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INCORPORATION OF ^{3}H-AMINO ACIDS INTO PROTEINS IN A PARTIALLY PURIFIED FRACTION OF AXOPLASM: EVIDENCE FOR TRANSFER RNA-MEDIATED, POST-TRANSLATIONAL PROTEIN MODIFICATION IN SQUID GIANT AXONS[1]

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Abstract

Transfer RNA (tRNA) has been demonstrated to be present in axons of both invertebrates and the higher vertebrates, but nothing is known of its role in the metabolism of the axon. The present experiments were performed to determine whether tRNA functions in axons as a participant in post-translational protein modification of endogenous proteins.

RNA was extracted from the axoplasm of squid giant axons and incubated with a variety of ^{3}H-amino acids, aminoacyl-tRNA synthetases (obtained from squid optic lobe), and an appropriate reaction mixture. All of the amino acids tested were bound to an RNA fraction, but this reaction did not occur when samples were incubated in the presence of ribonuclease or in the absence of axoplasmic RNA. When radioactive RNA was chromatographed by polyacrylamide gel electrophoresis, the radioactivity comigrated with known tRNA markers, suggesting the presence of ^{3}H-aminoacylated tRNA. Aminoacylation of RNA could also be demonstrated by incubating fresh axoplasm with labeled amino acids and a reaction mixture, minus exogenous aminoacyl-tRNA synthetases. These findings indicate the presence in axoplasm of a variety of species of aminoacyl-tRNAs as well as their corresponding synthetase enzymes.

In the latter experiment no radioactivity was found associated with the protein fraction. This was also the finding when ^{3}H-aminoacylated tRNA was either injected directly into the axon or incubated with extruded axoplasm. Thus, under the conditions described above, there is no evidence of transfer of amino acids from tRNA to proteins. In other experiments, axoplasm was pooled to a volume of 50 to 100 μl, homogenized gently, and centrifuged at 150,000 × g for 1 hr. Some of the high speed supernatant was incubated with labeled amino acids and an appropriate reaction mixture, and the remainder was passed through an S-200 Sephacryl column before incubation with the same reaction mixture. There was no incorporation of amino acids into protein in the high speed supernatant fraction. However, in the S-200 purified fraction ^{3}H-labeled Arg, Lys, Tyr, Leu, and Asp were all incorporated into proteins in amounts of 44, 30, 7, 5 and 3.5 times heat-inactivated controls. The reaction is not inhibited by Ca^{2+} or Ca^{2+}-activated proteases, but appears to be dependent on the presence of tRNA. The addition of amino acids to protein is not protein synthesis since the reactions occurred in a partially purified fraction of the 150,000 × g supernatant, a fraction devoid of ribosomes and free amino acids. Thus, we conclude that the reactions we are observing represent the post-translational addition of a range of amino acids to proteins in axoplasm, and that these reactions are usually inhibited by a factor(s) yet to be described.

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The giant stellate axon of the squid contains approximately 0.02% (w/v) of RNA (Lasek et al., 1973). Gel electrophoretic analysis of axoplasmic RNA indicates that the predominant RNA species present is 4 S RNA (Black and Lasek, 1977; Giuditta et al., 1980). Other experiments have shown that axonal 4 S RNA has the properties of transfer RNA (tRNA) (Black and Lasek, 1977; Giuditta et al., 1977). The application of indirect techniques to examine RNA in axons of vertebrates has led to the general conclusion that 4 S RNA is likely to be present in all axons, both central and peripheral, and that, during growth of the axon, the amount of 4 S RNA transported axonally into the nerve is greatly increased (reviewed by Ingoglia and Zanakis, 1982). It also is likely that the 4 S RNA in vertebrate axons, like that in squid giant axons, is tRNA (M. F. Zanakis, B. Eskin, and N. A. Ingoglia, submitted for publication). The function of tRNA in axons is not known.

It has been proposed that axons are capable of synthesizing their own proteins (Giuditta et al., 1968, 1982; Koenig and Adams 1982), but this view has been frequently challenged (Barondes, 1974; Black and Lasek, 1977; Lasek et al., 1977). Even if protein synthesis does occur in axons, this would still leave unanswered the question of why axons contain such an inordinate amount of tRNA compared with other RNAs. One of the possibilities which has been raised repeatedly is that tRNA in axons is acting in the absence of ribosomes and messenger RNAs as a component of a system whereby amino acids are added to the amino terminal end of proteins (Droz and Koenig, 1971; Lasek et al., 1973; Black and Lasek, 1977; Ingoglia, 1978; Gunning et al., 1979). tRNA-dependent, post-translational protein modification has been described in a variety of biological tissues (reviewed by Soffer, 1980), including brain (Barra et al., 1973), but in eukaryotes only Arg-tRNA has been reported to be capable of participating in this reaction.

The purpose of the present experiments was to determine whether tRNA-dependent, post-translational protein modification occurs in the axoplasm of squid giant axons and whether, as has been reported in rat brain (Barra et al., 1973), the reaction is specific for arginine.

Materials and Methods

Chemicals. L-[2,3-^{3}H]Arginine (21 Ci/mmol), L-[4,5-^{3}H]leucine (40 to 60 Ci/mmol), L-[4-^{3}H]proline (15 to 30 Ci/mmol), L-[*ring*-3,5-^{3}H]tyrosine (40 to 60 Ci/mmol), L-[2,3-^{3}H]aspartic acid (15 to 25 Ci/mmol), L-[4,5-^{3}H] lysine (60 to 80 Ci/mmol), and 2-[^{3}H]taurine (20 to 40 Ci/mmol) were purchased from New England Nuclear Corp. Cycloheximide, ribonuclease A (bovine pancreatic), proteinase K, and calf liver aminoacyl-tRNA synthetase were obtained from Sigma Chemical Co.

All experiments were performed on squid (*Loligo pealii*) obtained at the Marine Biological Laboratories, Woods Hole, MA. Squid were housed in tanks with running sea water and axoplasm was obtained from the giant axon of the stellate nerve by techniques described previously (Baker et al., 1962; Giuditta et al., 1968). In all dissections great care was taken not to contaminate the axoplasm with extraneous materials from squid tissue or from sea water. Axoplasm was either used immediately (see experiments described in Fig. 1, and Table II) or pooled and stored in 1.2-ml Eppendorf tubes at −20°C. In experiments in which a 150,000 × *g* supernatant fraction was assayed (see Figs. 4 to 6, Tables IV to VI), axoplasm was stored at −20°C in a solution of 50% glycerol containing 10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM 2-mercaptoethanol, and 10 mM KCl. This procedure was used to preserve the activity of the aminoacyl-tRNA synthetases.

In one experiment, injections of radioactive materials directly into the axon were performed according to techniques described previously (Lerman et al., 1969).

Preparation of tRNA from axoplasm. Axoplasm was pooled and frozen until a volume of approximately 500 μl (469 mg) was reached. It was then homogenized in 1.0 ml of 50 mM Tris-HCl, pH 9.0 (to deacylate tRNAs). The homogenate was centrifuged at 27,000 × *g* for 20 min at 4°C. After washing the pellet, the supernatant was centrifuged again at 105,000 × *g* for 3 hr at 4°C. Sodium dodecyl sulfate and proteinase K were added to the high speed supernatant fraction to a final concentration of 0.5% and 100 μg/ml, respectively. The sample was then incubated for 1 hr at 30°C with gentle shaking to allow the proteinase K to digest proteins. An equal volume of phenol equilibrated with the homogenizing buffer and containing 0.1% 8-hydroxyquinoline was added, and proteins and lipids were removed by successive extractions with phenol, phenol:chloroform (4:1, v/v) and chloroform:isopropanol (1%). RNA was precipitated from the aqueous fraction by addition of ammonium acetate (final concentration, 0.3 M) and 2.5 vol of ice-cold absolute ethanol. The sample was stored at −20°C overnight and centrifuged at 17,000 × *g* for 60 min. The supernatant was discarded. The precipitate was dried under nitrogen and dissolved in 250 μl of 10 mM Tris-HCl, pH 7.4. This procedure resulted in the recovery of 47 μg of tRNA with a 260/280 nm absorbance ratio of 3.6.

Preparation of crude aminoacyl-tRNA synthetase from squid optic lobes. In one experiment (see Table II), aminoacyl-tRNA synthetase was prepared from squid optic lobes. Approximately 5 gm of tissue were homogenized in 5 ml of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.1 mM $MgCl_2$, 80 mM 2-mercaptoethanol, and 170 mM sucrose (buffer S). A 105,000 × *g* supernatant fraction was prepared and layered on a column of DEAE-Sephadex at 4°C. The column had been equilibrated previously with a buffer containing 10 mM Tris-HCl, pH 7.4, 20 mM KCl, 1 mM $MgCl_2$, and 15% glycerol. Proteins were eluted with a similar buffer containing 0.25 M KCl. The eluent was monitored for absorbance at 280 nm, and fractions showing maximum absorbance were pooled, brought to a concentration of 50% with glycerol, and stored at −20°C.

Preparation of ^{3}H-Aminoacylated tRNAs. Aminoacylation of optic lobe tRNA with [^{3}H]Arg, [^{3}H]Lys, or [^{3}H] Asp was carried out using aminoacyl-tRNA synthetase obtained from bovine liver. Optic lobe tRNA was prepared according to the same technique as described above for axoplasm. Reaction mixtures (final volume = 250 μl) contained calf liver aminoacyl-tRNA synthetase (250 units), optic lobe tRNA (approximately 40 μg), 10 mM Tris-HCl, pH 7.3, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5

mM ATP, 1 mM CTP, and 25 μCi of [^{3}H]Arg, [^{3}H]Lys, or [^{3}H]Asp. Samples were incubated for 30 min at 35°C and placed in ice to stop the reaction, and ammonium acetate was added to a final concentration of 0.3 M. ^{3}H-Aminoacylated tRNA was precipitated with 2.5 vol of ice-cold ethanol and stored overnight at −70°C. Samples were then centrifuged at 27,000 × *g* for 1 hr. The supernatant was decanted, and the precipitate was dried and dissolved in 25 μl of Millipore filtered distilled H_2O. The radioactivity associated with free amino acids, tRNA, and protein was determined by a procedure described below, and the distribution of counts in each fraction is given under "Results" (see Table II).

Polyacrylamide gel electrophoresis. When ^{3}H-amino acids were incubated with RNA extracts obtained from axoplasm, and in the presence of aminoacyl-tRNA synthetases, labeling of RNA occurred which appeared to represent aminoacylation of RNA (see Table I). To verify this finding, some of the radioactive product was layered onto a 10% polyacrylamide gel and electrophoresis was carried out as described previously (Ingoglia and Tuliszewski, 1976). Gel slices of 2.0 mm were cut, dissolved in Protosol (New England Nuclear Corp.), and counted in a liquid scintillation spectrometer.

Analysis of tissue radioactivity. In all experiments, reactions were stopped by adding ice-cold 5 to 10% trichloroacetic acid (TCA) to the reaction tubes and immediately transferring them to ice. Radioactivity, which was soluble in three or four washings of cold 5% TCA, was considered as the "free amino acid" fraction. Radioactivity associated with RNA (all experiments are conducted in axoplasm and/or in a 150,000 × *g* supernatant of axoplasm; thus it is likely that this RNA is tRNA) is that radioactivity which is insoluble in cold TCA but is solubilized by heating the TCA-insoluble material to 95°C for 20 min in 5% TCA. Protein-associated radioactivity is the insoluble radioactivity following hot TCA treatment. In experiments described in Figure 1 and Table I, radioactivity was determined by the filter fiber assay described by Mans and Novelli (1961). In all other experiments radioactivity was determined by precipitation and centrifugation. In all experiments cold and hot TCA-insoluble pellets were washed three to four times with cold 5% TCA before solubilization and counting.

Preparation of 150,000 × g supernatant fraction and fractionation by S-200 column chromatography. Axoplasm, stored at −20°C in buffer S, was homogenized in ice in a 1.2-ml conical Eppendorf tube using a pestle made of dental cement. The homogenate was transferred to a high speed polypropylene centrifuge tube, and the Eppendorf tube was washed with 60 μl of 10 mM Tris-HCl, pH 7.4 (homogenizing buffer), containing 0.25 M sucrose and 6 mM 2-mercaptoethanol. Centrifugation was performed at 150,000 × *g* for 60 min at 4°C. Following centrifugation, the supernatant was removed and then either analyzed directly for the ability to incorporate ^{3}H-amino acids into protein or purified further by column chromatography.

Seventy-five to 100 μl of the high speed supernatant was passed through a Sephacryl S-200 (Pharmacia) column (2.5 mm × 300 mm). Elution was carried out with the homogenizing buffer and 150-μl fractions were collected at a flow rate of 6.90 ml/hr. Samples were collected until absorbance at 260 and 280 nm reached base line.

Fractions were pooled to represent three portions of equal volumes of the eluent. Proteins of known molecular weights were chromatographed separately and the approximate molecular weights of each fraction were determined. Fraction I contained material of $M_r > 120{,}000$, fraction II contained material of M_r between 90,000 and 60,000, and fraction III contained material of M_r between 40,000 and 25,000 (see Fig. 3).

Results

Assays for the presence of aminoacyl-tRNA synthetases in axoplasm

Previous experiments have reported the presence of aminoacyl-tRNA synthetase activity in axoplasm using a mixture of ^{3}H-amino acids (Black and Lasek, 1977). Because it has been reported that, in brain, only arginine can be added to proteins in the presence of tRNA but in the absence of ribosomes (Barra et al., 1973), we attempted to determine whether only Arg-tRNA and its corresponding tRNA synthetase enzyme were present in axoplasm.

Axoplasm was extruded from four to six giant axons pooled and homogenized in 100 μl of buffer (Tris-HCl, 0.1 M, pH 7.5). Homogenates were incubated in a mixture containing 100 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 5 mM ATP, and 5 μCi of each amino acid, at 30°C for 0 min (control) or 30 min (experimental). Reactions were stopped by the addition of 2.5 vol of ice-cold 5% TCA, and radioactivity associated with nucleic acids was determined by a filter fiber assay as described under "Materials and Methods."

In each of the reactions, significantly more radioactivity was incorporated into nucleic acids at 30 min compared with 0 min controls (Fig. 1). These results suggest that each of the amino acids tested (and not just arginine) is capable of binding to nucleic acids in axoplasm and, therefore, that each of the tRNA species and their corresponding aminoacyl-tRNA synthetases are natural components of squid axoplasm.

To be certain that the reaction we were measuring was the aminoacylation of tRNA, RNA was extracted and purified from axoplasm, and crude exogenous aminoacyl-tRNA synthetase was prepared from squid optic lobes (see "Materials and Methods").

RNA was incubated with aminoacyl-tRNA synthetase and the following reaction mixture: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 1 mM CTP, 22.5 mM 2-mercaptoethanol, 121 mM KCl, 15% glycerol, and the radioactive amino acid (2 μCi/50 μl of reaction mixture). Reactions were carried out for 20 min at 30°C with or without RNA and with RNA but preincubated in the presence of bovine pancreatic ribonuclease (19.2 μg/ml). Radioactivity associated with nucleic acids was determined by the filter fiber assay. The absence of RNA from the reaction mixture reduced the incorporation of ^{3}H-amino acids into nucleic acids by greater than 80%, and the addition of ribonuclease inhibited the reaction by approximately 90% (Table I). The results indicate

that the binding of ^{3}H-amino acids to nucleic acids in squid axoplasm is RNA dependent and is blocked by ribonuclease. Both findings are consistent with the assumption that the reaction we are examining is the aminoacylation of tRNA.

As further proof that this is the case, some of the post-reaction nucleic acids from the "(+) tRNA" fractions were re-extracted with phenol, precipitated with ethanol in 0.3 M NH_4Ac, dissolved in acetate buffer (50 mM, pH 5.3, containing 0.5% sodium dodecyl sulfate), and layered on 10% polyacrylamide tube gels. Electrophoresis was carried out for 4.5 hr at 5 mA/gel, and calf liver tRNA markers were used to localize tRNA migration on the gel. When gels were analyzed for optical density (tRNA marker) and radioactivity (presumptive [^{3}H]aminoacyl-tRNA), peaks were found to coincide (Fig. 2).

This is further evidence that some of the radioactivity we are examining is ^{3}H-aminoacylated tRNA. These experiments indicate that the tRNAs and the corresponding aminoacyl-tRNA synthetases for the amino acids tested are present in the axoplasm of the giant axon of the stellate nerve.

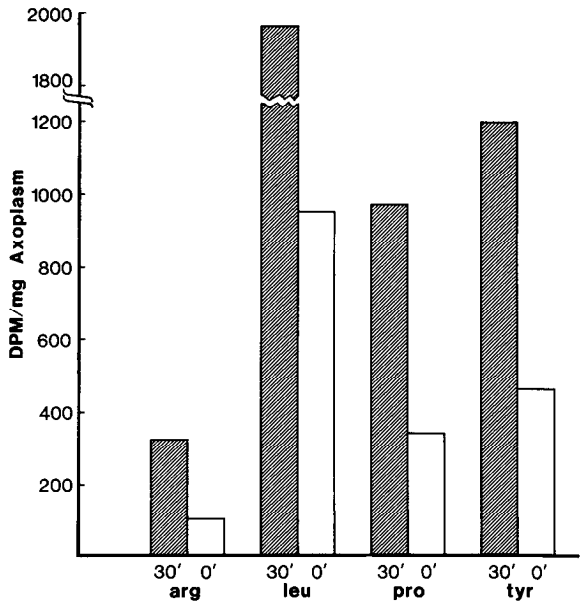

Figure 1. Radioactivity associated with a nucleic acid fraction following incubation of squid axoplasm with ^{3}H, Leu, Pro, or Tyr and the reaction mixture described under "Results." Significantly greater amounts of ^{3}H-amino acids are associated with nucleic acids in those samples incubated for 30 min compared with controls.

Assay for the transfer of amino acids from tRNA to proteins

In vitro experiments. In the experiments presented in Figure 1, a hot TCA-insoluble (presumably protein) fraction was obtained and assayed for radioactivity. Negligible counts were associated with this fraction, suggesting the lack of transfer of amino acids from aminoacylated tRNA to protein. We reasoned, however, that perhaps the low levels of labeled amino acids associated with tRNA in those experiments were insufficient to demonstrate a transfer of label to protein. Thus, experiments were undertaken in which we prepared relatively high specific activity ^{3}H-aminoacylated tRNA and either incubated it with extruded axoplasm or injected it directly into the giant axon itself.

Tritiated Arg-, Lys-, and Asp-labeled tRNAs were prepared as described under "Materials and Methods."

TABLE 1

Incorporation of ^{3}H-amino acids into the nucleic acid fraction of samples containing RNA extracted from axoplasm and the reaction mixture described under "Results"

	Arg	Leu	Pro	Tyr
(+) RNA	92,973[a]	28,547	11,660	21,337
(−) RNA	14,243	3,270	1,850	4,880
(+) RNase	3,000	1,297	1,053	3,580

[a] Values are dpm/sample.

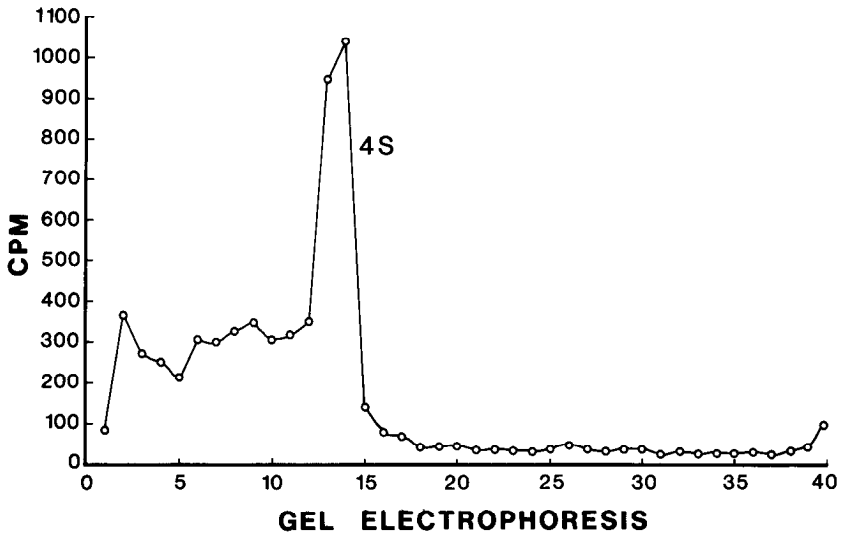

Figure 2. Gel electrophoretic profile of reaction product of [^{3}H]tyrosine incubated with axoplasmic RNA and a reaction mixture described under "Results." The radioactivity peak comigrates with a calf liver 4 S RNA marker.

TABLE II

Incubation of [^{3}H]Arg, [^{3}H]Lys, and [^{3}H]Asp transfer RNAs with axoplasm extruded from the giant axon

Amino Acid	Fraction	Radioactivity Added to Incubation Medium (0 min)		Radioactivity Following 2-Hr Incubation at 20°C	
		DPM	%	DPM	%
[^{3}H]Arg	Free a.a.[a]	85,891	76	105,715	99
	tRNA	25,677	23	220	1
	Protein	873	1	106	1
[^{3}H]Lys	Free a.a.	134,324	77	177,747	99
	tRNA	39,950	23	1,916	1
	Protein	1,140	1	45	1
[^{3}H]Asp	Free a.a.	255,336	89	284,138	99
	tRNA	29,727	10	2,084	1
	Protein	263	1	61	1

[a] a.a., amino acid.

Fresh axoplasm was obtained from giant axons collected in 1.2-ml Eppendorf tubes and immediately chilled in ice. Five microliters of axoplasm were transferred to another Eppendorf tube and gently mixed, until it formed a relatively uniform suspension, with 10 μl of a reaction mixture containing 10 mM Tris-HCl, pH 7.3, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM ATP, and 5 mM CTP. The reaction was initiated by the addition of 2 μl of ^{3}H-aminoacylated tRNA, and incubations were carried out at 19°C for 2 hr. Reactions were stopped by the addition of 250 μl of ice-cold 10% TCA, and radioactivity in free amino acid, nucleic acid, and protein fractions was determined by a centrifugation assay as described under "Materials and Methods." To determine the distribution of radioactivity in the radioactive material added to the incubation tubes, identical reactions were prepared except that, in these samples, reactions were stopped by addition of cold 10% TCA immediately upon addition of the labeled tRNAs.

The radioactive material added to the reaction mixture contained large amounts of ^{3}H-labeled free amino acids along with ^{3}H-aminoacylated tRNAs (Table II). This is likely to be due either to an incomplete removal of labeled free amino acids during preparation of the ^{3}H-aminoacylated tRNA or to deacylation of the tRNA after synthesis. Following 2 hr of incubation, virtually all of the radioactivity was found in the TCA-soluble (free amino acid) fraction, with no labeled material in the protein fraction (Table II). Thus, incubation of extruded axoplasm with ^{3}H-aminoacylated tRNA results in deacylation of tRNA but not in the incorporation of ^{3}H-amino acids into protein.

In situ experiments. Next, we attempted to demonstrate the transfer of amino acids from tRNA to proteins by injecting ^{3}H-aminoacylated tRNAs directly into the giant axon. ^{3}H-aminoacylated tRNAs were prepared as described for the previous experiments, precipitated with ice-cold ethanol, and stored at −20°C. Prior to intra-axonal injection, samples were centrifuged at 15,000 × *g* for 1 hr. The RNA pellet was dried thoroughly and then dissolved in 10 μl of an artificial axoplasm solution (compliments of S. Brady). The giant axon was removed from the squid, and approximately 1 μl of ^{3}H-aminoacylated tRNA was injected intra-axonally as described under "Materials and Methods." Electrical activity was monitored before and after injections; in all cases axons were capable of conducting action potentials after injections had been made. Axons containing ^{3}H-aminoacylated tRNAs were then transferred to incubation chambers (described by Lasek et al., 1977) and incubated for 1 hr at 19°C in Millipore filtered sea water. Axoplasm was extruded, and axoplasm and sheaths (containing axolemma and adhering adaxonal glia) were analyzed separately for radioactivity by TCA precipitation and centrifugation.

As in the previous experiments, the radioactivity injected into the axon was composed of free ^{3}H-labeled amino acids and ^{3}H-aminoacylated tRNA (Table III). In all experiments (three with Arg, two with Lys, and two with Asp), variable amounts of radioactivity in axoplasm remained in the tRNA fractions following incubation. However, in none of the experiments was radioactivity transferred to the protein fraction. Rather, all of the radioactivity liberated from tRNA was recovered in the free amino acid fraction. Thus, injection of ^{3}H-amino acids and ^{3}H-aminoacylated tRNA into the giant axon does not result in the incorporation of any of the label into protein.

Sheaths were homogenized in 50 μl of distilled H_2O, and radioactivity was extracted in the same manner as described for axoplasm. Variable amounts of radioactivity were transferred from axoplasm to the sheath, but in all experiments a significant fraction of the radioactivity was found associated with the protein fraction. Although

TABLE III

Distribution of radioactivity in axoplasm and sheath following the intra-axonal injection of [^{3}H]Arg, [^{3}H]Lys, or [^{3}H]Asp transfer RNA

	Radioactivity Injected into Axoplasm	Exp.	Radioactivity in Axoplasm after 1-Hr Incubation	Radioactivity in Sheaths after 1-Hr Incubation
	dpm		*dpm*	
[^{3}H]Arg				
Free amino acids	7,580	1	9,045	152
		2	9,240	76
		3	5,940	110
tRNA	810	1	0	0
		2	55	0
		3	15	0
Protein	0	1	0	70
		2	0	67
		3	0	70
[^{3}H]Lys				
Free amino acids	7,625	1	14,050	4,419
		2	22,925	1,964
tRNA	8,130	1	4,185	76
		2	5,810	33
Protein	123	1	0	176
		2	33	130
[^{3}H]Asp				
Free amino acids	11,410	1	11,760	781
		2	14,450	5,929
tRNA	5,790	1	1,850	0
		2	2,570	362
Proteins	0	1	0	70
		2	21	73

there are several interpretations of these data, the most likely one is that some free ^{3}H-labeled amino acids in the axon escaped from the axon and were taken up by glia. A portion of the ^{3}H-amino acids in glia was then utilized for protein synthesis. This finding, coupled with the fact that these axons were all capable of conducting action potentials following intra-axonal injections, indicates that the preparation was metabolically active during the experiment. These data strengthen the conclusions reached concerning the intra-axonal metabolic machinery, i.e., that under the conditions described, the incorporation of amino acids (either from a free amino acid pool or from aminoacylated tRNA) into protein does not occur in the giant axon of the stellate nerve of the squid.

Experiments carried out on the 150,000 × g supernatant of axoplasm. Axoplasm was collected and pooled in 1.2-ml Eppendorf tubes containing 100 μl of a solution of 50% glycerol and 50% Tris-HCl (100 mM, pH 7.3), 100 mM $MgCl_2$, and 50 mM dithiothreitol. Two separate tubes, each containing 60 to 100 μl of axoplasm, were collected and stored but not frozen at −20°C.

One hundred microliters (containing approximately 50 μl of axoplasm) of the stored samples were removed and gently homogenized in an Eppendorf tube and centrifuged at 150,000 × *g* for 60 min, as described under "Materials and Methods." A portion of the 150,000 × *g* supernatant fraction was then layered onto a S-200 column and 150-μl samples were collected as described under "Materials and Methods." Absorbance at 260 nm (indicating the presence of nucleic acids, mostly tRNA in this preparation) and 280 nm was determined for each sample, and samples corresponding to three areas of the elution profile were pooled (Fig. 3). Aliquots of all fractions were removed for protein determinations as described previously. Aliquots (15 μl) of each of these fractions, and of the 150,000 × *g* supernatant fraction were incubated in separate reaction tubes with a variety of ^{3}H-amino acids and a reaction mixture containing 5 mM $MgCl_2$, 5 mM ATP, 100 mM KCl, 30 mM Tris-HCl, pH 7.4. Each reaction mixture contained between 2 and 90 pmol of ^{3}H-amino acid in a total volume of 33 μl. Incubations were carried out for 20 min at 37°C. Samples were assayed in triplicate, and nonspecific binding was determined by boiling control tubes for 20 min before incubation with labeled amino acids. Radioactivity in tRNA and protein fractions was determined by TCA precipitation and centrifugation, and total proteins were determined by a modification (Schacturle and Pollack, 1973) of the Lowry assay (Lowry et al., 1951).

Figure 4 illustrates the radioactivity associated with the protein fraction following each of these reactions. Values obtained for fractions I to III (see Fig. 3) have been pooled and are represented here as total activity of the eluate of the S-200 column. In the 150,000 × *g* supernatant fraction, no difference in radioactivity in the protein fraction was found between experimental groups and heat-inactivated controls. Thus, as in the previous two experiments, we were not able to demonstrate the incorporation of amino acids into protein, in this case in the 150,000 × *g* supernatant fraction of axoplasm (Fig. 4). However, when the S-200 eluent was assayed, significant differences in experimental versus control radioactivity were found for all amino acids tested except taurine. Taurine was used here as a control for nonspecific binding because large amounts of taurine are known to be present in axoplasm (Rubinson and Baker, 1979), but taurine, a sulfonic amino acid whose biological role in axoplasm is not fully understood, is not incorporated into protein in any system studied thus far (Huxtable and Barbeau, 1976). Thus, the S-200 eluent of the 150,000 × *g* supernatant of axoplasm is capable of incorporating all of the carboxylic amino acids tested (two basic, two neutral, and one acidic) into protein. In most experiments the greatest amount of activity was found in the void volume (fraction I), but in some experiments, activity could be found in all fractions (Fig. 5). In one case (see Lys (1), Fig. 5) the activity was greatest in fraction III.

Other experiments were performed to determine

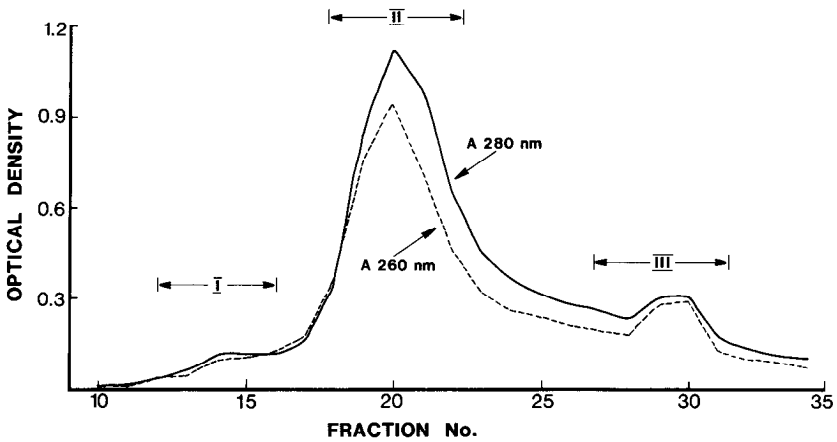

Figure 3. A_{260} and A_{280} of fractions collected from the purification of the 150,000 × *g* supernatant of axoplasm on a Sephacryl S-200 column. Experiments in which protein standards of known molecular weights were chromatographed, under similar conditions as described for axoplasm, indicate that the molecular weights of substances collected in each fraction are approximately: I > 120,000, II = 90,000 to 60,000, III = 40,000 to 25,000.

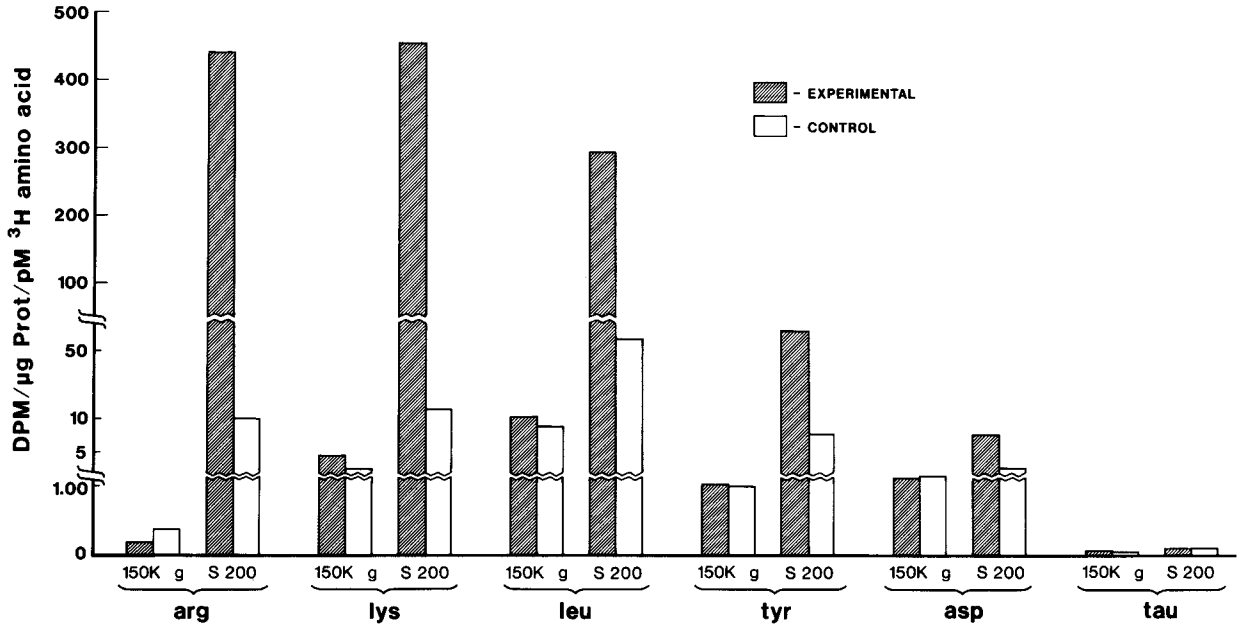

Figure 4. Incorporation of a variety of ^{3}H-amino acids into protein in the 150,000 × *g* supernatant fraction of axoplasm and samples collected following chromatography on S-200 Sephacryl columns. Control samples were heat inactivated prior to addition of ^{3}H-amino acids, and each value is the mean of three replicate assays. No significant difference between experimental and control samples was observed in any of the 150,000 × *g* samples or in the incubations with the sulfonic amino acid taurine. Significant differences in the samples collected following chromatography were recorded for all carboxylic amino acids tested. Values are shown as dpm per microgram of protein per picomole of ^{3}H-amino acid in each reaction mixture.

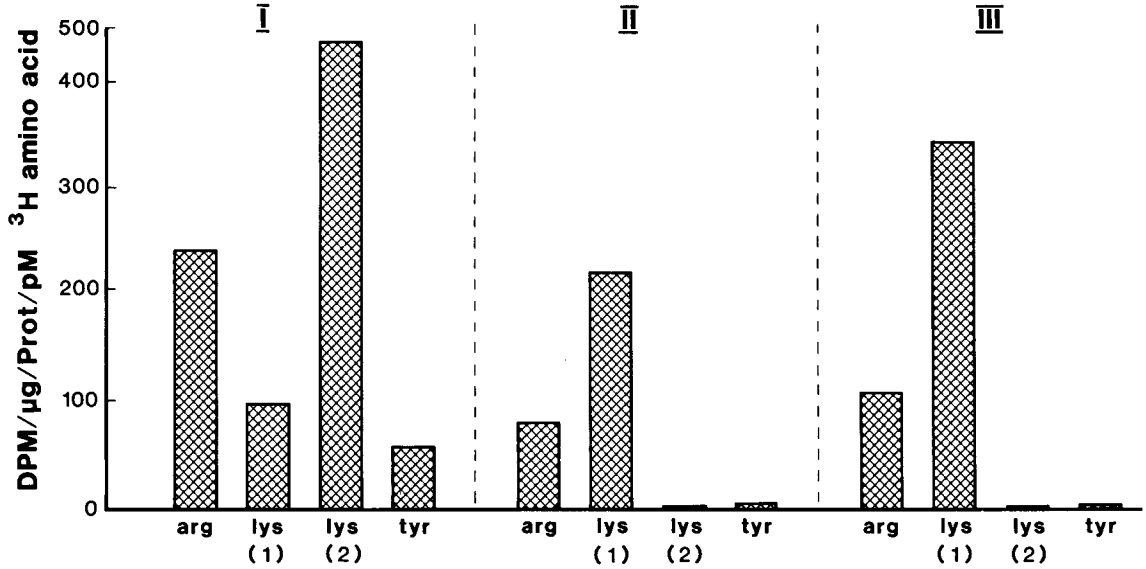

Figure 5. Distribution of radioactivity associated with protein following incubations as described for Figure 4, in fractions of an S-200 Sephacryl column (see Fig. 3). Note that in most cases the greatest activity is in fraction I, but that the lower molecular weight fractions (II and III) also contain significant levels of activity (values are expressed as experimentals minus heat inactivated controls).

whether, after these reactions, the radioactivity associated with the hot TCA-precipitable material was still present as ^{3}H-amino acids. Following incubation with [^{3}H]lysine, some of the reaction product was washed thoroughly with cold 5% TCA and dissolved in 0.5 ml of 2 N KOH, and 2 mg of bovine serum albumin were added as a protein carrier. The solution was transferred to a Pyrex tube and brought to 6 N HCl. The sample was then frozen in liquid nitrogen flushed with nitrogen gas, sealed under vacuum, and then heated to 120°C for 24 hr to hydrolyze proteins. Hydrolyzed samples were then evaporated to dryness, dissolved in 1.0 ml of 0.1 N HCl, and chromatographed on a Beckman 119CL automatic amino acid analyzer according to standard procedures. One-milliliter samples were collected and radioactivity was determined by liquid scintillation counting. The labeled substrate was treated identically, except that it was not heated prior to analysis. When the elution profile

TABLE IV

Effect of Ca^{2+}, Na^+, and leupeptin on the incorporation of [^{3}H]Arg into protein in the 150,000 × g supernatant and the FI fraction of an S-200 column[a]

Fraction	Experimental	Control[b]	E-C
	dpm/μg of protein		
150,000 × *g*			
Control	14.8	20.1	−5.3
+ EGTA (1.0 mM)	18.0	32.5	−14.5
+ Leupeptin (250 μg/ml)	28.9	30.9	−2.0
FI, S-200 Column			
Control	670	42	628
+ NaCl (5 mM)	525	29	496
+ $CaCl_2$ (5 mM)	773	40	733

[a] Incubation conditions were as described for experiments in Figure 4.

[b] Control samples were heat inactivated as described in Figure 4.

of the labeled substrate was compared with that of the hydrolyzed reaction product, the two profiles were found to be identical, and both were eluted with the position expected for lysine. These data indicate that the radioactivity incorporated into the TCA-insoluble fraction of the reaction mixture was still present as [^{3}H]Lys.

We have also investigated the nature of the association between radioactive amino acids and the hot TCA-precipitable material. Because of the scarcity of squid axoplasm, these experiments have been performed in samples treated exactly as described above for squid but derived from homogenates of rat sciatic nerves. Like squid axoplasm, sciatic nerve preparations are capable of incorporating a variety of amino acids into proteins in the S-200 eluent but not in the high speed supernatant from which the S-200 fraction is derived (M. F. Zanakis, G. Chakraborty, and N. A. Ingoglia, manuscript in preparation). Some of the hot TCA-insoluble material was washed with chloroform:methanol (4:1), but no radioactivity was liberated by this procedure. In other experiments performed with [^{3}H]Lys, the reaction product was washed four times with cold 5% TCA, and TCA crystals were removed with ice-cold ethanol. The precipitate was then treated with proteinase K (100 μg/ml) for 30 min at 37°C. Greater than 90% of the precipitable radioactivity was liberated into a TCA-soluble fraction by this procedure, indicating that the labeled amino acid is bound to protein (M. F. Zanakis, G. Chakraborty, and N. A. Ingoglia, manuscript in preparation).

Thus, the evidence indicates that the reaction we are measuring represents the covalent incorporation of amino acids into endogenous acceptor proteins. Since the incorporation of amino acids into proteins does not occur in the high speed supernatant, it appears that the addition of amino acids to protein in axoplasm is inhibited or masked in the 150,000 × *g* supernatant of axoplasm, but that the inhibition is lost when the material is fractionated by S-200 chromatography.

We attempted next to determine whether the inability to observe the incorporation of amino acids into protein in the supernatant was due to the presence of a Ca^{2+}-activated inhibitory agent or to the presence of Ca^{2+}-activated proteases which might hydrolyze newly formed proteins (Pant and Gainer, 1980). Aliquots of the 150,000 × *g* supernatant were incubated as described in Figure 4, or under conditions in which 1.0 mM EGTA or 250 μg/ml of the protease inhibitor leupeptin (obtained from W. Troll) were added prior to the addition of [^{3}H]arginine. Neither treatment was capable of improving the incorporation of [^{3}H]arginine into protein in the 150,000 × *g* supernatant (Table IV). The addition of 5 mM NaCl or 5 mM $CaCl_2$ to FI samples also did not significantly inhibit the incorporation of [^{3}H]arginine into protein. Thus, the factor responsible for blocking this reaction in the 150,000 × *g* supernatant, and presumably in the intact axon, is not Ca^{2+} or Na^+, or a Ca^{2+}-activated protease. The reaction in FI could be blocked, however, by the addition of small amounts of the 150,000 × *g* supernatant fraction (Fig. 6). Thus, combining 1.5 μl of supernatant with 13.5 μl of fraction I (10% dilution) was sufficient to inhibit the reaction by approximately 85% (Fig. 6).

Finally, we examined the susceptibility of the reaction to the action of ribonuclease and cycloheximide. Since all reactions were performed on a 150,000 × *g* supernatant fraction, we assume that if ribosomes or ribosomal structures were present at all in axoplasm, they would not be contained in our assay system. Yet when we examined the effect of cycloheximide on this reaction, we found a significant inhibition of the incorporation of ^{3}H-amino acids into protein at concentrations of both 0.3 and 1.0 mM in the FI fraction (Table V). At concentrations of 1.0 mM the inhibition was complete, but it should be noted that in each experiment cycloheximide also inhibited the incorporation of ^{3}H-amino acids into RNA by 17%, 100%, and 91%, respectively. This finding and the fact that these are high speed supernatant fractions indicate that cycloheximide is probably not exerting its effects by its presumed mechanism as an inhibitor

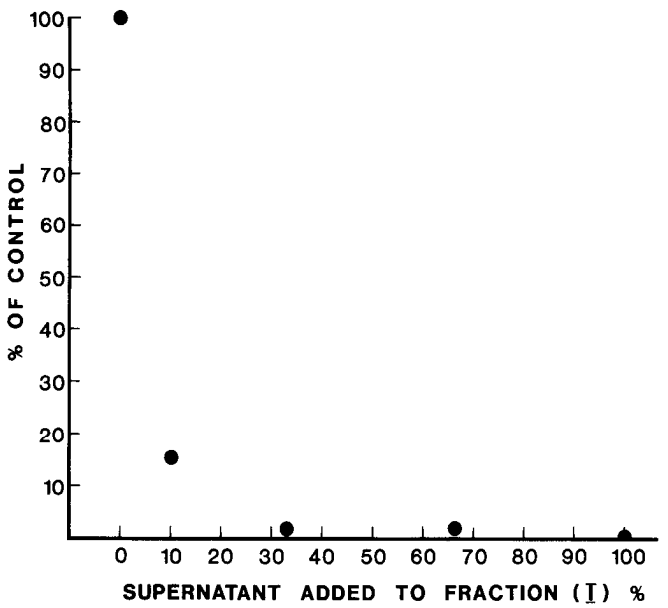

Figure 6. Inhibition of the incorporation of [^{3}H]Lys into protein in the FI fraction of an S-200 column by the addition of varying amounts of the 150,000 × *g* supernatant to the FI fraction. Incubation conditions were as described in the legend to Figure 4.

TABLE V

Effect of cycloheximide on the incorporation of ^{3}H-amino acids into protein in the eluent of an S-200 Sephacryl column[a]

Amino Acid	Control[a]	Cycloheximide treated[a,b]	Percentage Inhibition
		0.3 mM	
[^{3}H]Lys	618	489	21 (17)[c]
		1.0 mM	
[^{3}H]Lys	3,828	0	100 (100)[c]
[^{3}H]Tyr	1,248	7	100 (91)[c]

[a] Cycloheximide was added to the reaction mixture prior to the addition of ^{3}H-amino acids, and reactions were conducted as described in Figure 4.

[b] Values are expressed as dpm/μg of protein of experimentals minus enzyme-inactivated controls.

[c] Indicates the percentage of inhibition of incorporation of [^{3}H]Lys or [^{3}H]Tyr into RNA.

TABLE VI

Effect of ribonuclease on the incorporation of ^{3}H-amino acids into protein in the eluent of an S-200 Sephacryl column[a]

Amino Acid	Control[b]	Ribonuclease treated[b]	Percentage Inhibition
		50 μg/ml	
[^{3}H]Arg			
FI	496	522	0
FII	176	291	0
FIII	115	6	95
		100 μg/ml	
[^{3}H]Tyr			
FI	1248	1033	17
FII	44	23	45
FIII	45	0	100

[a] Samples were preincubated in the presence of ribonuclease for 10 to 15 min at 37°C. ^{3}H-Amino acids were added and incubations were continued for an additional 20 min at 37°C.

[b] Values are expressed as dpm/μg of protein of experimentals minus enzyme-inactivated controls.

of ribosomal-dependent protein synthesis but may have a more generalized effect on the reaction.

Other experiments were performed to determine whether these reactions were sensitive to ribonuclease. Preincubation of the reaction mixture with 50 μg/ml of ribonuclease had no effect on the incorporation of ^{3}H-Arg into RNA or protein except in the lowest molecular weight fraction (III) of the S-200 column (Table VI). When the concentration of ribonuclease was increased to 100 μg/ml, inhibition of the reactions, although variable, was observed in all fractions. In both experiments the greatest inhibition was in fraction III, the lower molecular weight fraction. We interpret these data as indicating that at least a portion of the reaction is RNA dependent, but that when RNA is present as part of a high molecular weight complex (as in fraction I), then it is protected from enzymatic digestion with ribonuclease.

Discussion

These experiments describe the incorporation of a variety of carboxylic amino acids into protein in a partially purified fraction of the 150,000 × *g* supernatant of squid axoplasm. The incorporation of amino acids into protein could not be demonstrated in extruded axoplasm (confirming experiments of Lasek et al., 1977), nor when ^{3}H-amino acids or ^{3}H-aminoacylated tRNA were injected directly into axons, nor in the unpurified 150,000 × *g* supernatant. However, when molecules of less than M_r ~ 25,000 were removed by S-200 chromatography, then all of the carboxylic amino acids tested were actively incorporated into a hot TCA-insoluble, presumably protein fraction. We assume that, *in vivo*, axoplasm contains the necessary elements for this amino acid incorporation, but that, under the conditions in which we and others have attempted to examine the reaction, it is inhibited by a single or multiple factors. The nature of this factor is not known, but it is not Ca^{2+}, nor is it a Ca^{2+}-activated protease which might destroy axonal proteins.

Because (*1*) the radioactivity is associated with a thoroughly washed hot TCA-precipitable pellet in experiments but not in heat-inactivated controls, (*2*) the carboxylic amino acids but not taurine are incorporated into the protein pellet, and (*3*) the radioactive amino acid can be liberated from the reaction product by treatment with a proteolytic enzyme, we conclude that what we are observing is the covalent incorporation of amino acids into protein. Thus, these experiments demonstrate the incorporation of a variety of amino acids into endogenous acceptor proteins of axoplasm.

The incorporation of ^{3}H-amino acids into protein could be the result of *de novo* protein synthesis or of the addition of amino acids to preformed proteins (protein modification). It is not likely that this is classical ribosome-dependent protein synthesis because axoplasm is generally considered to be devoid of ribosomes, and, even if some ribosomes were present, they would have been removed by centrifugation as would other membranes or membrane fragments. Thus, it appears that amino acid incorporation is occurring in the absence of ribosomes and membranous material. Inhibition of the reaction with cycloheximide (Table V) could be taken as evidence for classical protein synthesis. However, in these experiments, cycloheximide blocked the aminoacylation of tRNA, as well as the amino acid transfer reaction, suggesting that cycloheximide had a more generalized effect on the reaction than is usually ascribed to it. We conclude that the reaction is inhibited by cycloheximide, but not as an inhibitor of ribosomal-dependent protein synthesis. Protein synthesis is also ruled out since fractions I to III are all free of unlabeled amino acids and, thus, the only amino acid present is the labeled substrate. We conclude that protein synthesis cannot be occurring in these reactions and that the reactions we are observing are the result of post-translational protein modification.

Protein modification by amino acid addition has been described in a number of experiments. Tyrosine is added to tubulin in a reaction which is not dependent on ribosomes, mRNA, or tRNA (Barra et al., 1980). Arginine can be added to the —NH_2 terminus of a number of proteins in eukaryotes, in a reaction which is ribosome independent, but tRNA dependent, and not affected by cycloheximide (reviewed by Soffer, 1980). Recently, a tRNA-dependent, ribosome-independent incorporation of leucine into protein has been described (Laughrea, 1982). All of these reactions occur in the high speed

supernatant of brain. The reactions we are reporting differ from those mentioned above in that, in our experiments, the reactions do not occur in the 150,000 × *g* supernatant unless it is fractionated, and, as far as we can tell, the reaction is not specific for any amino acids. We have measured the activity of two basic, two neutral, and one acidic amino acid and have found that, although the level of incorporation was variable (see Fig. 4), all were capable of being incorporated into proteins. It seems likely that the reactions described in this paper are true for all carboxylic amino acids.

Although the evidence is not conclusive that the reactions we are studying are dependent on tRNA, ribonuclease did inhibit the reactions under several experimental conditions (Table VI). Thus, at 50 μg/ml, ribonuclease had no effect on the reaction except in fraction III, the low molecular weight fraction. At 100 μg/ml, a similar trend was observed with some inhibition in fraction I and complete inhibition in fraction III (Table VI). These results can be explained if one considers the likely form of tRNA in the living axon. In all cells as well as in squid axon (see Figs. 3 and 5 and discussion below), tRNA appears to exist as part of a high molecular weight complex of nucleic acid and protein (Bandyopadhyay and Deutscher, 1971). Gentle treatment of a tissue homogenate followed by centrifugation and column fractionation retains a fraction of molecules with M_r > 120,000. These fractions contain tRNA, the synthetases necessary for aminoacylation, and the transferases necessary for transfer of amino acids to protein. We propose that the reason that ribonuclease was not (or was less) effective in fraction I compared with fraction III (Table VI) is that, as part of a high molecular weight complex in axoplasm, tRNA is protected *in vivo* from inactivation by ribonuclease. When this complex is broken down as in fraction III, or if the tRNA is completely removed from its protein "shield" by phenol extraction (see Table I), then it is susceptible to the action of ribonuclease. This interpretation of the data is consistent with the long half-life for tRNA in axons in general (Ingoglia, 1982), and with the likelihood that tRNA in squid axons, as in other neurons, is derived by axonal transport and thus must exist in axoplasm for extended periods of time without degradation by endogenous ribonuclease. The conclusion that at least a portion of the reactions described in this study is tRNA dependent is also consistent with our demonstration of the presence in axoplasm of multiple species of aminoacylated tRNAs and their associated aminoacyl tRNA synthetases (Fig. 1, Table I).

Other experiments have been performed in rat sciatic nerves and support the conclusion that at least a portion of the aminoacylation of proteins is tRNA dependent. In the first of these, a fraction comparable to FI of squid axoplasm was obtained from segments of rat sciatic nerves. This fraction (containing molecules of M_r > 120,000) was subjected to phenol extraction and ethanol precipitation. The precipitate was then dissolved in Tris-HCl buffer containing sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis. Optical density profiles at 260 nm showed absorption peaks which were identical to *Escherichia coli* tRNA markers. These data indicate that this fraction contains 4 S (transfer) RNA (G. Chakraborty, unpublished findings). In another experiment the removal of tRNA from FI by DEAE ion exchange chromatography resulted in the loss of 93% of the aminoacylation of protein activity, and the readdition of this fraction back to the protein fraction resulted in the recovery of 64% of the original activity (M. F. Zanakis, unpublished findings). These experiments indicate that at least a portion of the aminoacylation of protein reactions we are observing is dependent on the presence of tRNA.

Thus, we conclude that the amino acid addition to protein observed in these reactions is likely to be a tRNA-dependent, ribosome-independent, post-translational addition of amino acids to axonal proteins.

We propose that all of the components of the reaction we are measuring, the aminoacyl-tRNA synthetases, tRNAs, protein transferase, and acceptor proteins, are part of a high molecular weight complex. Each of these components by themselves have, in general, M_r < 100,000. If they existed separately in axoplasm, reactions would not be present in fraction I since this fraction includes only substances of M_r > 120,00. Thus, as has been demonstrated in other tissues (Bandyopadhyay and Deutscher, 1971) including brain (Vadeboncoeur and La Pointe, 1980), the elements required for aminoacylation of tRNA exist in axoplasm of squid as part of a high molecular weight complex. In axoplasm, we now extend that finding to include the elements necessary for transfer of amino acids to protein.

Proteins are transported axonally in at least two distinct waves. tRNA appears to be transported as part of the slower wave (M. F. Zanakis, B. Eskin, and N. A. Ingoglia, submitted for publication), specifically as part of the faster component of the slow wave, slow component b (Hoffman and Lasek, 1975). Aminoacyl-tRNA synthetases and protein transferases are soluble proteins, and soluble proteins are in general also transported as part of slow component *b*. Recently, turtle optic axons have been observed under the electron microscope following quick freezing techniques for preserving the axoplasm without chemical fixation. The authors describe a granular material associated with neurotubules likely to correspond to proteins in slow component *b* which are larger than 10 nm, and "... are unlikely to be single cytoplasmic proteins; instead they could represent several proteins associated in a particle" (Schnapp and Reese, 1982). It is possible that the high molecular weight tRNA-protein complex we observe in the present experiments represents part of the granular material described by Schnapp and Reese (1982).

Recent experiments have revived earlier claims that elements present in axons are able to incorporate amino acids into proteins (Alvarez, 1982; Benech et al., 1982; Giuditta et al., 1982; Koenig and Adams, 1982). Viewed with respect to the present experiments, it seems reasonable that what was being measured in these experiments was the post-translational addition of amino acids to protein and not *de novo* protein synthesis. This is an intriguing possibility since, in all of the experiments in vertebrates, the incorporation of ^{3}H-amino acids into protein was observed under conditions where the axons were growing (either in culture (Koenig and Adams, 1982) or after a crush injury). Thus, it may be that one of the physiological conditions in which this reaction is

unmasked in axons is during growth. This conclusion is also consistent with the finding that large increases in the axonal transport of tRNA are coincident with growth and regeneration of axons (reviewed by Ingoglia and Zanakis, 1982).

Thus, we currently interpret our data as indicating that axons contain the necessary components (tRNAs and associated enzymes) for the post-translational addition of a variety of amino acids to axonal proteins (post-translational protein modification), and that under certain conditions, the reactions are inhibited by a soluble factor(s) in axoplasm, but that the removal of these factors, perhaps in response to injury, disinhibits the reaction, allowing for the addition of amino acids to acceptor proteins. We speculate that the addition of amino acids to proteins in axons may have important roles in other areas where post-translational protein modification of axonal proteins has been reported; i.e., in processing of neurofilament proteins (Nixon et al., 1982), and in synapse formation and stabilization (Skene et al., 1982).

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