axons. As shown in Table 1, at concentrations of 0.2 mg/ml, puromycin and cycloheximide produced an inhibition in excess of 90 per cent, while chloramphenicol was less effective, producing approximately 80 per cent inhibition at a concentration of 0.5 mg/ml. A comparable degree of inhibition was also produced on the incorporation of amino acids into the protein fraction of the axonal envelope, although this system was somewhat less sensitive to the action of cycloheximide.

The incorporation of radioactive amino acids in the protein fraction of the axoplasm and of the envelope and the marked sensitivity of this process to puromycin, cycloheximide, and chloramphenicol appear to have established the occurrence of protein synthesis in the squid axon separated from the cell body and its nucleus. This finding is of interest to the fundamental problem of whether the proteins in the organelles of the periphery, such as, e.g., the plasma membrane, may be formed on the spot or are supplied by axonal flow or by the endoplasmic

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TABLE 1. *Percentage inhibition of the incorporation of labeled amino acids into the protein fraction of extruded axoplasm.*

	Inhibition (%)
Puromycin	87.5–97.0
Cycloheximide	95.0–96.0
Chloramphenicol	70.5–81.0*

The incorporation was measured as the percentage of total counts recovered in the protein fraction. Each axon was preincubated for 1 hr in buffered sea water containing the inhibitor (0.2 mg/ml) and then transferred for an additional hour to the same medium containing the mixture of labeled amino acids. Control axons were preincubated in sea water.

* The concentration of chloramphenicol in the second experiment was 0.5 mg/ml.

reticulum. Similar conclusions have recently been reached for the giant axon of *Dosidicus sigas*[13] and of *Loligo vulgaris*.[14]

The contribution of neuronal and nonneuronal structures to the much greater incorporation detected in the axonal envelope cannot be readily ascertained with the technique used, in view of the complex organization of this envelope which includes, besides the outer axoplasmic layer and the axolemma, a sheath of Schwann cells and a variable amount of contaminating connective tissue. However, the occurrence of many mitochondria close to the plasma membrane and the relatively high rate of respiration of the envelope after extrusion of most of the axoplasm seems to favor the assumption that at least a marked fraction of the protein synthesis observed in the envelope is due to the neuronal elements.[15, 16]

Some indication that the radioactive proteins of the axoplasm and of the axonal envelope were not identical was obtained by examining their distribution between a soluble and a particulate fraction derived from both these compartments. After incubation of giant axons for two hours in buffered sea water containing C^{14}-labeled amino acids, the axoplasm and the envelope were separated, each sample being homogenized in 0.32 *M* sucrose, 5 mM Tris buffer pH 7.8 and centrifuged at 25,000 × *g* for 20 minutes. Essentially all the radioactivity incorporated into the axoplasm (93%, average of two expts.) was recovered in the supernatant phase, whereas 60 per cent of that incorporated in the envelope was associated with the particulate fraction. Such difference might also be taken to argue in favor of two distinct systems of protein synthesis.

The accumulation of radioactive proteins in the axoplasm during the course of incubation of isolated giant axons with labeled amino acids might be attributed to the activity of at least three systems of protein synthesis present, respectively, in the axonal mitochondria, in the surrounding Schwann cells, or in a presumably nonmitochondrial axonal structure. Future work should distinguish between these possibilities.

Summary.—Incubation of isolated giant axons of *Loligo pealii* in buffered sea water containing a mixture of radioactive amino acids resulted in the incorporation of a significant amount of radioactivity into the protein fraction of the extruded axoplasm and of a much greater amount in that of the envelope. The process was strongly inhibited by puromycin, cycloheximide, and chloramphenicol. Most of the radioactive proteins of the axoplasm were recovered in a soluble fraction obtained by centrifugation, while the majority of those of the envelope was associated with a particulate fraction.

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* This work has been supported, in part, by the National Institutes of Health (grants NB-33271 and NB-07070), and by the National Science Foundation (grant NSF-GB-4844).

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