[1] Rowlinson, J. S., *Mol. Phys.*, **7**, 349 (1964); *ibid.*, **8**, 107 (1964).

[2] Henderson, D., and S. G. Davison, these PROCEEDINGS, **54**, 21 (1965).

[3] Hill, T. L., *Statistical Mechanics* (New York: McGraw-Hill, 1956).

[4] Reiss, H., H. L. Frisch, and J. L. Lebowitz, *J. Chem. Phys.*, **31**, 369 (1959).

[5] Percus, J. K., and G. J. Yevick, *Phys. Rev.*, **110**, 1 (1958).

[6] Wertheim, M. S., *Phys. Rev. Letters*, **10**, 321 (1963); *J. Math. Phys.*, **5**, 643 (1964).

[7] Thiele, E., *J. Chem. Phys.*, **39**, 474 (1963).

[8] Green, H. S., *J. Chem. Phys.*, **19**, 955 (1951).

[9] de Boer, J., and A. Michels, *Physica*, **5**, 945 (1938).

[10] Henderson, D., *Mol. Phys.*, in press.

[11] Hirschfelder, J. O., C. F. Curtiss, and R. B. Bird, *Molecular Theory of Gases and Liquids* (New York: John Wiley and Sons, Inc., 1954).

[12] Barker, J. A., and J. J. Monaghan, *J. Chem. Phys.*, **36**, 2564 (1965).

[13] Michels, A., W. De Graaff, T. Wassenaar, J. M. H. Levelt, and P. Louwerse, *Physica*, **25**, 25 (1959).

DEMONSTRATION OF AXONAL FLOW BY THE MOVEMENT OF TRITIUM-LABELED PROTEIN IN MATURE OPTIC NERVE FIBERS*

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The original demonstration 20 years ago, by one of the authors (Paul Weiss[1]), that axoplasm is continuously being generated in the nerve cell body and then conveyed into and down the nerve fiber, was based on the observation that locally constricted nerve fibers develop a permanent configurational asymmetry relative to such artificial bottlenecks: upstream dilation, downstream shrinkage. The axon appeared as a feeder column carrying major macromolecular systems from their exclusively near-nuclear production sites in the cell soma to distant peripheral sites, where they served (a) within the axon, as replacements for catabolic losses, and (b) as transmitters, neurohumors, and perhaps trophic agents for terminal discharge. The daily advance of the column was experimentally determined to be of the order of the millimeter.[2] From the very start, (a) was considered to involve primarily traffic of proteins. This was supported by the fact that the observed rate of advance matched roughly the calculated protein depletion rate of peripheral nerve. It also agreed well with the concurrently discovered dominance of the nerve cell nucleus in protein synthesis.[3]

For a more detailed quantitative study of axonal flow the radioactive tracer technique obviously recommended itself as method of choice. We had applied it successfully in the early days of biological isotope use for measuring the rate of proximodistal flow of fluid in the endoneurial spaces[4]—about one millimeter per hour, i.e., one magnitude faster than the axonal flow. Attempts to label the axon itself, however, with P^{32} (refs. 5–7) or C^{14}, remained for some time disappointing in

that they added little beyond reconfirming the existence of cellulifugal axonal transport.[8,9] Some of the shortcomings were (1) the lack of strictly localized applicability of the isotope source to nerve cell bodies, and (2) the inseparability of the relative shares contributed by axons and their surrounding cells to bulk measurements of radioactivity. The former difficulty can be partly obviated by topical injections of isotopes directly into spinal cord or brain.[10] For differentiation between axon and sheath cell radioactivity. one had to turn from bulk counters to the high-resolution technique of radioautography of microscopic sections.

Our earlier experiments with four C^{14}-labeled amino acids gave inconclusive counts.[8] The subsequent experiments of Droz and Leblond,[11] using tritiated leucine as label, however, produced a decisive advance: these authors succeeded in tracing radioautographically the proximodistal advance of the protein-incorporated label within the axons of isotope-injected rats of the predicted order of a millimeter per day. Their success has led to our next step, described in this paper, which introduces (1) a more rigorously localized application of the label to nerve cells; and (2) the use of symmetrical paired nerve sources in the same animal as prototype for future studies on the effect of various agents on the rate of axonal flow: one side for testing, the other side serving as control.

Materials and Methods.—The pairs of nerves chosen were the optic nerves of nine young white mice of about 30 gm of weight. The cells of origin of the optic nerve lie in the innermost layer of the retina, thus bordering directly on the posterior chamber of the eye (vitreous body). Any solution injected into the latter will therefore bathe the optic ganglion cells. Leakage beyond the retina is barred by the fibrous sclera surrounding the eyeball. Because of this confinement to the deposit site, the isotope can be used in minute quantities, which obviates the diffuse labeling of the rest of the animal.

The tritiated tracer was 5DL leucine-H^3 (1 mc/ml), diluted 2:1 with a watery solution of methylene blue (for a visual check of the distribution of the injected mass). Each eye received 3 μl (= 2 μc) of this mixture through a fine glass micropipette attached to a screw-driven micro-syringe.

For the injection, the cornea of the anesthetized mouse was punctured near the limbus, reducing the intraocular pressure and providing an opening for the pipette. The latter was then thrust obliquely through the iris and the suspensory ligament of the lens into the vitreous body (Fig. 1). Halfway in, the measured quantity of solution was expressed and the pipette was then withdrawn. The injected mass remained confined within the eyeball.

One animal was sacrificed 3 hr after the injection, the other eight animals at one-day intervals thereafter. At autopsy blood samples were taken for flow counter determination of diffuse background radioactivity. Both eyes were resected with their optic nerves back to the chiasma, placed on paper for straightening the nerves, and then fixed in Bouin's fluid for one day. Embedding,
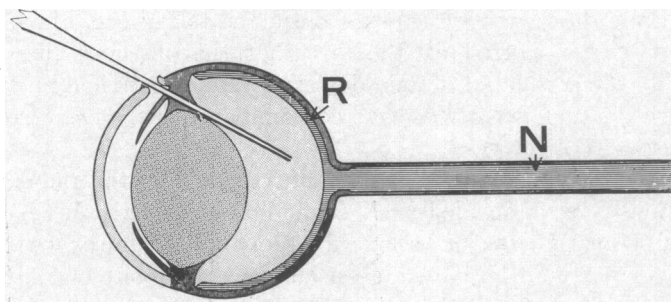

Fig. 1.—Diagram of injection site. *R*, retina; *N*, optic nerve.

sectioning, staining, and radioautography were carried out according to Kopriwa and Leblond.[12] One nerve of each pair was sectioned longitudinally, the other transversally. The photographic emulsion of the coated sections was developed after 8 or 15 days of exposure.

The distribution of protein-incorporated tritiated leucine along the nerve was determined by counts of radiation-reduced silver grains. For longitudinal sections, this was done by enumerating standard sample fields directly under the microscope at 1000 × magnification. Cross sections were projected on a screen and all discernible grains were counted.

For a critical measurement of a lengthwise shift of radioactivity along the nerve with time, a fixed reference mark is crucial. We have chosen the sharp exit point of the optic nerve from the bulb. Even so, the varying distances between this point and the ganglion cells, where the incorporated label starts its travel, introduce an unavoidable blurring factor. Since uncontrollable variability of dosage, uptake, and technique (histologic and photographic) precludes interindividual comparisons in absolute units, the data of all eyes have been normalized, setting the highest count along any given nerve as 100% and expressing all other counts on the same nerve as fractions of that value.

Results.—As early as 3 hr after an injection, label was found to have been incorporated in the nerve cell bodies of the retina. The continuous absence of silver grains over the tunic (sclera, eye muscles, and extraocular connective tissue) (Fig. 2), as well as over the hypothalamic tissue near the optic tract (Fig. 3), proves the initial confinement of the injected mass to the source. Peripheral blood samples proved that only a minimal amount of the injected free amino acid had escaped into the circulation. This, at its highest, was only slightly above background noise level and was as low 3 hr after injection as during the following 4 days, when label freed from protein breakdown could have reappeared in the blood. There were, however, silver
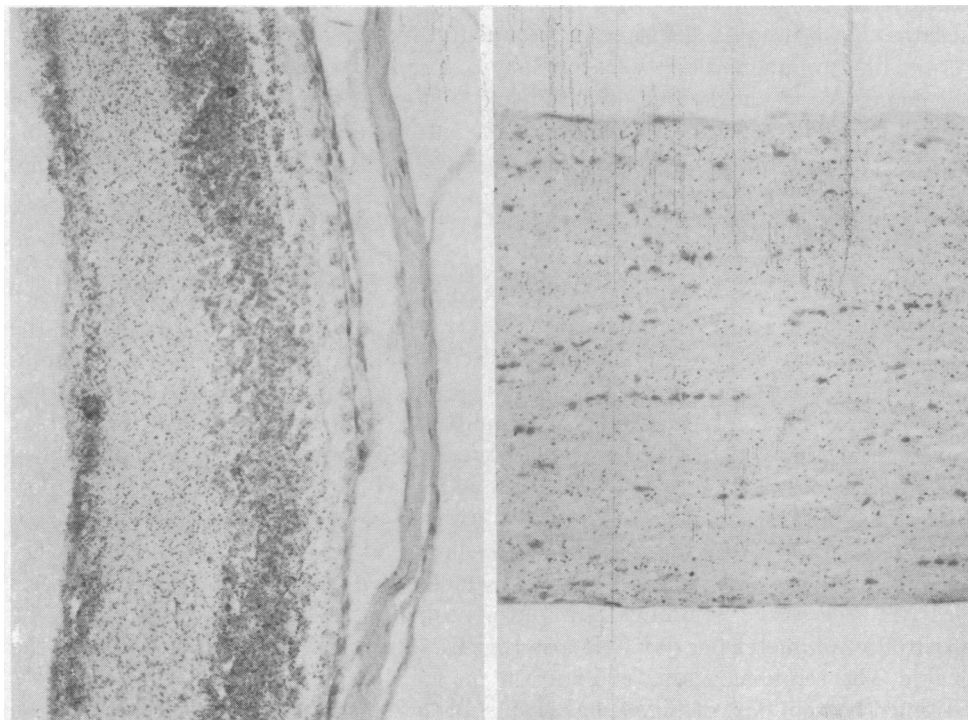

FIG. 2.—Distribution of silver grains over the retina (*left*) and optic nerve (*right*), 3 days after injection, showing the absence of label over the scleral coat.
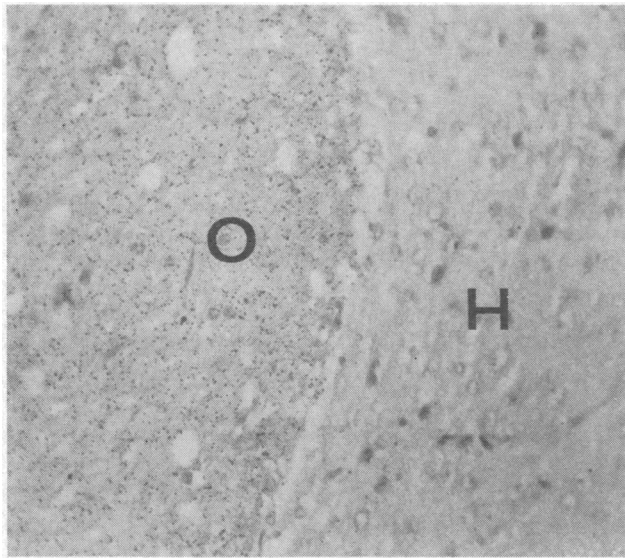
Fig. 3.—Section through the optic tract (*O*) near the chiasma 7 days after injection, showing absence
of silver grains over the surrounding hypothalamic tissue (*H*).

grains over the optic nerve (Fig. 1). Since axons do not take up labeled amino
acid,[13-15] this early nerve record must be ascribed to uptake by glia and connective
tissue cells of label that had diffused between the optic fibers within the dural
sheath. At 3 hr, this diffusion and nonneural incorporation is limited to the near-
retinal first millimeter of nerve, dropping off sharply (stippled area in Fig. 4). Since
the counts for the next three days, plotted in Figure 4, show essentially the same
initial slope, we conclude that most of the extra-axonal diffusate has thus become
fixed. Its distribution, therefore, can serve as base line for the intra-axonal
recordings. (The 5-day curve has been omitted because activity in both eyes of
that mouse was inordinately low.)

According to Figure 4, the distribution of labeled material shifts systematically
down the optic nerve with time elapsed since injection. One notes a daily increase
in the concentration of label at the 5-mm level, while at the more proximal levels, the
density begins to recede in reverse order (at 1 mm: $1 < 2 > 3 > 4 > 6$; at 2 mm:
$1 < 2 < 3 > 4 > 6$; at 3 mm: $1 < 2 < 3 < 4 > 6$; at 4 mm: $1 < 2 < 3 < 4 < 6$).
Grain counts for the 7- and 8-day cases indicated that the wave crest had passed
beyond the 5-mm length of nerve between eye and chiasma. As can be seen in
Figure 3, the labeled material at this stage has already entered the intracentral por-
tion of the optic tract.

Correcting for the fixed extra-axonal "background" by plotting the "above-back-
ground" activity only, one obtains the set of curves of Figure 5. This reveals
clearly a progressive retinofugal shift of the peak of intra-axonal label at a rate of the
order of a millimeter per day. It also reveals, however, a gradual flattening of the
peak. The semidiagrammatic Figure 6, in which the 10 per cent "noise level"
(stippled bottom part of Fig. 4) has been subtracted, shows both the progress of the
wave and the gradual widening of its crest. One also notes a marked asymmetry in
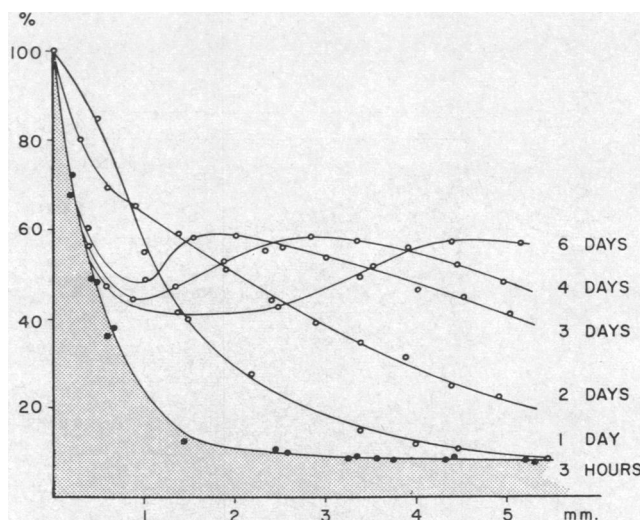the wave form, the trailing side being steeper than the leading one.

Fig. 4.—Density variation of silver grains along six optic nerves fixed at different days after injection. (Explanation in text.)

The conclusions to be drawn from these features are the following. The lag on day 1 of about $1/2$ mm between nerve exit and peak is, of course, due to the different distances between the exit points of the optic fibers and their cells of origin in the retinal hemisphere. The further flattening of the gradient, especially on the trailing side, indicates that the intra-axonal transport proceeds at different rates in different axons. The picture is not unlike that of a horse race, the field starting off staggered (with handicaps) and dispersing in accordance with the individual speeds.

Discussion.—In conclusion, the two objectives listed in paragraph 3 of this paper have been essentially accomplished: (1) The primary injection had remained confined intraocularly. There was some secondary interstitial penetration into the optic nerve, the rate of which corresponds closely to the proximodistal flow of *endoneurial* (extra-axonal) fluid recorded in peripheral nerve[4] (>1 mm per hour); it may represent what has been described[16] as "swift somato-axonal" transport, but to judge from the steep decline of the curve, its labeled amino acid content is rapidly exhausted by incorporation in the endoneurial and sheath cells. (2) The normalized
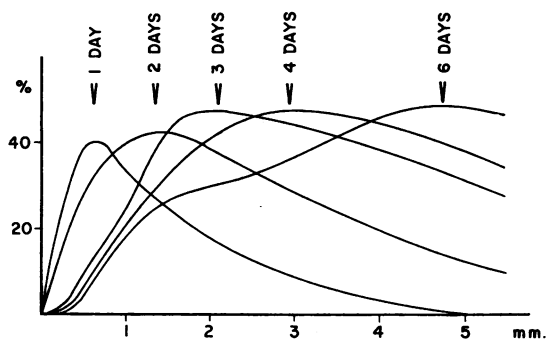

Fig. 5.—Distribution of intra-axonal activities after subtracting the extra-axonal activity at 3 hr (stippled area in Fig. 4).
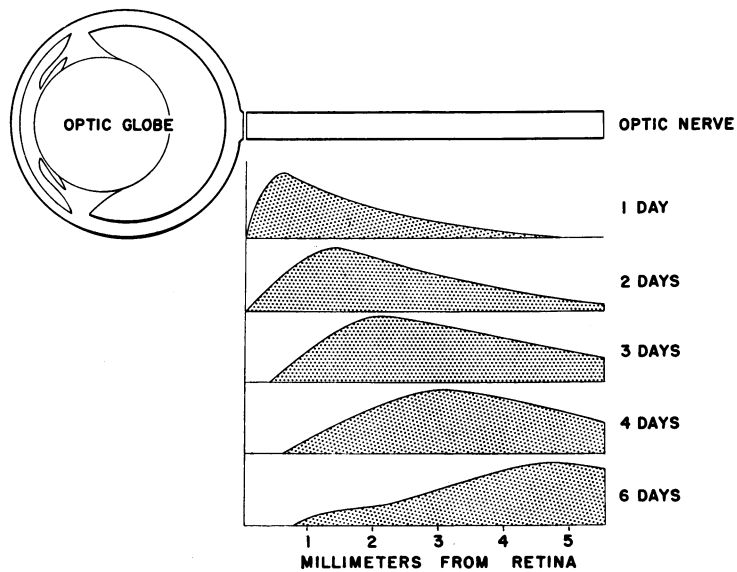
FIG. 6.—Changes in the form of the advancing wave on successive days.

curves representing the intra-axonal flow patterns in the two optic nerves of any one animal fixed on a given day are sufficiently similar to enable one to use one eye as control for the other one in experiments on the effects of extraneous factors on the rates of neuronal synthesis and axonal flow (e.g., light versus darkness). Experiments to produce such bilateral asymmetries are under way.

The isotope technique, by its very nature, cannot decide whether the cellulifugal advance of the labeled protein connotes transport of protein in solution *within* the axon or a translatory movement *of* the axon as a semisolid column containing the labeled protein in structural fixation, or even a combination of both modes of convection. Therefore, it is important to stress once more that other techniques have furnished crucial evidence for the movement of the axon as a whole.[2, 17] The earlier evidence has recently been further strengthened by the demonstration that the axonal mitochondria are swept along in the axonal stream.[18]

The rate of reproduction of neuroplasm in the ganglion cell body can then be estimated from the rate of its drainage by axonal flow. For a nerve cell of 20 μ in diameter, i.e., a volume of 4000 $\pi/3$ μ^3, with an axon of 2 μ caliber and a daily advance of 1 mm, the daily drainage would be 2000 $\pi\mu^3$, or approximately $1^1/_2$ times the volume of the cell. Considering nuclear and other relatively more stable cell structures, this represents a *minimum* value.

Summary.—Localized labeling of the proteins of retinal ganglion cells by tritiated leucine injected intraocularly has confirmed and further quantified the cellulifugal advance of the axonal column (axonal flow) at the predicted *average* rate of the order of a millimeter per day. The fact that the wave flattens as it progresses indicates that the *actual* flow rate is different in different fibers.

[1] Weiss, Paul, and Hallowell Davis, *J. Neurophysiol.*, **6**, 269–286 (1943); Weiss, Paul, *Anat. Record*, **86**, 491–522 (1943).

[2] Weiss, Paul, and H. B. Hiscoe, *J. Exptl. Zool.*, **107**, 315–395 (1948).

[3] Hydén, Holger, *Acta Physiol. Scand.*, **6**, (Suppl. 17), 5–136 (1943).

[4] Weiss, Paul, Hsi Wang, A. C. Taylor, and M. V. Edds, Jr., *Am. J. Physiol.*, **143**, 521–540 (1945).

[5] Shepherd, E. H., dissertation, U. of Chicago (1951).

[6] Samuels, A. J., L. L. Boyarsky, R. W. Gerard, B. Libet, and B. Brust, *Am. J. Physiol.*, **164**, 1 (1951).

[7] Ochs, S., and E. Burger, *Am. J. Physiol.*, **194**, 449–506 (1958).

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

[9] Weiss, Paul, in *Symposium on the Effect of Use and Disuse of Neuromuscular Functions*, ed. E. Guttman (Prague, 1963), p. 171.

[10] Ochs, S., D. Dalrymple, and G. Richards, *Exptl. Neurol.*, **5**, 349–363 (1962).

[11] Droz, B., and C. P. Leblond, *J. Comp. Neurol.*, **121**, 325–346 (1963).

[12] Kopriwa, B. M., and C. P. Leblond, *J. Histochem. Cytochem.*, **10**, 269 (1962).

[13] Amano, cited in ref. 11 on p. 334.

[14] Utakoji, T., and T. C. Hsu, *J. Exptl. Zool.*, **158**, 181–201 (1965).

[15] Rahmann, H., *Z. Zellforsch.*, **66**, 878–890 (1965).

[16] Miani, N., *J. Neurochem.*, **10**, 859–874 (1963).

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

[18] Weiss, Paul, and P. A. Pillai, these PROCEEDINGS, **54**, 48–56 (1965).

SURVIVING CYTOPLASTS IN VITRO

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Enucleated fragments of the unicellular alga *Acetabularia* have been shown by Hammerling[1] to be capable of growth and differentiation. We describe here a further simplification of this biological system. From enucleated *Acetabularia* cells we have obtained and are studying the characteristics of small droplets of cytoplasm. These droplets appear to contain all the components of a live cell except for the nucleus and the outer cell walls. The droplets retain many complex vital processes for as long as several weeks. We propose to call these "live" droplets "cytoplasts."

Cytoplasts differ from protoplasts in that the latter structures contain nuclei and lack only the outer cell walls. Kuroda[2] has studied the protoplast droplets from *Nitella* cells; the protoplasts exhibited active motion of cytoplasmic fibrils on their surfaces and survived for up to 50 hr. Cocking[3] and also Ruesink and Thimann[4] have studied protoplasts from *Avena* coleoptiles and from tomato roots; the protoplasts were prepared by enzymatic digestion of the cell walls and survived for only a few hours. Allen[5] obtained droplets of cytoplasm from amoeba which continued their typical streaming motion for about 70 min.

In this paper, methods are described to obtain and maintain active cytoplasts of *Acetabularia*. Preliminary observations are noted on some of their properties.