NEURONAL DYNAMICS AND AXONAL FLOW, II. THE OLFACTORY NERVE AS MODEL TEST OBJECT

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Neuroplasm (specifically, nerve protein) is continuously being reproduced in the cell body of the neuron ("perikaryon") and conveyed cellulifugally down the nerve fiber at an average daily rate of a few millimeters.[1] Ever since the discovery of this "axonal flow,"[2] there has been a pressing need for quantitative studies of the physical and chemical dynamics involved. Isotope labeling of the axonal protein stream at its source appeared as the method of choice.[3] Labeling of whole animals has suffered from the low signal-to-noise ratio, that is, the difference between the advancing radioactive nerve content and the general background of radioactivity in the body. In order to obtain higher resolving power, labeling had to be confined to a strictly circumscribed nerve source, leaving the rest of the body uncontaminated and blank.

As described in the first paper of this series,[4] this has been achieved by injecting H^3-leucine into the interior of the eye.[5] The labeled amino acid was rapidly incorporated into the proteins of the retinal nerve cells and the "hot" axonal flow down the optic fibers from retina to brain registered sharply over the "cold" tissue background. For brevity, we shall continue to refer to tissues possessing or lacking radioactive markers as "hot" or "cold," respectively.

The present note reports an even simpler method. Its advantage lies in the choice of a nerve source located in the body surface, hence easier of access than the centrally located nerve cells used in our previous experiments. The only nerve answering this description in vertebrates is the olfactory nerve. Its perikarya lie in the sensory epithelium that lines the bilateral nasal cavities. The axons issuing from those cells course anteroposteriorly, first in small fascicles, then jointly as the two bilateral stems of the olfactory nerve which, on approaching the forebrain, merge into a single median trunk and terminate in the so-called olfactory bulb on telodendrons of the mitral cells of the forebrain. Although our experiments included both mice and toads, this description and the following report pertain solely to the amphibian group.

The experiments were predicated on the assumption that labeled amino acid introduced into one of the nasal cavities would penetrate into the sensory perikarya in the lining and be incorporated into their proteins. Protein traffic to the brain could then be measured directly, the nerve of the opposite, untreated side serving as "cold" control. This expectation has been confirmed by the results.

Materials and Methods.—In 17 adult toads (*Bufo Americanus*), a small pellet of Gelfoam soaked in tritiated leucine (stem solution: 0.026 mg of L-leucine-4,5-H^3 in 1 ml sterile saline) was inserted into the right nostril which was then sealed, mostly by a thin layer of vacuum grease. After 2, 5, 11, 14, and 18 days, the animals were then decapitated in intraperitoneally administered urethane anesthesia. After fixation in Bouins for several days, their heads were sectioned at 8 μ, mostly transversally. Every 200 μ, five consecutive sections were mounted, deparaf-

finized, hydrated, air-dried and coated by dipping into Kodak NTB-3 nuclear track emulsion; then dried, kept light-tight in the refrigerator for 14–16 days, developed (Kodak D-170), fixed, rinsed, stained (Cason modification of Mallory), and mounted for microscopy and photomicrography.

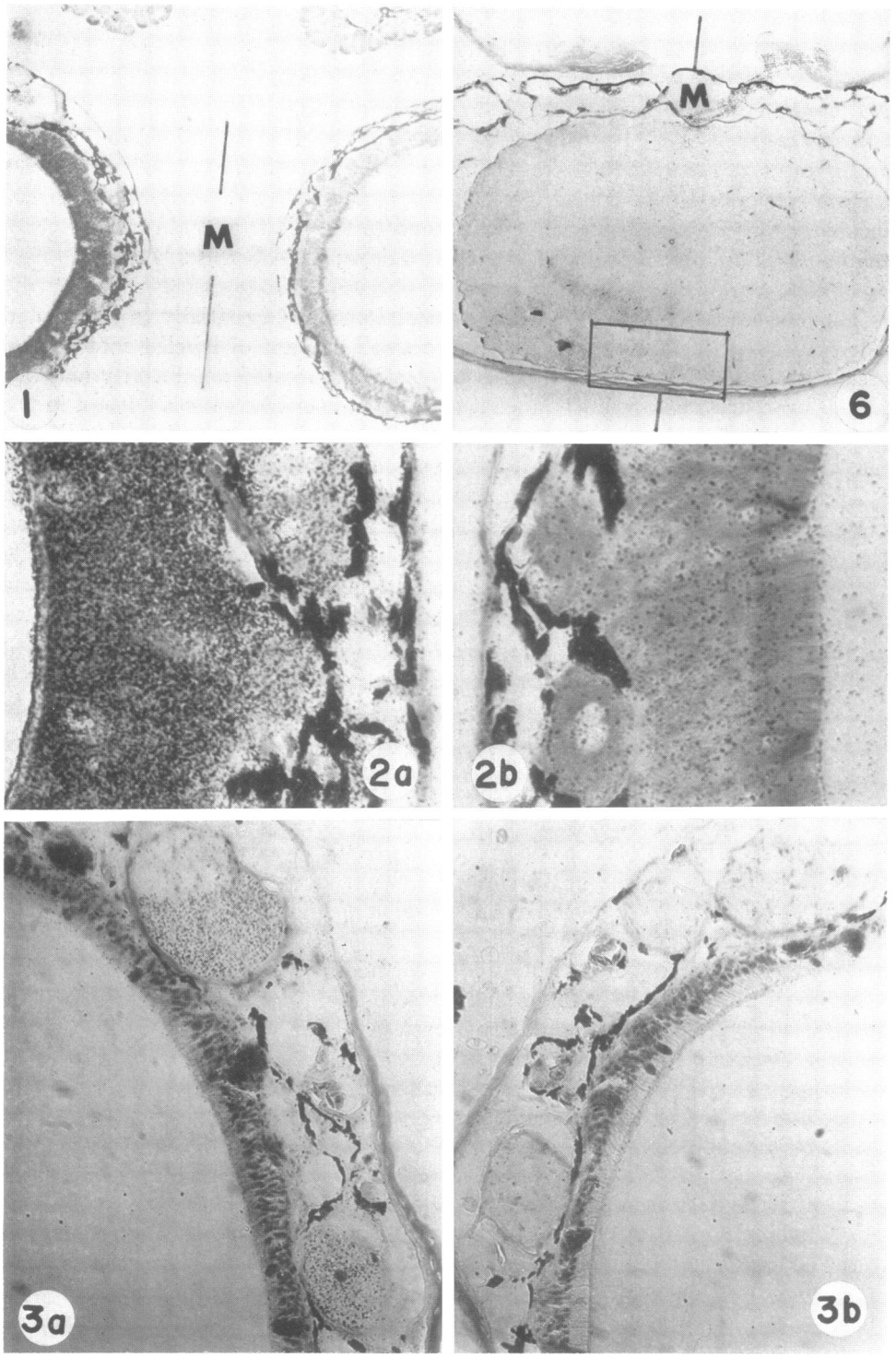
Results.—Figures 1–11 give representative samples of the obtained radiograms. In Figures 1, 6, 7, 8, and 10, the whole median part (*M*, midline) of each given cross section is reproduced, whereas Figures 2, 3, 4, 5, and 9 show pairs of sample sectors located symmetrically to the left (*a*, hot) and to the right (*b*, cold control) of the midline. Times after labeling: Figs. 1–7, 5 days; Figs. 8 and 9, 11 days; Figs. 10 and 11, 14 days.

The labeled protein has in all cases remained confined essentially to the treated side. In a few instances (e.g., Fig. 2*b*), a small amount of labeled matter had escaped from the treated into the communicating untreated nasal cavity and was found incorporated in a few epithelial cells and the exuded mucus but not in the nerve fibers. By 11 days, some scattered silver grains appeared over cells, indicative of vascular recycling of label from secondarily degraded protein.

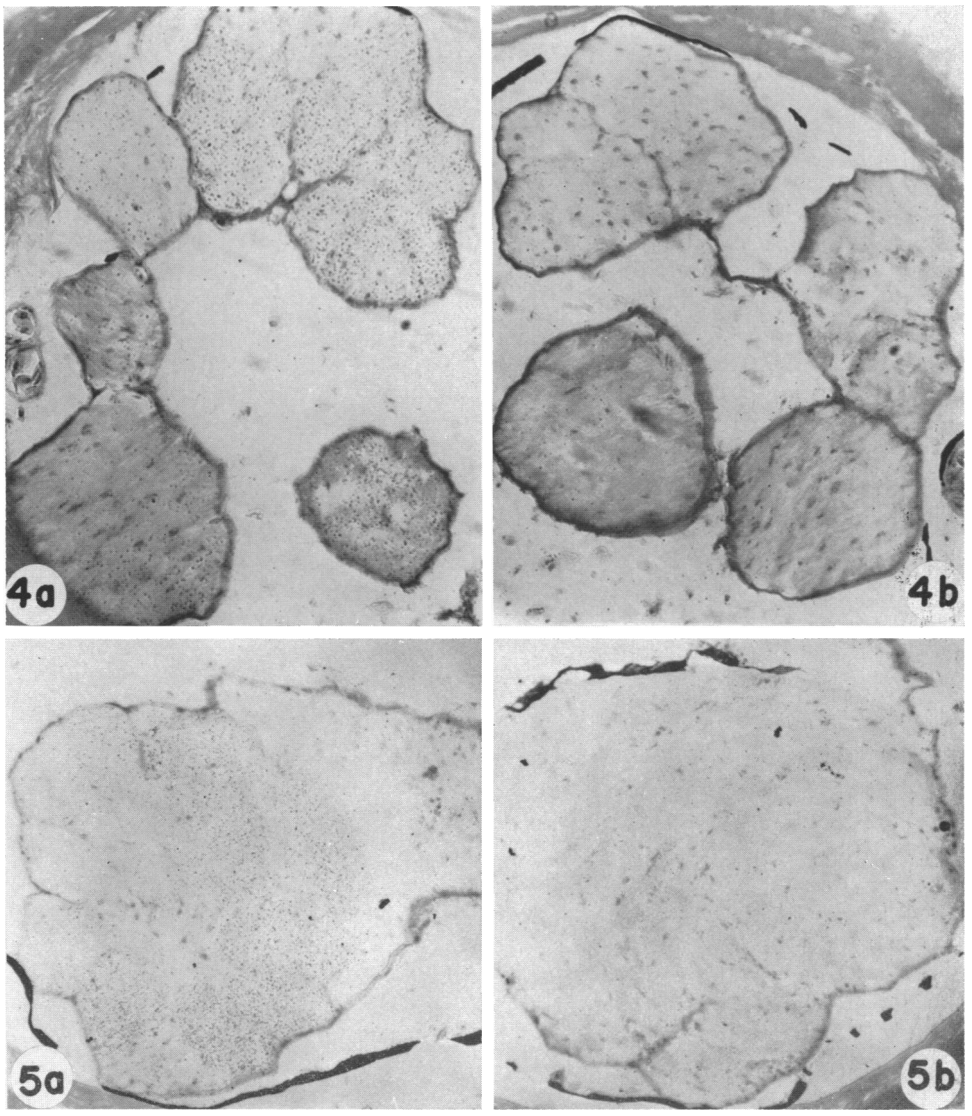
At five days, radioactivity in the nasal epithelium on the hot side is still high (Figs. 1 and 2*a*). Figure 2 illustrates the exit of (pigment-coated) nerve roots from the epithelium. Proceeding towards the brain (Fig. 3), the axons on the hot side (Fig. 3*a*) are heavily labeled, contrasting sharply with their cold counterparts (Fig. 3*b*). Note that the upper nerve fascicle in Figure 3*a* shows a sharp demarcation line between a labeled and an unlabeled portion, the latter evidently having originated from a sector of sensory epithelium beyond the confines of the label-charged gelatin plug. This sharp projection of the peripheral receptor mosaic into the fasciculation of the nerve branches has been substantiated by all our observations (see farther cerebral sections in Figs. 4, 5, 7, 9). Figure 4 shows the beginning merger of the separate olfactory branches into two major stems. In Figure 5, farther posteriorly, the two stems are already enclosed in a common meningeal sheath; here the labeled protein is confined mainly to the medioventrally located fiber group. Still closer to the brain, the bilateral nerves appear fully merged (Fig. 6). The medioventral region of Figure 6, reproduced at higher magnification in Figure 7, shows the remarkably sharp border between the hot and cold fiber populations to either side of the midline.

At the entrance into the olfactory bulb, the course of the fibers is no longer straight anteroposterior. Fascicles splay out, as seen in Figure 9. The sharp distinction between hot and cold fascicles on the treated side is quite conspicuous. It emphasizes the potential value of this technique for the detailed tracing of the central course of nerve tracts to their endings.

Figures 10 and 11 present frontal (longitudinal) sections. Figure 10 is a lengthwise slice, showing two symmetrical components of the olfactory nerves still adjacent to the nasal sacs, the one on the labeled side hot, and the one on the control side blank. Figure 11 is the synaptic zone of the hot nerve at its termination in the olfactory bulb in the forebrain. The cells of the glomeruli are seen as large grey patches. It is evident that the advance of the labeled protein has stopped abruptly at this level. Accordingly, no transynaptic transfer of either axonal proteins as such or of any of their degradation products containing leucine has taken place up to the time of sacrifice.

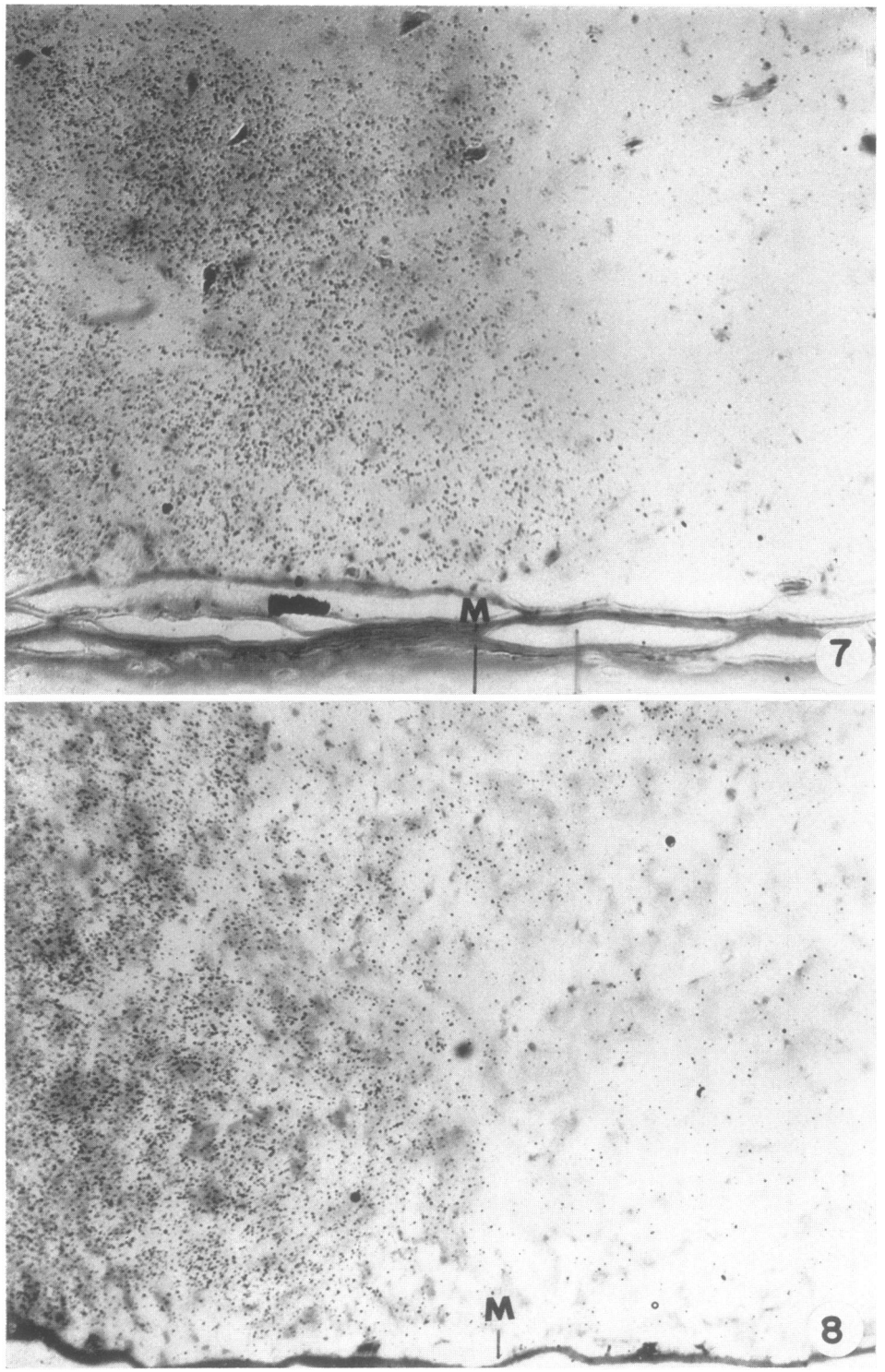


FIGS. 1–3, 6.

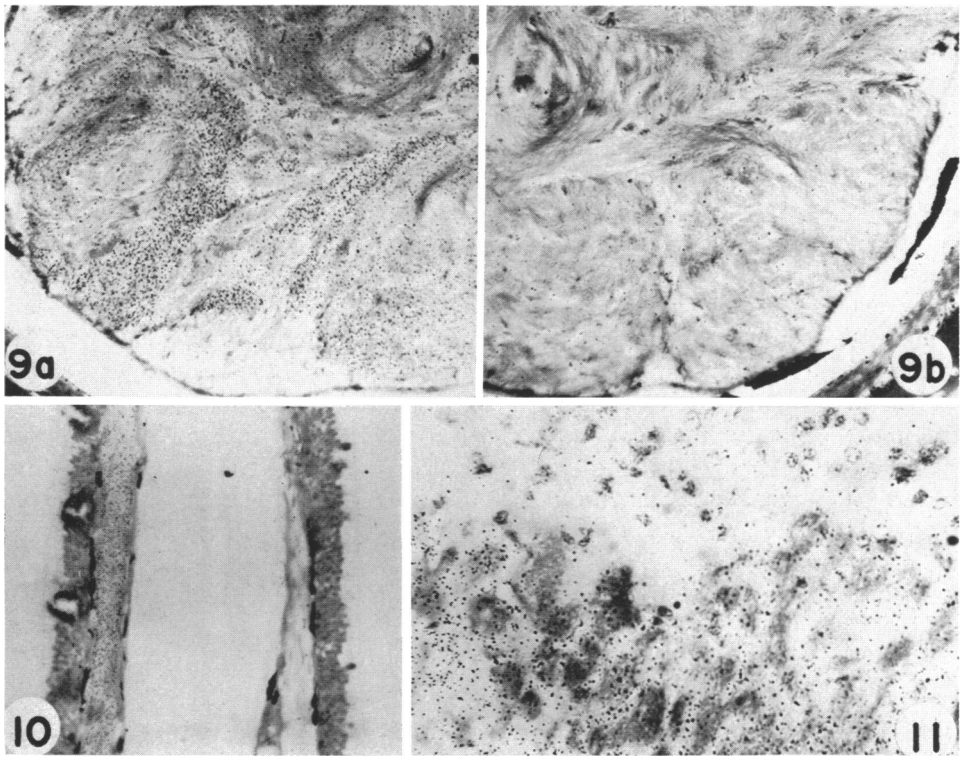


FIGS. 4 AND 5.

Discussion.—The results of the whole series have been remarkably uniform. The above illustrations are therefore fully representative. They lead to the following conclusions: Tritiated amino acid introduced into the nasal cavity is promptly absorbed into the cells of the lining, including the sensory nerve cells interspersed in it whose axons are the olfactory nerve fibers. As the labeled amino acid did not penetrate into deeper layers (see Figs. 3–5), all label observed subsequently in the nerve fibers can be conclusively referred to sources in the nasal epithelium. The presence in the same nerve branch of both labeled and unlabeled fascicles, sharply delineated (Figs. 3–5), corroborates this conclusion. It also opens a new techniqus for the mapping of the peripheral distribution of nerves.



FIGS. 7 AND 8.



FIGS. 9–11.

The fact that at five days the axonal markings had moved down the whole length of the olfactory nerve to the brain (about 1 cm) proves that the axoplasm had again advanced at an average rate of at least one millimeter per day. Allowing for the 15°C temperature difference between toads and warm-blooded animals (at a Q_{10} of approximately 3), this would compare to a convection rate in the mouse of about 5 millimeters per day, quite within the range established by earlier investigations. Exact quantitative determinations will have to await the results of the laborious grain counts to be reported at a later date.

The data here reported should be viewed not in isolation but in the context of the past findings on axonal flow. It must be particularly emphasized that one is dealing here not primarily with protein moving in solution in liquid channels, but with a *cellulifugal convection of the whole axonal content*,[2] serving as feeder line for the replenishment of its own catabolically degraded macromolecular constituents, including enzymes, structural components, mitochondria, as well as a vehicle for neurosecretions, transmitter substances, and perhaps "trophic" agents of central origin. The axonal movement is actuated by a microperistaltic wave in the axonal surface and myelin sheath. Its rate, determined by cinemicrography of mouse nerve fibers,[6] is remarkably constant, with a mode of 16 minutes per pulse. This is compatible with the observed rate of axonal propulsion. Combining all the available data, one arrives at a value for the rate of renewal of protein in the perikaryon destined for axonal flow of approximately 100 per cent per day.

Extensive electron microscopic studies in our laboratory, moreover, indicate the presence of liquid channels, particularly neurotubules, within which transport at a much higher rate than that of the coherent axonal column can proceed.[7]

Summary.—The cells of origin of the olfactory nerve fibers have been successfully labeled with tritiated leucine in toads and mice without contamination of the surrounding tissues. The amino acid became promptly incorporated in the proteins of the cell bodies. These labeled proteins could then be traced autoradiographically in their convection down the olfactory nerve fibers to their terminations in the forebrain. The observed rate of movement is compatible with the previously established average rate of axonal flow of a few millimeters per day. This connotes a total synthesis of exportable protein in the cell body of about 100 per cent per day, making the nerve cell one of the fastest growing cells of the body, which adds a significant new dimension to our concepts of adaptability of neurons in memory, neuropathic changes, regeneration, and neurotrophic actions. No transmission of labeled substance beyond the primary neuron was observed. Aside from its value for systematic quantitative investigations of reproduction of neuroplasm and axonal flow, the technique opens a new way for the tracing of nerve fiber connections from source to destination in normal and experimentally modified specimens. It also reemphasizes the value of the neuron as a unique object for fundamental studies in cell biology.[8]

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[1] Weiss, P., in *Regional Neurochemistry*, ed. S. S. Kety and J. Elkes (Oxford: Pergamon Press, 1961), p. 220.

[2] Weiss, P., and H. B. Hiscoe, *J. Exptl. Zool.*, **107,** 315 (1948).

[3] Weiss, P., in *Symposium on the Effect of Use and Disuse of Neuromuscular Functions*, ed. E. Guttman (Prague, 1963), p. 171; Lubinska, L., in *Mechanisms of Neural Regeneration*, ed. M. Singer and J. P. Schadé (Amsterdam: Elsevier, 1964), p. 1.

[4] Taylor, A. C., and P. Weiss, these PROCEEDINGS, **54,** 1521 (1965).

[5] Less rigorous confinement was previously obtained by injection of P_{32} into the spinal cord (Ochs, S., and E. Burger, *Am. J. Physiol.*, **194,** 499 (1958). Tritiated leucine for nerve labeling was introduced by Droz, B., and C. P. Leblond, *Science*, **137,** 1047 (1962).

[6] Weiss, P., A. C. Taylor, and P. A. Pillai, *Science*, **136,** 330 (1962).

[7] This presumably explains the faster rate of distad convection reported, for instance, for phospholipids (Miani, N., in *Mechanisms of Neural Regeneration*, ed. M. Singer and J. P. Schadé (Amsterdam: Elsevier, 1964), p. 155, and catecholamines (Dahlström, A., and K. Fuxe, *Z. Zellforsch.*, **62,** 602 (1964)).

[8] Comparable to *Acetabularia*, e.g., Haemmerling, J., *Symp. Soc. Exptl. Biol.*, **17,** 127 (1963).