SYNTHESIS OF GLYCOPROTEINS IN A SINGLE IDENTIFIED NEURON OF *APLYSIA CALIFORNICA*

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ABSTRACT

Incorporation of L-[^{3}H]fucose into glycoproteins was studied in R2, the giant neuron in the abdominal ganglion of *Aplysia*. [^{3}H]fucose injected directly into the cell body of R2 was readily incorporated into glycoproteins which, as shown by autoradiography, were confined almost entirely to the injected neuron. Within 4 h after injection, 67% of the radioactivity in R2 had been incorporated into glycoproteins; at least 95% of these could be sedimented by centrifugation at 105,000 *g*, suggesting that they are associated with membranes. Extraction of the particulate fraction with sodium dodecyl sulfate (SDS), followed by gel filtration on Sephadex G-200 and polyacrylamide gel electrophoresis in SDS revealed the presence of only five major radioactive glycoprotein components which ranged in apparent molecular weight from 100,000 to 200,000 daltons. Similar results were obtained after intrasomatic injection of [^{3}H]*N*-acetylgalactosamine. Mild acid hydrolysis of particulate fractions released all of the radioactivity in the form of fucose. When ganglia were incubated in the presence of [^{3}H]fucose, radioactivity was preferentially incorporated into glial cells and connective tissue. In contrast to the relatively simple electrophoretic patterns obtained from cells injected with [^{3}H]fucose, gel profiles of particulate fractions labeled with [^{14}C]valine were much more complex.

INTRODUCTION

Glycoproteins are known to be important constituents of cellular membranes. In addition to providing binding sites for antibodies, lectins, and viruses, they are also thought to be involved in intercellular recognition and adhesion (1). In nervous tissue, glycoproteins may play a role in the specific interaction between neurons, between neurons and glial cells (2, 3), and within the neuron they may mediate the interactions between the membranes of vesicles and of other subcellular organelles. While it is well established that glycoproteins are synthesized in a complex series of reactions involving the endoplasmic reticulum and the Golgi apparatus (4), little is known about how they are processed into neuronal membranes.

Nerve cells vary greatly in structure and function, and it is likely that these variations are reflected in the composition of glycoproteins in their membranes. Moreover, nervous tissue contains metabolically active glial cells and connective

tissue elements. While biochemical properties of tissues containing homogeneous populations of cells may be studied by averaging the activities of the entire population, bulk samples of nervous tissue reveal a heterogeneous and complex distribution of glycoproteins (5). Synthesis of glycoproteins and their subsequent processing into membranes might best be approached by studying these reactions in individual neurons.

We describe the synthesis of glycoproteins in the cell body of R2, the giant neuron of the abdominal ganglion of *Aplysia californica*, and show that these proteins are few in number and are primarily associated with membranes. Ambron et al. (6) show that the newly synthesized glycoproteins are rapidly transported along the major axon of the cell. We chose to work with this identified cholinergic neuron because the large size of its cell body (400–800 μm in diameter) makes it easy to isolate for biochemical analysis, and also allows direct intrasomatic injection of a variety of precursors, including L-[^{3}H]fucose and [^{3}H]*N*-acetylgalactosamine (Fig. 1). We were not able to introduce fucose or *N*-acetylgalactosamine specifically into R2 by incubating ganglia in the presence of the precursors, since *Aplysia* neurons are invested with glial sheaths (7) which cannot be removed (8). Because glial cells are active in incorporating sugars into glycoprotein, neurons isolated after incubation contain a mixture of neuronal and glial macromolecules. Injected precursors, however, are incorporated into glycoproteins almost entirely within the injected neuron. A preliminary report of these studies has been published (9).

MATERIALS AND METHODS

Aplysia californica, weighing 60–120 g, were supplied by Dr. R. C. Fay (Pacific Bio-Marine Supply Co., Venice, Calif.) and were maintained at 15°C in well-aerated aquaria of Instant Ocean (Aquarium Systems Inc., Eastlake, Ohio). Seaweed was included as food. Radioactive compounds were purchased from New England Nuclear, Boston, Mass., and were checked for purity by paper chromatography.

Preparation of Radioactive Solutions for Injection

1 mCi of either L-[^{3}H 1, 5, 6]fucose (4.8 Ci/mmol) or [G-^{3}H]*N*-acetyl-D-galactosamine (25 Ci/mmol) was dried under nitrogen in the bottom of an acid-washed conical test tube. The residue was dissolved in 1–2 μl of water and transferred, a portion at a time, to a small (500 μm) depression in a plastic petri dish. Each portion was allowed to evaporate before the next was added. In this way we were able to confine the radioactivity to a very small area. The bottom of the dish was then covered with 200 Dielectric fluid (350 cs, Dow Corning Corp., Midland, Mich.) and the material stored at −20°C. For injection water was added to the depression. By varying the amount of water or the volume taken up in the injection micropipette, we could vary the amount of radioactivity injected over a wide range.

Intrasomatic Injection of R2

Injections and subsequent incubations were carried out in an artifical sea water supplemented with amino acids and vitamins (10) and sterilized by filtration. The intact central nervous system, consisting of the abdominal ganglion and the circumenteric ganglia (11), was removed and pinned in a chamber for intracellular recording and for stimulation of nerves (12). Penetration and injection of R2 was performed as previously described (10). In these experiments, the volume injected was no greater than 3 nl, which is about 5% of the volume of the R2 cell body (8), and contained from 19,000 to 450,000 cpm.

We did not use cells which were damaged during penetration or injection. Any of the following conditions was sufficient to reject a cell: a continuous injury discharge, an action potential less than 65 mV, or a resting potential less than −45 mV. R2 is normally a silent cell with an action potential of 89 ± 2 mV (n = 27)[1], and a resting potential of −56 ± 1.2 mV (n = 27). Although our criteria for rejection are somewhat arbitrary, we did not wish to inject cells which were electrophysiologically unsound. Fewer than 1 out of 10 cells impaled were rejected.

General uptake of [^{3}H]fucose from the bath was minimized by perfusing the nervous system continuously during injection and 15 min thereafter. At the end of the period of perfusion, ganglia were transferred to 20 ml of fresh, supplemented sea water and incubated at 15°C for 4 or 10 h. Cells incubated for 10 h were impaled again at the end of the incubation period; every cell had an acceptable resting and action potential, as already defined. After incubation, ganglia were washed, placed on a brass block, and rapidly frozen with solid CO_2. The position of the cell body of R2 was marked with ink and the right connective severed just distal to the mark. Usually the connective was cut just proximal to the bag cell region (Fig. 1). Occasionally, however, the cell body occupied a more caudal position. When this occurred, the rostral part of the ganglion was removed with the connective. The branchial nerve and left connective were severed at their points of emergence from the ganglion. Since we will show that the incorporated radioactivity essentially was restricted to the injected neuron, the cell

[1] All values reported are ± standard error of the mean followed by the number of experiments in parentheses.

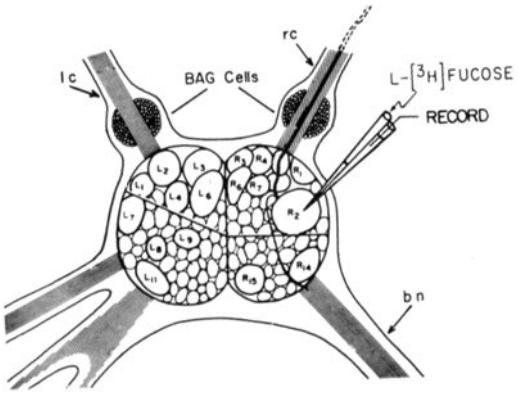


FIGURE 1 Diagram of the dorsal surface of the abdominal ganglion of *Aplysia californica* (12). The cell body of R2 is impaled by an injection and recording electrode. The major axon of R2 exits from the ganglion through the right connective (*rc*), while the branchial nerve (*bn*) contains the minor axon. The left connective (*lc*) does not contain any process of R2.

body was not dissected from the ganglion in every experiment. Ganglia, nerves, and isolated cell bodies were thoroughly homogenized at 0°C in ground glass tissue grinders (Micrometric Instruments, Cleveland, Ohio) containing 50 mM Tris-HCl (pH 7.6).

Incubations in [^{3}H]Fucose and [^{14}C]Valine

Abdominal ganglia were pinned through the connective tissue sheath to silicone plastic (Sylgard, Dow Chemical Corp.) and incubated at 15°C with 22 μM [U-^{14}C]valine (24 mCi/mM) or 21 μM [^{3}H]fucose in a 100 μl drop of Instant Ocean containing 50 mM Tris-HCl buffer (pH 7.6), streptomycin (0.1 mg/ml), and penicillin G (200 U/ml) (13). The drop was changed every 30 min for up to 4 h. For longer incubations, ganglia were placed in 1 ml of supplemented artificial sea water containing the antibiotics and 21 μM [^{3}H]fucose. After incubation, tissues were washed for 40 min with sea water. The cell body of R2 was isolated from the ganglion by dissection in sea water (8) and was homogenized at 4°C in 50 mM Tris-HCl (pH 7.6) together with approximately 0.5 mg unlabeled *Aplysia* nervous tissue.

Fractionation of Labeled Nervous Tissue and Cells (Fig. 2)

All procedures were carried out at 4°C unless otherwise stated. Homogenates of cell bodies or nerves were centrifuged at either 20,000 *g* for 20 min or 105,000 *g* for 45 min. The resulting pellet was washed three times with 50 mM Tris-HCl (pH 7.6), and the supernates were combined. Radioactivity in both supernate and pellet were analyzed.

SUPERNATE FRACTION: Samples of the supernate were removed for counting and the remaining solution made 5% in TCA, 0.5% in PTA[2] (each weight per volume). The precipitates were collected and washed on glass fiber pads (Whatman GF/C) and tritium was counted by scintillation at 25% efficiency (13). Carbon-14 was counted in a gas flow detector at 23% efficiency.

PELLET FRACTION: Pellets were extracted for 15 min at 70°C with either 3% SDS (recrystallized from ethanol) or 1% SDS, 0.14 M 2-mercaptoethanol in 10 mM sodium phosphate, pH 7.2. Extracts were centrifuged at 20,000 *g* for 10 min at room temperature. A sample of the supernate was removed for counting on the glass fiber pads and the counts were corrected for self-absorption. Unextracted radioactivity in the residue was solubilized for counting with 90% aqueous formic acid. Treatment of the formic acid residue with 1 N HCl (100°C; 45 min) did not release additional radioactivity. The sum of the radioactivity in (*a*) TCA-PTA precipitate, (*b*) SDS extract, and (*c*) formic acid extract was taken as the total labeled glycoprotein content of a tissue or cell. In some experiments, SDS-extracted glycoproteins were reduced and alkylated (14). At 4 h after an injection, 80% of the somatic content of [^{3}H]glycoprotein could be sedimented by centrifugation at 20,000 *g*, and most of our earlier studies were carried out at this force. To achieve a more complete separation of particulate and soluble material we now centrifuge the homogenates at 105,000 *g*. At this force, 95% of the labeled glycoproteins are sedimented.

Analysis of SDS Extracts

Gel filtration of SDS-extracts was carried out at room temperature on Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with 1% SDS. Column fractions were lyophilized and then reconstituted to one-tenth their original volume with 6 M urea and 0.01 M 2-mercaptoethanol, containing 100–200 μg of carrier ovalbumin. Radioactive proteins were precipitated by adding acetone at 0°C to a final concentration of 94%. This procedure removes unbound SDS while precipitating more than 90% of the labeled protein. The precipitate was collected by centrifugation at 4°C and thoroughly dried under a stream of nitrogen. SDS gel electrophoresis was carried out by the method of Vinuela et al. (15) as modified by Weber and Osborn (16). Gels were prepared in 5 × 75-mm glass tubes coated with Photo-Flo (Eastman Kodak Co., Rochester, N. Y.). The gel solution consisted of 7.5% acrylamide, 0.1% SDS, 0.27% (wt/vol) ethylene diacrylate, 0.075% (wt/vol) tetramethylethylenediamine, and 0.07% (wt/vol) ammonium persulfate in 0.1 M sodium phosphate (pH 7.2). Gels were prerun at 2 mA/gel for 1 h before addition of samples.

[2] *Abbreviations used in this paper:* PTA, phosphotungstic acid; SDS, sodium dodecyl sulfate.

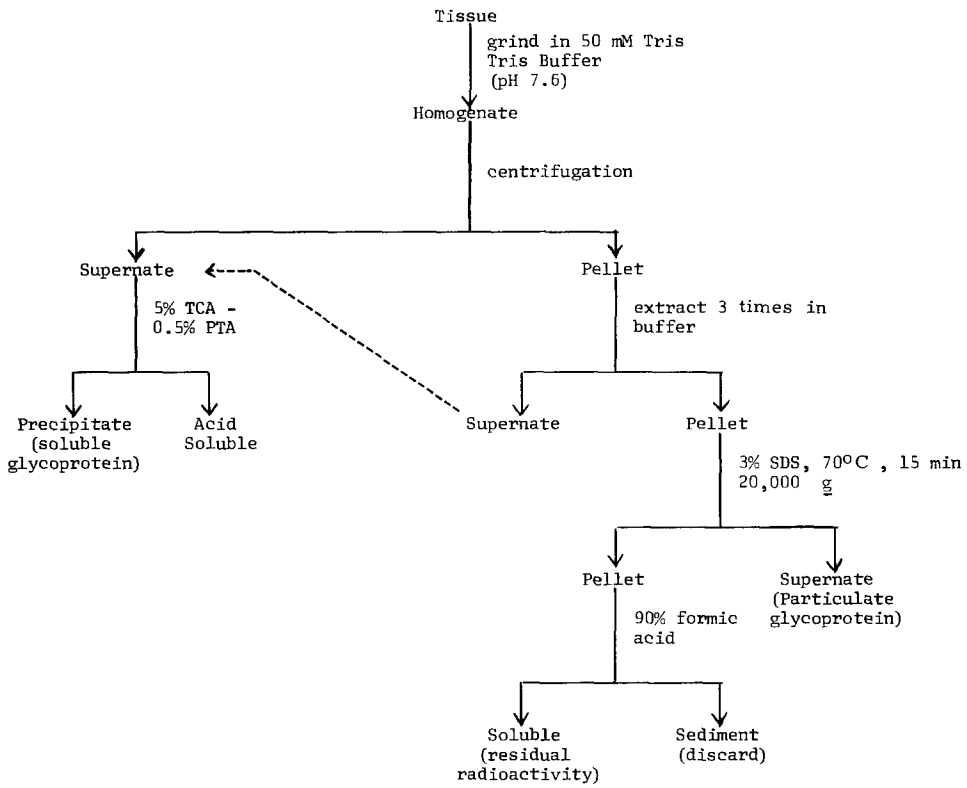


FIGURE 2 Fractionation: separation of particulate and soluble glycoproteins.

Acetone-precipitated proteins were dissolved at a maximum concentration of 2 mg/ml by heating at 70°C for 15 min in 1.0% SDS, 0.14 M 2-mercaptoethanol, 0.01 M sodium phosphate buffer (pH 7.2) containing 25% glycerol (sample buffer). Bromphenol blue and 10 μg dansylated bovine serum albumin (17) were added to samples as internal standards. The dansylated monomer and dimer of albumin could be readily detected under ultraviolet light after electrophoresis. A plot of the log molecular weight against distance migrated for the dansylated monomer, dimer, trimer, and tetramer of albumin resulted in a straight line (16).

Electrophoresis was carried out at 6.5–7.0 mA/gel in 0.1 M sodium phosphate, (pH 7.2), 0.1% SDS until the bromphenol blue reached approximately 1 cm from the anode. After the positions of the standards were marked with ink, the gels were frozen on solid CO_2 and cut into 1-mm sections using a Mickle Gel Slicer (Brinkman Instruments, Inc., Westbury, N. Y.). Each slice was dissolved in a glass scintillation vial by heating with 1.3 ml 10% ammonium hydroxide at 60–70°C and counted at 18% efficiency after addition of 10 ml scintillation fluid containing Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.).

Autoradiography and Microscopy

Tissues were prepared for autoradiography after fixation in buffered 6% glutaraldehyde, postfixation in buffered 1% osmium tetroxide, and embedding in Epon. 2-μm thick sections mounted on glass slides were coated with L4 emulsion (Ilford Ltd., Essex, England) by dipping, and were exposed in a dry atmosphere at room temperature for 5–21 days. They were developed in Kodak D-19 at 68°C for 4 min, and examined by light microscopy.

Analytical Methods

IDENTIFICATION OF [^{3}H]FUCOSE IN HYDROLYSATES OF LABELED PARTICULATE MATERIAL: Acid hydrolysis was carried out at 100°C either in 1 N HCl for 45 min (resulting in complete solubilization of the radioactive material) or in 1 N H_2SO_4 for 3 h (resulting in 85% solubilization). HCl was removed by lyophilization and H_2SO_4 by ion ex-

change (18). [^{3}H]fucose in the hydrolysates was identified by descending paper chromatography on Whatman No. 1 paper in three solvent systems (by volume): solvent system A, 1-butanol-ethanol-water, 10:1:2 (19); solvent system B, ethyl acetate-pyridine-water, upper phase, 100:40:100 (20); solvent system C, ethyl acetate-pyridine-acetic acid-water, 5:5:1:3 (21). The mobilities of various monosaccharides, expressed as percent of migration of fucose (100) were: solvent A, mannose 61; glucose and galactose 40; solvent B, glucosamine 20; galactose 48; mannose 80; *N*-acetylglucosamine 79; solvent C, N-acetyl galactosamine 92. Reducing sugars were detected using silver nitrate (22). Radioactivity was eluted with water and counted in Triton X-100 scintillation fluid.

PROTEOLYTIC DIGESTION: Proteolytic digestion was carried out at 37°C for 72 h on 105,000 *g* particulate fractions from injected cells. The pellets were suspended in 0.5 ml of 0.15 M Tris-HCl (pH 7.8), 1.5 mM $CaCl_2$, containing 100 μg pronase (Calbiochem, San Diego, Calif., 45 proteolytic U/mg) which had been preincubated for 30 min to destroy any other enzyme activities which might contaminate the pronase preparation. After 24 h and 48 h of incubation, we added an additional 50 μg preincubated buffered pronase and 25 μl M Tris-HCl (pH 7.8).

ADSORPTION TO CHARCOAL: 5–10 mg of activated, acid-washed Norite (Fisher Scientific Co., Springfield, N. J.) were added to centrifuged tissue extracts acidified to pH 4 and containing 0.4 μmol of AMP as carrier (23). The charcoal was collected on cellulose acetate filters (Millipore Corp., Bedford, Mass.) and washed with 0.05 N acetic acid. Radioactivity was eluted from the charcoal with 50% aqueous ethanol containing 1% NH_4OH, and the elute collected by filtration through a second filter. Recovery of GDP-fucose was not tested because we did not have a sample of the authentic material; under these conditions, however, small amounts of labeled UDP-glucose were nearly quantitatively adsorbed and eluted. Material labeled with [^{3}H]fucose which was adsorbed to charcoal was characterized further by thin-layer chromatography (24) on plastic sheets (20 × 20 cm) coated with cellulose MN 300 impregnated with polyethyleneimine (Brinkman Instruments, Inc.). GDP-[^{3}H]fucose in 105,000 *g* supernates was also identified by paper chromatography in 0.1 M amonium acetate (pH 7.0): ethanol, 8:7 (solvent system D [25]), and by high voltage paper electrophoresis in pyridine-acetic acid at pH 4.7 (8).

RESULTS

Intrasomatic Injection of [^{3}H]Fucose

DISTRIBUTION OF INJECTED RADIOACTIVITY WITHIN THE GANGLION: L-[^{3}H]fucose injected into the cell body of R2 was readily incorporated into glycoproteins. Not all of the injected radioactivity remained within the cell, however. With amounts of fucose greater than 10 pmol, 40% of the radioactivity initially injected could be found in the bath. Approximately 65% of the fucose which was destined to escape did so during the first hour of incubation. General uptake of labeled fucose from the bath was estimated in every experiment by examining the left connective for radioactivity. This nerve, which does not contain any of the processes of R2 (12), contained only 0.1–0.5% of the total radioactivity in R2.

To ensure that the radioactivity which escaped from R2 was not incorporated into glycoproteins by other cells, we used autoradiography to examine the distribution of the labeled macromolecules within a ganglion containing an injected R2. Label was localized to the injected cell; there was essentially none over glia, connective tissue, or neighboring neurons (Fig. 3 *a*). A more extensive autoradiographic analysis has been reported by Thompson et al. (26) and will be published in greater detail. A further indication for localization of label to the injected neuron was the observation that by 4 h after injection 95% of the radioactivity in the ganglion was isolated by dissecting out the cell body of R2.

INCORPORATION OF THE INJECTED RADIOACTIVITY INTO GLYCOPROTEIN: During a period of 4 h after injection, 66.8% of the [^{3}H]fucose remaining within R2 was incorporated into glycoprotein (Table I). The actual extent of incorporation was directly proportional to the amount of [^{3}H]fucose within the cell up to approximately 35 pmol (Fig. 4). This amount corresponds to an intracellular [^{3}H]fucose concentration of 0.5 mM, estimated using 66 nl as the volume of R2 (8). At greater concentrations, incorporation reached a plateau, suggesting that some step in the utilization of fucose had become saturated. Still higher concentrations appeared to inhibit incorporation (Fig. 4). All of the values reported in tables and in the other figures of this paper were determined using cells containing less than 40 pmol.

Fucose is incorporated into a variety of polysaccharides (27) and is known to be a component of mucous secretions in *Aplysia* and several other marine animals (28). Pronase digestion of labeled macromolecules from R2, however, indicated that fucose was predominantly, if not entirely, incorporated into glycoproteins: less than 25% of the digest remained large enough to be excluded

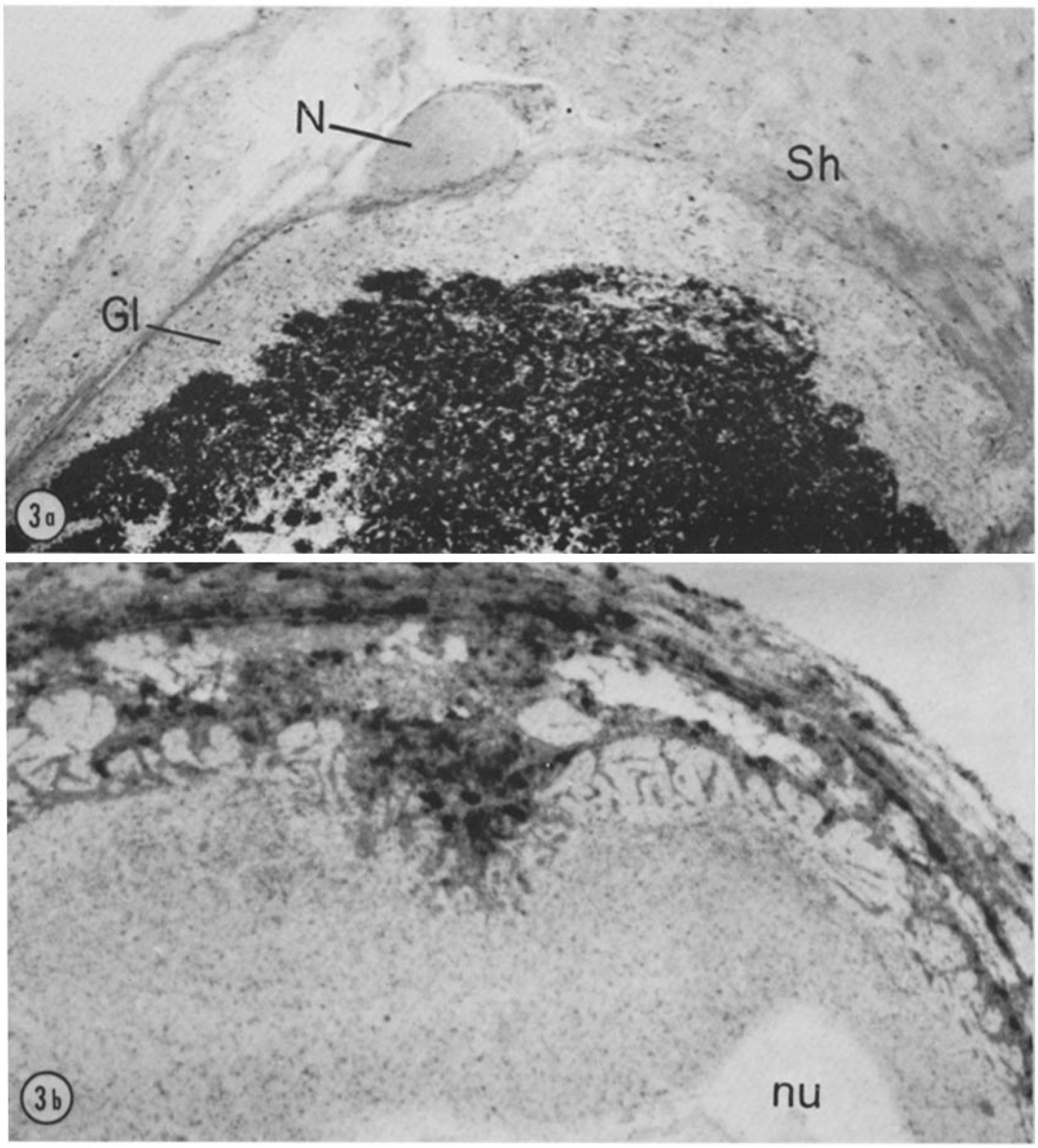


FIGURE 3 Light microscope autoradiograph of portions of an R2 cell body. (*a*) Intrasomatic injection of [^{3}H]fucose. After injection the ganglion was bathed in supplemented sea water for 4 h. Silver grains are located almost exclusively over cytoplasm with little labeling of surrounding glia (*Gl*) or connective tissue sheath (*Sh*); *N*, cell body of unidentified neuron. × 564. (*b*) Incubation in [^{3}H]fucose. The abdominal ganglion was incubated for 4 h in supplemented sea water containing 21 μM [^{3}H]fucose. Silver grains are located primarily over glia and the connective tissue sheath which surrounds R2. *Nu*, nucleus. × 345.

during gel filtration on Sephadex G-50, whereas approximately 85% of the undigested control was excluded (Fig. 5). The total included radioactivity from the digest was collected and passed through a column of AG-1-X2 (acetate form) to remove SDS (29). More than 90% of the radioactivity remained bound to the column, and this was quantitatively eluted at pH 4.7 with 0.1 M pyridine-acetate.

Analysis of this material by paper chromatography in solvent system B and by paper electrophoresis at pH 4.7 revealed the presence of at least seven compounds, presumably glycopeptides.

TABLE I

Fate of [^{3}H]Fucose Introduced into the Cell Body of R2 by Injection and Incubation

	Distribution of radioactivity (Percent of total radioactivity associated with cell body)		
	A Injected 4 h (6)	B Incubated 4 h (8)	C Incubated 18 h (3)
Total acid soluble radioactivity	33.8 ± 1.4	67.6	72.4
Soluble glycoproteins	12.9 ± 1.7	5.5	8.5
Particulate glycoproteins (SDS extract)	52.6 ± 2.0	23.6	15.8
Residual radioactivity	1.3 ± 0.2	3.2	3.3
Total macromolecules	66.2 ± 1.5	32.3	27.6
Total radioactivity (cpm/cell)	7,500–70,000	2,410	8,108

Cells or ganglia were fractionated at 20,000 *g* for 30 min (see Materials and Methods and Fig. 1). Total macromolecular radioactivity is the sum of the radioactivity in soluble and particulate glycoproteins and in the residue. Injection: R2 was injected with [^{3}H]fucose. The six ganglia containing the injected cells were homogenized and analyzed separately. Incubation: ganglia were incubated with [^{3}H]fucose. The cell bodies were dissected from the ganglia. In order to obtain sufficient radioactivity the indicated number of cell bodies were combined before fractionation and analyzed together. Under our conditions of counting, 2200 cpm was equivalent to 1 pmol.

Characteriation of Newly Synthesized Glycoproteins

PARTICULATE GLYCOPROTEINS: Centrifugation at 20,000 *g* of a homogenate from an R2 cell body 4 h after injection sedimented 54% of the total somatic radioactivity (Table I) and 80% of

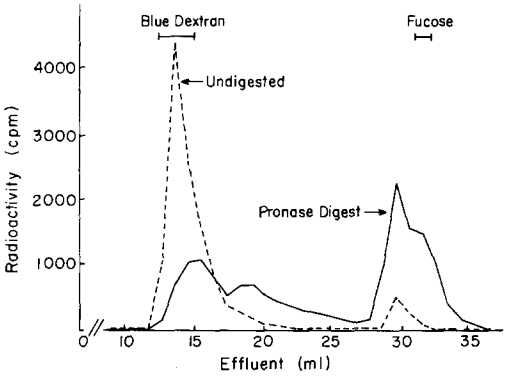


FIGURE 5 Pronase digestion of fucose-labeled particulate fraction. 4 h after injection the abdominal ganglion was fractionated at 105,000 *g* for 45 min. The pellet was extracted with SDS and the extract was divided in half. Pronase digestion was carried out for 72 h on one acetone precipitate while the other served as control. The control and digest were lyophilized and filtered on a column (0.9 × 58 cm) of Sephadex G-50 using 1% SDS at a flow rate of 5 ml/h. The column was standardized with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and [^{3}H]fucose. Fraction size was 0.9 ml.

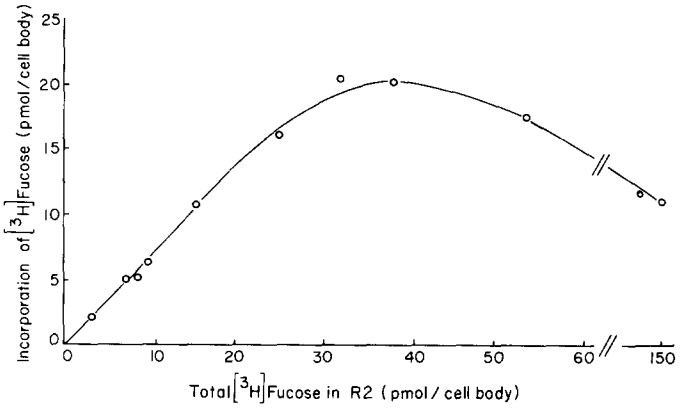


FIGURE 4 Incorporation of [^{3}H]fucose into glycoprotein after intrasomatic injection. 4 h after injection the abdominal ganglion containing the cell body of R2 was homogenized and fractionated. Total incorporation of [^{3}H]fucose into glycoprotein was determined as described in the legend to Table I. Total [^{3}H]fucose in the cell was the sum of radioactivity in macromolecules and in the acid soluble fraction.

the total [^{3}H]glycoprotein. This pellet would be expected to contain fragments derived from the various membrane systems of the cell. When we examined the total, newly synthesized proteins in this fraction using [^{14}C]valine, a complex labeling pattern was found (see below). Since it was our intention to characterize membrane proteins in R2, we sought to reduce this complexity by using [^{3}H]fucose to label glycoproteins selectively. As expected, we found that [^{3}H]fucose labeled far fewer proteins than did [^{14}C]valine. To increase resolution of newly synthesized glycoproteins, we first sorted them into three size fractions by gel filtration on Sephadex G-200 (Fig. 6).

The material of lowest molecular weight (totally included fraction, C, Fig. 6), amounted to approximately 15% of the total radioactivity extracted from the 20,000 *g* pellet, and could not be recovered by subsequent precipitation with acetone. Although this material has not yet been studied further, its solubility in organic solvents suggested that it was lipid or proteolipid. Glycolipids containing fucose have been previously reported (30), and a proteolipid from human myelin contains carbohydrate moieties (31).

The high and intermediate molecular weight fraction (excluded and partially included fractions, A, B, Fig. 6) together contained 85% of the total radioactivity in the 20,000 *g* pellet. All of the radioactivity in these two fractions was quantitatively recovered by acetone precipitation. The radioactive proteins in the precipitates were analyzed by SDS gel electrophoresis. In all, five major radioactive components were resolved (Fig. 7): four of these (peaks I, II, III, and IV) were found only on the electropherogram from the excluded fraction (Fig. 7 A). Peak V was the major component in the partially included fraction (Fig. 7 B), although it was also present as a minor peak in the profile from the excluded fraction. Reduction and alkylation (14), which should minimize aggrega-

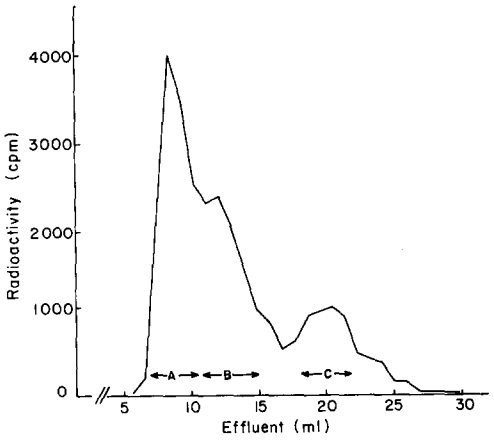


FIGURE 6 Gel filtration of SDS extract of particulate fraction. The 20,000 *g* pellet was extracted with 3% SDS containing Blue Dextran 2000. The extract was placed on a column (1 × 35 cm) of Sephadex G-200 equilibrated with 1% SDS and with a flow rate of 3 ml/h. Fraction size was 0.9 ml. Recovery of radioactivity was 100%. Arrows indicate the fractions which were combined for further analysis.

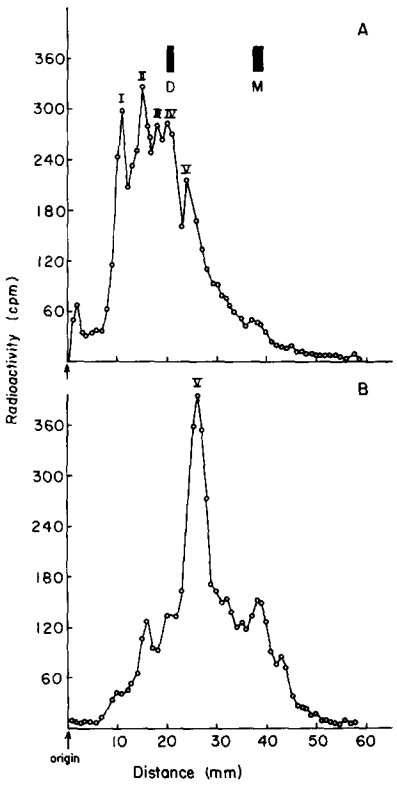


FIGURE 7 SDS polyacrylamide gel electrophoresis of glycoproteins labeled with fucose previously fractionated by gel filtration. Effluent samples were combined into two fractions, A and B, as shown in Fig. 6 and the glycoproteins quantitatively recovered by acetone precipitation. The precipitates were dissolved and reduced in sample buffer. Electrophoresis was performed for 4 h at 6.5 mA/gel at room temperature. Gels were cut into millimeter segments and the radioactivity in each segment counted as described in Methods. *M*, monomer; *D*, dimer (of beef serum albumin).

TABLE II

Estimated Molecular Weights of Glycoproteins Labeled with [^{3}H]Fucose and [^{3}H]N-Acetylgalactosamine

Label	Percent acrylamide	Estimated molecular weight (daltons) I	II	III	IV	V
Fucose	5	209,000	200,000	158,000	138,000	122,000
Fucose	7.5	182,000	152,000	136,000	123,000	107,000
N-Acetylgalactosamine	7.5	180,000		138,000	124,000	104,000

Labeled glycoproteins were extracted from particulate fractions with SDS, reduced, and separated by polyacrylamide gel electrophoresis in SDS. Molecular weights were estimated from their electrophoretic migration using the monomer, dimer, and trimer of beef serum albumin as standards (16).

tion, did not alter the patterns obtained. These relatively simple glycoprotein patterns were obtained reproducibly from both 20,000 *g* and 105,000 *g* pellets from injected cells which were not removed from ganglia before extraction for the electrophoretic analysis. We observed no differences, however, between patterns from injected cell bodies isolated by dissection and injected cells left within the ganglion. This is an indication of the minimal contribution made by [^{3}H]fucose which had escaped from the injected neuron and which might have been incorporated into other components of the ganglion.

All of the particulate glycoproteins were large, with molecular weights greater than 100,000 daltons (Table II). Since glycoproteins that have large carbohydrate moieties are known to migrate anomalously in SDS gel electrophoresis (32), we analyzed data from 5% and 7.5% gels as recommended by Banker and Cotman (33). Components II, III, IV, and V showed the same relationship between size and free electrophoretic mobility (Mo) seen with soluble proteins. Component I, however, had an anomalously high retardation coefficient (K_R) relative to its estimated free electrophoretic mobility. This analysis is tentative, however, since we used data from only two concentrations of acrylamide. A more detailed analysis is probably premature, because we are still uncertain whether any of the components are single glycoproteins.

Although the relative proportions of the components varied from cell to cell, peaks I and V were always prominent. Electrophoretic patterns obtained from extracts of the cell body 10 h after injection were virtually identical to those from 4-h cells (Fig. 8); by 15 h, however, the pattern had changed as a greater proportion of some of the components moved out of the cell body into the axon (6).

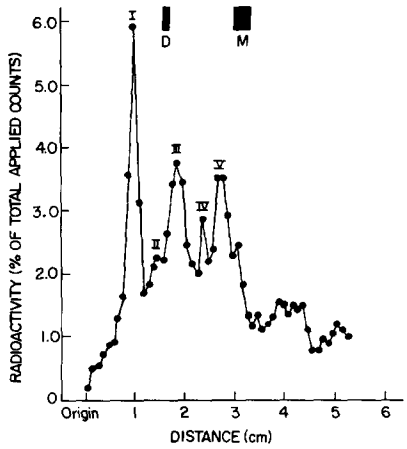


FIGURE 8 SDS polyacrylamide gel electrophoresis of particulate glycoproteins from an R2 10 h after injection. Fractionation was at 100,000 *g*; the pellet was extracted into sample buffer containing 0.5% SDS and electrophoresed.

A pattern of labeled glycoproteins similar to that obtained with fucose was found 10 h after injecting [^{3}H]*N*-acetylgalactosamine into the cell body of R2. The high specific radioactivity of this sugar enabled us to examine a greater number of fractions resulting after gel filtration (Fig. 9). Although peak II appeared to be absent, it may be contained in the shoulder of peak I (Fig. 10 A). The relative proportions of the other components were similar to those labeled with fucose (compare Fig. 10 to Fig. 7), suggesting that both precursors are incorporated into the same glycoproteins.

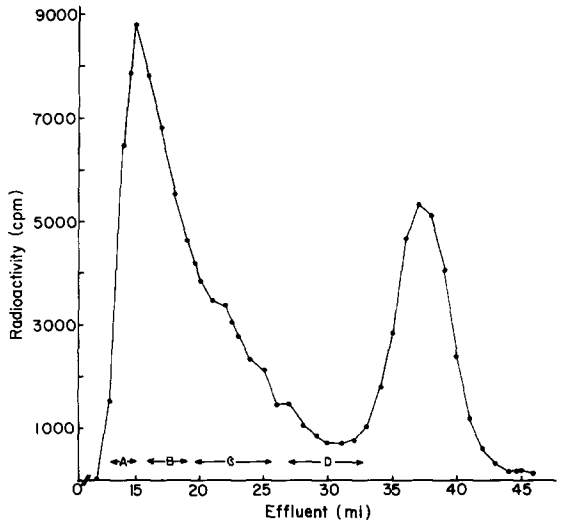
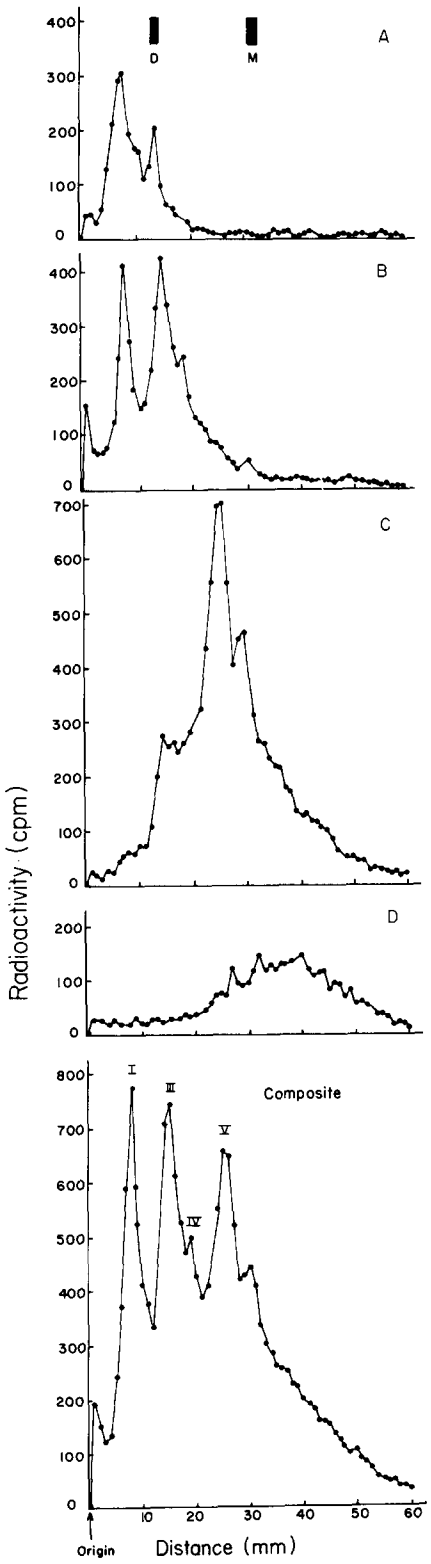


FIGURE 9 Fractionation of [^{3}H]*N*-acetylgalactosamine-labeled particulate glycoproteins on Sephadex G-200. The 105,000 *g* pellet from an R2 10 h after injection was extracted with 3% SDS. Gel filtration was performed on a column (0.9 × 58 cm) of Sephadex G-200 as described in the legend to Fig. 6. Arrows indicate the fractions which were combined for further analysis.

The electrophoretic behavior of some proteins is irreversibly altered by organic solvents (34). Since we precipitated samples with acetone, we compared the electrophoretic patterns obtained from material precipitated by acetone with those from pellets extracted directly with SDS in sample buffer. There were no differences in either the distribution or the appearance of the five components. Another problem in the analysis of membrane proteins is the presence of endogenous proteases which can degrade samples during solubilization in SDS (35). In order to avoid this difficulty we used high concentrations of SDS and heated the samples. Nevertheless, to check for possible proteolytic digestion, we incubated a washed membrane pellet at pH 5.5 for 1 h at 37°C. The electrophoretic pattern obtained from this sample was identical to that obtained from samples extracted directly into SDS.

EXTRACTION WITH DEOXYCHOLATE: One purpose for washing the particulate fraction exhaustively with hypotonic buffers is to prevent trapping of soluble membrane components within vesicular structures which are either normally present in the tissue or artifactually produced from broken fragments of membrane during homogenization. To obtain further evidence that the electrophoretic patterns obtained represent integral membrane glycoproteins, we extracted the particulate fraction from an injected R2, first with hypotonic buffer (10 mM sodium phosphate, pH 7.2) and then with sodium deoxycholate. (The deoxycholate-to-protein ratio was approximately 0.3.) This treatment would be expected to release trapped cytoplasm and other loosely bound constituents and has been used to release bound proteins from microsomes (36, 37). During a 2-h extraction at room temperature only 12.3% of the radioactivity in the particulate fraction was solubilized by deoxycholate. When the unextracted radioactivity was analyzed by gel electrophoresis, the resulting pattern was essentially identical to the control which had not been treated with the detergent.

IDENTIFICATION OF [^{3}H]FUCOSE IN HYDROLYSATES FROM THE PARTICULATE FRACTION OF THE CELL: L-fucose has been shown to be metabolically stable in a variety of vertebrate tissues (38, 39, 40). Comparable studies, however, have not been carried out in invertebrates. We found that all the radioactivity in the glycoproteins could be recovered as fucose when the particulate fraction from an injected R2 was hydrolyzed either in HCl or in H_2SO_4. Analysis of the hydrolysates by paper chromatography in solvent systems A, B, and C resulted in the detection of only a single radioactive compound. This material, [^{3}H]fucose similarly treated with acid, and the standard fucose cochromatographed in



each system; they were completely separated from glucosamine, glucose, galactose, mannose, *N*-acetylglucosamine, and *N*-acetylgalactosamine. Other radioactive sugars in the hydrolysate would have been detected if they contained 5% as much label as did fucose.

Radioactivity in R2, which was not incorporated into macromolecules or retained as fucose, was found in two compounds tentatively identified as fucose-phosphate and GDP-fucose. These were isolated from the totally included radioactivity obtained by gel filtration on Sephadex G-50 of a 105,000 *g* supernate from an R2 4 h after injection. The compounds were characterized by paper chromatography in solvent system D: 61% of the radioactivity in the totally included Sephadex fraction was identified as fucose; about 15% migrated close to glucose 6-phosphate, and was presumably fucose-phosphate. Radioactivity in this compound also migrated as a monophosphate ester during high voltage paper electrophoresis at pH 4.7. The rest of the radioactivity occupied the position on chromatograms relative to marker GMP expected of GDP-fucose (41).

In order to obtain further evidence for the presence of GDP-fucose, we treated the soluble fraction from R2 cell bodies 4 h after injection with charcoal, and found that 6.4 ± 2.1% (n = 5) of the total somatic radioactivity was absorbed. Thin-layer chromatography on polyethyleneimine plates of this adsorbed material, which was removed from charcoal under alkaline conditions, separated two charged compounds. Most of the radioactivity migrated far from any of the nucleotide sugar reference substances, and probably was fucose-phosphate, the breakdown product expected from hydrolysis of GDP-fucose (42). The rest of the radioactivity migrated as expected of GDP-fucose (24). No radioactivity was found in the region of UDP-glucose, suggesting that fucose does not enter the general pathways of carbohydrate catabolism.

FIGURE 10 SDS polyacrylamide gel electrophoresis of glycoproteins labeled with *N*-acetylgalactosamine previously fractionated by gel filtration. Effluent samples were combined into four fractions. A–D, as shown in Fig. 9. The acetone-precipitated glycoproteins were extracted in 1% SDS, reduced, and aliquots were electrophoresed. The composite profile was constructed by adding the amounts of radioactivity in corresponding gel slices after corrections were made to normalize sample sizes.

Incubation of Ganglia with [^3H]Fucose

Fucose injected into the cell body of R2 was incorporated into macromolecules almost entirely within the injected neuron. In contrast, a 4-h incubation in the presence of 20 μM [^{3}H]fucose resulted in a distribution of label throughout all the components of the ganglion. Most of the labeling occurred over glial cells and connective tissue, although some was also incorporated into the cytoplasm of R2 (Fig. 3 *b*). Cell bodies, however carefully dissected from ganglia, are known to be contaminated with glial cells and connective tissue (8). Thus an R2, labeled with fucose by incubation and then isolated by dissection, would contain a mixture of neuronal and nonneuronal [^{3}H]glycoproteins. After 4 h of incubation 32% of the radioactivity was incorporated into macromolecules (Table I, B), less than half of the fraction found in an injected R2 (Table I, A). When the duration of incubation was lengthened to 18 h, more fucose was taken up, but still only about 30% was incorporated (Table I, C). The maximum amount of [^{3}H]fucose associated with an incubated R2 was considerably less than that introduced by even a small injection.

There also appeared to be a difference between the types of newly synthesized glycoproteins in incubated and injected cells. Using gel electrophoresis, we compared the total particulate fraction labeled with fucose by incubation (Fig. 11) with the pattern obtained after injection (Fig. 7). The two patterns were similar from the 17th to the 30th mm, which contain the proteins of intermediate size, peaks III, IV, and V. The appearance of apparently similar proteins in both injected and incubated cells was not surprising, since at least some of the radioactivity in the incubated cell was neuronal and since neurons and glial cells probably have some glycoproteins of the same type. From the origin to the 16th mm, the region of the electropherogram which contains larger proteins, the patterns were different. Peak I, which was a major feature in the profile from an injected cell (Fig. 7) was not seen in a pattern from incubated cells. In addition, a component with an apparent molecular weight of 158,000 was prominent in the profile from the incubated cell (arrow, Fig. 11). This component, which was absent from injected cells, presumably is not neuronal.

Incubation of Ganglia with [^{14}C]Valine

[^{14}C]valine is readily incorporated into particulate macromolecules but, in contrast to fucose, the profiles on gel electrophoresis were far more complex. To obtain an estimate of the total number of newly synthesized proteins, we prepared the particulate fraction from R2 cell bodies dissected from ganglia which had been incubated in [^{14}C]valine for 4 h. It has previously been shown that during incubation in the presence of labeled amino acid most of the radioactivity is incorporated into neuronal perikarya although some is incorporated into glial cells and connective tissue (43, 13, Thompson and Schwartz, unpublished results). Therefore we did not introduce the amino acid by injection. Approximately 60% of the total radioactivity in R2 was incorporated into particulate macromolecules, a value similar to that obtained with injected fucose (Table I). We subjected the extracted material to gel filtration and the proteins in the high, intermediate, and low molecular weight regions were then separated by gel electrophoresis (Fig. 12). The overall distribution of the proteins on the gels after electrophoresis reflected the order of their emergence from the Sephadex column. Much of the valine was incorporated into the high molecular weight proteins and several of these had mobilities similar to the fucose-labeled glycoproteins shown in Figs. 7 and 8.

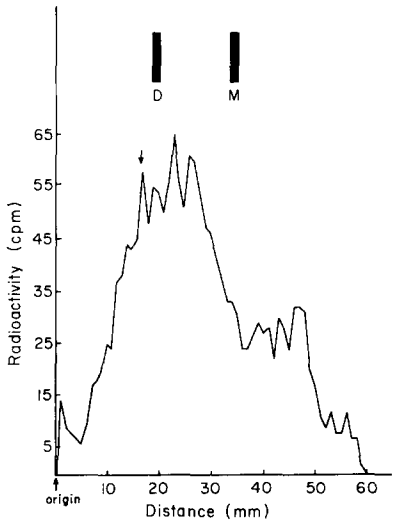


FIGURE 11 SDS polyacrylamide gel electrophoresis of labeled particulate glycoproteins from R2 cell bodies after incubation in the presence of [^{3}H]fucose. Three abdominal ganglia were incubated for 18 h. Cell bodies were dissected from the ganglia, combined, and fractionated at 20,000 *g*. The particulate fraction was extracted with sample buffer and electrophoresed.

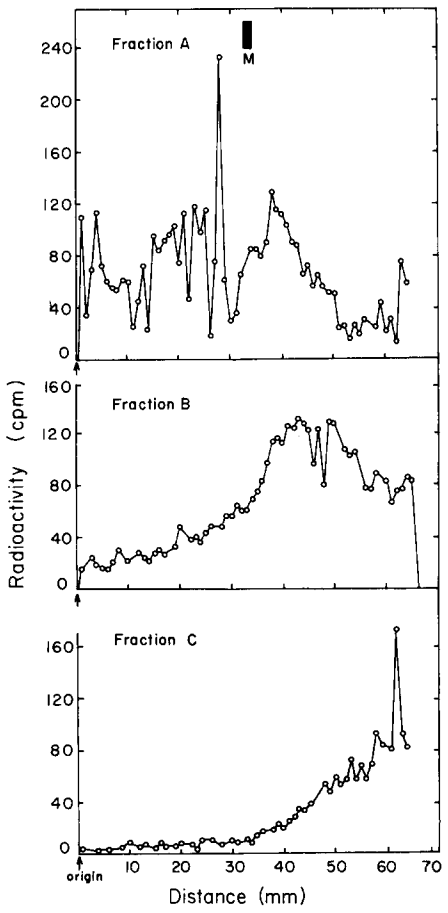


FIGURE 12 SDS polyacrylamide gel electrophoresis of labeled particulate proteins from R2 cell bodies after incubation in the presence of [^{14}C]valine. Six abdominal ganglia were incubated for 4 h. Cell bodies were dissected from the ganglia, combined, and fractionated at 20,000 *g*. The pellet was extracted with SDS and filtered on a column (1 × 30 cm) of Sephadex G-200. The labeled proteins in the excluded, partially included, and totally included fractions were combined and analyzed by SDS gel electrophoresis as described in the legend to Fig. 7.

DISCUSSION

Glycoproteins are associated with the external surface of neurons and have also been shown to be components of vesicle and mitochondrial membranes (44, 45). Despite the importance of these macromolecules to the neuron, little information is available either on the synthesis of neuronal glycoproteins, or on the means by which they become inserted into membranes of the nerve cell. One major obstacle has been the heterogeneity of nervous tissue.

Fucose Metabolism in a Single Identified Neuron of Aplysia

Incorporation of injected [^{3}H]fucose into glycoproteins in *Aplysia* appears to take place by a sequence of reactions similar to that proposed for other animals (46). The initial steps in this pathway require the phosphorylation of fucose followed by the formation of GDP-fucose, the substrate for the transfer reaction. We have established by paper chromatography that fucose is the only labeled sugar present in the soluble fraction from injected cells. Labeled fucose-phosphate and GDP-fucose were tentatively identified in this same fraction. They were characterized by adsorption to charcoal, by paper and thin layer chromatography in several systems, and by high voltage electrophoresis. Conclusive identification of fucose-phosphate and GDP-fucose was not achieved, however, since we did not have the authentic compounds as references.

The last step in the fucosylation of proteins in tissues of vertebrates is the enzymatic transfer of the fucosyl residue from GDP-fucose to the carbohydrate moiety of the nascent glycoprotein (47). Since fucose is found only in the terminal position of carbohydrate side chains (48), this transfer is likely to be one of the last steps in the synthesis of glycoproteins. Autoradiography has shown that incorporation of fucose occurs in the membranes of the Golgi apparatus (49, 50). Using autoradiography in the light microscope we observed labeling over the cytoplasm after injecting R2 (Fig. 3 A). More extensive studies using light and electron microscope autoradiography in L10, another identified neuron of *Aplysia*, showed that the principal site of fucosylation in *Aplysia* is the Golgi region (26 and Thompson, Schwartz, and Kandel, manuscript in preparation).

When nervous tissue was incubated in the presence of relatively high concentrations of [^{3}H]fucose, we found that radioactivity was incorporated primarily into glial cells and connective tissue. This difference in distribution of label might be the consequence of some barrier to the entrance of fucose to neuronal cytoplasm. Thus, radioactivity which escaped from the injected neuron into the perfusate would be poorly utilized by other neurons. Some of the radioactivity which escaped may be retained within the ganglion to be incorporated, however. Although it is difficult to obtain a quantitative estimate of the extent of labeling outside of the injected neuron, autoradiographic

and biochemical evidence indicates that background incorporation was very small, and could be neglected.

Characterization of Newly Synthesized Glycoproteins

In vertebrate nervous tissue fucose is incorporated primarily into glycoprotein (39, 40). The fate of fucose in molluscs has not been widely studied, however. Since *Aplysia* and other marine molluscs feed on seaweed known to contain polymers of fucose (27), they may utilize fucose more extensively than do vertebrates. For example, in *Aplysia*, McMahon et al. (28) found fucose to be a major constituent of a mucus-like polysaccharide secreted into the genital tract. It was necessary, therefore, to show that the labeled macromolecules were glycoproteins.

We found that 4 h after injection, 67% of the total radioactivity in the cell body was in macromolecular form (Table I), and that 95% of this material sedimented at 105,000 *g* in 50 mM Tris-HCl (pH 7.6). Radioactivity incorporated into particulate macromolecules was recovered entirely as fucose after complete acid hydrolysis. The bulk of the particulate radioactivity was characterized as glycoprotein by its susceptibility to proteolytic digestion with pronase (Fig. 5). Approximately 15% of the particulate radioactivity was extracted in either acetone or in chloroform-methanol, and might possibly be fucolipid or proteolipid.

The largely particulate nature of the labeled glycoproteins indicated that they are associated with membranes. Freeze thawing, washing with mildly alkaline hypotonic buffers, and extraction with sodium deoxycholate are procedures known to release enzymes and other proteins contained in vesicular structures and to free protein adsorbed to membranes (36, 37). We found that most of the incorporated radioactivity remained insoluble after subjecting the particulate material to these procedures. Presumably these insoluble glycoproteins are integral parts of membranes derived from various cellular structures. In support of these biochemical observations, Thompson et al. (26 and manuscript in preparation) have found that label was distributed over neuronal membranes including the Golgi apparatus, vesicles, mitochondria, and the external membranes of both L10 and R2 after intrasomatic injection of [^{3}H]fucose.

Only a small proportion of the injected fucose was incorporated into soluble glycoproteins of R2 (Table I), as in mouse brain (39) and the goldfish visual system (40). A preliminary characterization of these glycoproteins in *Aplysia* indicates that they are of lower molecular weight than particulate glycoproteins. They presumably include the lysosomal hydrolases, many of which in other animals are known to be glycoproteins (51).

In contrast to the total proteins of R2 labeled with [^{3}H]leucine (52, 53), particulate glycoproteins labeled with [^{3}H]fucose or [^{3}H]*N*-acetylgalactosamine appeared to be all of high molecular weight and to be few in number. Essentially all of these glycoproteins were extracted by hot SDS. Gel filtration of the extracts followed by electrophoresis on polyacrylamide gels in SDS resulted in the separation of only five major radioactive components (Figs. 8, 9). It is possible that the five fucose-labeled components, which were consistently observed, are aggregates of a smaller number of glycoproteins. We feel that this is unlikely, for the following reasons: (*a*) the particulate glycoproteins were extracted and kept in an excess of SDS or SDS and urea, conditions which are known to dissociate proteins; (*b*) reduction and alkylation (14), which should minimize aggregation, did not alter the patterns obtained on gel electrophoresis; (*c*) peak V predominates in the profile of the intermediate molecular weight fraction (Fig. 7 B), thus this component neither aggregates nor dissociates under the conditions of extraction; (*d*) only one additional peak appeared (in the high molecular weight region of the gel) during electrophoresis as described by Fairbanks et al. (34). This system contains borate, which should facilitate separation of glycoproteins by producing charged complexes with polysaccharide side chains.

The pattern of newly synthesized [^{3}H]glycoproteins was considerably simpler than the pattern of total particulate protein labeled with [^{14}C]valine in R2 (Fig. 12). Even though the glycoprotein pattern obtained from R2 was relatively simple, we have not yet identified the biochemical or physiological functions of these distinctive neuronal [^{3}H]glycoproteins. Because they are well-resolved and are limited in number, however, this task may be possible.

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