FAST AND SLOW COMPONENTS IN AXONAL TRANSPORT OF PROTEIN

BRUCE S. McEWEN and BERNICE GRAFSTEIN

From The Rockefeller University, New York 10021

ABSTRACT

(*a*) After injection of labeled leucine into the eye of goldfish, radioactive protein rapidly accumulates in the contralateral optic tectum in the layer containing the synaptic endings of the optic fibers. This material reaches the tectum 6–12 hr after the isotope injection, a fact which indicates that the rate of transport is at least 40 mm per day. (*b*) This rapidly transported material has been shown to consist exclusively of protein, in which the label remains attached to leucine. (*c*) Inhibition of protein synthesis in the retina prevents the appearance of the transported protein in the tectum, but inhibition of protein synthesis in the tectum does not. Substances having some of the same properties as leucine, such as cycloleucine and norepinephrine, are not transported to the tectum. These experiments all indicate that the transported protein is synthesized in the retina. However, inhibition of retinal protein synthesis after this protein has been formed does not interfere with the transport mechanism itself. (*d*) The fast component consists of about 85% particulate material. It may be distinguished from a slowly moving component, transported at 0.4 mm per day, which contains about 5 times as much radioactivity as the fast component, and which consists of 60% particulate matter and 40% soluble protein.

INTRODUCTION

Flowing down the axon of every nerve cell there is a continuous stream of materials that have been synthesized in the nerve cell body. The rate at which these materials are transported was first established in 1948 by Weiss and Hiscoe (29) in their classic experiments on constricted nerves. After having observed that the fibers above a constriction swelled because of the build up of transported materials that could not get past the constricted region, Weiss and Hiscoe removed the constriction, and found that the "bolus" of accumulated material moved down the axon at a rate of 1 mm per day. In subsequent experiments performed in a number of laboratories including Weiss's own, radioactive tracers were used to provide direct evidence of the transport phenomenon (5, 19, 26, 27), but even with these more sophisticated methods there was very little need to revise the original estimate of the rate of transport. Although there have been occasional reports of values as high as 11 mm per day (11), values of 1–5 mm per day have more generally been found in mammalian nerves (16).

Recently, there have been indications that a much more rapid movement of newly synthesized material can also occur in axons (2, 4, 7, 8, 10, 13, 14, 18, 21, 24). One of the earliest reports came from Miani (18), who used radioactive phosphate to label phospholipids and radioactive amino acids to label proteins. His results indicated rates of transport of up to 70 mm per day.

Grafstein (8), studying the transport of radioactive protein in the fish optic system, obtained evidence of two rates of protein movement, one slow and the other fast. We obtained the slow rate, about 0.4 mm per day, by estimating the rate of movement of radioactivity in the optic nerve, and this rate undoubtedly corresponds to

the classical axoplasmic flow discovered by Weiss and Hiscoe (29). The fast rate, however, was found to be more than 25 times faster. It applied to material which accumulated in the layer of the optic tectum containing the synaptic endings of the optic nerve fibers. McEwen, working independently with different objectives and techniques, also observed the rapid movement of radioactive protein from the eye to the optic tectum.

This paper is a report of a collaboration between our two laboratories in examining some of the characteristics of the fast and the slow transport. These experiments show that the fast component moves even faster than we originally believed and that it is synthesized by the retina. We have also found that this fast component consists mainly of particulate material, i.e. protein attached to or part of cell particles or organelles, while the slow component consists of a high proportion of soluble proteins in addition to particulate elements. These experiments lead us to propose that the rapidly transported component represents a mechanism for the transfer of certain substances from the cell body to the synaptic endings, whereas the slow transport provides replacement materials for the axoplasm. Some of the material in this paper has been reported in a preliminary form elsewhere (17).

EXPERIMENTAL PROCEDURE

Goldfish (*Carassius auratus*) 3–4 in. in length were obtained from a local dealer. For some experiments, large "pond" fish 6–8 in. in length were also used because of the larger size of the optic nerve.

Injections into the posterior chamber of the eye of unanesthetized fish were made with a 10-μl Hamilton syringe. A polyethylene sleeve over the fixed needle allowed only 2–3 mm of the needle to enter the eye. The injection was made into the eyeball at the border of the retina, and during the injection the tip of the needle behind the lens was visible through the cornea. Each fish was injected in the right eye with 2–4 μl of the isotope solution (1 μc/μl), and in the left eye with an equal volume of unlabeled leucine (13.1 γ/μl). The injection procedure rarely produced bleeding inside the eye, and immediately after the injection the fish responded normally to visual stimuli such as a sudden movement of the hand over the aquarium.

Isotope for most experiments was leucine-4,5-^{3}H (5–35 c/mmole) obtained from New England Nuclear, Boston, or Nuclear Chicago, Desplaines, Ill. Acetoxycycloheximide (AXM) was a gift from Dr. T. J. McBride of the J. L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, N.J., and was produced there with the support of National Institutes of Health contract No. PH 43-64-50.

Radioactively labeled fish were decapitated and the brain was rapidly exposed. For radioautography, the brain was fixed for up to 48 hr in Bouin's solution, and then processed in the usual way (12). For analysis of the fresh tissues, the telencephalon and underlying cartilage forming the base of the skull were cut away, and the optic nerves were fully exposed. We removed the nerves by cutting them at the tectum and 0.5 mm from the eyeball, then separating them at the chiasma. The midbrain with the optic tecta was lifted out of the skull and placed under a dissecting microscope, and the tecta were peeled away from the tegmentum.

Radioactive analyses were performed according to one of the following methods:

METHOD 1: For determination of total radioactivity in tissue sample, the fresh tissue was placed on dried, weighed pieces of lens paper, dried in an oven at 100°C overnight, and weighed on a semimicro balance. For assaying the vitreous humor, measured samples of the vitreous were withdrawn with a micropipette and dried on cotton pledgets. The samples were then incinerated for scintillation counting by Gupta's technique (9), and counted in a Packard Model 3375 Liquid Scintillation spectrometer. Results were expressed as counts per minute or disintegrations per minute per mg dry weight of tissue (or per microliter of vitreous humor). The counting efficiency was around 41% for tritium. This technique was used in experiments with both tritiated leucine and norepinephrine.

METHOD 2: For assaying TCA-precipitable radioactivity separately from that in free amino acid or other acid-soluble materials, fresh tissue samples were fixed in 3 ml of 10% trichloroacetic acid (TCA) for 24 hr at 4°C. The fixed tissue was removed from the TCA and then dried, weighed, and counted as in method 1. Aliquots, 0.5 ml, of the TCA supernatant were counted in Bray's solution (3) at 18% efficiency. Results were expressed as disintegrations per min per mg dry weight or as the difference in specific activity between left and right nerves or tecta, divided by the specific activity of the right (control) tectum. This normalization procedure permits comparison of results from different experiments despite variations in the specific activities of the injected materials. This technique was used in experiments with labeled cycloleucine as well as leucine.

METHOD 3: For separating soluble proteins from particulate material, fractionation of nerves and tecta was performed by homogenizing pooled tissues from three or four fish in 0.6 ml of 0.25 M sucrose, in a motor-driven Teflon-glass homogenizer. The homogenates were centrifuged at 200,000 *g* for 45 min in 0.8-cc tubes in the SW50 rotor of the Spinco Model L ultracentrifuge. The pellet of particulate material was taken up in 100 μl of 1 N NaOH. Soluble protein was

precipitated from the supernatant by the addition of 0.2 ml of 50% TCA at 4°C. The TCA precipitate was taken up in 100 μl of 1 N NaOH. 5–20-μl aliquots of the NaOH extracts were analyzed for protein by the method of Lowry et al. (15), and 20–40-μl aliquots of the extracts were dried on cotton, combusted, and counted by Gupta's method (9) at 41% efficiency. Aliquots, 0.2 ml, of the TCA supernatant were counted in Bray's solution at 25% efficiency.

METHOD 4: For analysis of TCA-precipitable material in pooled tecta from four or five fish treated in the same way, samples were homogenized in 3 ml of ice-cold distilled water, and 0.8 ml of 50% TCA was added to bring the final concentration to approximately 10%. The TCA-precipitable material was collected by centrifugation and washed three times with cold 10% TCA. For most experiments, the precipitate was dissolved in 1 N NaOH for protein determination and combustion analysis of radioactivity.

For the purification of total protein and subsequent acid hydrolysis and chromatography of amino acids, the following additional steps were employed. The washed TCA precipitate was resuspended in 2 ml of 10% TCA and heated at 90°C for 10 min. After centrifugation, the pellet was treated two times at room temperature with 3 ml of 3:1 chloroform: methanol, and one time each with absolute ethanol and ether. The final lipid-free pellet was then subjected to hydrolysis in 12 N HCl at 90–100°C for 48 hr. The hydrolyzate was evaporated to dryness in a stream of air, dissolved in distilled water, and evaporated twice more, redissolved in a small volume of distilled water, and chromatographed on Silica Gel F plates (E. Merck, distributed by Brinkmann Instruments, Inc., Westbury, N.Y.) with use of butanol/acetic acid/water (8:2:2) for one-dimensional development, and 96% ethanol/34% ammonia (7:3) followed by butanol/acetic acid/water (8:2:2) for two-dimensional development (25). Amino acid spots, visualized by ninhydrin staining, were scraped from the plate into scintillation vials, eluted with 0.5 cc of 0.1 N HCl overnight, and counted in Bray's solution. Elution of radioactivity from the silica gel was 75% complete; to control for this, aliquots of hydrolyzate were evaporated on other plates but not separated, stained with ninhydrin, eluted with 0.5 cc of 1 N HCl, and counted. For presentation of the data, all recoveries were corrected for the incomplete elution.

RESULTS

Localization in the Optic Tectum of Rapidly Transported Protein

Throughout the present experiments, we have adopted the technique of injecting the labeled amino acid into one eye and comparing the resulting distribution of radioactivity in the two sides of the brain. Since the optic nerves in the goldfish cross completely at the optic chiasma, material transported down the nerve from the labeled eye is conveyed exclusively to the contralateral optic tectum. Thus, the rapidly transported protein component could be detected in the contralateral tectum, confined to the layer containing the synaptic endings of the optic fibers (8). Grain counts from radioautographs show that in this layer there is nearly three times as much radioactive protein on the contralateral side as on the ipsilateral side (Fig. 1), while the other layers do not show any statistically significant differences on the two sides.

Another way to measure this accumulation of radioactive protein is to compare the total radioactivity in the tecta (Fig. 2). Fig. 2 *a*, *c*, and *d* shows the distribution of total radioactivity in the tecta and in four other brain regions at various times following intraocular injection of tritiated leucine. The radioactivity in the tectum (left) connected to the injected eye (right) is considerably higher than in the other tectum, presumably reflecting the difference in accumulation of labeled protein in the synaptic layer.

Source of Radioactivity in Other Brain Structures after Intraocular Injections

90 min after the injection of tritiated leucine into the posterior chamber of the goldfish eye, only 10% of the injected dose remains in the vitreous humor (Fig. 3). Only about 4% was found to be incorporated into protein in the retina and presumably only some fraction of this would be transported. Labeled protein in the lens contained 1% of the total radioactivity. If we make a generous assumption that another 5% of the radioactivity can be accounted for as free amino acid in the retina and lens and that 5% is lost by direct diffusion to the other tissues of the eye and orbit, then we are led to the conclusion that, by 90 min after the injection, as least 75% of the injected radioactivity has entered the circulation. In the blood, this amino acid is available for incorporation by most of the tissues of the body, including the cell bodies of neurons throughout the nervous system. It is against this widely distributed background of radioactivity that a difference between the two tecta appears as a consequence of the transport of material down the nerve from the injected eye. That this is the correct interpretation is further illustrated in Fig. 2 *a* and *b*. In this ex-

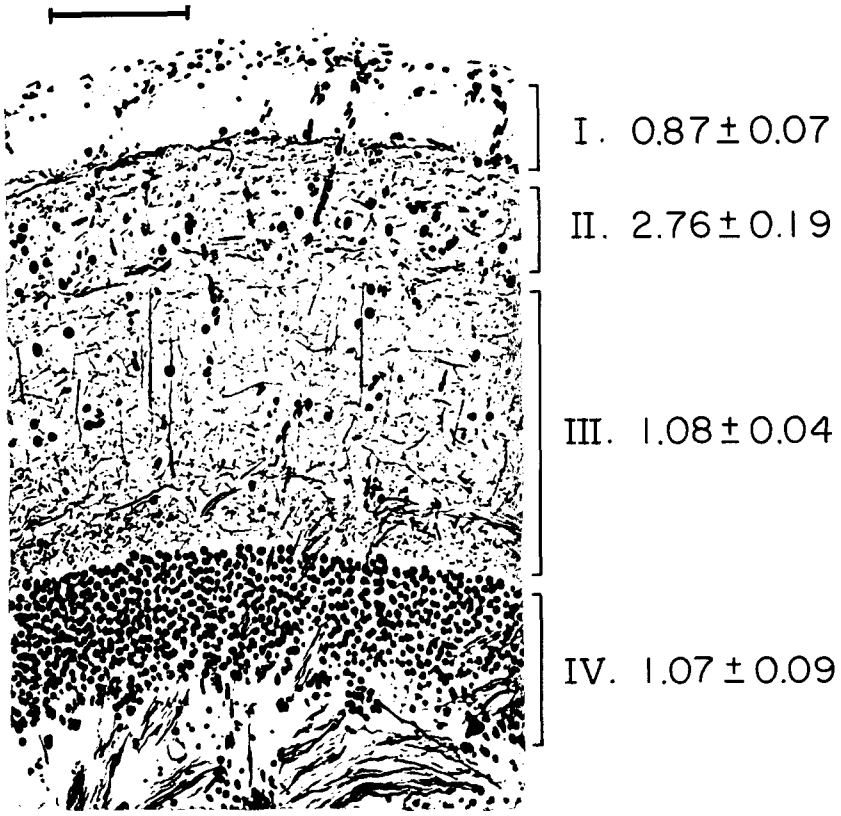

FIGURE 1 Silver grain counts in various layers of optic tectum, from radioautograms of tecta from fish sacrificed 24 hr after injection of labeled leucine. Counts expressed as mean ratio of count in left tectum to count in right tectum ±SE of the mean. Counts made in: *I.* Subpial molecular layer; *II.* Layer of synaptic endings of optic nerve fibers; *III.* Intermediate layer, containing various cell and fiber strata; *IV.* Granule cell layer. In each of seven animals, readings were taken on four different radioautographic sections, over an area of 37,000 μ^2 in each layer. Only the ratio in layer *II* is statistically significant ($p < 0.01$). The layers are indicated on a photograph of a Bodian's-stained section 6 μ thick. The horizontal bar represents 100 μ.

periment, identical amounts of radioactivity were injected either into the right eye (*a*) or into the peritoneal cavity (*b*). With both routes of injection, similar levels of radioactivity were observed 24 hr later in all brain structures except the left optic tectum.

Time Course of Appearance of the Rapidly Moving Material

The rate of accumulation of TCA-precipitable radioactive material in the two optic tecta after the injection of radioactive leucine into the right eye is shown in Fig. 4 *a*. This accumulation includes both material transported down the nerve and material labeled by local background incorporation. On both sides, the material builds up over a period of about 12–18 hr, but the arrival of the material transported along the nerve, as indicated by the difference in radioactivity between the two tecta, occurs over a rather shorter period of time. In the experiment illustrated, the plateau of the transported radioactivity was reached 6–8 hr after the injection. When the results of a whole series of experiments were normalized and graphed (Fig. 4 *b*), the plateau was seen to be attained about 12 hr after the injection.

These results indicate that the rapidly moving component has a rate of transport down the nerve well in excess of previous estimates in which the earliest time point studied was 24 hr after the in-

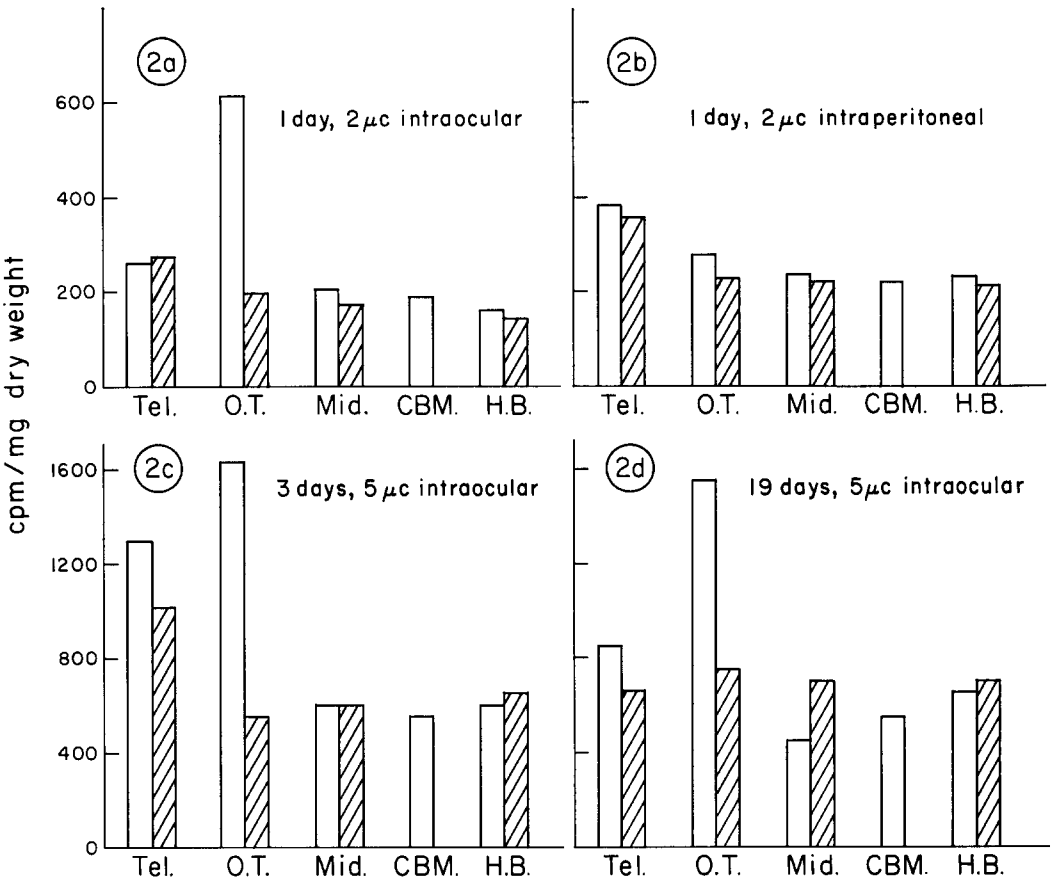

FIGURE 2 "Specific" radioactivity (counts per minute/milligram dry weight) of left and right halves of four regions of the fish brain and cerebellum after injection of leucine-4,5-^{3}H into right eye. Total radioactivity was measured by method 1. Data presented are for single fish and were selected as representative of a large series of determinations. *Tel.* telencephalon; *O.T.*, optic tectum; *Mid.*, midbrain beneath optic tectum; CBM., cerebellum; H.B., hindbrain including vagal lobes.

jection (8). In the goldfish optic nerve, which is about 10 mm long, the rate of movement must be on the order of 40 mm per day if the transport is to be essentially complete by 6–12 hr after the injection. This rate is considerably greater than the rate of 5–7 mm per day that has been reported by Rahmann (24).

The amount of transported material remained constant until at least 30 hr after the injection. In some experiments, there appeared to be a tendency for values to fall off by about 20% at 36 hr (Fig. 4 *b*), but determinations made at later times after the injection (e.g., 11 days in Fig. 7) indicated relatively little further loss. In other experiments (Grafstein, B., and J. Rausch. Unpublished results.), the bulk of the fast component has been found to have a half-life of around 100 days.

Site of Synthesis of Rapidly Moving Component

Early in these studies, it was necessary to rule out the possibility that the fast component was synthesized locally in the synaptic layer of the tectum from free amino acid that had arrived from the eye. An early indication that this is not the case was the observation that, for at least 6 hr after the intraocular injection of tritiated leucine, there is no left-right difference in the TCA-soluble

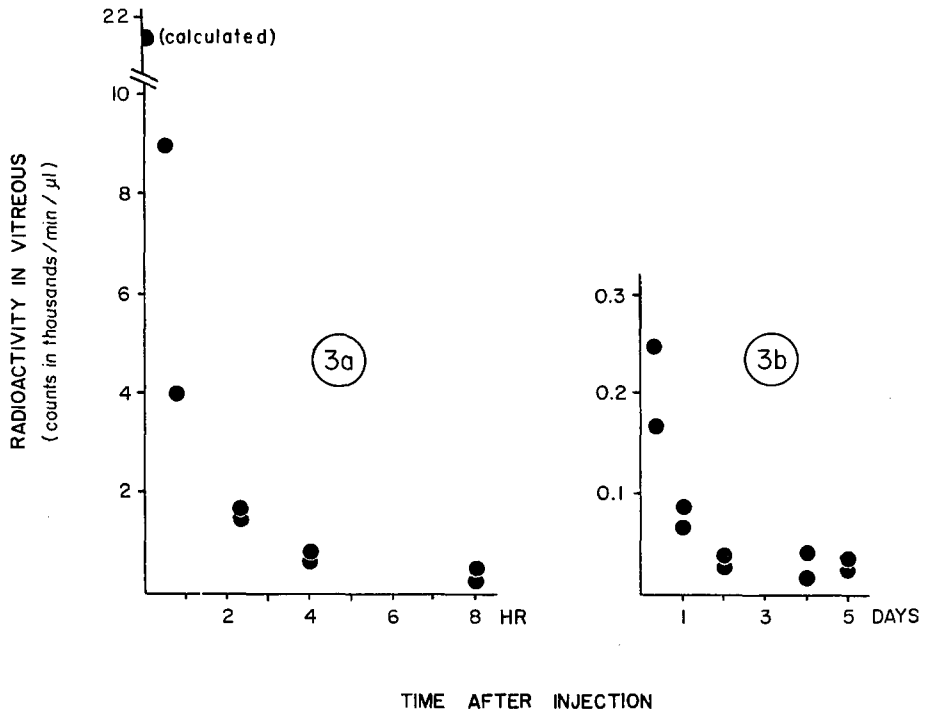

FIGURE 3 Radioactivity in vitreous humor as a function of time after injection of leucine-4,5-^{3}H into the eye. *a*, results for up to 8 hr after the injection; *b*, for 8 hr–5 days. Note different scales of the ordinates. The point at "zero" time was calculated from the dilution of the known volume of injected material in the estimated volume of the eye.

TABLE I

Radioactivity in Tectum 6 hr after Intraocular Injection

Injected substance	Specific activity in tectum		$\frac{L-R}{R}$	Average
	Left	Right		
	cpm/mg dry wt			±SD
*Cycloleucine-1-^{14}C	423	406	0.04	
	680	630	0.08	0.03 ± 0.06
	358	371	−0.04	
‡Norepinephrine-7-^{3}H	168	211	−0.21	
	191	270	−0.29	
	346	294	0.18	−0.02 ± 0.26
	261	179	0.46	
	284	322	−0.12	
	241	281	−0.14	
*Leucine-4,5-^{3}H	894	914	−0.02	
	843	968	−0.13	−0.03 ± 0.08
	789	752	0.05	

* Radioactivity measured in TCA supernatant from each tectum.

‡ Total radioactivity measured by method 1.

pool in the optic tectum (Fig. 4 *b* and Table I). Since we have observed that the TCA-soluble pool represents 95, 68, and 82% of the total radioactivity in the fish brain at 1, 3, and 6 hr, respectively, after injection of tritiated leucine, it is evident that protein synthesis in the fish brain does not immediately utilize precursor as it enters the brain. This makes it extremely unlikely that free leucine could enter the optic tectum from the nerve and be immediately incorporated into protein without being detected first as free amino acid in the tectum. Further evidence against the transport of free amino acid via the nerve into the tectum was obtained by experiments in which labeled cycloleucine or norepinephrine was injected into one eye. Cycloleucine is an analogue of leucine which is actively transported into cells but is not incorporated into protein (20); norepinephrine becomes tightly bound to storage granules in adrenergic neurons, but, since the optic fibers are not adrenergic, it probably remains largely extracellular. It might be expected that if a special circulatory pathway, or an intra- or extracellular diffusion process exists for the transfer of small molecules to the contralateral optic tectum, then we would see one or both of these substances arriving in the tectum in substantial amounts. However, neither one did appear in the contralateral tectum in excess of its concentration on the ipsilateral side (Table I). We therefore have no reason to believe that free amino acid can traverse the optic nerve to the contralateral optic tectum.

The most conclusive demonstration that the labeled TCA-precipitable radioactive material which appears in the contralateral optic tectum is synthesized in the retina and not in the tectum was obtained by the selective inhibition of protein synthesis at these sites with acetoxycycloheximide (AXM). The inhibitor was injected either into the right eye or into the cranial cavity 2 hr before tritiated leucine was injected into the right eye. Pooled samples of left and right tecta from four or five fish treated in the same way were analyzed for radioactivity and protein. Transport of the fast component to the contralateral tectum was measured as the difference in specific activity of TCA-precipitable radioactivity between the left and right tecta and is expressed as per cent of values for groups of four or five control fish (which had received no AXM). Incorporation of tritiated leucine into TCA-precipitable material of the right retina, expressed as per cent of values for control fish, was used as an indication of the extent of inhibition of incorporation at the presumed site of synthesis of the fast component. Incorporation of leucine into TCA-precipitable material of the right tectum (which does not receive the radioactive fast component), expressed as per cent of values for control fish, was used as an index of the extent of inhibition of "background" incorporation of amino acid arriving in the left and right tecta from the blood. It can be seen in Fig. 5 that the amount of transported material appearing in the left tectum was proportional to the amount of incorporation in the right retina and was not related to the amount of "background" incorporation in the right tectum. For example, in experiment 1, in which 0.2 γ of AXM was injected intracranially, so that incorporation in the tectum was strongly inhibited, retinal incorporation was relatively unaffected, and the arrival of the transported material was not interfered with. By contrast, in experiment 4, in which 0.05 γ of AXM was injected into the eye, the transported protein failed to appear at all, although tectal incorporation in the right tectum was completely unaffected; retinal protein synthesis, on the other hand, was very severely inhibited. It might be argued in this case that the intraocularly injected AXM had, in fact, been transferred along the nerve to the contralateral tectum. Under these conditions, however, it would be expected that incorporation into the left tectum would be reduced below the level in the right tectum, giving a large "negative" value for the "transported" protein component. In fact, this value is very close to zero in experiment 4 and above zero in experiment 3 (Fig. 5).

Effects on Rapid Transport of Delaying the Injection of AXM into the Eye

To test whether the synthesis of rapidly moving protein and its movement down the axon are separate processes, we determined whether inhibition of retinal protein synthesis also blocked the actual mechanism for moving the protein to the optic tectum. The injection of AXM into the eye was made at various intervals after the injection of tritiated leucine into the same eye, so that a certain amount of rapidly moving protein was synthesized before the inhibition of protein synthesis set in. Accumulation of rapidly moving material in the contralateral optic tectum was determined 24 hr after the isotope injection. Fig. 6

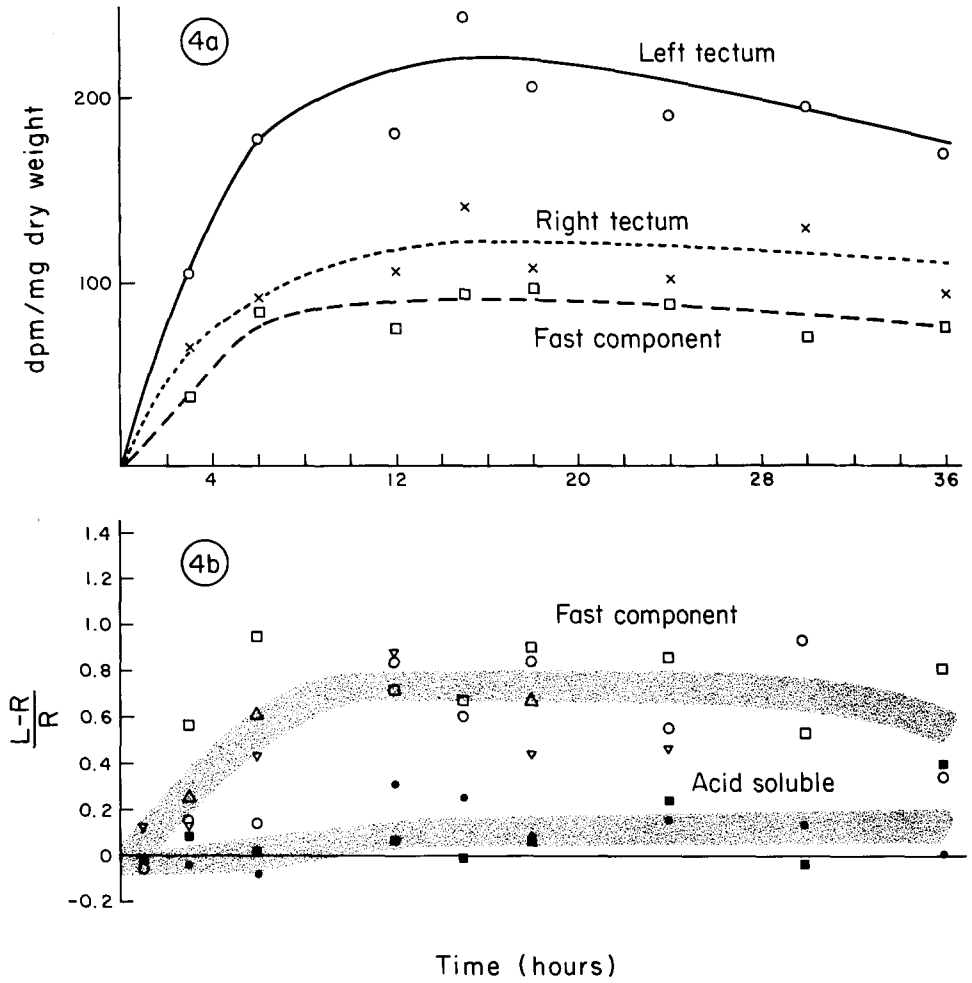

FIGURE 4 *a*, Time-course of incorporation of labeled leucine into TCA-precipitable material of left and right tecta, and accumulation of the rapidly transported component in the left tectum (measured as the difference between left and right tecta) after the injection of leucine-4,5-^{3}H into the right eye. (TCA-insoluble radioactivity was measured by method 2.) Pooled material from two fish was used for each time-point. *b*, Time-course of arrival in left tectum of the fast protein component (open symbols) and of free amino acid and other TCA-soluble material (closed symbols). Data expressed as the normalized parameter of transport, $(L - R)/R$. The various symbols represent different experimental series, in each of which three to four fish were used for a given time-point.

shows that the appearance of labeled rapidly moving protein was interfered with only if AXM was administered within about 3 hr after the isotope injection. Thus, the effect of AXM is apparently manifested only during the period of active protein synthesis. Even if determinations were made 6 hr after the isotope injection (Table II), that is, during the period when the AXM was maximally active and the accumulation of transported material was most rapid, the inhibitor had no effect on the subsequent appearance of the transported material in the tectum, provided it was injected near the end of the synthesis period, which, according to these experiments, lasts for about 3 hr after the isotope injection. Thus, inhibition of protein synthesis has no effect on the rapid transport mechanism itself.

Demonstration that the Radioactivity in the Fast Component Belongs to Protein

In view of the almost complete inhibition of the synthesis of the rapidly moving component ob-

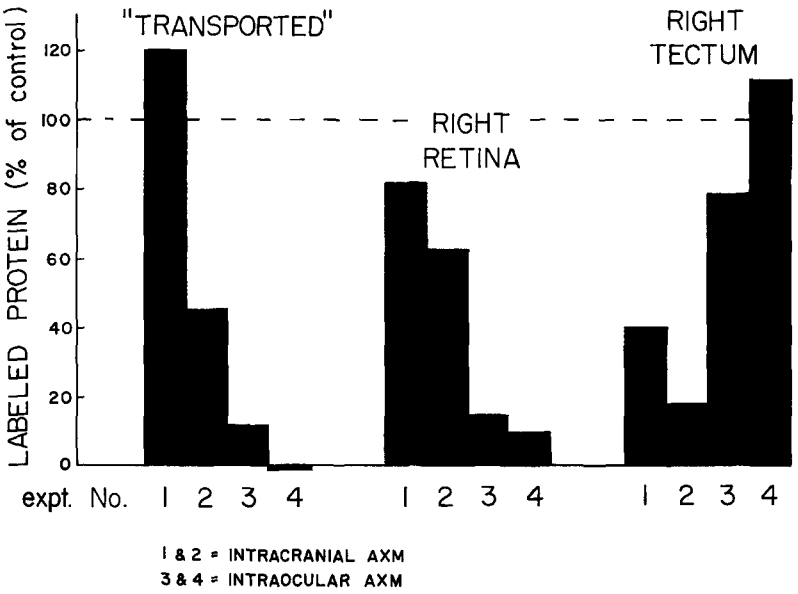

FIGURE 5 Comparison of the amount of rapidly transported material, the amount of labeled protein in the *right* retina, and the amount of labeled protein in the *right* tectum after inhibition of protein synthesis by AXM. (The right tectum is not receiving the labeled rapidly moving material and is used as a control for the level of "background" incorporation.) All values are expressed as per cent of control levels determined in fish without AXM. AXM was injected intracranially (0.2γ in experiment 1 and 0.4γ in experiment 2) or intraocularly (0.05γ in experiments 3 and 4) 2 hr before the intraocular injection of labeled leucine into the *right* eye. Labeled protein in retina and tecta was measured 24 hr after the isotope injection. The transported material was measured as the difference in specific activity between the two tecta. For measurements on the tecta, method 4 was used. The retinas were fixed in Bouin's solution, freed of vitreous humor and choroid, then dried, weighed, and analyzed for radioactivity by combustion.

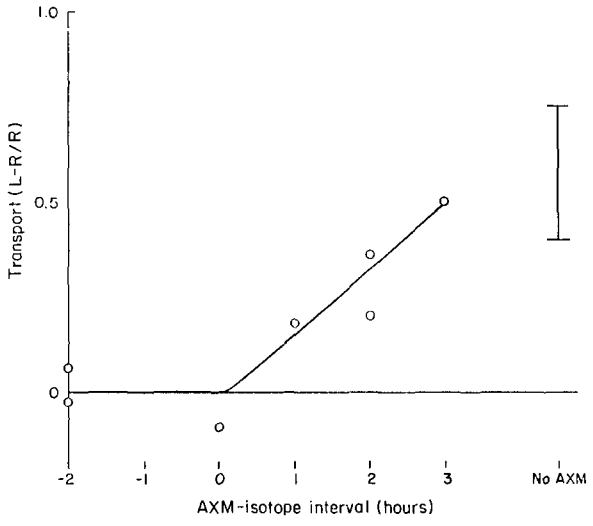

FIGURE 6 Effect on rapid transport of AXM injections before or after the injection of leucine-4,5-^{3}H into the same eye. The arrival of fast-moving material in the optic tectum was measured by method 2 24 hr after leucine injection. Abscissa, interval between AXM injection and isotope administration. Negative times mean that AXM was injected first. Ordinate, normalized parameter of transport $(L - R)/R$.

tained by injection of AXM into the eye, it seems very likely that it is indeed some type of protein that contains the radioactive label. We have carried out an experiment to show that this is so, and furthermore that the label resides exclusively in leucine. Four fish were injected in the right eye with 100 μc each of l-leucine-4,5-^{3}H dissolved in 5 μl of saline and in the cranial cavity with 0.3 γ of AXM. The fish were sacrificed 6 hr later, and the left and right tecta were treated for removal of acid-soluble material and lipids as described in method 4. The results of this experiment are presented in Table III. The intracranial AXM suppressed "background" incorporation in the tecta to such an extent that more than 70% of the TCA-precipitable radioactivity in the left tectum was due to the transported material. In agreement with our previous results, there was no left-right difference in the TCA-soluble pool; nor was there a left-right difference in the hot TCA extract or the lipid extract. All three of these fractions must have originated from radioactive leucine which entered the brain from the systemic circulation and not from transport along the nerve. The rapidly transported component consisted entirely of protein. Furthermore, acid hydrolysis of the protein followed by chromatography of the amino acids revealed that more than 95% of the label could be recovered in the leucine/isoleucine spot (Table III).

TABLE II

Effect on Fast Component of AXM Administration 2 hr after Isotope

	Specific activity		$\frac{L-R}{R}$
	Left	Right	
	cpm/mg dry wt		
Control	178	91	0.95
	149	131	0.14
	375	260	0.44
	178	111	0.60
Average ±SD			0.53 ± 0.29
0.05 γ AXM intraocular	2090	1310	0.59
	1770	1270	0.39
	1380	1110	0.24
	213	118	0.80
	454	371	0.22
Average ±SD			0.45 ± 0.22

Rapid transport was measured 6 hr after injection of leucine-4,5-^{3}H by Method 2.

Some Differences in Composition between Rapidly Moving and Slowly Moving Proteins

By homogenizing and centrifuging in several aqueous media the optic nerves and tecta from goldfish injected in one eye with tritiated leucine, we have been able to show that the rapid and the slow components are qualitatively very different from each other (Table IV and Fig. 7). For the study of the rapidly moving component, left and right nerves and tecta from goldfish injected in one eye with radioactive leucine 6–25 hr before sacrifice were homogenized in isotonic sucrose and

TABLE III

Identification of Radioactivity in the Fast Moving Component as Protein

Fraction	Left tectum		Right tectum		$\frac{L-R}{R}$
	Total dpm recovered	Specific activity	Total dpm recovered	Specific activity	
1. Soluble in cold 10% TCA	66,423	42.2	74,838	41.0	0.03
2. Soluble in hot 10% TCA	2,590	1.64	3,204	1.75	−0.06
3. Lipid extract	2,245	1.43	2,688	1.47	−0.03
4. Protein	23,500	14.9	8,100	4.4	2.4
5. Radioactivity with Rf of leucine/isoleucine	22,200 (95%)	—	7,800 (96%)	—	—

Four fish, injected in the right eye with 100 μc of L-leucine-4,5-^{3}H, were sacrificed at 6 hr, and pooled left and right tecta were analyzed by method 4. For purposes of comparing radioactivity in each extract, all counts per minute were corrected to 100% efficiency with the automatic external standardization and a calibration curve. "Specific activity" refers to total disintegrations per minute in each fraction divided by total protein in the fourth fraction.

TABLE IV

Solubility Characteristics of Fast and Slow Components

	Per cent of total in particulate fraction		
	1	2	3
Fast component, radioactivity			
Nerve, 6–25 hr	82 ± 11 (n = 8)		
Tectum, 6–25 hr	88 ± 12 (n = 9)		
Tectum, 11 days		80	90
Slow component, radioactivity			
Nerve, 11 days		64	70
Nerve, 44–47 days		68	52
Tectum, 44–47 days		60	51
Optic nerve, total protein	71 ± 4 (n = 8)		
Optic tectum, total protein	52 ± 8 (n = 6)		

Experiments were performed by method 3. The first column presents the mean ±SD of from six to nine experiments on pooled material from two to four fish. Columns 2 and 3 denote two separate experiments on pooled material from two to four fish. The data in column 2 are the same as those illustrated in Fig. 7.

centrifuged at 200,000 *g* for 45 min (see Experimental Procedure). In the nerve, 82% of the rapidly transported material, calculated as the left-right difference in specific radioactivity, was recovered in the centrifugal pellet, and in the tectum 88% was thus recovered. In a further experiment, tecta were homogenized in isotonic saline containing 0.1% Triton-X-100 and centrifuged. Of the rapidly transported material, 72% was again recovered in the centrifugal pellet, which indicates that this protein is very tightly bound to or part of a subcellular structure or organelle.

When the interval between isotope injection and sacrifice is increased to 11 days, we may expect the slowly moving component to have advanced into the segment of the optic nerve between the eye and chiasma, but not yet to have reached the optic tectum (8). This particular time interval has enabled us to study the slow component in the nerve and the fast component in the tectum of the same fish. From the distribution of radioactivity at this time (Fig. 7), we found that in the tectum the label still resided predominantly in particulate material, since 85% was recovered in the centrifugal pellet. However, the labeled material in the nerve, which now constituted the slowly moving component, had a strikingly different composition, namely 67% particulate and 33% soluble protein. 44–47 days after the isotope injection, slowly moving material is found in both the optic nerve and tectum. As in the optic nerve at 11 days, this material had a characteristically different composition from that of the fast component: 60 and 56% particulate material in the nerve and tectum, respectively. Thus, the average values for the slow component are 61% particulate and 39% soluble protein, as against 85 and 15%, respectively, for the fast component.

The solubility characteristics of the rapid and slow components are compared (in Table IV) with the particulate:soluble distribution of the total protein in the nerve and tectum. It can be seen that the fast component has a higher proportion of particulate material than is contained in the heavily myelinated optic nerve, while the slow component shows a somewhat higher proportion of soluble protein than the optic tectum.

Another important difference between fast and slow components is one of the amount of radioactive material involved. From Fig. 7, for example, it can be seen that the amount of radioactive protein in the tectum at 44 days is about three times that at 16 hr. In other such experiments, values of five to ten times that at 16 hr were obtained. From this result, it is evident that the slow component contains considerably more radioactivity than the fast component.

DISCUSSION

In the present experiments, a clear distinction has been made between the fast and the slow movement of radioactively labeled protein in the optic nerve of the goldfish. These fast and slow components were distinguished on the basis of the following criteria: (*a*) The slow component moves at a rate of 0.4 mm per day while the fast component moves about 100 times as fast. (*b*) The slow component contains about five times as much radioactivity as the fast component and consists of approximately 60% insoluble and 40% soluble material; the fast component is predominantly insoluble (i.e. particulate).

The rate of 40 mm per day that we have here

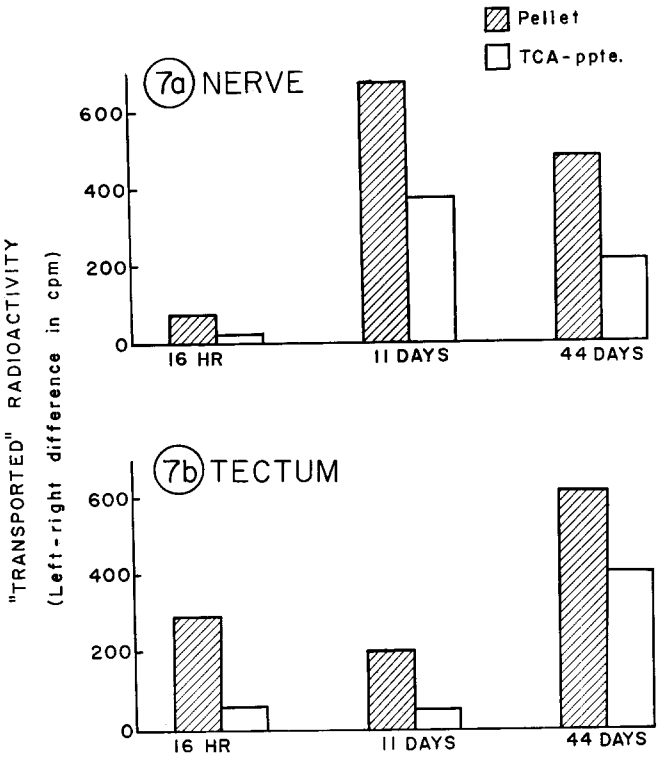

FIGURE 7 Distribution of radioactivity between soluble protein (open bar) and particle-bound protein (hatched bar) at various times after injection of labeled leucine. Determinations were performed by method 3.

estimated for the rapidly moving protein component is probably a conservative estimate of the rate at which the substances involved actually move along the nerve. It is based on the time at which the specific activity of the transported material in the tectum reaches a plateau, i.e. transport is essentially complete. A better approximation of when the peak of rapidly moving radioactivity reaches the tectum might be the midpoint of the rising part of the transport curve, which occurs between 3 and 6 hr. Another factor which tends to make even this estimate too long is that the synthesis period for radioactive protein in the retina lasts around 3 hr. This tends to make the transport curve reach its plateau later than it would if synthesis occurred in a true pulse lasting only a few minutes. When these factors are taken into account, the actual transport rate might be twice our present estimate, or even greater. Another consideration in the comparison of our value for the rate of movement with those values obtained in other preparations is the temperature. If we assume a Q_{10} of two, the rate at 20°C, at which the present experiments were conducted, must be multiplied by a factor of 3.3 for conversion into the equivalent rate at 37°C. Thus, we appear to be dealing with a rate of transport equivalent to several hundred mm per day in mammalian nerves. This finding is part of the growing body of evidence indicating that the rapid type of transport is a phenomenon common to many, if not all nerves. In the optic nerve of the metamorphosing bullfrog tadpole, a protein component has been observed to move at rates of at least 10–22 mm per day (7). In hypoglossal nerves, Miani (18) has found rates of protein and phosphate transport of about 70 mm per day. In adrenergic nerves of the mammalian nervous system, the rate of accumulation of norepinephrine at a constriction in the nerve indicates a rate of movement of this material down the axon of 70–200 mm per day (4, 14). In dorsal spinal roots of the cat and in sciatic nerve of the rat, rates of protein migration of up to 400 mm per day have been observed by Lasek (13), and Ochs and his coworkers have made similar observations (21). The most extreme case of rapid movement yet reported is the observation of Jasinski et al. (10) that neurosecretory material in neurons of the preoptic nucleus of the goldfish moves down the

axon at rates of 2 mm per min, which corresponds to 2.9 m per day!

To construct any hypothesis about how these rapid rates of movement are achieved, it is essential that we should be clear about what the nature of the transported material is. Our present experiments have clearly shown that it must be the protein itself, and not free amino acid, that is transported, since (*a*) labeled free leucine does not appear in the contralateral tectum in such amounts and at such early times after injection as to indicate that its transport has occurred, (*b*) cycloleucine and norepinephrine, substances with some properties like those of free leucine, are not transported to the tectum, and (*c*) the appearance of the transported material depends on protein synthesis in the retina, but is independent of protein synthesis in the tectum. We have also shown here that the radioactive material that is transported is largely particulate in nature. This makes unreasonable the supposition that the material has been transported extracellularly, e.g. by the "endoneurial flow" mechanism demonstrated by Weiss et al. (31), since it is difficult to imagine that *particulate* matter might be extruded by the neuron, move down the nerve through the extracellular space, and re-enter the neuron at the neuron terminals. Lasek has also provided some evidence against the possibility that the protein transport is occurring extracellularly (13).

We are, therefore, led to think that in the case of the rapidly moving protein we are dealing with an intracellular organelle. This conclusion has been reached also by workers dealing with the rapid transport phenomenon in other nerves (4, 13, 14) and, in fact, it has been shown by direct observations of cultured neurons that particles of 0.1–1 μ in diameter move through the axoplasm at rates of up to 9 μ per sec, which would be equivalent to 780 mm per day (2, 23). In this case, since observations could be made over only a short stretch of axon, it is not known whether such particles could travel the whole length of the axon. Also, since the particles were seen moving in both directions along the axon, it is not clear what their net rate of movement in one direction would be. Nevertheless, we are reassured to know that such rapid movement does occur, even though we might have expected that it would be opposed by frictional and electrical forces of enormous magnitude. It is at present difficult to imagine what the intracellular machinery might be that provides the motive force under these conditions.

Our present studies do not enable us to directly identify the rapidly transported particle, but electron microscopic studies to this end are currently being planned. Our only clue so far is that, although it reaches the optic tectum very quickly, the rapidly moving component appears to have a long lifetime in the synaptic layer. Even at 25 days after the injection, the rapid component was not appreciably diminished in amount compared to much earlier time intervals (8). It is evident, from the rapid movement and slow turnover, that only a small fraction of the total material is being synthesized each day. A model for the kind of substance which may be involved is the catecholamine-storage granule, since this granule moves rapidly down the axon and yet has a long lifetime in the synapse (4). In the optic nerves, where catecholamines do not, as far as we know, play a role in synaptic transmission, a similar storage granule containing the appropriate transmitter might be involved. Although an alternate possibility that the mitochondria constitute the fast component cannot be completely ruled out, the experiments of Barondes (1) on rat brain strongly suggest that mitochondria are part of the slowly moving material. In his experiments, mitochondrial proteins from rat brain synaptic endings were not maximally labeled until 9–10 days after the isotope injection. Weiss and Pillai (30) have also presented evidence that the mitochondria move as part of the slow transport.

In her thorough and thoughtful review article on axoplasmic flow, Lubinska (16) has emphasized the possibility that transport in the nerve may occur in both directions. This view is not incompatible with our present observations which could be explained on the assumption that the plateau of accumulation of transported protein in the tectum represents the attainment of a uniform level, throughout the neuron, of a labeled component which moves rapidly in both directions and has a long lifetime. (We are indebted to Mr. D. Forman for working out this idea with us.) However, no direct evidence for the retrograde flow yet exists.

Our experiments involving inhibition of protein synthesis have shown that, whatever the mechanism is that produces the actual movement of materials in the rapid transport system, this mechanism does not depend on protein synthesis, and is not blocked as a side effect of the inhibitor action. Other workers have come to the same conclusion about the slow transport mechanism (20, 22).

Nevertheless, it is clear that, in the case of the slow component of the protein transport, we are dealing with different material, and undoubtedly a completely different mechanism of transport, as Weiss has explained (28). Because the slowly moving material has not yet been found to move beyond the nerve fibers into the synaptic layer, it is tempting to consider the possibility that this material is related to the neurofilaments and neurotubules, which do not usually penetrate into the synaptic terminals. One piece of evidence in line with this idea is Droz's report (6) that, in dorsal root ganglion cells labeled with tritiated amino acid, the radioactivity appearing in the axons was most concentrated in the axoplasm rich in neurofilaments and neurotubules. There seems to be some contradiction in the claim of Ochs et al. (20) that the slowly moving material is largely soluble. We have calculated from their data, however, that whereas an average of 58% of the labeled material that they are dealing with is in the soluble fraction ("S" in their Table 1), an average of 28% of this is TCA-soluble material ("% sup." in their Table 3), which we presume to be free amino acid and polypeptide. Thus only about 41% of their total labeled material is soluble protein. Since particulate matter accounts for 42% of the radioactivity in their original homogenate, we interpret their results to indicate that the labeled protein contains approximately equal proportions of soluble and particulate matter. This is sufficiently close to our own values of about 40% and 60%, respectively, to suggest that it is quite likely that we are dealing with the same material. It should be pointed out that about half of the protein of neurotubules is solubilized under conditions similar to those in our experiments (Shelanski, M. Personal communication.). Therefore, both the soluble and particulate radioactive proteins in these nerves might originate from the neurotubules.

If our hypothesis[1] that the slowly moving material consists of neurotubules and neurofilaments is correct, it would still be necessary to account for the renewal of the other proteins of the axoplasm. We are, therefore, planning experiments which will distinguish between the possibility that this slowly moving radioactivity represents one class of axonal proteins and the possibility that it represents the renewal of the entire axoplasm, which would be more closely in line with Weiss's demonstration of the peristaltic propulsion of the axon contents (28).

It is clear at the same time that, whatever the nature of the slowly moving material, the nerve cell has a special mechanism for the transfer of selected substances from the cell body to the synapse at a much faster rate. The existence of such a mechanism opens the possibility that the nervous system can adjust rapidly to changes in physiological activity by synthesizing substances in the cell body and transferring them to the synapses.

Dr. McEwen would like to express his appreciation to Dr. N. E. Miller for his support and encouragement. Dr. Grafstein is grateful to Dr. Paul A. Weiss for making available some of his facilities and equipment, as well as for his interest and encouragement. We would like to acknowledge the expert technical assistance of Mr. Sam Briseno, Mrs. Vernall G. Daniels, Miss Linda Plapinger, Miss Jacqueline Rausch, and Mrs. Leslie Schwartz. Mr. David Forman made many useful suggestions during the course of the investigation and helped catalyze many helpful discussions.

This research was supported by grant No. NB 07080 from the National Institutes of Health to Dr. McEwen, by National Science Foundation grant No. GB 4208 to Dr. Grafstein, by National Institutes of Health grants No. MH 13189 to Dr. N. E. Miller, and No. CA 10096 to Dr. Paul A. Weiss.

Received for publication 20 February 1968, and in revised form 8 April 1968.

REFERENCES

1. BARONDES, S. 1966. *J. Neurochem.* **13**:721.
2. BURDWOOD, W. O. 1965. *J. Cell Biol.* **27**:115A.
3. BRAY, G. 1960. *Analyt. Biochem.* **1**:279.
4. DAHLSTRÖM, A., and J. HÄGGENDAL. 1967. *Acta Physiol. Scand.* **69**:153.
5. DROZ, B., and C. P. LEBLOND. 1963. *J. Comp. Neurol.* **121**:325.
6. DROZ, B. 1967. *J. Microscop.* **6**:201.
7. GOLDBERG, S., and M. KOTANI. 1967. *Anat. Record.* **158**:325.
8. GRAFSTEIN, B. 1967. *Science.* **157**:196.
9. GUPTA, G. N. 1966. *Analyt. Chem.* **38**:1356.
10. JASINSKI, A., A. GORBMAN, and T. J. HARA. 1966. *Science.* **154**:776.
11. KOENIG, H. 1958. *Trans. Am. Neurolog. Assn.* **83**: 162.

[1] We would like to acknowledge the participation of Dr. Michael Shelanski, of Albert Einstein College of Medicine, in developing this idea with us.

12. KOPRIWA, B., and C. P. LEBLOND. 1962. *J. Histochem. Cytochem.* **10**:269.
13. LASEK, R. J. 1968. *Brain Research.* **7**:360.
14. LIVETT, B. G., L. B. GEFFEN, and L. AUSTIN. 1968. *Nature.* **217**:278.
15. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
16. LUBINSKA, L. 1964. *In* Mechanisms of Neural Regeneration. Progress in Brain Research. M. Singer and J. P. Schade, editors. **13**:1.
17. MCEWEN, B. S., and B. GRAFSTEIN. 1968. *In* Metabolism of Nucleic Acids and Proteins and the Function of the Neuron. Z. Lodin, editor. Excerpta Medica Foundation. 225.
18. MIANI, N. 1963. *J. Neurochem.* **10**:859.
19. OCHS, D., D. DALRYMPLE, and G. RICHARDS. 1962. *Exptl. Neurol.* **5**:349.
20. OCHS, S., J. JOHNSON, and M. H. NG. 1967. *J. Neurochem.* **14**:317.
21. OCHS, S. 1967. *Neurosciences Research Progr. Bull.* **5**:337.
22. PETERSON, R. P., R. M. HERWITZ, and R. LINDSAY. 1967. *Exptl. Brain Research.* **4**:138.
23. POMERAT, C. M. 1961. *Intern. Rev. Cytol.* **11**:307.
24. RAHMANN, H. 1967. *Naturwissenschaften.* **54**:174.
25. RANDERATH, K. 1966. Thin Layer Chromatography. Academic Press Inc., New York. 110.
26. TAYLOR, A. C., and P. WEISS. 1965. *Proc. Natl. Acad. Sci. U.S.* **54**:1521.
27. WEISS, P. 1961. *In* Regional Neurochemistry. S. Kety and J. Elkes, editors. Pergamon Press, London. 220.
28. WEISS, P. 1967. *Neurosciences Research Progr. Bull.* **5**:371.
29. WEISS, P., and H. B. HISCOE. 1948. *J. Exptl. Zool.* **107**:315.
30. WEISS, P., and A. PILLAI. 1965. *Proc. Natl. Acad. Sci. U.S.* **54**:48.
31. WEISS, P., H. WANG, A. C. TAYLOR, and M. V. EDDS, JR. 1945. *Am. J. Physiol.* **143**:521.