AXOPLASMIC TRANSPORT IN THE CRAYFISH NERVE CORD*

By H. L. Fernandez† and P. F. Davison‡

Department of Biology, Massachusetts Institute of Technology, Cambridge

Communicated by Francis O. Schmitt, June 30, 1969

Abstract.—Axoplasmic proteins in the crayfish nerve cord were labeled by the incorporation of high specific activity ³H-leucine that was injected into one of the abdominal ganglia. The labeled proteins moved caudally as a sharply defined peak at 1.1 mm/day. The level of radioactivity in the cord decreased slowly as the peak approached the tail. From the sharpness of the peak and the low decrement of label with distance it is deduced that the axoplasm is probably a gel, and some of it is not consumed as it is transported along the axon but reaches the terminal and, perhaps, the synaptic regions.

Weiss and his co-workers showed that part of the cytoplasm of vertebrate neurons is transported continuously from the perikaryon along the axon at 1–3 mm/day.¹ These findings have now been confirmed and extended by many workers in studies on mammals, birds, and amphibia. In addition to this slow transport, the faster movement of proteins, phospholipids, vesicles, and other particulates has been observed in various preparations. The evidence for this primarily somatofugal axoplasmic traffic has been reviewed recently.¹⁻³

In order to explore the mechanisms underlying axoplasmic transport the characteristics of movement of radioactively labeled protein in the axons of a suitable poikilothermic animal, the crayfish, were studied. Certain of our findings, particularly the sharp localization of the label in the cord, are reported here; other observations on the effects of cold and colchicine on transport and an examination of the proteins transported will be published elsewhere.⁴

Materials and Methods.—(a) Experimental animals: Three- to five-inch crayfish Procambarus clarkii girard and Cambarus sp., 25–35 gm, from the Lemberger Co. (Oshkosh, Wisconsin) were maintained in aerated aquaria in shallow tap water at 19 to 21°C and were fed daily. (b) Injection solutions: The solvent in all the solutions was Van Harreveld saline⁵,⁶ buffered at pH 7.6. Sufficient Bromophenol blue (LaMotte Chemical Prod.) was added to monitor visually the progress of the injection.

High specific activity 1-4,5-³H leucine (60 c/mM) (Schwartz Biochemicals) served to label protein. ¹⁴C-cycloleucine (4.25 me/mM) from New England Nuclear Corp., a non-metabolite, was used to measure nonspecifically bound isotope, and cycloheximide from Sigma Chemical Co. was used at a concentration of 200 μg/ml as a protein synthesis inhibitor. The radioactive compounds were evaporated to dryness and dissolved to a final concentration of 1 μc/μl (checked by counting a measured volume).

To assess the fate of solutions injected into the ganglion samples of a solution of the fluorescent dye Procion Yellow M, 4RS (ICI America, Inc.) were injected and the fluorescence was observed under a dissecting microscope over several hours.

(c) Injection procedure: A simplified diagram of the crayfish nervous system and of the injection technique are shown in Figure 1. The crayfish were lightly anesthetized with CO₂ and placed supine, partly immersed in water, in an illuminated dissecting chamber where they were restrained by rubber bands. The abdominal cord was exposed through a 4-mm square window cut in the ventral shell adjacent to the rib overlying the second or third abdominal ganglion. A glass pipette of 2 to 3 μ diameter tip filled with ³H-leucine solution was steered clear of the ventral artery and inserted through the outer

512
ganglionic sheath. From a micrometer syringe, 0.2–0.4 μl was injected while the site was continuously washed with physiological saline. The micropipette was withdrawn, a piece of premoistened Gelfoam absorbable gelatin (Upjohn Co.) was laid over the exposed area, and the flap in the selerum was closed and sealed by vaseline. More than 90% of the animals survived this operation over the usual testing periods (1–15 days). By injecting samples of the leucine solution into counting bottles it was found that the injected volume was reproducible to ±10%.

In a small number of experiments the crayfish cords were ligated caudal to the injection site at the time of the injection.

(d) Sampling of tissue: The distribution of ³H l-leucine was measured by carefully dissecting out the length of the cord, free from the abdominal artery and ganglionic nerve roots, from the first to the sixth abdominal ganglion. The cord then was pulled through a 3–4 cm length of Teflon tubing (i.d. = 0.12 cm, Bel-Art Prod.) that was then filled with a liquid vaseline-mineral oil mixture (1:2) from a syringe and frozen at −16°C. The rigid tube containing the cord now was serially sectioned at 1- or ½-mm intervals by a modified Minot Rotatory Microtome.

e) Liquid scintillation counting: The cord segments were freed from the Teflon sleeve and then unincorporated leucine was eliminated by a series of washes each in 3 ml of 10% trichloroacetic acid according to the following schedule; (1) at 50°C for 30 min; (2) at room temperature for 15 hr; (3) at 50°C for 30 min. Each sample then was air dried for 2 hr and digested according to the techniques of Hansen and Bush⁸ in 0.5 ml of N.C.S. solubilizer (Amersham/Searle, Illinois), 0.6 N solution in toluene (1) for about 12 hr at room temperature; (2) for 6 hr at 50°C; and (3) for 12 hr at room temperature. After dilution in 10 ml of toluene-based scintillant (Liquifluor, Pilot Chemicals) the samples were counted on a Nuclear-Chicago scintillation counter. Counting efficiencies measured by internal standards ranged from 23 to 26%. Tests for quenching⁹ showed little variation among samples and corrections were unnecessary. The background count (25–35 cpm) was measured by counting cord segments from un.injected animals.

In experiments to assess the distribution of injected ³H-leucine the unincorporated amino acid was assayed in cord segments by omitting the TCA washes. Volumes of hemolymph also were dried, digested, and counted. The fate of the leucine after 24 hr was checked by grinding and macerating separately three whole crayfish and counting TCA-precipitable and soluble tritium in an aqueous extract of the tissues and in the aquarium water.
(f) Methodology controls: The radiochemical purity of $^3$H-L-leucine was proved by paper chromatography in several solvent systems. The effectiveness of the TCA wash procedure was checked with segments of cord cut from uninjected animals and incubated with $^3$H-leucine (0.2 $\mu$C), together with chloramphenicol and cycloheximide added to inhibit protein synthesis. After the trichloroacetic acid washes, all the label was removed to a background level.

Results.—Consequences of intraganglionic injections were studied by injecting solutions of fluorescent Procion Yellow. By far the greater part of the small volumes (0.2–0.4 $\mu$l) injected into the ganglia of the crayfish remained localized in the cord after the injection needle was withdrawn.

Over the following 30 minutes, dye was redistributed in both directions along the cord by epineurial flow and it could not be detected after one hour.

The fate of the radioisotope injected in the ganglion was studied in a series of 29 animals in which, at intervals after the injection, the hemolymph was sampled, as well as segments of the cord and other tissue. The rate of disappearance of label from the blood (Fig. 2) indicates that the $^3$H-L-leucine uptake into cells occurs rapidly; within two hours the radioactivity in the circulating hemolymph attains a constant level. The radioisotope incorporation into the ganglionic proteins is maximal after approximately one hour and then much is rapidly lost from the cord as a whole (Fig. 2); it would appear that there are proteins turning over rapidly within the cord or in local connective tissue. Within 12 hours the only labeled protein in the cord is in the injected ganglion and much of that will move subsequently down the cord (Fig. 3).

Twenty-four hours following a $^3$H-L-leucine injection 30 per cent of the radioisotope has been excreted from the animal and 60 per cent is found in the water-soluble proteins extracted from the total tissues of the animal. Much of the remainder (8% of the total) appears as TCA-soluble material (not necessarily leucine but possibly metabolic by-products of leucine); by difference little of the isotope is bound to water-insoluble tissue residues. The only significant peak of radioactivity detectable in the whole cord 24 hours after injection is the TCA-precipitable material in the injected ganglion (0.03% of the total counts injected).

Fig. 2.—The level of incorporated $^3$H-leucine in the injected ganglion (●) and free $^3$H-leucine in 5$\mu$l hemolymph (average of five samples) (○) at successive times after intraganglionic injection of 172,000 cpm $^3$H-leucine. The level of isotope in the hemolymph implies a rapid dispersal of the leucine presumably after epineurial distribution along the cord, and then its depletion presumably by protein synthesis throughout the tissues of the animal. The rapid rise and fall in the level of incorporated leucine in the ganglion indicates the very rapid turnover of proteins there. Usually 50–70% or more of the counts remaining after 12 hr are in the proteins that move into the axon (see Fig. 3). Average background 35 cpm.
Microinjection directly into the ganglion exposed a limited cellular population to high specific activity label. Intra-abdominal injection of the same label resulted in 100-fold higher counts in muscle and hemolymph and correspondingly few in the cord.

When 200 μg/ml cycloheximide (an inhibitor of protein synthesis)\textsuperscript{10} was injected into the ganglion or administered intra-abdominally one hour before the injection of isotope, there was an 80 per cent depression of incorporation levels from normal demonstrating that the incorporation of \textsuperscript{3}H-leucine into TCA-precipitable material represents protein biosynthesis. In corroboration, after injection of \textsuperscript{14}C-cycloleucine, which is known to be taken up by cells but does not become incorporated into protein,\textsuperscript{13} no radioactivity was detectable in the cord.

From 1–17 days after injection, animals were sacrificed and cords were sectioned and counted. A sharp peak of radioactivity was detected invariably traveling caudad and confined within one of the 1 or 1/2 mm segments into which the cord was cut. This progressive movement of the labeled protein along the cord is illustrated in Figure 3 which shows a representative set of experiments consistent with a transport velocity of 1.1 ± 0.2 mm/day. The level of isotope incorporation along the cord, in Schwann cells, or in connective tissue, from precursors diffusing down the cord from the injection site or circulating in the hemolymph, was not measurable.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.jpg}
\caption{Representative distributions along the crayfish cord of \textsuperscript{3}H-leucine incorporated into protein. At different times after leucine injection the animal was sacrificed and the tritium in each 1-mm segment was measured. The cord is shown diagrammatically over each histogram and the injected ganglion is marked with a V. The ganglia are from left to right, the 2nd, 3rd, 4th, 5th, and 6th abdominal ganglia; the 6th is also referred to as the caudal ganglion. The background counts are indicated by the dotted line. The injected segment is shown hatched; an irreproducible fraction of the input counts remained in the injected ganglion, some perhaps in nonneuronal cells. In the fourth experiment, \textsuperscript{3}H-leucine was injected into the same ganglion 8 and 3 days prior to killing.}
\end{figure}
The possibility that the detected radioactivity distribution pattern could be the result of a nonperikaryal macromolecular synthesis occurring in axons or satellite cells along the cord was ruled out by the fact that in eight experiments no significant amounts of labeled protein were found one or more days after injections into interganglionic segments of the cord.

Other aspects of the experimental system were studied in small series of experiments: (a) Five long-term experiments (over 10 days) with double ligatures showed accumulation of labeled protein at the proximal side of the first ligature, whereas no label was found at the distal side nor around a second separate tie distal to the first. (b) Six experiments with $^{14}$C-cycloleucine injections, in which subsequently the segments of cord were not washed with trichloroacetic acid showed that the isotope was symmetrically distributed about the site of injection in a Gaussian fashion. In contrast the incorporated $^3$H-L-leucine moves as a sharp peak showing no diffusional broadening. (c) In ten experiments, each with two sequential injections (3–7 days apart) into the same ganglion two peaks of radioactivity were found with a peak-to-peak distance consistent with a movement of approximately 1 mm/day (Fig. 3.) Usually the second peak was markedly smaller than the first.

The appearance of the incorporated isotope in a single segment of cord could, of course, have been mimicked by unincorporated leucine that was not washed out. The significance of counts incorporated in any segment is convincing, therefore, only when the consistency of the results is appreciated. This consistency is amply brought out by the data in Figure 4. The movement of labeled protein is at the rate of $1.1 \pm 0.2$ mm/day at 19–21°C; that rate is similar to the velocities estimated by many other workers on other animal species.$^{2, 3}$

In these and short-term (1–48 hours) experiments no peak of radioactivity moving faster than 1.1 mm/day was detected although such peaks have been reported recently from a number of other laboratories.$^{2, 3, 13, 14}$ When the cord was ligated 7–10 mm caudal to the site of injection, an accumulation of radioactivity was detected at the proximal side of the ligature; this build-up was not detectable between 12 and 20 hours after injection but was found over periods from 20–48 hours, after which it declined. Thus, some material is transported faster than 1.1 mm/day.

Since the intraganglionic injection procedure enables a reproducible level of label to be introduced into the neuronal population an assessment of the dec-
rement of the label along the crayfish cords was made. The isotope cannot be continuously monitored along any one crayfish cord, therefore a series of animals was injected with equal volumes of a common solution of $^4\text{H}$-leucine and the incorporated activity was measured in animals killed at various intervals. The radioactivity resident in the injected ganglion and in the transported peak is plotted as a function of distance along the cord in Figure 5.

**Discussion.**—These experiments on the crayfish have shown that in this animal, as in many others including invertebrates, radioactively labeled amino acids are rapidly incorporated into protein in nervous tissue and some of this protein is transported along the nerve cord at a rate close to 1 mm/day. In the crayfish the radioisotope movement was uniformly caudal from the second, third, fourth, or fifth abdominal ganglia; however, some of the emerging axons are directed rostrally so the failure to observe label moving there is puzzling. These experiments do not show explicitly that the migrating population of radioactive protein molecules is moving within axons, and autoradiographic experiments are in progress to decide this point. Nevertheless, it is probable that these proteins are intraxonial for the following reasons: (a) our findings are similar to those of other workers on transported proteins that have been demonstrated to be within the axon; (b) uptake must occur in a ganglion and not in a connective for the peak to be seen; (c) leucine is incorporated into protein (as shown by the inhibition by cycloheximide, and the failure of cycloleucine to be incorporated) presumably by an intracellular process; and (d) the linear movement and sharply restricted distribution of label is incompatible with distribution of the label by a flow (see below) or diffusion process in or without an axon.

The most striking result of these experiments, the sharp distribution maintained by the labeled material in its passage along the cord, is in contrast to the findings of most workers. The most commonly observed distribution is a broad spread of radioactivity advancing from the site of injection; however, recently some reports have included illustrations of peaked distributions at certain times within nerves. We postulate that the condition that made possible our narrow distribution of label was the use of a very small quantity (0.2-0.4 $\mu$L) of high specific activity leucine that was briefly contained within the ganglia and available to the neurons before it became diluted with local leucine and dispersed.
in the circulation. If the quantity of leucine was increased (10–100 times), possibly flooding the local pools, the distribution at a subsequent time was broader along the cord and frequently trailed back to the site of injection.

On the assumption that the migrating peak of incorporated leucine was in fact moving intra-axonally in a population of neurons, an important conclusion may be drawn from these experiments. The transportation process clearly is not one involving laminar flow of dissolved protein. Under laminar flow in a cylinder, a pronounced velocity gradient occurs across the stream and the highest flow rate is twice the average while the boundary layers are stationary; a rapid dispersion of any initially compact label, therefore, must result. The sharp localization of the label in our experiments indicates that the protein does not flow along the axon in a laminar stream but is moved, either as a gel with a uniform migration front, or by some fixed or slowly moving structures within the axon that maintain a uniform rate across the axon. The mediation of transport by the neurofilaments or microtubules in a neuron has been postulated.

If the axoplasm is a gel there must exist channels through which selected materials are moved since, as the ligation experiments show, some constituents move faster than the gel. The counts detected at the ligature indicate arrested transport rather than local isotope uptake by injured cells because no label accumulated on the distal side of the ligature or around a second, distal ligature; and no build-up was detected until 20 hours elapsed, and then the tritium was not in the epineurium but widely distributed in the body.

The data embodied in Figure 5 showing the decrease in labeling with movement along the cord cannot be interpreted precisely because we do not know which axons are labeled. Some of the decrease between 5a and 5b could reflect axons that originate in the third and terminate in the fourth ganglion; some could represent catabolism of the protein in the axon. The decrease between 5b and 5c is sufficiently small to suggest that even if the disappearance represents entirely consumption en route and none of these labeled axons terminate in the fifth ganglion, then the length of the axons beyond the sixth ganglion is unlikely to be enough to complete the consumption of all the labeled protein. We deduce that a significant fraction of moving protein will reach the end of the axon. The function of this protein, like that turned over rapidly in the soma (Fig. 2) is unknown.

The label remaining in the injected ganglion may represent long-lived protein in the perikarya or in local connective tissue and glial cells. In view of the small decrease of label between 5b and 5c, we suspect that the large decrease in label between 5a and 5b seems to imply that some of the axons terminated in the fourth ganglion. The failure to find any counts in this ganglion suggests that the residence time of slowly moving proteins that reach the axon terminus is not more than a day or two. In contrast, observations on the rapidly transported amine storage granules suggest that they have a long life.

Further studies on this system will be undertaken to decide if the label decreases along a single connective, and also to compare those proteins that start the journey with those that reach the terminal regions of the axon since the latter may contain proteins concerned not only with trophic and transmitter function
but also with other neuronal activity, perhaps transynaptic, that presently is only the subject of speculation.

This investigation was initiated as a result of discussions with Professor Francis O. Schmitt and we gratefully acknowledge the stimulus and inspiration provided by his energy, perception and prescience. We are indebted to Dr. I. Nadelhaft and Dr. R. Nystrom for discussions in the course of this work and for careful criticisms of the manuscript. We also thank Dr. F. C. Huneeus for the use of the modified microtome he developed and for assistance and discussion, Mrs. Maritza J. Fernandez for preparing the figures, and Mr. Dean Roller for technical assistance. This study was supported by grant NB-00024 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service. H. L. F. was the recipient of a Fulbright-Hays State Department Grant (Program no. 33031, Student no. 700230).

* Part of this work was submitted by H. L. F. in partial fulfillment of the requirements for an M.S. degree in the Biology Department at the Massachusetts Institute of Technology, January 20, 1969.
† Present address: Department of Physiology and Cell Biology, The University of Kansas, Lawrence, Kansas 66044.
‡ Present address: Department of Fine Structure Research, Boston Biomedical Research Institute, 20 Staniford Street, Boston, Massachusetts 02114.
1 Weiss, P., in Symposium of the International Society of Cell Biology, ed. S. H. Barondes (New York: Academic Press, Inc., in press), vol. 7.
3 Barondes, S. H., and F. Samson, Neurosciences Res. Prog. Bull., 5, 307 (1967).
5 Grafstein, B., in Advances in Biochemical Psychopharmacology, ed. E. Costa and P. Greengard (New York: Raven Press, in press).
4 Fernandez, H. L., F. C. Huneeus, and P. F. Davison, manuscript in preparation.
6 Van Harreveld, A., Proc. Soc. Expl. Biol., 34, 428 (1936).
7 Lockwood, A. P. M., Comp. Biochem. Physiol., 2, 241 (1961).
8 Hansen, D. L., and E. T. Bush, Anal. Biochem., 18, 320 (1967).
9 Bush, E. T., Nuclear Chicago Corporation Tech. Bull., 13, (1964).
10 Campbell, M. K., H. R. Mahler, J. W. Moore, and S. Tewari, Biochem., 5, 1174 (1966).
11 Brink, J. J., R. E. Davis, and B. W. Agranoff, J. Neurochem., 13, 589 (1966).
12 Weiss, P., H. Wang, A. C. Taylor, and M. V. Edds, Amer. J. Physiol., 143, 521 (1945).
13 Ochs, S., J. Johnson, and M.-H. Ng, J. Neurochem., 14, 317 (1967).
14 Ochs, S., M. I. Sabri, and J. Johnson, Science, 163, 686 (1969).
15 Karlsson, J. O., and J. Sjöstrand, Brain Res., 11, 431 (1968).
16 Smith, B. H., Ph.D. Thesis, Massachusetts Institute of Technology (1967).
17 Davison, P. F., The Neurosciences—Second Study Program (New York: The Rockefeller University Press, in press).
18 Schmitt, F. O., and F. Samson, Neurosciences Res. Prog. Bull., 6, 117 (1968).
19 Schmitt, F. O., these PROCEEDINGS, 60, 1092 (1968).
20 Dahlström, A., and J. Häggendal, Acta Physiol. Scand., 69, 153 (1968).