# *NEURONAL DYNAMICS AND AXONAL FLOW, III. CELLULIFUGAL TRANSPORT OF LABELED NEUROPLASM IN ISOLATED NERVE PREPARATIONS**

By Paul Weiss

THE ROCKEFELLER UNIVERSITY

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The perpetual reproduction of neuroplasm in the mature nerve cell body and its cellulifugal transport down the axon[1] is a highly complex phenomenon. Its quantitative study by isotope labeling calls for the pinpointing of the label to the nerve cell body (perikaryon) without contaminating the surroundings. As reported in two earlier communications, localized application to the cells of origin of the optic[2] and olfactory[3] nerves proved successful because of their sequestered sites. Other nerve sources, e.g., ganglia, are not located so favorably for treatment *in situ*. The method of choice for those less secluded ones is experimental isolation for the labeling procedure, as described in the following note.

Sensory nerves were excised with their ganglia of origin, the ganglionic end was immersed in isotope solution, shielding the nerve against contamination, and after thorough washing, the whole preparation (i.e., tagged ganglion with its supposedly untagged nerve attached) was transplanted subcutaneously into an unlabeled animal. (The advantage of restrictive labeling is partly offset by the unavoidable trauma and temporary interruption of vascular supply of treated tissue.) In one series, the labeled preparations were reared in a nutrient medium *in vitro*, instead of subcutaneously.

*Materials and Methods.*—Intercostal spinal ganglia with their nerves attached were excised from young mice and grouped in pairs. The ganglionic ends of both were then immersed in a pit containing 0.01–0.02 ml of tritiated 5-DL-leucine solution (1 mc/ml). A barrier of nonwettable silicon grease and vaseline was placed directly behind the ganglia to preclude radioactive contamination of the peripheral nerves by surface seepage. The individual nerves measured 0.1–0.2 mm in diameter and 6–8 mm from ganglion to distal cut. After periods varying from 15 minutes to 5 hours (see below), they were washed repeatedly in Earle's balanced salt solution. Then the ganglion of one nerve of each pair was cut off. Thereafter each pair was again treated alike; both nerves were threaded side by side under the skin of an untreated mouse and sacrificed 1, 3, 4, 5, 7, and 14 days later. Some mice received several pairs (Table 1).

The pairing of nerves, treated identically except for the postlabeling deganglionation of one in each pair, made possible a direct comparison between primary diffusion into the nerve during immersion treatment and the expected subsequent displacement of labeled material directly attributable to the presence of ganglion cell bodies.

Radioactivity in the retrieved nerves was determined by a liquid scintillation counter according to Gupta.[4] After amputation of the single remaining ganglion in each pair, each nerve was cut by a special calibrated microguillotine into 2-mm segments (volume per segment 0.03–0.05 cu mm). Counts are given throughout in readings per 10 minutes. The ganglion and the serial 2-mm segments distally are

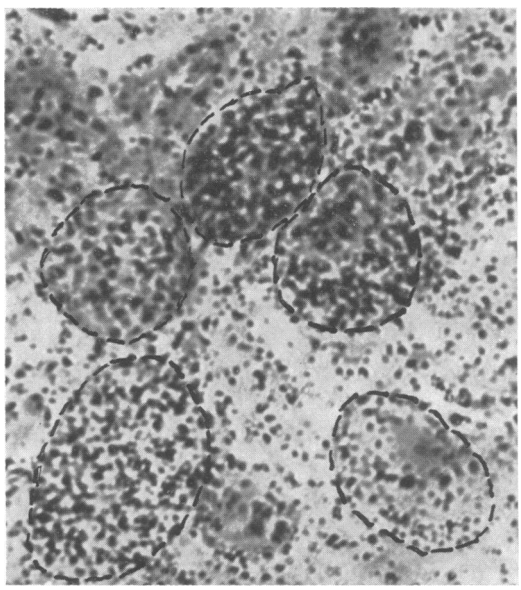

FIG. 1.—Autoradiograph of 4-$\mu$ section through ganglion fixed immediately after 2 hr immersion in $H^3$-leucine solution. Dotted lines trace contours of five ganglion cells showing different degrees of incorporation of label.

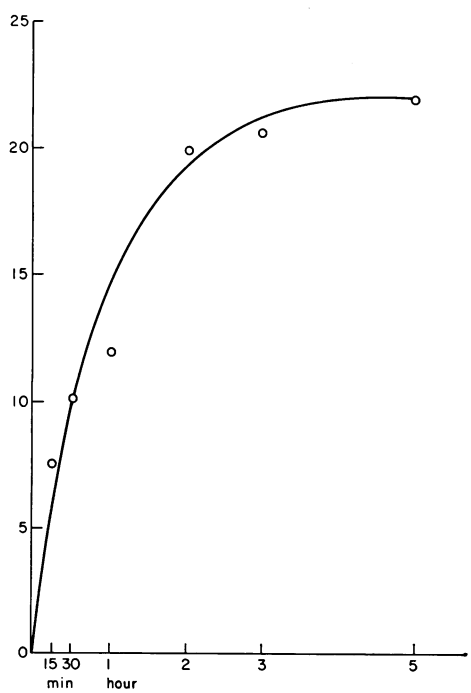


FIG. 2.—Time course of incorporation of $H^3$-leucine in intercostal ganglia immersed in labeling solution (afterwards kept for 1 day as subcutaneous grafts in "cold" host). Ordinate = counts in thousands.

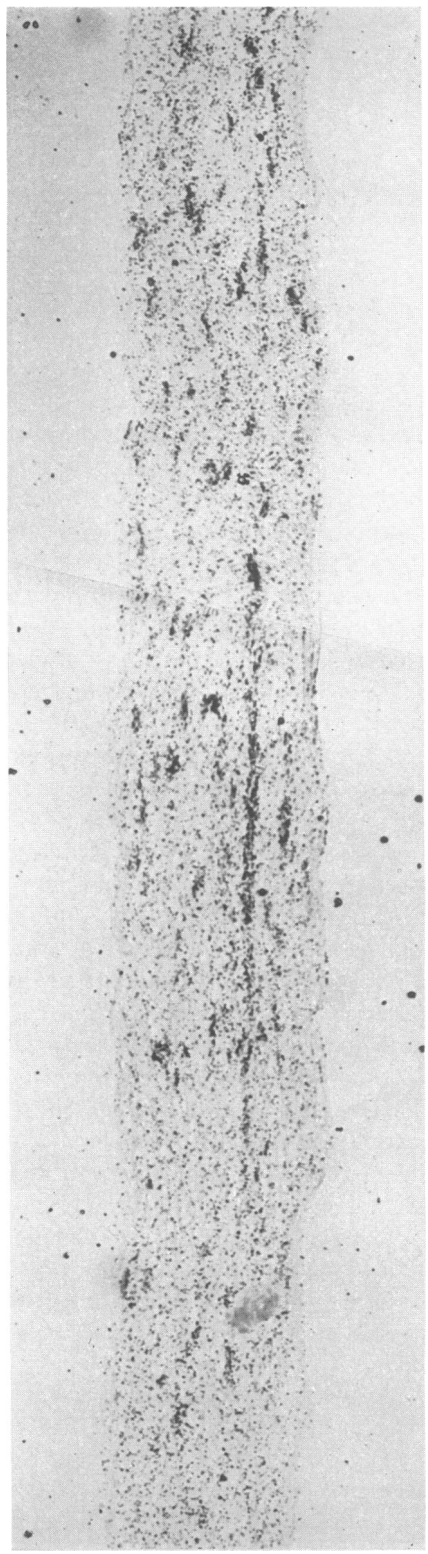

TABLE 1

TALLY OF EXPERIMENTS

| Serial number | Number of host mice | Number of labeled donor nerves | Days left in host |
|---|---|---|---|
| 3632 | 2 | 8 | 1 |
| 3633 | 2 | 6 | 3 |
| 3634 | 1 | 8 | 1 |
| 3635 | 1 | 6 | 1 |
| 3636 | 3 | 9 | 3, 7, 14 |
| 3637 | 3 | 6 | 1, 4, 7 |
| 3638 | 5 | 29 | 1, 4, 7 |
| 3640 | 4 | 37 | 1, 4, 7 |
| 3642* | 10 | 60 | 1, 4, 7 |
| 3643 | 4 | 12 | 5 |
| 3644 | 3 | 12 | 4 |
| 3645 | 3 | 12 | 4 |
| 3646† | — | 50 | 1, 4, 7 |
| Total | 41 | 255 | |

* All nerves deganglionated and immersed whole into labeling solution.
† *In vitro* experiments.

identified as G, $s_1$, $s_2$, $s_3$, and $s_4$. The whole extraganglionic portion of the preparation will be referred to as "S."

In some cases, autoradiographs of equidistant 4-$\mu$ sample sections were also made and evaluated by counts of silver grains in the coating emulsion.

*Results.*—(*a*) Autoradiographs of sections of control ganglia immersed in labeling solution for two hours, washed, and fixed immediately showed a relatively sparse background population of silver grains over the tissue against which some nerve cell bodies stood out sharply by their intense radioactivity, the intensity varying markedly from cell to cell (Fig. 1). The results show that two hours' immersion in the isotope is sufficient for heavy labeling of protein in perikarya; that uptake and incorporation times differ considerably between neighboring ganglion cells; and that free amino acid has been satisfactorily washed out.

(*b*) Optimum immersion time was ascertained by determining total radioactivity in one-day transplants (expts. 3634 and 3635, Table 1) whose ganglia had been dipped for periods of from 15 minutes to 5 hours. As seen in Figure 2, uptake and incorporation have reached over 30 per cent saturation in 15 minutes, and 90 per cent in 2 hours. The total radioactivity incorporated in specimens immersed for 2 hours was determined as roughly 10 per cent of that of an equal volume of the original leucine solution.

(*c*) The capacity for direct uptake of leucine by the ganglion-free parts of nerves (S) was determined for 23 and 19 nerves immersed for 15 minutes and for 2 hours, respectively. The mean activities per nerve of the 15-minute and 2-hour specimens were 8.058 and 25.570, respectively, i.e., in the same ratio of approximately 1:3 as in (*b*).

(*d*) The degree of radioactive contamination of the nonimmersed, extraganglionic nerve portion S was determined by autoradiography of nerve washed and fixed immediately after two-hour immersion of its ganglion. Incorporated lable was present in the endoneurial spaces, particularly in blood vessels (Fig. 3). Grain

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FIG. 3.—Autoradiograph of distal nerve portion close to exit from ganglion, fixed immediately after the latter had been immersed for 2 hr in isotope solution. Note heavy concentration of silver grains over blood vessels and lighter activity in other endoneurial tissue. Proximodistal direction from top to bottom.

density declined steeply in a proximodistal gradient; spread in flow channels became indistinct 2.7 mm distally to the ganglion but some scattered grains were still found farther down (see below). The form of this endoneurial seepage, predictable from our experience with intraocular injections,[2] is shown in Figure 4, con-

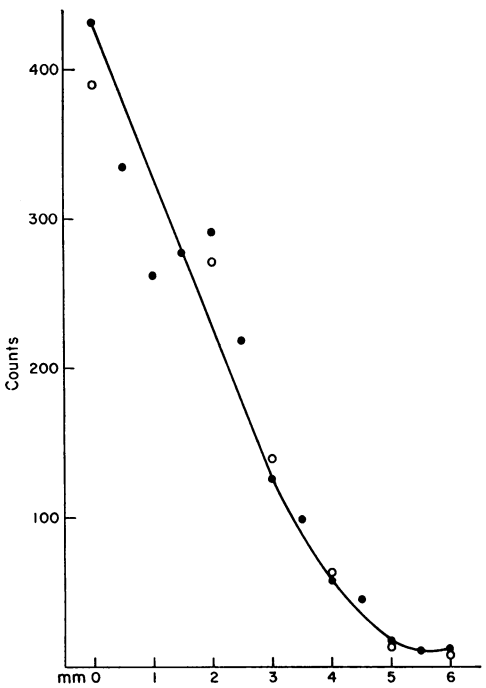


FIG. 4.—Proximodistal decline of primary infiltrate into nerve from ganglion (2 hr $H^3$-leucine-immersed, then 1 day subcutaneously), determined from two independent sets of counts of silver grains plotted as ordinates over distance from ganglion.

structed from two separate sets of counts (solid circles and open circles) of silver grains at 0.5-mm intervals along the nerve axis (corrected for minor local variations in section width), each counted strip containing *ca.* 40,000 $\mu^3$ of nerve substance. The grain count over the first sample strip at the exit from the ganglion averaged 450 grains, had declined by 50 per cent at 2 mm farther distally, and reached background level at 5 mm. This initial infiltration of the nerve can evidently be ascribed to the regular proximodistal convection of endoneurial fluid at the rate of a few millimeters per hour, demonstrated previously[5] and shown to continue for hours even in excised nerves. All further increments of radioactivity in the nerves S during their subsequent residence in a host animal or *in vitro* express secondary acquisitions of labeled material from the ganglia. These changes are summarized in the following, comparing always the grafted nerve, which had been left in possession of its labeled ganglion cells as source of radioactive neuroplasm, with its companion nerve deganglionated prior to grafting and serving as control base of initial infiltration.

(*e*) In all ganglionated nerves, appreciable amounts of radioactive matter have continued to shift from the ganglia into the more distal ("postganglionic") portions S, both in subcutaneous grafts and *in vitro*. Changes in the distribution pattern within the postganglionic stretch S were less definite, partly because of the variable longitudinal shrinkage of the isolated nerves, which averaged 8, 12, and up to 33 per

cent for days 1, 4, and 7, respectively, thus making contents of 2-mm standard samples not strictly comparable between different days. The first significant postgrafting shifts were recorded in the four-day specimens. A sample set (3644, six nerve pairs; 3645, five nerve pairs) is reproduced in Figure 5, in which radioactiv-

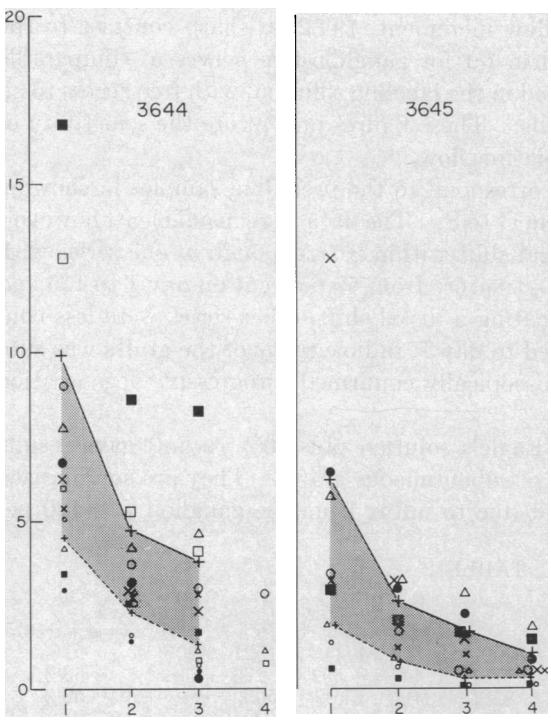


FIG. 5.—Activity counts in nerve pairs grafted subcutaneously for 4 days *with* and *without* their "hot" ganglia, the former identified by larger symbols, the latter by identically shaped smaller ones. Means for each set (+,+) are connected by lines, the shaded area between means for the ganglionated (+) and deganglionated (+) sets representing the 4-day increment of radioactivity in the former.

ity counts in consecutive 2-mm segments (ganglionic counts omitted) have been plotted for the ganglionated nerves and their deganglionated controls. The shaded areas give the mean differences between ganglionated and deganglionated sets, i.e., the four-day increments of the radioactive content in the postganglionic portions S of those nerves left in possession of their cell bodies over that of their otherwise comparable deganglionated controls. Distribution of radioactivity in the deganglionated controls has remained essentially stationary. Initial seepage on the first day (Fig. 4) accounts for a ratio of activities between consecutive segments $s_1$, $s_2$, and $s_3$ of 100:43:10. On the fourth day, the ratios in the deganglionated control nerves (Fig. 5) were still about the same, i.e., 100:51:29 and 100:44:22, respectively, revealing no major translocation within the control nerves.

(*f*) All surplus registered in the ganglion-connected nerves over their deganglionated mates during the postlabeling period must have moved in from the ganglionic source by axonal flow. At four days, this increment, calculated for series 3644 and 3645 from the data underlying Figure 5, amounted to 140 and 250 per cent, respectively. Actual protein transfer was calculated as follows. At four days, the mean increments in S of the two ganglionated nerves were 9,500 and 7,500, while the counts of G averaged 63,780 and 34,870. Uncorrected, this would correspond to a three-day shift from G to S of 13 and 18 per cent, respectively.

Considering that the ganglionic count includes both nerve-cell and satellite-cell activity in a ratio (determined from autoradiograms) of roughly 55:45, these figures rise to 24 and 32 per cent, i.e., a daily move of 8–11 per cent of the proteins of the nerve cells into their axons.

(*g*) Mean activities per nerve in the specimens of (*e*) were, for the primary infiltrate, 1,036, and for the axonal flow increment, 1,682, in sharp contrast to the much higher value of 25,570 found in (*c*) for ganglion-free nerves of comparable length that had been totally immersed in the labeling solution, with free access to all Schwann's and other intraneural cells. These figures underscore the sensitivity of the method for the recording of axoplasmic flow.

(*h*) The other four-day results correspond to the preceding samples in showing massive influx of active material from G to S. The data were insufficient, however, to prove a definite further centrifugal shift within S. Although in one series with 29 nerves (3638), the ratio of $(s_2 + s_3):s_1$ rose from 57 per cent on day 1 to 126 and 152 per cent on days 4 and 7, indicating a distal shift, other series were less conclusive. In all experiments extended to day 7, radioactivity of the grafts was substantially diminished, due to (microscopically confirmed) progressive degeneration of the transplanted ganglion cells.

(*i*) The nerves reared *in vitro* (Eagle's solution plus 10% serum) gave results essentially in agreement with those of subcutaneous grafts. They are summarized in Table 2. The average increment due to influx from the ganglion was 120 per

TABLE 2

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| Day | State | No. of nerves | G | $s_1$ | $s_2$ | $s_3$ | $s_4$ | S/G | $(s_2 + s_3 + s_4)/s_1$ |
| 4 | With ganglion | 11 | 67,611 | 6,909 | 3,349 | 1,413 | 1,521 | 20% | 91% |
| | Deganglionated | 10 | — | 3,787 | 1,447 | 552 | 276 | — | 60% |
| | Increment | | — | 82% | 134% | 156% | 450% | — | 52% |
| 7 | With ganglion | 10 | 62,473 | 4,091 | 1,582 | 966 | 769 | 11% | 81% |
| | Deganglionated | 10 | — | 3,513 | 937 | 347 | 232 | — | 43% |
| | Increment | | — | 17% | 69% | 180% | 230% | — | 88% |

cent on day 4, and 48 per cent in the seven-day group. The increment by day 4 of 7.130 represents 17 per cent of the ganglionic nerve cell protein, calculated as in (*f*) as 55 per cent of the total ganglionic count and corrected for the drain into the nerve. This connotes a daily G to S movement of about 6 per cent of cell protein, slightly less than in the subcutaneous transplants. The seven-day cases are within range, but unreliable because of the extensive degeneration of neurons in the interim. The further proximodistal shift within the postganglionic nerve portion was more marked than in the subcutaneous grafts, as is evidenced by the steady rise of the incremental percentages from $s_1$ to $s_4$ (Table 2, columns 5–8), and in the gain of the activity ratios of the more distal segments, $s_2 + s_3 + s_4$, over the most proximal ones, $s_1$ (Table 2, column 10) of 52 per cent by day 4 and 88 per cent by day 7. Without necessarily putting reliance on the quantitative accuracy, the general trend seems assured by these data.

*Conclusions and Summary.*—In sum, the results lead to the following conclusions: (1) The localized labeling by tritiated leucine of ganglionic protein in excised isolated nerve preparations is feasible. (2) There is initial time-limited seepage of incorporated label through endoneurial channels, which has been recorded quantita-

tively (up to several millimeters per day). (3) Intraneuronal protein transport through axonal flow from ganglion to peripheral nerve continues in isolated preparations after subcutaneous transplantation and explantation *in vitro*. (4) Daily translocation of intraneuronal protein from ganglion to nerve is between 6 and 11 per cent. On the tenuous assumptions that protein concentration in axons is roughly 4 per cent, that most nonproteinaceous compounds also come from the perikaryon, and that most axonal water is structurally bound, this would indicate a daily movement down the axon of between $1^1/_2$ to 3 times the volume of ganglionic mass. For a nerve cell of 40 $\mu$ diameter with an axon of 6 $\mu$ diameter, this would amount to an advance of axonal flow of the order of 1 mm per day, which agrees with our previous determinations. (5) Some indications of proximodistal movement within the nerve portion itself were also obtained. (6) Besides providing confirmation and better quantification for the phenomenon of neuroplasmic flow, the method seems uniquely suited for short-term mass experiments.

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