CELL-TO-CELL TRANSFER OF GLIAL PROTEINS TO THE SQUID GIANT AXON

The Glia-Neuron Protein Transfer Hypothesis

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ABSTRACT

The hypothesis that glial cells synthesize proteins which are transferred to adjacent neurons was evaluated in the giant fiber of the squid (*Loligo pealei*). When giant fibers are separated from their neuron cell bodies and incubated in the presence of radioactive amino acids, labeled proteins appear in the glial cells and axoplasm. Labeled axonal proteins were detected by three methods: extrusion of the axoplasm from the giant fiber, autoradiography, and perfusion of the giant fiber. This protein synthesis is completely inhibited by puromycin but is not affected by chloramphenicol. The following evidence indicates that the labeled axonal proteins are not synthesized within the axon itself. (*a*) The axon does not contain a significant amount of ribosomes or ribosomal RNA. (*b*) Isolated axoplasm did not incorporate [^{3}H]leucine into proteins. (*c*) Injection of RNase into the giant axon did not reduce the appearance of newly synthesized proteins in the axoplasm of the giant fiber. These findings, coupled with other evidence, have led us to conclude that the adaxonal glial cells synthesize a class of proteins which are transferred to the giant axon. Analysis of the kinetics of this phenomenon indicates that some proteins are transferred to the axon within minutes of their synthesis in the glial cells. One or more of the steps in the transfer process appear to involve Ca^{++}, since replacement of extracellular Ca^{++} by either Mg^{++} or Co^{++} significantly reduces the appearance of labeled proteins in the axon. A substantial fraction of newly synthesized glial proteins, possibly as much as 40%, are transferred to the giant axon. These proteins are heterogeneous and range in size from 12,000 to greater than 200,000 daltons. Comparisons of the amount of amino acid incorporation in glia cells and neuron cell bodies raise the possibility that the adaxonal glial cells may provide an important source of axonal proteins which is supplemental to that provided by axonal transport from the cell body. These findings are discussed with reference to a possible trophic effect of glia on neurons and metabolic cooperation between adaxonal glia and the axon.

It has often been suggested that adjacent cells communicate with one another via the actual intercellular transfer of molecules including macromolecules. The possibility that molecular transfer may play a role in intercellular regulation has taken on added credibility since the discovery and

conclusive demonstration of the passage of small molecules such as inorganic ions and marker dyes across low resistance junctions between electrically coupled cells (3). These junctions will pass molecules up to 1,200 daltons (59). Thus, while electrotonic coupling has given substance to the idea that cells may interact directly via the exchange of small molecules, study of this phenomenon has left open the question of whether the broad class of informational macromolecules such as proteins and RNA traverse the membrane barriers separating the cytoplasm of adjacent cells.

Many experiments have been carried out in an attempt to add support to the hypothesis that newly synthesized macromolecules are transferred from one cell across the intervening extracellular space into an adjacent cell. One of the widely employed approaches to this problem makes use of radioactive precursors to label the macromolecules of a "sending" cell so that any transferred macromolecules might be detected in an adjacent "receiving" cell. The essential obstacle to this approach is the obvious possibility that radioactive precursors can enter the receiving cell and be incorporated by the synthetic machinery which is present in this cell (24). This problem has been overcome partially in experiments by employing dispersed cells in tissue culture and specific inhibitors of macromolecular synthesis (36). Although these experiments provide evidence for the transfer of proteins and RNA between cells of an established cell line in tissue culture, the possible extension of this experimental paradigm to the study of intact tissues is difficult.

An alternative approach to the study of macromolecular transfer between cells which are contained in organized tissues is offered by neurons and their axonal extensions. This is the case, because the protein-synthesizing machinery of mature neurons appears to be contained exclusively in the neuron cell body and dendrites of the neuron, and does not extend into the axon. This view is supported by a variety of evidence, including the restriction of ribosomes to the cell body and their apparent absence from the axon. Thus, the axon provides an unusual situation in which a large fraction of the cell's cytoplasm is separated from the primary protein synthetic machinery by distances of the order of centimeters or even meters. Although a well-developed mechanism, axonal transport, provides for the supply of macromolecules to the axon from the distant cell body, it has been suggested that the axon might also be supplied with some macromolecules by the specialized glial cells which surround it (40, 60). We have carried out a series of studies aimed at testing this latter hypothesis in the giant axons of squids. These axons are large enough to carry out biochemical analyses, which are currently not possible in smaller vertebrate and invertebrate axons.

One aspect of our studies concerns the metabolism of the squid giant axon in vitro. These studies confirmed and extended previous observations (20) indicating that squid giant fibers separated from their neuron cell bodies are capable of incorporating labeled amino acids into proteins, some of which appear in the axon (42). The appearance of these labeled proteins in the axon raised an interesting question regarding the site at which these proteins were synthesized. Since the giant fibers were separated from their neuron cell bodies, the proteins which appeared in the axon must have been synthesized locally at the level of the axon, either in the glial cells surrounding the axon or in the axon itself. Our previous studies of axoplasmic RNA indicated that the axon lacks the necessary machinery for protein synthesis (5, 41). Thus, the alternative possibility that labeled axonal proteins are synthesized in the glial cells surrounding the axon and transferred to the axon gained support. In this paper we extend our studies, using both extrusion of the axoplasm and autoradiography to study the glia-neuron protein transfer hypothesis. An adjoining paper presents experimental evidence obtained by perfusing the giant fiber with artificial axoplasm (18).

MATERIALS AND METHODS

Squid with mantle lengths ranging between 15 and 25 cm were maintained in a running seawater tank and used within 48 h of capture. The paired giant fibers associated with the stellar nerves were carefully dissected from squid which had been killed by decapitation. The fibers were tied at each end and any remaining small fibers removed over a distance of 4 cm from the proximal ligature. We cleaned only 4 cm of the axon because it is difficult to routinely clean more distal regions of the giant fiber without damaging it. Care was taken not to pull off any of the small branches which emanate from the giant axon. Holes in the axon can be detected by the presence of white spots in the axoplasm, apparently due to entry of Ca^{++} into the axon. Axons with large white spots were discarded. The temperature during the dissection and subsequent incubation was 18–21°C. We found that if the temperature was higher, the squid giant fibers would not take up the labeled amino acids from the incubation media and the amino acid incorporation into proteins was reduced by 90% or more.

The axons were incubated in a trough containing 0.5-1.0 ml filtered seawater. The troughs were constructed from cleaned microscope slides on which an elliptical barrier was formed with petroleum jelly. Evaporation was minimized by placing the trough in a petri dish with a piece of moistened tissue paper.

To obtain valid comparisons between giant fibers we found that it was essential to compare paired fibers from the same animal. The interanimal variation of amino acid incorporation into the giant fibers was often very great when squid from a single catch were compared and even greater when different catches were compared. However, the variability between pairs from individual animals was reasonably small. Therefore, we used paired controls in all of the studies in which conditions were experimentally altered.

Incubation media consisted of Millipore-filtered seawater (Millipore Corp., Boston, Mass.). Labeled amino acids were added by rapidly drying the amino acid in a test tube under vacuum and adding filtered seawater. Drugs such as puromycin were added as solids to filtered seawater and the pH was adjusted to 7.6. In the experiments in which the ionic composition of the media was varied, we employed artificial seawater (ASW) with the following compositions: 425 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 10 mM Tris HCl, pH 7.6. The alterations of the ionic composition of this solution are listed in the legends of Figs. 6 and 7.

The incubations were terminated after 15 min to 4 h by immersing the giant fiber in filtered seawater at 4°C. Excess seawater was removed from the giant fiber by touching it to a microscope slide several times. Axoplasm was extruded, with a length of polyethylene tubing, from the proximal 3 cm of fiber measured on a ruler under a microscope. Great care was taken not to contaminate the axoplasm with material which moves along the surface of the sheath in association with the polyethylene tubing. The axoplasm was quickly drawn up into a capillary tube and transferred to a homogenizer (4°C) and the 3-cm length of extruded sheath was transferred to a separate homogenizer.

A note should be made about the terminology which we employ to describe the giant axon. Isolated giant fiber refers to a giant fiber from which most of the adhering small fibers have been cleaned. The giant fiber contains the giant axon which is surrounded by an inner layer of adaxonal glial cells (Schwann cells) and an outer layer of connective tissue (44, 66, 67; see also Fig. 1). Extrusion separates the axoplasm from the sheath. Since a small part of the axoplasm and the entire axonal plasma membrane (axolemma) remain behind with the sheath, the word sheath is a bit of a misnomer. However, it has come into conventional usage in extrusion experiments and we continue to use it (albeit realizing that it must be qualified).

The axoplasm and sheath were routinely homogenized in 0.25 ml of 50 mM Tris HCl buffer, pH 7.4, in a glass-glass microhomogenizer. A 0.1-ml aliquot was analyzed for hot TCA-precipitable radioactivity on Whatman 3 MM filters (43), and a 0.1-ml aliquot was added directly to scintillation cocktail for total radioactivity. The efficiencies of counting for the filters and the total radioactivity were both 35%. Therefore, the data were corrected for the size of the aliquot and expressed as hot TCA-precipitable cpm or TCA-soluble cpm (total cpm-hot TCA-precipitable cpm). In our earlier work (42) we expressed the incorporated radioactivity relative to the proteins in the sample. We have found that expression of the data relative to a unit length of giant fiber compares favorably with the expression relative to protein content. Comparisons of protein content of axoplasm and sheaths from 3-cm long paired giant fibers showed differences within the range of error of the Lowry method. Therefore, except where noted, all of the data have been expressed as cpm/3-cm giant fiber.

To analyze proteins on polyacrylamide gels, the axoplasm and sheaths from two or more giant fibers were pooled and homogenized in 0.5 ml of 50 mM Tris HCl, pH 7.4. These samples were separated into a soluble fraction and pellet by centrifugation at 27,000 *g* for 30 min at 4°C. For sodium dodecyl sulfate (SDS)-polyacrylamide gels, the samples which contained 1% SDS and

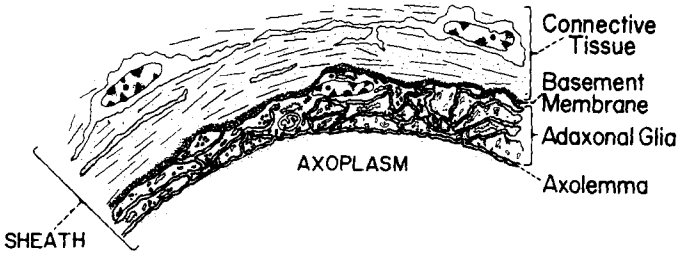


FIGURE 1 A schematic drawing of a sector of the squid giant axon. This figure was constructed from our own unpublished electron micrographs and those of others. The complexity of the sheath is illustrated. It contains an outer connective tissue layer composed of collagen and fibroblasts which is separated from the inner adaxonal glial layer by a thick basement membrane. The adaxonal glial cells extend many finger-like processes from the cell body and these processes envelop the axon. The glial cells contain numerous elements of the rough endoplasmic reticulum and membrane profiles.

5% 2-mercaptoethanol were heated at 90°C for at least 2 min. Gels were prepared and run according to Neville (46). In some of the experiments, L-[^{3}H]leucine and L-[^{14}C]leucine were mixed together and the counts per minute for each isotope were obtained by the dual channel method. The soluble fraction was also analyzed by isoelectric focusing on polyacrylamide gels.

In Vivo Experiment

Injections of 0.1 ml of 10 μCi of L-[^{3}H]leucine filtered seawater were made bilaterally into the connective tissue space which surrounds the stellar nerve. The stellar nerve can be visualized in a live, intact squid by emptying the seawater from the mantle, transilluminating it through the dorsal surface, and looking in through the mantle opening. The injections were made with a Hamilton microlite syringe (Hamilton Co., Reno, Nev.) with a 27-gauge needle which was inserted through the mantle opening. The entire injection procedure took only 1 or 2 min, after which the squid were quickly resuscitated by flushing the mantle cavity with seawater.

Biochemical Materials

The following radioactive chemicals were employed: L-[4.5-^{3}H]leucine (30–50 Ci/mmol; L-[^{14}C]leucine (270 mCi/mmol); L-[3-^{3}H]arginine (15 Ci/mmol); L-[3-^{3}H]-glutamic acid (20 Ci/mmol) and L-[^{35}S]methionine (200 Ci/mmol). Puromycin (A grade), cyclohexamide (B grade), and chloramphenicol (B grade) were obtained from Calbiochem (San Diego, Calif.). Reagent grade chemicals and distilled water were used to make all of the chemical solutions. Seawater was obtained from the running seawater table in the laboratory.

RESULTS

Inhibition of Amino Acid Incorporation into Proteins of the Squid Giant Fiber

Our previous studies indicated that the protein synthesis inhibitors puromycin and cycloheximide reduced the incorporation of amino acids into proteins of the squid giant fiber (42). No significant difference was found between axons incubated in chloramphenicol and their contralateral controls (Table I). On the other hand, puromycin reduced the acid-precipitable radioactivity in the axoplasm and sheath to 2% of the control levels, but had no demonstrable effect on the levels of free [^{3}H]-leucine in either the axoplasm or sheath. The results of the experiments with chloramphenicol and puromycin reinforce our previous results and those of others indicating that the incorporation of [^{3}H]leucine into proteins of the squid giant fiber occurs via protein synthesis on eukaryotic ribosomes. In contrast to our previous results on unpaired axons (42), the paired control paradigm indicates that cycloheximide is not a completely effective inhibitor of protein synthesis in the squid giant fiber, since 0.1 mg/ml cycloheximide reduced protein synthesis in the sheath by only 50% when compared with paired controls. Cycloheximide does not completely inhibit protein synthesis in *Aplysia* ganglia (57), suggesting that this protein synthesis inhibitor may not be effective on intact molluscan neurons.

Kinetics of Amino Acid Incorporation into Sheath and Axoplasmic Proteins

Fig. 2 illustrates the time-course over which labeled proteins appear in the sheath and axoplasm when they are separated by mechanically extruding the axoplasm. Labeled proteins were detectable in both the sheath and axoplasm after 15 min of incubation in L-[^{3}H]leucine (Fig. 2), and the incorporation rates of both these compart-

TABLE I

Effects of Protein Synthesis Inhibitors on the Appearance of Labeled Proteins in the Isolated Squid Giant Fiber

	Control			Experimental			
Experimental condition	Axoplasm	Sheath	A/S	Axoplasm	Sheath	A/S	N
			%			%	
TCA-precipitable cpm (× 10^{-3}) per giant fiber							
Chloramphenicol	13.7 (1.6)	87.7 (20)	16.4	12.0 (2.5)	89.9 (20)	13.4	4
Puromycin	–	–	–	0.12 (0.02)	0.86 (0.13)	16.7	6
TCA-soluble cpm (× 10^{-3}) per giant fiber							
Chloramphenicol	151	233		132	412		4
Puromycin	–	–		150	204		6

In the chloramphenicol experiments, each experimental fiber was paired with its contralateral homologue from the same animal. The fibers treated with puromycin were obtained from separate animals but were incubated in parallel with the chloramphenicol controls. The concentration of chloramphenicol and puromycin were each 100 μg/ml. The experimental fibers were preincubated in media containing the drug for 10 min followed by 120 min in media containing both the drug and L-[^{3}H]leucine. Control fibers were incubated similarly in media which did not contain the drug. All of the values are averages of the number of experiments indicated by N with SEM in parentheses.

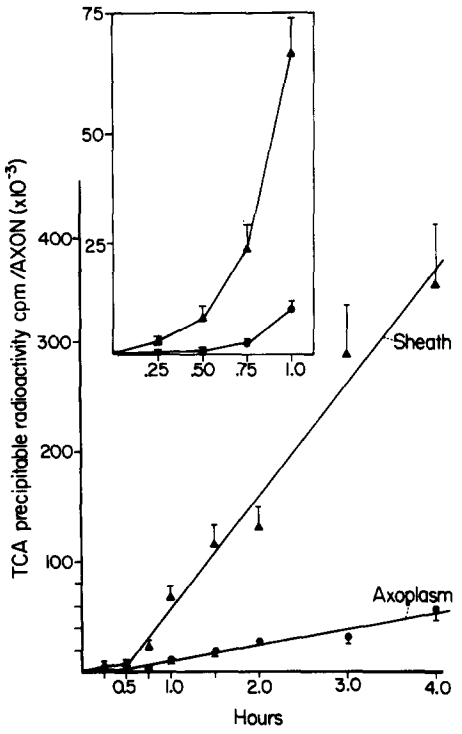


FIGURE 2 Illustration of the kinetics of L-[^{3}H]leucine incorporation into the proteins of axoplasm and sheath of isolated squid giant fibers. The isolated giant fibers were incubated in filtered sea water containing 25 μCi/ml of L-[^{3}H]leucine for the periods indicated. Axoplasm was separated from the sheath by extrusion, and the hot TCA-precipitable radioactivity was measured. Each point is the mean of at least five determinations, and the standard error of the mean is shown for each point. The amounts of radioactivity in the axoplasm at 15 and 30 min are not well resolved in the figure. The mean values for eight experiments were 258 (±50) cpm at 15 min and 963 (±210) cpm at 30 min.

ments were linear after about 0.5 h of incubation.

Fig. 3 illustrates the corresponding TCA-soluble radioactivity in the axoplasm and the sheath. The TCA-soluble radioactivity in the sheath reached a high level at the early intervals and continued to increase throughout the experiment. In contrast, the soluble radioactivity in the axoplasm increased more gradually and became asymptotic after 1–2 h. The differences in these kinetics in the axoplasm and sheath are probably a reflection of differences in the contents of these compartments. While the axoplasm is a purely intracellular compartment, the sheath contains extracellular space and a variety of cell types. The unusual kinetics of the soluble counts in the sheath probably results from the presence of extracellular space which would equilibrate rapidly with the precursor in the incubation media.

Autoradiography

The appearance of labeled proteins in the axoplasm of the squid giant axon has been demonstrated by autoradiography, and the ratio of labeled protein found in the axoplasm versus the sheath (axoplasm/sheath ratio) was similar in the autoradiographic and extrusion experiments (42). Furthermore, the number of silver grains was reduced to background by puromycin but not by chloramphenicol. The results of the autoradiographic studies indicate that the labeled proteins that appear in the axoplasm in extrusion experiments were not artifactual contaminants which arise from the sheath during the extrusion process (42).

Fig. 4*a* and *b* illustrate the appearance of labeled proteins in the sheath and axoplasm of a giant fiber which was only partially cleaned so that the smaller fibers around it remained attached. Another giant fiber is shown from which the smaller fibers were removed and which is comparable to the fibers used in the extrusion studies (Fig. 4*c*). The silver grains are particularly abun-

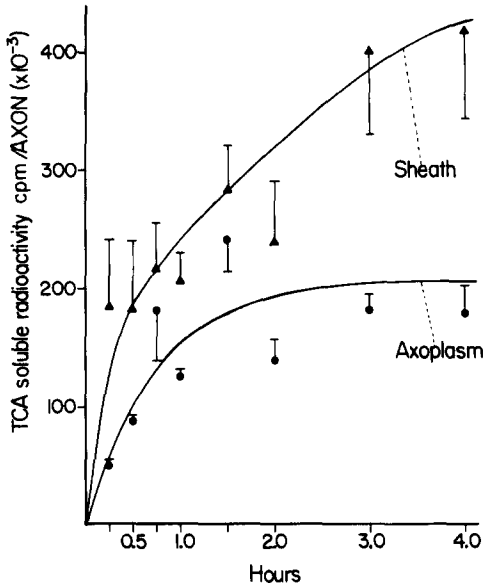
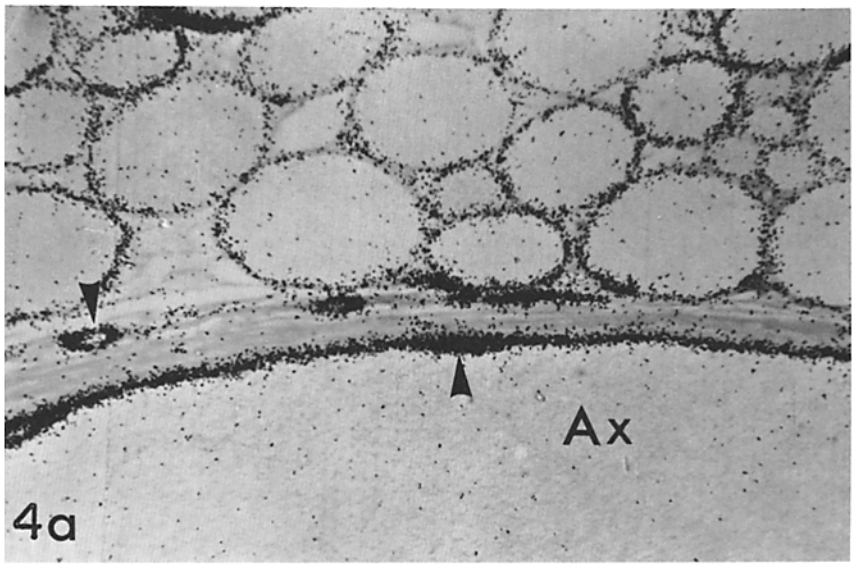
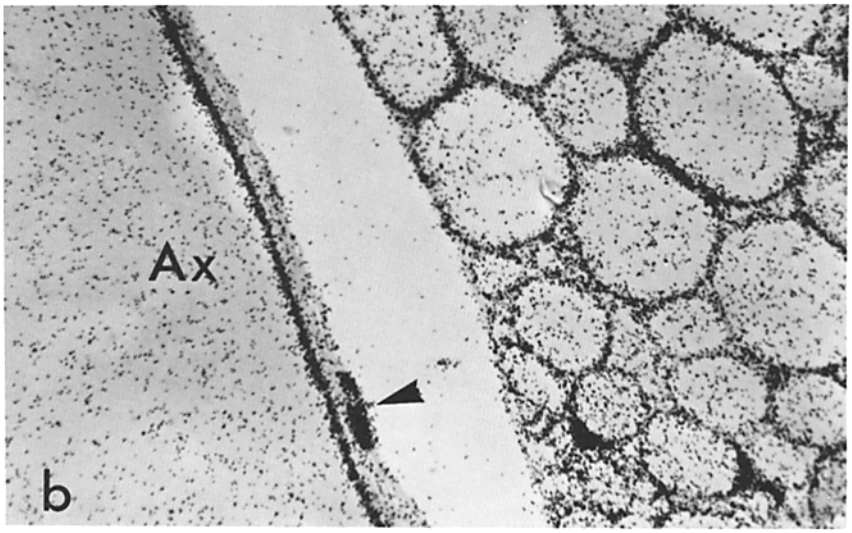


FIGURE 3 Illustration of the temporal appearance of soluble radioactivity in the axoplasm and sheath of squid fibers incubated in L-[^{3}H]leucine. These data were obtained from the giant fibers used in Fig. 2 and were calculated by taking the difference between the total radioactivity and hot TCA-precipitable radioactivity. For other details, see Fig. 2 and Materials and Methods.


Ax
4a


Ax
b


c

dant over the adaxonal glia cells which directly surround the giant fiber and smaller fibers. The outer portion of the sheath of the giant fiber, which is composed mainly of a collagen-containing connective tissue matrix and a few scattered cells, is more diffusely labeled than the inner adaxonal glial layer. The scattered cells which are present in the outer portion of the sheath can be seen since they are very heavily labeled and appear as dense spots in the autoradiograph (Fig. 4*a* and *b*). The diffusely distributed grains present in the outer sheath probably correspond to the small processes of the fibroblasts in the connective tissue matrix. Grains can also be seen over the axoplasm of the small fibers as well as the giant fibers, indicating that the appearance of labeled proteins in the axoplasm is not peculiar to the giant fiber.

The autoradiographs have also provided other information. It was previously noted that the adaxonal portion of the sheath is not uniformly labeled around its circumference when labeled giant fibers are viewed in transverse section (42). Using this information, it was possible to design another test of the glia-neuron transfer hypothesis. If the hypothesis has merit, it is predicted that more labeled proteins will be transferred to the axoplasm adjacent to heavily labeled regions of the sheath than will be transferred to axoplasm adjacent to lightly labeled regions of the sheath. In fact, autoradiographic grain counts appear to fulfill this prediction (Table II). In all six of the randomly chosen sections which were analyzed, the number of grains over axoplasm subjacent to heavily labeled regions of the sheath exceeded that of grains over axoplasm subjacent to lightly labeled regions of the sheath.

TABLE II

Comparison of Radioautographic Grains over Axoplasm subjacent to Heavily Labeled and Lightly Labeled Regions of the Sheath

Minutes of incubation	60		120		180	
Section no.	1	2	1	2	1	2
	Grains/10,000 μm^2 of axoplasm					
Heavily labeled sheath	140	51	145	434	546	826
Lightly labeled sheath	35	15	106	344	349	450

Three giant fibers were incubated in [^{3}H]leucine, one for each of the incubation periods which are indicated. Two transverse sections were taken from each axon for analysis and are indicated by the superscripts 1 and 2. Grains were counted over regions of axoplasm subjacent to either heavily labeled areas of the sheath or lightly labeled areas. The amount of radioactivity in the heavily labeled regions of the sheath exceeded that of the lightly labeled regions by a factor of three or more. Grain counts were made with a 25-μm^2 ocular micrometer at ×1,000. Each figure represents the total grains found by traversing the micrometer over a square field which was 10,000 μm^2. This field was positioned so that one edge was parallel to the sheath. However, the edge was always at least 5 μm from the sheath in order to avoid counting any grains which arose directly from the adaxonal glia. Background was estimated by counting all of the grains in a 2,000 μm^2 field of the section adjacent to the giant nerve fiber. All of the counts shown were corrected for background which ranged between 8 and 28 grains/10,000 μm^2. Note that in every case the number of grains over axoplasm subjacent to the heavily labeled region of the sheath exceeds that in the complementary area of axoplasm next to a lightly labeled region of the sheath.

Amino Acid Incorporation in Isolated Axoplasm

To determine whether the axoplasm has a significant protein synthetic capacity, freshly extruded axoplasm was incubated for 90 min with L-[^{3}H]leucine at a final concentration approximately twice that present in the axoplasm of intact giant fibers incubated for 90 min. A small amount of hot TCA-precipitable radioactivity appeared in isolated axoplasm (Table III). This precipitable radioactivity was ~3% of that which appeared in the axoplasm of intact giant fibers, and it was not significantly affected by puromycin.

It might be argued that the process of extrusion damaged the axoplasm and thus reduced its ability to incorporate amino acids into protein. However, isolated sheaths retained their capacity for normal protein synthesis even after they had been subjected to the process of extrusion (Table III). The lack of protein synthesis in isolated axoplasm is

FIGURE 4 Light microscope autoradiographs of transverse sections through squid giant nerve fibers incubated in L-[^{3}H]leucine for 2 h. The fibers were fixed in paraformaldehyde, dehydrated, and embedded in Epon. Previous experiments with puromycin have demonstrated that this procedure eliminates all of the soluble radioactivity from the nerve fiber. In (*a*) and (*b*) the small nerve fibers were left attached to the giant nerve fiber (*Ax*). In (*c*) which is a low-power, darkfield micrograph, the small fibers were removed before the incubation. Silver grains are particularly abundant over the adaxonal glial layer which directly surrounds the giant axon (*Ax*) and the smaller axons in (*a*) and (*b*). The outer connective tissue layer of the sheath is more lightly labeled except for the heavily labeled connective tissue cells of the sheath (arrows). The axoplasm of both the giant axon and smaller axons contains label far in excess of background levels which can be seen in the artifactual space between the giant fiber and small fibers (*b*). Note that the small fibers were completely removed from the cleaned fiber shown in (*c*).

TABLE III

Comparison of Amino Acid Incorporation into Proteins of Isolated Axoplasm or Sheath with Incorporation into Intact Giant Fibers

	Axoplasm			Sheath	
	Isolated axoplasm		Intact giant fiber	Isolated sheath	Intact giant fiber
	Control	Puromycin			
	cpm			*cpm*	
	495	310	7,550	74,200*	67,500
	150	235	9,271	95,670*	27,750
	152	57	9,805	69,550*	68,010
	262	125		25,690	
	387	217		6,880	
				11,540	
Mean	289	188	8,872	56,700	54,420

Isolated axoplasm was obtained from the giant fibers by extrusion. 3–5 μl of axoplasm obtained from an individual fiber was incubated for 90 min in a 5-μl microwell, in which a 1-μl solution containing 1 μCi of L-[^{3}H]leucine had previously been dried. In the puromycin experiments the microwells also contained 2 μg of dried puromycin. TCA-soluble radioactivity in the isolated axoplasm was found to be comparable to that in the axoplasm from intact giant fibers. Mean values for TCA-soluble radioactivity in isolated axoplasm and axoplasm obtained from incubated giant fibers were 416,000 cpm and 228,000 cpm, respectively. Isolated sheaths were obtained by laying the giant fibers onto a piece of tissue paper soaked with sea water and extruding the axoplasm. The isolated sheaths were then incubated for 90 min in seawater containing L-[^{3}H]leucine by the method described for whole giant fibers. Axoplasm from the three isolated sheaths denoted by asterisks was discarded because it was contaminated with seawater. Axoplasm from the remaining sheaths was collected for incubation. However, in order to insure that the axoplasm was not contaminated with seawater, these sheaths may have been briefly dehydrated. This factor could explain the relatively low incorporation of those isolated sheaths. Intact giant fibers were also incubated for 90 min and the axoplasm and sheath were prepared in the usual way.

probably not the result of limited energy stores or a missing metabolite provided by the sheath. The axoplasm contains substantial amounts of amino acids and carbohydrates, and these metabolites can be converted into ATP by enzyme systems known to be present in axoplasm (10, 54). Furthermore, Larrabee and Brinley (38) have shown that isolated axoplasm from the squid giant axon incorporates ^{32}P into phospholipids as effectively as axoplasm in intact giant fibers. Although this latter study indicates that isolated axoplasm retains its capacity to carry out some synthetic processes, the lack of protein synthesis in our experiments on isolated axoplasm remains a negative result and is subject to the criticism which applies to such results. Therefore, a more direct experiment was designed to determine whether endogenous axonal protein synthesis contributes significantly to the appearance of newly synthesized axonal proteins in the squid giant fiber.

Absence of an Effect of RNase on the Appearance of Labeled Proteins in the Axoplasm of Squid Giant Fibers

Since RNase destroys the necessary components involved in protein synthesis, it can be used as an inhibitor of protein synthesis. Furthermore, since RNase is a protein, we reasoned that it would remain inside the giant axon, if it was injected into the axoplasm. Thus, any inhibitory effects of intra-axonal RNase would be limited to the axon and not involve the surrounding glial cells which represent the putative source of the newly synthesized axonal proteins. This logic led us to choose RNase as a selective inhibitor of protein synthesis rather than the more commonly employed inhibitors. Injection of RNase into the axon through a cannula inserted from one end of the axon had no apparent effect upon the appearance of labeled proteins in the axoplasm even though the internal concentration of RNase inside the axon was calculated to be 0.1 mg/ml (Table IV). The lack of an effect of RNase on the appearance of labeled proteins in giant axons has been verified in our studies of the perfused giant axon (18). These results stand in sharp contrast to the dramatic effect produced when similar concentrations of RNase were injected into *Aplysia* giant neuron cell bodies. The synthesis of proteins in *Aplysia* neurons, which actively engage in protein synthesis, was inhibited by ~90% (unpublished results). Thus, the use of RNase to inhibit intracellular protein synthesis appears to be valid; and the lack of an effect of RNase upon the appearance of labeled protein in the giant axon provides strong supporting evidence for the hypothesis that the newly labeled axonal proteins are synthesized at some site outside the confines of the axonal plasma membrane.

In Vivo Incorporation of [^{3}H]Leucine into Squid Giant Fibers

To ascertain whether the information obtained

from the isolated giant fibers in vitro is relevant to the system in vivo, comparisons of the incorporation of labeled precursors into proteins of the giant fiber have been made in vivo and in vitro. The results of these comparisons indicated that there was no significant difference between the relative amounts of incorporation into proteins of the sheath and axoplasm in these two circumstances (Table V). The absolute amounts of both soluble and incorporated radioactivity in the giant fibers labeled in vivo were 1,000-fold less than in the in vitro fibers. This difference most probably reflects the significant dilution of the labeled precursor which was injected into the vascular space surrounding the giant fiber in the in vivo experiments. However, it is noteworthy that the axoplasm/sheath ratios, which we believe reflect the amount of labeled protein transferred from sheath to axon, were similar in both in vivo and in vitro experiments.

TABLE IV

Injection of Ribonuclease into Squid Giant Fibers

	TCA-precipitable cpm ($\times 10^{-3}$) per giant fiber		
	Axoplasm	Sheath	A/S
			%
Control	26.4	131.6	21
RNase 1	21.30	124.5	17
RNase 2	25.29	133.3	19

Squid giant fibers were incubated for 2 h in artificial seawater containing [^{3}H]leucine as described in Materials and Methods. Two giant fibers were injected with RNase by inserting a cannula in one end of the axon and injecting artificial axoplasm containing RNase.

The internal concentration of RNase was estimated to be 100 μg/ml. Axoplasm was extruded from the giant fibers in the usual way, and the TCA-precipitable radioactivity was measured accordingly. The control data are mean values for nine giant fibers.

Since the cell bodies remained intact and were connected to the giant axons in the in vivo experiments, the possibility arises that the labeled proteins appearing in the axon were conveyed in this instance by axonal transport. However, this possibility is unlikely for at least two reasons. First, the isotope was injected into the vascular space surrounding the giant fiber at a distance 2–3 cm from the ganglion, in order to expose the giant fiber maximally to the precursor and the ganglion as little as possible. Second, we have made a number of attempts to demonstrate axonal transport in the squid giant fiber and have been unable to obtain evidence for transport at the rates necessary to label the giant axon appreciably several centimeters from the cell bodies 3 h after injection of the precursor. Thus, these results indicate that the information obtained in our studies of isolated giant fibers in vitro have relevance to the intact system.

Analyses of Labeled Polypeptides Appearing in the Giant Fiber

Previous analyses of the labeled proteins in the axoplasm and sheath on SDS-polyacrylamide gels have shown these polypeptides to be heterogeneous with respect to molecular weight (42). Although some similarities were found between the labeled proteins of the axoplasm and sheath, significant differences were also noted. To better compare the proteins of the axoplasm and sheath, a double-label experiment was carried out. We compared [^{3}H]leucine-labeled axoplasmic proteins with ^{14}C-labeled sheath proteins run concurrently on individual SDS gels. Since the glia-neuron transfer hypothesis presupposes that the labeled polypeptides of the axoplasm should also be present in the sheath, one of the purposes of this experiment was to test this aspect of the hypothe-

TABLE V

Incorporation of Amino Acids into Proteins of Squid Giant Fibers Exposed for 3 h to Precursor In Vivo and In Vitro

	In vivo			In vitro		
	Axoplasm	Sheath	A/S	Axoplasm	Sheath	A/S
	cpm		%	*cpm* $\times 10^{-2}$		%
TCA-precipitable	380	1,735	18.8	308	2,890	10.7
TCA-soluble	895	608		1,806	4,028	

In the in vivo experiments, 10 μCi of [^{3}H]leucine were injected bilaterally into the vascular space surrounding both giant axons of live squid. The axons were dissected from the animals 3 h after injection, and radioactive proteins in the axoplasm and sheath were measured by the technique employed in the standard in vitro experiment. The values are means calculated for five in vivo giant fibers and eight in vitro giant fibers.

sis. Examples of possible homologies between axoplasmic and sheath proteins can be seen in the profile of soluble proteins having molecular weights between 12,000 and 45,000 daltons (Fig. 5). Several other examples of apparent homology are illustrated in the proteins of the pellet, notably at 6,000, 43,000, and between 65,000 and 150,000 daltons. However, the complexity of the profiles and the large number of overlapping peaks found on these SDS-gels rule out the possibility of using this method to evaluate critically the prediction that all of the labeled polypeptides of the axoplasm are also present in the sheath.

The patterns of labeled proteins produced by the incorporation of L-[^{3}H]leucine were compared with those produced by the incorporation of an acidic amino acid (L-[^{3}H]glutamate) and the incorporation of a basic amino acid (L-[^{3}H]arginine). Each profile differed somewhat from the others, as might be expected, since the amount of these individual amino acids is disproportinately high in certain proteins (Fig. 6). These experiments with

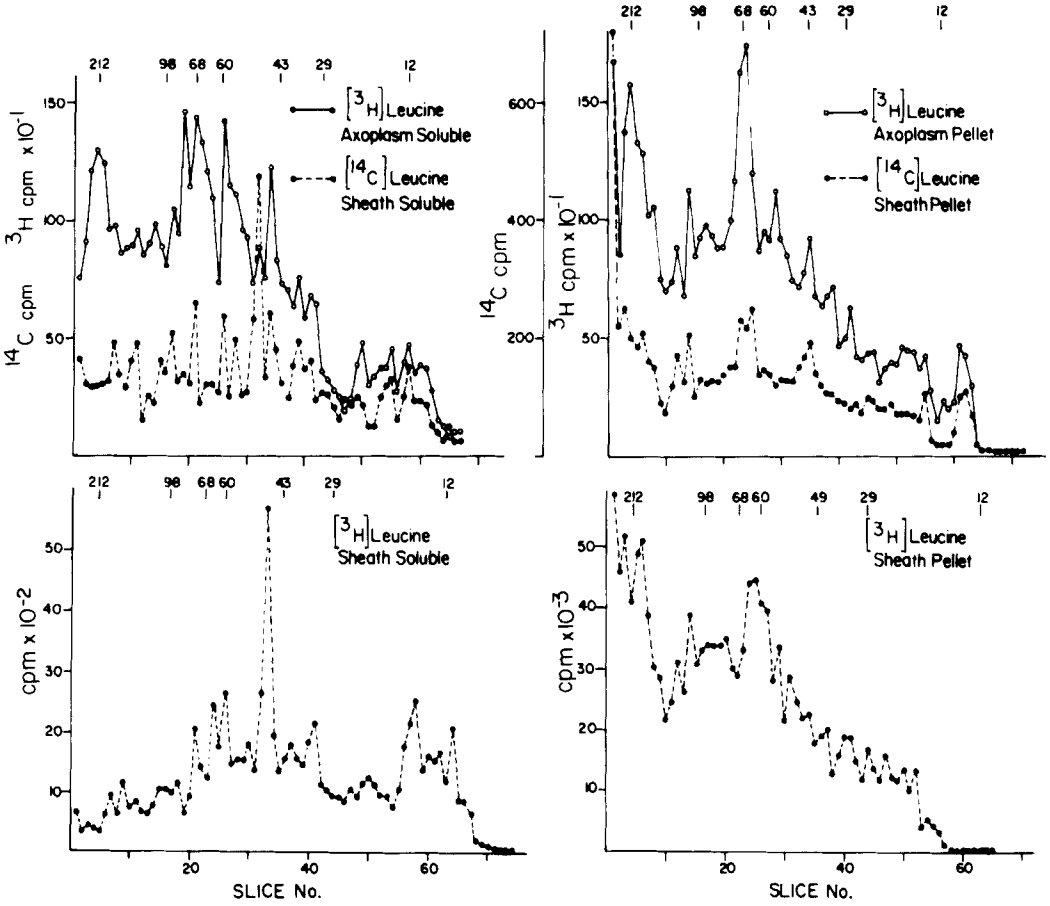


FIGURE 5 Comparison of the proteins which appear in the sheath and axoplasm of squid giant nerve fibers incubated in L-[^{3}H]leucine (250 μCi/ml) or L-[^{14}C]leucine (100 μCi/ml). Four giant fibers were incubated for 3 h and the axoplasm was separated from the sheath by extrusion. The axoplasm and sheath were homogenized separately in 0.25 ml of 50 mM Tris, pH 7.4, and centrifuged for 2 h at 27,000 *g*. Although 80% of the labeled axoplasmic proteins were soluble, only 30% of the sheath proteins were soluble. In the upper panels, ^{3}H-labeled axoplasm proteins were mixed with ^{14}C-labeled sheath proteins and compared on the same gels. The gels were sliced at 1-mm intervals and ^{3}H and ^{14}C were determined by the double channel method to correct for spillover. The lower panels illustrate the more heavily labeled ^{3}H-sheath proteins for comparison with the ^{14}C-proteins in the upper panel. The molecular weight markers in thousands which are noted at the top of each panel were calculated from a series of seven standard proteins run on parallel gels.

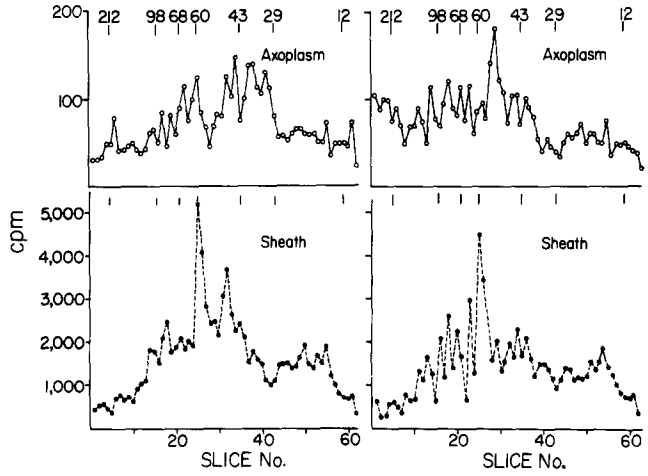


FIGURE 6 SDS-polyacrylamide gel electrophoretograms comparing axoplasmic and sheath proteins labeled with either L-[^{3}H]glutamate (right hand panels) or L-[^{3}H]arginine (left hand panels). Only the soluble proteins are shown. Incubation conditions and preparative procedures are identical to those described in Fig. 5.

amino acids other than leucine, such as glutamate, arginine, and methionine, indicate that the appearance of labeled proteins in the axoplasm is not peculiar to the use of leucine as a precursor. The axoplasm/sheath (A/S) ratios for giant fibers incubated in each of these amino acids for 90 min were similar to those for [^{3}H]leucine and ranged from 10 to 20% (unpublished results).

One important feature of the SDS-gel profiles is the obvious dissimilarity between the profiles of the axoplasm compared to the sheath. For example, the soluble sheath fraction evidences labeled peaks which contain a disproportionate amount of the total radioactivity in this fraction. Examples of such peaks are seen at 50,000 daltons in Fig. 5 and 60,000 daltons in Fig. 6. These differences in the profiles of labeled proteins found in axoplasm and sheath may reflect a selectivity in the transfer of proteins from the sheath to the axoplasm. Alternatively, the differences may result from the complex morphology of the sheath. For example, the outermost connective tissue layer of the sheath which is separated from the axon by a thick basal lamina (Fig. 1) may not interact significantly with the axon. In regard to this question of specificity in the transfer mechanism, we investigated the possibility that labeled proteins might leak nonspecifically from the sheath into the media and subsequently be taken up nonspecifically from the media into the axon. For example, it has been demonstrated that radioactively labeled bovine serum albumin is taken up by the squid giant axon and appears in the axoplasm (21).

When giant fibers were incubated with [^{3}H]-leucine for 3 h, proteins appeared in the incubation media and amounted to <50% of the labeled proteins appearing in the axoplasm. When these labeled proteins in the media were analyzed on SDS gels (Fig. 7), the profile was completely different from that of the labeled proteins of the axoplasm (Fig. 5). Therefore, it is unlikely that a substantial fraction of the labeled proteins which were found in the axoplasm resulted from the uptake of labeled proteins that were present in the incubation media.

Alterations of the Ionic Composition and Osmolarity of the Incubation Media

Since the axolemma must play some part in the transfer of proteins from the glial cells to the axon, it seemed plausible that alterations of certain extracellular ionic concentrations could affect the transfer of proteins between the sheath and the axon. Ca^{++} is important in a variety of phenomena involving plasma membranes, for example, electrotonic coupling, pinocytosis, exocytosis, and the fusion of myoblasts. Replacement of Ca^{++} in the experimental media with Mg^{++} resulted in a significant reduction of the axoplasm/sheath ratio; however, reduction of the extracellular Ca^{++} had no measurable effect on either the amount of incorporation of [^{3}H]leucine into proteins of the sheath or the amount of soluble radioactivity in the axoplasm or the sheath (Table VI). Having obtained this result, we examined the effects of Co^{++}, a

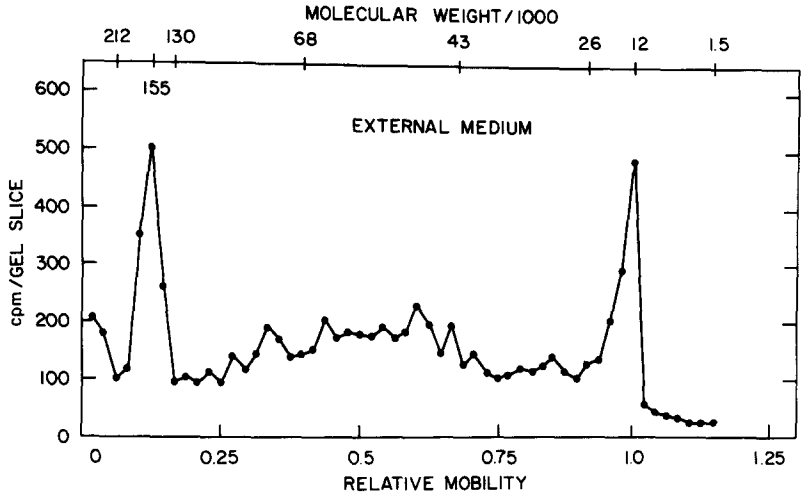


FIGURE 7 Illustration of the labeled proteins which appear in the external medium during the incubation of giant nerve fibers. The incubation medium was collected from a preparation in which two axons were incubated for 3 h in 50 μCi/ml [^{3}H]L-leucine. The medium was dialyzed for 48 h against four changes of 1,000 ml of distilled water (4°C). The sample was lyophilized and analyzed on SDS gels as described in Materials and Methods (see Fig. 4 for other details). The profile of these proteins is markedly different from that of the axoplasmic proteins seen in Fig. 4.

TABLE VI

Effects of Divalent Cations on the Appearance of Labeled Proteins in the Isolated Squid Giant Fiber

	Control			Experimental			
Experimental condition	Axoplasm	Sheath	A/S	Axoplasm	Sheath	A/S	N
			%			%	
TCA-precipitable cpm ($\times 10^{-3}$) per giant fiber							
Ca^{++} free	24.8 (3.7)	189 (26)	14.1	12.6 (2.6)§	163 (7.6)	7.56§	7
60 mM Co^{++}	22.4 (6.5)	179 (40)	12.2	.48 (.08)*	32.3 (5.3)*	1.56‡	4
10 mM Co^{++}	16.2	114	14.2	2.23	44.1	5.05	3
60 mM Ca^{++}	43.3 (4.0)	237 (19)	18.7	20.1 (3.1)*	189 (48)	12.6	5
TCA-soluble cpm ($\times 10^{-3}$) per giant fiber							
Ca^{++} free	231	703		270	644		7
60 mM Co^{++}	159	406		103	391		4
10 mM Co^{++}	89.3	206		90.8	321		3
60 mM Ca^{++}	385	428		116*	319		5

Ionic concentrations for the control media were those listed in the Materials and Methods. The experimental media differed from the control media as follows, Ca^{++} free: 10 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$; 60 mM Co^{++}: 10 mM $CaCl_2$ and 50 mM $MgCl_2$ were replaced by 60 mM $CoCl_2$; 10 mM Co^{++}: 10 mM $CaCl_2$ was replaced by 10 mM $CoCl_2$; and 60 mM Ca^{++}: 50 mM $MgCl_2$ was replaced by 50 mM $CaCl_2$. Each experimental fiber was paired with its contralateral homologue from the same animal and the results expressed as an average of the number of experiments indicated by N with SEM in parentheses. Statistical comparisons of the paired data were made using a paired *t* test. The values for *P* were > 0.05 except where indicated.

* $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.005$.

divalent cation which competes for Ca^{++}-binding sites. Substitution of Co^{++} for either Ca^{++} or both Ca^{++} and Mg^{++} greatly inhibited incorporation of proteins into the sheath and produced greater reduction of the axoplasm/sheath ratio than did the removal of Ca^{++} from the media (Table VI). The reduction of L-[^{3}H]leucine incorporation in the giant fibers incubated in Co^{++} does not appear to result from a reduction in the total soluble L-[^{3}H]leucine pool. However, this latter interpretation must be qualified, because the total soluble radioactivity does not necessarily reflect the specific amino acid pools directly associated with protein synthesis in the glial cells. Replacement of Mg^{++} by Ca^{++} had no significant effects on the levels of L-[^{3}H]leucine incorporation in the sheath but did reduce the soluble radioactivity and the labeled proteins appearing in the axoplasm (Table VI). The A/S ratio appeared to be less than the control values but the difference was not statistically significant.

Replacement of Na^+ with Li^+ reduced the amount of labeled proteins appearing in the sheath, but had no effect on the A/S ratio (Table VII). Complete replacement of external Na^+ with an uncharged molecule, sucrose, also reduced L-[^{3}H]leucine incorporation in the sheath; and in this condition both the A/S ratio and the soluble radioactivity appearing in the axoplasm were reduced. The osmolarity of the media was increased in one set of experiments, with the view of shrinking the glial cells from the axon membrane in order to reduce interactions between the glia and the axon. This condition had a drastic effect on the incorporation of L-[^{3}H]leucine into the sheath but only a small effect on the A/S ratio, which is of questionable significance (Table VII). Addition of colchicine, which interferes with microtubule formation and has been shown to inhibit secretion from some cells (53) but not others (48, 63), had no effect on the A/S ratio (Table VII). An attempt was also made to interfere with the transfer process by adding bovine serum to the media, since it has been shown that albumin is taken up from the media by the giant axon (21). However, the presence of albumin had no effect on any of the parameters measured (Table VII). The results of these experiments, such as the replacement of Ca^{++} with Mg^{++}, indicate that the appearance of proteins in the axoplasm can be selectively inhibited without affecting protein synthesis in the sheath. They are consistent with the glia-neuron transfer hypothesis and provide a basis for the future study of the mechanisms involved in the transfer of proteins between adjacent cells.

DISCUSSION

The concept that glial cells assist in the support of

TABLE VII

Effects of Monovalent Cations, Osmolarity, and BSA on the Appearance of Labeled Proteins in the Isolated Squid Giant Fiber

	Control			Experimental			
Experimental condition	Axoplasm	Sheath	A/S	Axoplasm	Sheath	A/S	N
			%			%	
TCA-precipitable cpm ($\times 10^{-3}$) per giant fiber							
Li^+/ONa^+	32.9 (3.7)	211 (26)	17.0	4.76 (.75)‡	36.3 (7.8)‡	15.5	6
Sucrose/ONa^+	27.4 (2.4)	152 (17)	18.7	3.66 (.57)‡	50.7 (4.8)‡	7.57*	4
Hyperosmotic sucrose	30.9 (3.2)	275 (44)	13.3	1.02 (.24)‡	18.1 (6.2)‡	9.31	5
BSA	11.3 (2.5)	88.3 (17)	13.7	8.23 (1.2)	60.8 (15)	14.4	5
Colchicine	7.42	48.7	15.0	4.50	28.1	16.1	3
TCA-soluble CPM ($\times 10^{-3}$) per giant fiber							
Li^+/ONa^+	79.1	362		109	282		6
Sucrose/ONa^+	275	444		151‡	436		4
Hyperosmotic sucrose	130	297		59.4*	117‡		5
BSA	51.4	61.0		49.7	108		5
Colchicine	195	621		150	381		3

Ionic concentrations for the control media were those listed in Materials and Methods. The experimental media differed from the control media as follows, Li^+/ONa^+: 425 mM NaCl replaced by 425 mM LiCl; Sucrose/ONa^+: 425 mM NaCl replaced by 425 mM sucrose; Hyperosmotic sucrose: 720 mM sucrose added to control media; and BSA competition: 20 mg/ml (0.17 mM) bovine serum albumin added to control media. Each experimental fiber was paired with its contralateral homologue from the same animal, and the results are expressed as an average of the number of experiments indicated by N with SEM in parentheses. Statistical comparisons were made on all the paired data by using a paired t test. The values for P were >0.05 except where indicated.

* $P < 0.05$.

‡ $P < 0.005$.

neurons both structurally and metabolically is one of the recurring themata in neurobiology. It was expressed by Golgi in 1883, quoted by Kuffler and Nicholls (37), and subsequently in an extreme version which held that the axons were formed by glial cells. This latter hypothesis was shown to be incorrect in great part by Ramón y Cajal (52) who convincingly demonstrated that the axon emanates from the neuron cell body. The proposition that the cell body is the trophic source of the axon has played an important role in the affirmation of the neuron doctrine. A contemporary proof of this theory is provided by the demonstration that axonal proteins are synthesized in the nerve cell body and transported within the axon (40). Although this historical trend has focused attention almost exclusively on the neuron cell body and axonal transport as the source of axonal proteins, a small but persistent literature has raised the possibility that proteins are supplied to the axon by mechanisms which exist locally at the level of the axon (4, 15, 20, 35, 42, 55, 56, 60, 61). These reports have convincingly demonstrated that amino acids are incorporated into axonal proteins in nerve fibers separated from their cell bodies and have awakened interest in the possibility that the glial cells might play a contributory role in the supply of macromolecules to the axon (see Singer, reference 60, for review). To test this possibility by using labeled precursors to follow the movement of proteins from the glial cells into the axon, it has been necessary to critically determine whether endogenous axonal protein synthesis contributes significantly to the appearance of labeled proteins in the axon.

Does Protein Synthesis Occur within the Squid Giant Axon?

Mitochondria are present in significant numbers within the squid giant axon and probably synthesize some proteins. Since chloramphenicol, an inhibitor of mitochondrial protein synthesis, has no effect on the appearance of labeled proteins in the giant fiber, we conclude that the mitochondria do not contribute significantly to amino acid incorporation in the giant fiber. The apparent absence of any significant contribution by mitochondria to the labeled proteins which appear in the squid giant axon is not surprising, since mitochondrial protein synthesis represents a very small fraction of the total protein synthesis in eukaryotic cells, and most of the mitochondrial proteins are synthesized on cytoplasmic ribosomes.

The alternative possibility that extramitochondrial protein synthesis in the axoplasm contributes to the synthesis of axonal proteins is also unlikely because there are few, if any, ribosomes in the axoplasm. Ribosomes appear to be confined almost exclusively to the cell body and dendrites of mature neurons (71). Although ribosomes are found in the neurites of developing neurons, the number of ribosomes decreases as these neurites develop into axons. This process appears to continue during later development since Zelena (71) has found a small number of ribosomes in the spinal ganglion cell axons of neonatal rats but very few in those of mature animals. The few axonal ribosomes which are found in postembryonic neurons are located near the cell body. The exclusion of the rough endoplasmic reticulum and free polysomes from the axon appears to be one of the important events that occur during the differentiation of the axon as compared to the dendrites of vertebrate neurons which contain rough endoplasmic reticulum.

Although very few ribosomes enter the mature axon and although they have not been found in the distal regions of the axon, RNA is present in the axon of a number of vertebrate and invertebrate neurons. Analysis of this RNA in the giant axons of the squid and a marine polychaete indicate that >90% is 4S RNA (41). Further analyses of this axoplasmic RNA in the squid indicate that it is physiologically active transfer RNA, since it can be charged enzymatically with amino acids (5). The presence of substantial amounts of transfer RNA in the axon raises questions about its source and its function in the axon. Whatever the role of this transfer RNA is, the absence of ribosomes and ribosomal RNA in the axon suggests that it does not participate in endogenous axonal protein synthesis.

The possibility that endogenous protein synthesis contributes significantly to the newly synthesized proteins which appear in the axon is also negated by our direct analyses of this question. When isolated axoplasm is incubted with labeled amino acids, protein synthesis is not observed. However, this experiment is subject to the following criticism. When axoplasm is extruded from the giant nerve fiber, a small but significant amount of axoplasm located next to the axolemma remains with the sheath. Therefore, it can be argued that a protein synthetic mechanism which remains tightly associated with the axolemma might have passed unnoticed in the experiments on extruded axoplasm. This possibility was ruled out by introduc-

ing RNase directly into the axon. Since RNase can eliminate protein synthesis by degrading transfer RNA, messenger RNA, and ribosomal RNA, the concentration of RNase achieved inside the axon (1.0 mg/ml in the perfusion studies) should have been orders of magnitude in excess of those required to inactivate any endogenous axonal protein synthesis. However, RNase had no effect on the incorporation of amino acids into axonal proteins of the giant fiber.

The appearance of labeled proteins in the axoplasmic perfusate from squid giant axons for periods of up to 8 h also argues against endogenous axonal protein synthesis. During the perfusion, the contents of the axon are continually washed out. It is probable that all the soluble components of the axoplasm such as transfer RNA and other factors required for protein synthesis are leached out of the axon by the substantial volume of the perfusate. As much as 7 ml of artificial axoplasm was perfused over a period of 8 h through a region of the axon which had a volume of ~1 μl. Yet the appearance of labeled proteins in the axon continued linearly throughout this period.

Although the possibility of endogenous axonal protein synthesis has frequently been raised in the literature (15, 20, 35), no evidence has been provided which convincingly supports this suggestion. None of the previous studies on protein synthesis in isolated nerve fibers have determined whether the newly synthesized axonal proteins were synthesized in the axon or in the glial cells. In all of these experiments, the glial cells remained intact around the axon during the incubation with labeled amino acid precursor.

The study of protein synthesis in synaptosomes represents an alternative paradigm which has been used to study endogenous axonal protein synthesis (2). Although synaptosome fractions are enriched with presynaptic axon terminals, they contain other subcellular components. A careful autoradiographic study of the incorporation of labeled amino acids into synaptosomes demonstrated that most of the incorporation can be ascribed to the postsynaptic elements and to synaptosomal mitochondria (19). Barondes (2) in his recent review of this subject has concluded that nonmitochondrial protein synthesis has yet to be convincingly demonstrated in synaptosomes.

Thus, both the evidence in the literature and our studies on the squid giant fiber indicate that endogenous axonal synthesis does not account for the bulk of the newly synthesized proteins which appear in the axoplasm of isolated nerve fibers. And, by the process of elimination, the adaxonal glial cells become the most logical site for the local synthesis of axonal proteins in the isolated squid giant nerve fiber. This logic does not provide a proof of the glia-neuron protein transfer hypothesis. However, by reducing the alternatives, it does provide important support for the hypothesis. Further support is provided by our experiments analyzing the characteristic properties of the synthesis and appearance of labeled proteins in the sheath and axon of the giant nerve fiber.

The Relative Amount of Newly Synthesized Glial Proteins Which Are Transferred to the Axon

If the glial cells surrounding the giant axon are the source of the labeled axonal proteins, then it necessarily follows that the amount of labeled proteins in the gial cells should exceed the amount of labeled protein in the axon, at least initially. The amount of labeled protein that appeared in the sheath was 5–10 times greater than that in the axon, at all of the intervals investigated (Fig. 3). Grain counts from autoradiographs of labeled giant fibers incubated for 1, 2, and 4 h indicate that ~50% of the labeled protein in the sheath is concentrated in the thin layer of adaxonal glial cells which directly surround the axon (Figs. 1 and 2). The amount of labeled protein in these glial cells was from 3 to 5 times the amount that appeared in the axon (42). These results demonstrate that the adaxonal glial cells actively engage in protein synthesis and can easily account for the labeled proteins that appear in the axon. If we assume that relatively little of the newly synthesized proteins are degraded during the period of incubation, then the amount of newly synthesized protein which is transferred from the glia to the axon can be estimated by the following relationship: cpm axoplasm/cpm axoplasm + cpm sheath $\times$ 100%. Approx. 8–20% of the labeled proteins which are synthesized in the nerve fiber appear in the axon. If these proteins are transferred principally from the adaxonal glial cells which contain ~50% of the newly synthesized protein of the sheath, then the adaxonal glial cells transfer as much as 40% of their newly synthesized protein to the axon.

To have some measure of the relative role that the glial cell plays in the overall economy of the neuron, it will be important to have an estimate of the relative contribution that these glial cells make to the axon, as compared to that from the neuron

cell body via axonal transport. The giant axon is a syncytium formed by a number of neuron cell bodies which are located in the stellate ganglion (70). Although axonal transport has yet to be demonstrated in the squid giant axon, it has been shown in other molluscan neurons (29). The relative contributions of newly synthesized proteins to the axoplasm by axonal transport from the cell bodies as compared to that from the glia can be roughly estimated by determining the relative incorporation of labeled amino acids into protein in the nerve cell bodies and the giant fiber. Such estimates must be viewed with caution because of the many factors involved in the labeling of proteins by radioisotopic precursors, e.g., rate of uptake and pool size.

Incubation of a 3-cm length of giant nerve fiber in [^{3}H]leucine for 2 h showed that ~25,000 cpm of newly synthesized protein has entered the axoplasm from the glial sheath (Fig. 2). In contrast, incorporation of [^{3}H]leucine into proteins of the total stellate ganglion, under the same incubation conditions, is ~56,000 cpm. Assuming that ~10% of this label is in neuron cell bodies associated with the giant axon, then only 5,600 cpm of newly synthesized protein is made by these neuron somata in 2 h. Thus, the rate of labeled protein entering the axoplasm from the glia in a 3-cm length of axon is fivefold greater than the rate of total incorporation of [^{3}H]leucine into the relevant population of neuron cell bodies. This comparison is highly conservative since in other nerve cells with a substantial extent of axonal transport, only a fraction of the total counts per minute incorporated into protein in the nerve cell bodies (40) is transported to the axon.

Glial cells may also contribute a significant amount of protein to the axons in other species. The relative amount of amino acid incorporation per milligram protein in crayfish peripheral nerve is twice the incorporation into the abdominal ganglion, and a significant fraction of the protein synthesized in the peripheral nerve appears in the motor axons (55). The amounts of amino acid incorporation per milligram protein into toad spinal ganglia and dorsal roots are equivalent (55), as are the rates of incorporation into amphibian peripheral nerve, brain, and liver (9). The amount of protein supplied to the axon by any individual glial cell, when observed in an autoradiograph of a cross section through that axon, at first glance may appear to be small. However, one need only recall that the volume of the axon of neurons such as a vertebrate anterior horn cell can be thousands of times greater than that of the cell body, and that the number of glia (in this case Schwann cells) which surround the axon can number in the thousands. Therefore, the total amount of protein contributed by all of the adaxonal glial cells along the length of the axon may represent an important additional source compared to that coming from the cell body.

Latency between Synthesis of Axonal Proteins in the Sheath and Their Appearance in the Axoplasm

If the glial cells surrounding the giant axon are the source of the labeled axonal proteins which appear in the isolated giant fiber, then it follows necessarily that the appearance of labeled proteins in the glial cells should precede their appearance in the axon. Our kinetic studies suggest that this interval is relatively short, of the order of a few minutes. For example, the appearance of labeled proteins in the sheath and axon is kinetically very similar. The curves in Fig. 2 are linear between 0.5 and 4.0 h, and it is noteworthy that if the linear portions of these curves are extrapolated to meet the abscissa, their intercepts are similar. Calculation of these intercepts by linear regression yields values of 24.9 min for the sheath and 25.7 min for the axoplasm. The similarity in these intercepts is consistent with the suggestion that the transfer of the labeled proteins to the axon occurs within a few minutes of their synthesis in the glial cells.

This suggestion is consistent with the morphology of the adaxonal glial layer which is only 5–10 μm thick. The glial cell processes are in close proximity with the axon, and the plasma membranes of the glial cells and the axon are separated by a narrow cleft of only 5–10 nm (66, 67). Adaxonal glial cells of the giant fiber have many of the morphological characteristics associated with certain types of protein-secreting cells, including a large number of vesicular profiles (66, 67). Many of these vesicular profiles are found in close proximity to the axon. The glial cells do not contain electron-dense, condensing vacuoles of the type seen in zymogen-secreting cells which store proteins for release in response to a stimulus. The morphology of glial cells is more like that of protein-secreting cells such as fibroblasts and hepatocytes. The lack of condensing vacuoles in the adaxonal glial cells is consistent with the sugges-

tion that the glial cells rapidly release their secretory proteins, rather than storing them for subsequent release.

Is the Transfer of Proteins from Glial Cells to the Axon a Physiological Process?

A number of lines of evidence indicate that the transfer of proteins from glial cells to the axon is a physiological process and not an artifact of our experimental system. (*a*) The integrity of the axon was assessed in perfusion experiments by eliciting and recording action potentials; and the axons continued to conduct action potentials throughout these experiments (18). (*b*) The linear kinetics of protein synthesis in the sheath indicate that the glial cells were not significantly damaged by the procedures employed in the in vitro experiments. (*c*) The similarity between the results of the in vitro experiments and the in vivo experiments demonstrates that the transfer of proteins from glial cells to the axon is a naturally occurring phenomenon which can be replicated in vitro.

The transfer of proteins from the glial cells to the axon may be a specific process. The differences that we have observed between the labeled proteins which remain in the sheath and those which appear in the axon (Figs. 5 and 6) may indicate that the glial cells synthesize a specific fraction of proteins that is earmarked for transfer to the axon. The proteins that enter the axon also differ substantially from those that are apparently released by the sheath cells into the incubation media.

Modulation of Local Axonal Protein Synthesis and Transfer

Our studies on giant fibers from squid collected during three seasons indicate that when paired giant fibers from individual squid were compared, the amount of protein synthesis and transfer were similar. However, when different squid were compared, the variation ranges over an order of magnitude The observed variation between squid may indicate that the amount of protein transfer is in some way governed by the physiological state of the nervous system.

One interesting possibility is that the synthesis and transfer of proteins in the giant fiber is related to the electrical activity of the axon (17). This possibility is made even more intriguing by the observation that the adaxonal glial cells of the giant axon are hyperpolarized by axonal action potentials and that this hyperpolarization may be mediated by acetylcholine (68, 69). Fischer and Litvak (16) measured the effects of a brief period of stimulation on amino acid incorporation into giant fibers. The stimulated fibers showed a significant increase in the percentage of cold TCA-precipitable radioactivity expressed relative to the total radioactivity. However, these results are subject to question, since chloramphenicol reduced the incorporation by 80%. Furthermore, the labeled amino acids were injected into the axon and the expression of the results as incorporated radioactivity as a percentage of total radioactivity does not provide information on the possible effects of stimulation on the amounts of soluble radioactivity in the sheath. Jethmol and Koenig (33) were unable to find an effect of electrical stimulation on the incorporation of amino acids into isolated mammalian axons. We have also measured amino acid incorporation in experiments on stimulated squid giant nerve fiber; however, the results were equivocal.

Although it is unclear whether electrical activity affects either protein synthesis or protein transfer in giant fibers, we and others (49) have demonstrated that gross alterations in the ionic milieu can affect both protein synthesis in the glial cells and the transfer process. For example, replacement of Ca^{++} appears to inhibit the transfer of proteins from the glial cells without affecting total protein synthesis in the glia. This observation raises the possibility that Ca^{++} plays some role in the transfer process. Ca^{++} appears to be important in protein secretion from a variety of cells and in rapid axonal transport in neurons (12). An alternative possibility is that the reduced external Ca^{++} selectively inhibits the synthesis of those proteins which are destined for transport from the glial cells. Since such inhibition would result in only a small reduction in the total synthesis of sheath proteins, more experiments are required in order to choose between the alternatives.

The Role of Proteins Transferred from Glia to Neurons

The functional properties of neurons and glial cells have evolved concomitantly, and these two cellular populations can be viewed as an integrated unit in which the participating elements are physiologically coordinated. It has long been thought that glial cells play a particularly important role as supporting cells for the neurons. This

view has logically followed from morphological studies demonstrating that the glial cells cover a considerable fraction of the neuronal surface. These glial investments serve to separate the neurons from one another and also from the circulatory system. The glia have a variety of specialized structural modifications with which they contact the neuronal surface, such as the myelin sheath. Studies of the developing nervous system indicate that glial cells may have some role in guiding the nerve cell bodies and their axons and dendrites, as these elements seek their appropriate positions in the nervous systems (28, 51). These examples of structural relationships between glia and neurons have led to the assumption that glia and neurons interact metabolically. A number of possible mechanisms, which might provide for the molecular coordination of these cells, have been suggested. For example, the glia and neurons might modulate one another by releasing biologically active molecules which interact with surface receptors on the adjacent cells. Another possibility is that the glia are responsive to the alterations of the ionic composition of the extracellular space that occur when the neuronal plasma membrane is depolarized. Some glia have been shown to behave like K^+ electrodes and their membrane potentials passively follow the neurons (37).

Another suggested role for glia which is of particular interest to the studies reported here, is that glia and neurons interact with one another by transferring macromolecules from the intracellular compartment of one cell to the intracellular compartment of neighboring cells. This suggestion has previously been based on inference from morphological observations and biochemical studies which demonstrate inverse changes in the RNA content of neurons and their associated glia (32, 50). See Kuffler and Nicholls (37) for a critical review. Recent studies on nerve growth factor (NGF) provide evidence which is consistent with this suggestion. Burnham et al. (7), Varon and Raiborn (64), and Varon and Saier (65) have found that ganglionic nonneuronal cells enriched with glia have an NGF-like competence with respect to the survival and physiological properties of isolated spinal ganglion cells. When sensory neurons dissociated from neonatal mouse spinal ganglia are cultured in vitro after removing the ganglionic glial cells, these neurons require the addition of NGF to the media in order to survive and elaborate neurites. The addition of glial cells to cultured neurons can substitute for this NGF requirement. One explanation that has been offered is that ganglionic glia provide an indigenous source of NGF or an NGF-like class of molecules (34). The effect of these NGF-like factors could result from the interaction of these molecules with surface receptors, rather than entering the neuronal cytoplasm. However, the recent studies of Hendry et al. (27), Paravicini et al. (47), and Stokel et al. (62) indicate that at least in some cases NGF may operate by an intracellular pathway involving retrograde axonal transport. This latter observation is particularly relevant to the possible role of protein transfer from glial cells to the axon because it indicates that proteins taken up by the axon or its terminals can modulate neuronal physiology at a considerable distance from their point of entry. This possibility, coupled with the observations by Varon and Raiborn (64) and Varon and Saier (65) that glial cells may have a trophic role in neuronal function, raises some interesting questions about the participation of adaxonal glia in the function of neurons that develop and maintain long axons. It seems logical that glial factors might play a proportionately larger role in neurons with long axons than in neurons with short axons, such as those that can be maintained in vitro.

In considering the role of the transfer of glial proteins to the axon, two sites of action are possible. One involves the neuron cell body and retrograde axonal transport (39). The other possibility is that the proteins transferred from the glial cells to the axon act locally within the axon near the site where they enter. This possibility that locally supplied axonal proteins supplement those proteins that are supplied by the cell body via axonal transport has gained credibility since the discovery by Hoy et al. (31) that crayfish motor axons remain functional for periods as long as 200 days or more after they are disconnected from their cell bodies. The prolonged survival of crayfish axons is unusual since other axons, including squid giant axons (58, 70), degenerate within a period of a few days after they are severed from the cell body. The prolonged survival of certain crayfish axons is not easily explained by a cessation in the turnover of proteins in the severed axon, because at least one of the axonal proteins, glutamic acid decarboxylase, which is required for synaptic transmission in the crayfish inhibitory motor fibers, apparently turns over in a matter of days in isolated fibers. This has led Sarne et al. (56) to suggest that this enzyme can be synthesized locally at the level of the axon, possibly in glial cells. The prolonged

survival of crayfish motor axons severed from their cell bodies could be explained if axonal proteins were supplied to the isolated axon by the surrounding glia. Labeled amino acids are incorporated into axonal proteins of isolated crayfish motor axons, and it is noteworthy that the glial cells surrounding severed motor axons hypertrophy and increase their rate of amino acid incorporation into protein after they are severed from the cell body (4, 55). The axons of the crayfish are exceptional, just as the squid giant axon is exceptional with regard to axonal diameter, but in biology it is often the exceptional case that provides the rule. This is exemplified by the important role that studies of the squid giant axon have played in elucidating the fundamental properties of the action potential. If, as the evidence suggests, glia transfer proteins to axons in the squid and in other neurons including vertebrate neurons, the crayfish motor axons may provide an extreme example of the contributory role which glia play in axonal maintenance.

These obervations raise what is certian to be one of the central questions regarding the role of proteins transferred from glia in axonal metabolism. What are the comparative roles of proteins supplied by the nerve cell body via axonal transport and those supplied by glia? There is not enough information about the proteins supplied by axonal transport and those supplied by glia to ascertain which of the components of axonal transport (slow, intermediate, or fast) might be supplemented by glial proteins. However, it is least likely that the glia supplement proteins that are provided by the slow component of axonal transport. The slow component of axonal transport contains the major cytoskeletal structures of the axon, i.e., neurofilaments and microtubules (30). These proteins are long lived and do not appear to turn over until they reach the axon terminal. The profile of labeled slow component proteins on SDS-polyacrylamide gels is relatively simple, having only five principal polypeptides. This contrasts with proteins transferred to the axon by glial cells which are heterodisperse on SDS-polyacrylamide gels. The complex profile seen in Fig. 5 is reminiscent of the equally complex pattern of proteins found in the fast component of axonal transport which has been observed in a variety of vertebrate neurons (1). However, there is at least one major difference between the proteins of the fast component and those transferred from glial cells. While 80% of the glial-transferred proteins remain soluble when they are subjected to centrifugation at 190,000 *g* for 2 h (0.25 M sucrose, 0.01 M Tris, pH 7.4), only 15–30% of fast component proteins in vertebrate neurons are soluble under similar conditions (8, 14).

If the glial cell proteins supplement those proteins conveyed by axonal transport, it is likely that there will be mechanisms that integrate these two sources of supply. In fact, the transfer of proteins from glia to neurons may not be a one-way street. It has been suggested that some axonally transported proteins are transferred from axons to their surrounding glial cells. This issue is clouded by the possibility that labeled amino acids may be released from the axons due to protein turnover and that these amino acids may subsequently be reincorporated into glial proteins (25). This problem of reincorporation has also plagued investigators attempting to assess the possibility that proteins which are axonally transported to axon terminals cross the synapse into the postsynaptic elements (13, 23) and between glial cells and nerve cell bodies (22). Although it has not been possible to demonstrate convincingly either protein transfer from neurons to glia, from neurons to neurons, or from glia to the neuron cell body, the evidence for protein transfer from glia to the axon provides a stimulus for further study of these other transfer routes.

One of the possible roles for the proteins transferred from glial cells to the axon is that they cooperate in the local metabolism of the axon. The concept of metabolic cooperation between cells involving an actual exchange of proteins is strongly supported by in vitro studies of cells from humans with inborn errors of metabolism. Neufeld (45) has demonstrated that the addition of normal fibroblasts to cultures of mutant fibroblasts from patients with Hurler's disease corrects the deficiency of these mutant cells. The corrective factor appears to be the enzyme alpha iduronidase which is deficient in Hurler homozygotes. Subsequent experiments suggest that the enzyme corrects the defect by entering the cell via specific pinocytosis and incorporation of the enzyme into lysosomes where it substitutes for the missing enzyme. This form of metabolic cooperation may also occur in vivo since heterozygotic carriers of Hurler's disease are phenotypically normal, even though half of their fibroblasts express only the mutant X-linked gene (11). This demonstration of metabolic cooperation involving the cell-to-cell transfer of enzymes raises the possibility that such

cooperation may be a general phenomenon in multicellular organisms and suggests that the transfer of proteins from glia to the squid giant axon is not a peculiar phenomenon which has evolved specifically to meet the unusual requirements of the neuron.

Mechanism of Protein Transfer from Glia to Neurons

A number of mechanisms exist in the literature that could readily account for the transfer of proteins from glia to the giant axon. One reasonable possibility is exocytosis of proteins from the glial cell into the extracellular space and pinocytosis by the axon. The morphology of the glia and axon is consistent with this possibility since both the glia and the axon contain numerous vesicular profiles located near their plasma membranes. The inhibition of transfer when the extracellular Ca^{++} concentration is lowered is also consistent with exocytosis and pinocytosis, since these processes are known to be Ca^{++} dependent. Active pinocytosis by neurons is well known and also occurs in the squid giant axon (21).

An alternative possibility is that glial proteins are transferred to the axon by a pinching-off process in which small portions of the glial cells are engulfed by coated vesicles which form at the axolemma. Examples of the inclusion of glial cytoplasm by coated vesicles have been reported in the squid giant axon and other neurons (26). These structures increase in number when Ca^{++} is added to the media and become less frequent when the Ca^{++} concentration is reduced.

A remaining but remote possibility is that the proteins are transferred across the plasma membrane of the glial cells and the axon. Gap junctions which allow small molecules to pass between electrically coupled cells do not appear to be candidates for the intercellular movement of proteins since they have not been found between the glia and the axon. However, mechanisms which do not involve any obvious membrane specialization appear to provide for the movement of proteins across membranes. For example, proteins synthesized on the rough endoplasmic reticulum apparently cross the membrane of the endoplasmic reticulum and enter the cisternae (6). Much more information about protein transfer will be required before we can choose between these possibilities.

CONCLUSIONS

The evolution of multicellular animals of continually increasing size and complexity has involved the concomitant increase in the length of axons. These long axons provide specific channels for the conduction of both electrical and molecular information between distant regions within these animals. The extension of the axon away from the neuron cell body required the adaptation of previously existing cellular mechanisms by which macromolecules are supplied in eukaryotic cells. For some reason, the protein synthetic machinery was not simply extended into the axon or, if it was, this does not appear to have been a successful means of providing for the axon, since no compelling evidence exists for protein synthesis in mature axons. In fact, the extension of the protein synthetic machinery into the dendritic processes of vertebrate neurons appears to have occurred relatively recently during the evolution of vertebrates from invertebrates. The neurons of invertebrates, with rare exceptions, do not have dendritic processes that extend directly from the cell body like the dendrites of multipolar neurons in vertebrates. In the invertebrates, the receptor neurites which are the equivalent of vertebrate dendrites branch from the axons rather than directly from the cell body.

During the evolution of neurons, two cellular mechanisms appear to have been adapted to supply proteins to the axon. One mechanism is axonal transport. This is a specialized form of cytoplasmic motility which shows many similarities with the motile mechanisms found in eukaryotic cells generally, including primitive unicellular organisms. Anterograde axonal transport extends the range over which proteins synthesized near the neuronal nucleus can be delivered to the outlying axonal cytoplasm. Furthermore, anterograde axonal transport is directly involved in the actual elongation of the axon from the cell body. Retrograde axonal transport provides the protein center of the neuron with feedback from the peripheral axoplasm. It also provides information to the cell body from the distant milieu which the axon contacts. The other mechanism by which proteins are supplied to the axon appears to involve intercellular transfer from the surrounding glial cells. This appears to be a commonly employed mechanism for the supply of proteins from one population of cells to another in multicellular organisms. These proteins may be directly involved in metabolic cooperation between cells or may represent specific modulators of cellular function as in the case of protein hormones such as NGF. It is not surprising that the vast potential which is present in

the endogenous transcriptional and translational machinery of the glia that surrounds axons has been tapped to supply the axon with proteins. Although we do not know the magnitude of the participatory role played by glia in the function of the axon and the neuron generally, the weight of the evidence supporting the glia-neuron protein transfer hypothesis suggests a significant role for this process.

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