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AXONAL TRANSPORT OF GLYCOPROTEINS AND GLYCOLIPIDS
IN THE GOLDFISH OPTIC SYSTEM

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

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Approval for publication

Bruce S. McEwen

Associate Professor

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PREFACE

It is impossible to acknowledge all the scientists and fellow students who contributed to my graduate education. However, I would like to give special thanks to Dr. Bernice Grafstein, who introduced me to axonal transport, and to Dr. Bruce McEwen, who gave me support and guidance throughout my thesis research. I want to thank Dr. Robert Ledeen of the Albert Einstein College of Medicine for his guidance of my work on gangliosides. I also wish to thank others who contributed greatly to my scientific education, but not specifically to this thesis, including Drs. Neal Miller, George Palade, Marion Murray, Harry Meindardi, Jaques Berthet, Jim Jamieson, and the Animal Behavior group. The reliable delivery of fish by Mr. T. O'Mara of the Madison Avenue Pet Shop was quite helpful. Timely, accurate typing by Eileen Gibson and Mary Costello was greatly appreciated. I am very thankful for having had the remarkable opportunity to study at The Rockefeller University. For much of my tenure I was supported by a National Science Foundation Graduate Fellowship, and their generous support is gratefully acknowledged. Finally, for helping to keep me feeling sane and human throughout this endeavor, I want to especially thank Janet Keithly, Steve Green and Karen Minkowski, Teg McAteer Stokes, CDS, the New York Choral Society, and WBAI-FM. I regret only that the pleasure and adventure of doing scientific research was diminished during this period by the awareness that the country which I love was senselessly prosecuting a cruel and unjustifiable war in Southeast Asia.

ABSTRACT

The goldfish retino-tectal pathway is a convenient experimental system for studying axonal transport. Radioactive precursors are injected into the eye, and radioactivity which is transported in the axons of the retinal ganglion cells can be studied as it moves in the optic nerve and optic tract to the optic tectum, where the optic fibers terminate. For instance, when labeled amino acids are injected into the goldfish eye, labeled proteins synthesized in the retinal ganglion cells are axonally transported in the optic fibers. Two main waves of protein are transported: most of the labeled protein moves slowly at a rate of about 0.4 mm/day, and much of this slowly transported protein is soluble. A smaller fraction of the transported protein moves much faster, at a rate of about 70-100 mm/day, and most of this rapidly transported protein is particulate. Free amino acids are not transported.

The axonal transport of proteins labeled with radioactive amino acids has been studied extensively. However, the axonal transport of glycoproteins labeled with radioactive sugars had not previously been examined. In the experiments reported here, [^3H]D-glucosamine and [^3H]L-fucose were injected into the goldfish eye. Both sugars are incorporated into macromolecules which are rapidly transported in the optic fibers. The transported glycoproteins move at the same rate as proteins labeled with radioactive amino acids, and this rate is about 70-90 mm/day. Very little of the transported glycoprotein is soluble; most of the transported macromolecules are tightly bound to sedimentable particles. In contrast to the results with amino acids, there is no evidence of a slow component. Inhibition of retinal protein synthesis with a small intraocular dose of acetoxycycloheximide (AXM) prevents the subsequent arrival of labeled transported macromolecules in the tectum, which is evidence that the labeled glycoproteins are synthesized in the retina.

Unlike amino acids, the sugar precursors also label a transported TCA-soluble component. Since it is small compared to the macromolecular

component, and lags behind the transported macromolecules, the transported TCA-soluble radioactivity does not appear to be a precursor for the local synthesis of the macromolecular component. [³H]Glucosamine also labels transported chloroform-methanol extractable materials.

[³H]Fucose is an especially favorable precursor for studying the axonal transport of glycoproteins, because it produces very little "background" labeling, and does not label lipids. More than 90% of the radioactivity which can be released from the transported labeled glycoproteins by acid hydrolysis moves during thin-layer chromatography with the mobility of free fucose. After an intraocular injection, [³H]fucose is incorporated into transported macromolecules for a much longer time than labeled amino acids, and as a result, transported [³H]fucosyl glycoproteins continue to arrive in the tectum after the transport of amino acid-labeled proteins has ceased.

N-[³H]acetyl-D-mannosamine, a precursor of the sialic acids, labels rapidly transported TCA-precipitable, TCA-soluble, and chloroform-methanol extractable materials. With N-[³H]acetylmannosamine, unlike [³H]glucosamine, there is more transported radioactivity in the chloroform-methanol extract than in macromolecules. After an intraocular injection of N-[³H]acetylmannosamine, rapidly transported radioactivity is found in highly purified gangliosides isolated from the tecta. Thus, gangliosides as well as glycoproteins are synthesized in the retinal ganglion cell body and then axonally transported.

These experiments do not exclude the possibility that some incorporation of carbohydrate into glycoproteins and gangliosides may occur in the axons and nerve endings. It is clear, however, that at least some glycoproteins and gangliosides are first synthesized in the neuron cell body, and then axonally transported.

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INTRODUCTION

Axonal Transport

Although the cell bodies of neurons are quite large compared to most other cells, the volume of the axon and endings is many times greater. For instance, the axon of a human motoneuron is about 150 times larger in volume than the cell body (Grafstein, 1969). Yet, some organelles that have important biosynthetic functions are found only in the cell body: the nucleus, free ribosomes, rough endoplasmic reticulum, and Golgi apparatus. Any products of these organelles that are needed for the maintenance and functioning of the axon and endings must be synthesized in the cell body and then transported down the axon. The movements of materials in nerve fibers has been termed axonal transport (also called "axoplasmic flow"). Axonal transport refers to intra-axonal movements of materials in either direction, but historically heavy emphasis has been placed on the centrifugal movements of materials synthesized in the neuron cell body and exported to the axon and endings. This thesis describes studies of the axonal transport of glycoproteins and glycolipids which are synthesized in the cell body of the retinal ganglion cell of the goldfish. The subject of axonal transport has been extensively reviewed (Weiss, 1961, 1963, 1969, 1970; Lubińska, 1964; Grafstein, 1969; Droz, 1969; Barondes, 1969; Lasek, 1970a; Dahlström, 1971) and has been the topic of several symposia (Barondes, 1967; Acta Neuropath., Supp. V., 1971). This review will concentrate on aspects most relevant to this thesis.

The observation that nerve fibers degenerate when their connection with the cell body is severed led several early workers to propose the necessity of some sort of axonal transport process (Scott, 1906; Parker, 1932; Gerard, 1932). However, the modern development of the concept and the first direct experimental evidence of axonal transport are due to Paul Weiss and his collaborators. Their early work is summarized in a classic paper by Weiss and Hiscoe (1948). They examined nerves which

had been forced to grow through a chronic constriction. The axons of the nerves were swollen just proximal to the constriction, while on the distal side of the cuff the diameter of the fibers was reduced. Weiss and Hiscoe deduced that this configuration could be explained if the axoplasm is constantly moving in a proximo-distal direction, and is "dammed up" at the constriction. When the constriction was removed, the dammed material moved down the axon. The advancing front moved at a rate of about 1 mm per day, which Weiss suggested was the normal rate of axonal transport. He proposed that the function of this "perpetual growth" of the neuron was to replace catabolized materials, especially proteins, which could not be synthesized in the axons and endings.

When nerves are cut or crushed, materials accumulate at the interruption in a manner which, as in the constricted nerves, appears to be due to the damming up of moving materials. Although few experimenters have used the constriction method of Weiss and Hiscoe, many have studied axonal transport by examining the accumulation of materials at a cut or crush (cf. Table VIII of Lubińska, 1964. Also Lubińska et al., 1964; Dahlström, 1965; Lasek, 1967; Niemierko and Lubińska, 1967; Kapeller and Mayor, 1967a; Zelená, 1968; Zelená et al., 1968; Banks et al., 1969; Johnson, 1970; Lubińska and Niemierko, 1971; Bray et al., 1971a). These studies have provided valuable information and insights. However, this method suffers from a serious drawback: it is difficult to tell which aspects of the observed phenomena are reflections of normal axonal transport mechanisms, and which are special reactions to the injury. Although the results of experiments on interrupted nerves are usually consistent with the results obtained with other methods, it is clearly desirable to find methods of studying axonal transport in normal undisturbed nerve fibers. Radioisotopic tracers make this possible.

Studies of Axonal Transport with Radioisotopes

Radiotracers have been used to study the axonal transport of some small molecules, including norepinephrine (Livett et al., 1968),

phospholipids (Miani, 1964), glutamate (Kerkut et al., 1967), [³H]colchicine (Grafstein et al., 1970) and γ -aminobutyric acid (Bondy, 1971). However, in most radiotracer studies of axonal transport, radioactive precursors have been used to label macromolecules that are synthesized in the neuron cell body and then axonally transported. The main technical problem in radiotracer studies of axonal transport is that of distinguishing transported radioactivity in axons and nerve endings from "background" label. The background radioactivity may be in glial cells or in other neurons in the tissue containing the axons and endings under study. It can also include radioactivity locally incorporated in the axons and endings themselves from precursors which have reached them by routes other than axonal transport, such as via the circulation. Some of the strategies which have been used for dealing with the problem of background labeling when measuring axonally transported radioactivity are listed below. Approaches 1, 7, and 9 were used in the work in this thesis:

1. Supplying the labeled precursor to the neuron cell bodies in a manner which minimizes the availability of precursor to other parts of the cell (Ochs and Burger, 1958; Taylor and Weiss, 1965; Weiss and Holland, 1967; Weiss, 1967; Lasek, 1968; Livett et al., 1968; Smith, 1971; Fernandez et al., 1970).

2. Microinjection of the precursor into single neurons (Globus et al., 1968; Lux et al., 1970; Lasher et al., 1970; Schubert et al., 1971).

3. Use of autoradiography to examine radioactivity in axons and endings specifically (Droz and Leblond, 1963; Droz, 1965, 1967a, 1969; Edstrom, 1966; Lasek, 1967; Grafstein, 1967; McEwen and Grafstein, 1968; Hendrickson, 1969; Lux et al., 1970; Droz and Koenig, 1971; Schonbach and Cuénod, 1971; Schonbach et al., 1971).

4. Use of cell fractionation techniques to isolate a synaptosomal fraction enriched in nerve endings (Barondes, 1964, 1966, 1968a; Zatz and Barondes, 1971a).

5. Microdissection of axons free from surrounding tissue (Edström, 1966; Jakoubek et al., 1970).

6. Examination of single neurons in culture (Utakoju and Hsu, 1965; Lasher et al., 1970).

7. Measurement of radioactivity in a symmetrical tissue which has the same background labeling but no transported radioactivity. The independently measured background level is then subtracted from the total radioactivity to calculate transported radioactivity (Taylor and Weiss, 1965; McEwen and Grafstein, 1968; Forman, 1971).

8. Selective suppression of background labeling with drugs or unlabeled precursor (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a; D. Forman, unpublished results).

9. Selection of precursors which produce very low background labeling (Elam and Agranoff, 1971a; McEwen et al., 1971).

A related experimental problem is the possibility that precursors might be locally incorporated into macromolecules inside the axon and endings. If the labeled precursor reaches the axon and endings via the circulation, the products will appear to have been transported when examined by methods 3, 4, and 5, but would not effect the results of other approaches, especially 7. However, if the precursor moves specifically along the axons by either intra- or extra-axonal routes, labeled macromolecules synthesized in the axon, endings, or glia would appear to have been transported when examined by all methods. In the case of the axonal transport of proteins labeled by supplying radioactive amino acids to the cell body, this possibility has been excluded by demonstrations that no significant amount of precursor moves along the axons (McEwen and Grafstein, 1968; Kidwai and Ochs, 1969; Elam and Agranoff, 1971a) and by experiments with protein synthesis inhibitors applied to various parts of the neuron (Ochs et al., 1967, 1970; Peterson et al., 1967; McEwen and Grafstein, 1968; Barondes, 1968a; Sjöstrand and Karlsson, 1969; Ochs and Johnson, 1969). Although local synthesis of protein in the axons and endings has been ruled out as a

source of artifact in particular types of studies of the axonal transport of proteins, there is still considerable controversy over the question of whether any protein synthesis exists in axons and endings, and if it does exist, what quantitative and functional significance it has (Reviewed by Droz, 1969; Koenig, 1969; Lasek, 1970a; Droz and Koenig, 1970).

Studies of the Axonal Transport of Proteins Labeled with Radioactive Amino Acids

When neurons are supplied with radioactive amino acids, a distinct wave of heavily labeled proteins can be detected moving down the axons. Weiss's original estimate of 1 mm/day for the rate of axonal transport has been repeatedly confirmed. Some representative measurements of this rate are given in Table I. Recently, several laboratories independently discovered that some materials are transported much more rapidly, about 100 times as fast (Miani, 1964; Grafstein, 1967; Goldberg and Kotani, 1967; McEwen and Grafstein, 1967; Dahlström and Haggendahl, 1966, 1967; Lasek, 1968; Livett et al., 1968; Ochs and Johnson, 1969). Although other components with intermediate rates have been suggested (Lasek, 1968; Karlsson and Sjöstrand, 1971a; Schonbach and Cuénod, 1971), the division of axonal transport into two major components, a slow component that moves about 1 mm/day and a rapid one that is about 100 times as fast, has generally proven to be valid and useful.

Slow axonal transport has been studied for a longer time than rapid transport, but less is known about the slow component, owing to the difficulties of studying such slow movements. The slow component includes many proteins which are soluble in the cytoplasm (McEwen and Grafstein, 1968; Bray and Austin, 1969; Ochs et al., 1967, 1969; Kidwai and Ochs, 1969, Sjöstrand and Karlsson, 1969, 1971a; Sjöstrand, 1970). Of special interest is tubulin, the major protein of microtubules (Weisenberg et al., 1968; Adelman et al., 1968), which moves in the slow component (Grafstein et al., 1970; James and Austin, 1970; McEwen et al., 1971; Karlsson and Sjöstrand, 1971b; Feit et al., 1971;

Table I. Rate of Slow Axonal Transport of Proteins Labeled with
Radioactive Amino Acids

| Rate mm/day | Experimental System | Reference |
|--------------------------------------|---|--|
| 0.4 (normal) 1.2 (regenerating) | Goldfish retinal ganglion cell, 20°C | Grafstein, 1967; Grafstein and Murray, 1969 |
| 1.0 | Mouse retinal ganglion cell | Taylor and Weiss, 1965 |
| 1.5 -2.0 | Rabbit retinal ganglion cell | Karlsson and Sjöstrand, 1971a |
| 1.0 -2.0 (adult) 4.0 -5.0 (young) | Rabbit retinal ganglion cell | Hendrickson and Cowan, 1971 |
| 1.0 -2.0 | Pigeon retinal ganglion cell | Schonbach and Cuénod, 1971 |
| 0.8 -1.0 | Cat retinal ganglion cell | Peterson <u>et al.</u> , 1967 |
| 1.2 -1.3 (adult) 2.9 (young) | Cat dorsal root ganglion cell | Lasek, 1968; Lasek, 1970b |
| 1.0 | Cat splenic nerve | Livett <u>et al.</u> , 1968 |
| 1.5 | Cat spinal motoneuron | Ochs and Johnson, 1969 |
| 2.0 -3.0 | Chicken spinal motoneuron | Bray and Austin, 1969 |
| 0.8 (adult) 1.5 (young) | Rat spinal motoneuron | Droz and Leblond, 1963 |
| 5.0 | Rabbit hypoglossal nerve | Sjöstrand, 1970 |
| 1.09 ± 0.02 | Crayfish nerve cord, 19-21°C | Fernandez and Davison, 1969; Fernandez <u>et al.</u> , 1970 |

Bondy, 1971). Weiss has proposed that the slow component represents the movement of the entire axoplasm as a coherent semisolid gel, and that this movement is propelled by a microperistaltic mechanism (Weiss, 1961; 1963, 1964, 1970; Weiss et al., 1962). Because only rapid axonal transport was examined in this thesis, the rapid component will be emphasized in this review.

Properties of Rapid Axonal Transport

1. Rate of rapid transport. The rate of rapid axonal transport which has been reported in mammals are in the range of 100-500 mm/day. Some representative measurements of the rate are shown in Table II. Some rapid rates have been reported which are faster (Jasinski et al., 1966) or slower (Jakoubek et al., 1970; Lux et al., 1970) than the rates in Table II. Since rapid axonal transport is an energy-dependent process with a Q_{10} significantly greater than 1, the slower rates reported in cold-blooded animals such as the goldfish are comparable to the faster ones measured in birds and mammals when corrections are made for the difference in temperature. Some of the diversity of the measured rates may be due to the different methods used; Ochs and his co-workers have found that the rate of rapid transport in the sciatic nerves of different mammalian species is essentially the same (unpublished results). The rate of rapid axonal transport is comparable with the fastest movements of intracellular particles which have been observed in other types of cells (Kamiya, 1959).

2. Axonally transported proteins are synthesized in the neuron cell body. It is well established that the peptide chains of both rapidly and slowly transported proteins are synthesized almost entirely in the neuron cell body (reviewed by Droz, 1969; Lasek, 1970a). When labeled amino acids are supplied to the neuron cell body, labeled proteins are later observed moving from the soma into the axons. When the axons and endings are supplied with radioactive amino acids, there is little or no incorporation (Edström, 1966; Jakoubek et al., 1970; Droz, 1969; Droz and Koenig, 1971). Even if the axon had a large

Table II. Rate of Rapid Axonal Transport of Proteins Labeled with Radioactive Amino Acids

| Rate mm/day | Experimental System | Reference |
|----------------------------|--|-------------------------------------|
| 70 - 100 | Goldfish retinal ganglion cell, 18-21°C | Elam <u>et al.</u> , 1971 |
| 150 (* and 40) | Rabbit retinal ganglion cell | Karlsson and Sjöstrand, 1971a |
| 240 (adult) 100 (young) | Rabbit retinal ganglion cell | Hendrickson and Cowan, 1971 |
| 100 - 500 (* and 20-60) | Pigeon retinal ganglion cell | Schonbach and Cuénod, 1971 |
| 401 ± 35 | Cat dorsal root ganglion cell | Ochs and Ranish, 1969 |
| 250 - 350 | Chicken spinal motoneuron | Bray and Austin, 1969 |
| 500 | Cat dorsal root ganglion cell | Lasek, 1968 |
| 120 | Cat splenic nerve | Livett <u>et al.</u> , 1968 |
| 300 | Rabbit hypoglossal nerve | Sjöstrand, 1970 |
| 400 | Rabbit vagus nerve | Sjöstrand, 1970 |
| 60 | Rat hypothalamoneuro- hypophyseal axons | Norström and Sjöstrand, 1971a, b |

* Rapid component of axonal transport reported to have two phases. The rate in parentheses is the rate of the slower phase.

protein synthetic capacity, in experiments where labeled amino acids are locally supplied to the neuron cell bodies, there is no transported free amino acid (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a; Bondy, 1971) or at most a very small amount which apparently is not incorporated into protein (Livett et al., 1968; Ochs et al., 1970). Further evidence that amino acids are not transported comes from the observation that cycloleucine, an amino acid which is taken up by cells but not incorporated into protein, is also not transported (Ochs et al., 1967, McEwen and Grafstein, 1968; Fernandez and Davison, 1969). Therefore the observed wave of labeled protein in the axons cannot be accounted for by local synthesis from transported precursor, and must be transported protein. Efforts to use radioactive amino acids to label proteins in the nerve endings which are then axonally transported to the cell body have given negative results (Schonbach and Cuénod, 1971).

3. Protein synthesis inhibitors prevent the synthesis of transported proteins but do not interfere with the axonal transport mechanism. Further evidence that axonally transported proteins are synthesized in the neuron cell body comes from experiments with drugs that inhibit protein synthesis, such as puromycin and acetoxycycloheximide. When protein synthesis is inhibited in the neuron cell body selectively, without inhibiting protein synthesis in the tissues containing the axons and endings, the appearance of labeled transported proteins in the axons is prevented. On the other hand, selective inhibition of protein synthesis in the region of the axons and endings does not affect the labeled transported component. Once the labeled proteins have been synthesized, their rapid axonal transport is independent of further protein synthesis, and proceeds normally in the presence of the inhibition produced by the drugs puromycin and acetoxycycloheximide (McEwen and Grafstein, 1968; Ochs and Johnson, 1969; Sjöstrand and Karlsson, 1969; Ochs et al., 1970). Slow axonal transport can also continue normally in the presence of protein synthesis inhibitors (Peterson et al., 1967; Ochs et al., 1967; Barondes, 1968).

4. The rapid component is smaller than the slow one. When labeled amino acids are supplied to the neuron cell body, much more radioactivity is incorporated into the slow component than into the fast one. For instance, in the chicken sciatic nerve there is 5 to 6 times as much radioactivity in the slow component as in the fast component (Bray and Austin, 1969). In mouse optic nerve the ratio is 5:1, although in the mouse superior colliculus the ratio is reduced to about 2:1 (B. Grafstein, personal communication). In the goldfish optic system the slow component is about 2 or 3 times as large as the fast one (McEwen and Grafstein, 1968; Grafstein and Murray, 1969; Grafstein et al., 1972a. See Figure 9).

5. Rapidly transported proteins are mainly particulate. Proteins in tissues can be separated into two classes by homogenizing the tissue in an aqueous medium and spinning exhaustively in an ultracentrifuge; the "soluble" proteins remain in the supernatant while the "particulate" proteins, those bound to subcellular particles, are found in the centrifugal pellet. Although this separation is extremely crude, it has been quite useful in studies of axonal transport. Rapidly transported proteins are mainly (at least 85%) particulate. A larger fraction of the slowly transported protein is soluble, a proportion that is about the same as the proportion of total tissue protein which is soluble (McEwen and Grafstein, 1968; Karlsson and Sjöstrand, 1969, 1971; Ochs et al., 1967; Bray and Austin, 1968; Kidwai and Ochs, 1969; Sjöstrand, 1970). Consistent with this finding is evidence that several specific proteins which are soluble are transported in the slow component (e.g. Choline acetylase: Hebb and Waites, 1956; Hebb and Silver, 1961. Tubulin: Grafstein et al., 1970; James and Austin, 1970; McEwen et al., 1971; Karlsson and Sjöstrand, 1971b; Feit et al., 1971) and that several specific proteins which are membrane-bound are transported in the rapid component (e.g. Acetylcholinesterase: reviewed by Lubińska, 1964. See also Lubińska et al., 1964; Niemierko and Lubińska, 1967; Kasa, 1968; Johnson, 1970; Lubińska and Niemierko, 1971; Lubińska, 1971. Dopamine- β -hydroxylase: Laduron and Belpaire, 1968. Adenyl cyclase: Bray et al., 1971a). It should be emphasized that besides the soluble protein, the

slow component also contains a large amount of particulate protein. There is evidence that mitochondria may move mainly in the slow component (Weiss and Pillai, 1965; Barondes, 1966).

It is not known whether the small amount of soluble protein transported in the rapid component is transported as a soluble protein free in the cytoplasm, or is adsorbed to or contained within a transported subcellular particle in a manner which is disrupted by water treatment. Attempts to selectively solubilize rapidly transported proteins from centrifugal pellets with detergents and other treatments have had little success (McEwen and Grafstein, 1968; Elam et al., 1971. Karlsson and Sjöstrand, 1971d, claim some success). The particulate nature of the rapid component suggests that the materials which move are membranous organelles.

6. Rapid axonal transport requires metabolic energy. Although the exact mechanism which propels rapid axonal transport is not understood, it is well established that it requires metabolic energy. Rapid transport promptly stops when a nerve is deprived of oxygen (Ochs and Ranish, 1970; Ochs and Hollingsworth, 1971; Ochs, 1971a) or when oxidative phosphorylation is inhibited by drugs such as dinitrophenol, cyanide, and azide (Sabri and Ochs, 1971; Ochs and Hollingsworth, 1971). Inhibitors of glycolysis such as iodoacetate are ineffective in stopping rapid transport directly, although they can stop it by depleting the supply of Krebs cycle intermediates (Ochs and Smith, 1971b; Sabri and Ochs, 1971).

7. The rate of rapid axonal transport is temperature dependent. The rate of rapid axonal transport is markedly temperature dependent (Elam and Agranoff, 1971a; Grafstein et al., 1972a; Ochs and Smith, 1971a). A Q_{10} of 2.0-2.3 has been measured in sciatic nerves in vitro (Ochs and Smith, 1971), and a Q_{10} of about 2.6 has been found in goldfish (Grafstein et al., 1972a). In the goldfish the rate of slow transport is relatively insensitive to temperature (Grafstein et al., 1972). Both rapid (S. Ochs, personal communication) and slow (Fernandez et al.,

1970) axonal transport stop entirely at temperatures near 0°C. The cessation of axonal transport at very low temperatures may be due to effects of temperature on the transport mechanism itself, on energy metabolism, or on microtubule structure.

8. The mechanism for rapid axonal transport is present throughout the entire length of the axon. When axons are crushed in more than one place, transported materials pile up at each interruption, demonstrating that the mechanism of rapid axonal transport is present throughout the entire length of the axon (Lubińska, 1964; Lubińska et al., 1964; Dahlström, 1965; Lasek, 1967; Kapeller and Mayor, 1967b; Banks et al., 1969; Lubińska and Niemierko, 1971). This is also true of the slow component (Weiss and Hiscoe, 1948). Rapid axonal transport can proceed normally in isolated segments of nerves in vitro (Ochs and Ranish, 1970; Ochs and Hollingsworth, 1971; Ochs and Smith, 1971a,b; Ochs, 1971a). Transported proteins accumulate at a region of local anoxia, and the block due to anoxia is reversible (Ochs, 1971a).

9. The mechanism of rapid axonal transport may involve the microtubules. Several authors have proposed that the mechanism of rapid axonal transport involves the microtubules (Schmitt, 1968; Davison, 1970; Schmitt and Samson, 1968; Wuerker and Palay, 1969; Smith et al., 1970; Ochs, 1971a, b; Samson, 1971). The main evidence for this view is that rapid axonal transport is stopped by drugs which disrupt microtubules, such as colchicine (Kreutzberg, 1969; Dahlström, 1968; Karlsson and Sjöstrand, 1969; Sjöstrand et al., 1970; Fernandez et al., 1970; Banks et al., 1971) and vinblastine (Banks et al., 1971; Fernandez et al., 1971; Hökfelt and Dahlström, 1971). These drugs also apparently disrupt slow axonal transport (James et al., 1970; Fernandez et al., 1970), but do not interfere with the accumulation of mitochondria at a crush or cut (Kreutzberg, 1969; Banks et al., 1971). It is interesting that these drugs stop axonal transport at concentrations which are too low to grossly disrupt the ultrastructure of the axonal microtubules, or reduce their number (Fernandez et al., 1970, 1971; Karlsson et al., 1971).

10. The rate of rapid axonal transport is independent of neural electrical activity. There is no difference in the rate of axonal transport in stimulated and unstimulated nerves (Jankowska et al., 1969; Lux et al., 1970; Ochs and Smith, 1971a) except in cases where overstimulation inhibits transport by depleting energy reserves (Ochs and Smith, 1971a). The rate is also not changed when nerve impulses are abolished by severing the connection with the cell body (Ochs and Ranish, 1969), by removing synaptic input (Geffen and Rush, 1968; Grafstein et al., 1972b) or by drugs such as tetrodotoxin (Ochs and Hollingsworth, 1971) or anaesthetics (Ochs et al., 1962; Ochs and Hollingsworth, 1971; Fink and Kennedy, 1971).

11. The rapid component of axonal transport seems to be concentrated in the nerve endings, while the slow component is more concentrated in the axons. While the generality of this observation is not yet established, several authors have noted that the rapid component of axonal transport seems to be more concentrated in the nerve endings than in the axons. On the other hand, the slow component may be more concentrated in the axons, and in some cases very little appears to reach the synaptic endings (Grafstein, 1967; Koenig and Droz, 1971; Schonbach and Cuénod, 1971; Grafstein et al., 1972a). The significance of this observation is also not clear. It could merely be a consequence of the particulate nature of the rapid component, and the larger proportion of soluble protein in the slow component: the ratio of surface to volume is high in the profusely branching nerve endings of some neurons, while the surface-to-volume ratio is much lower in the cylindrical axons. Thus if the particulate proteins are mainly in membranes, and if soluble proteins are mainly in the cytoplasm, one would expect relatively more rapidly transported proteins in the terminal regions and relatively more slowly-transported soluble protein in the axons. On the other hand, the difference in concentration could be related to the different chemical constituents of the axons. Significant amounts of rapidly transported radioactivity may be in organelles which are found mainly in the synapses (such as synaptic vesicles, or

synaptic junctional-complex proteins [Bloom and Aghajanian, 1968]) while some of the slowly transported radioactivity may be in materials which do not enter the synapses (e.g. microtubules).

12. The rate of axonal transport changes during growth and regeneration. Although the rate of axonal transport is not effected by neural electrical activity, it may change during growth and regeneration. In young animals the rate of rapid transport is slower than in adults and increases during maturation (Hendrickson and Cowan, 1971) while the rate of slow transport is faster in young animals (Droz and Leblond, 1963; Lasek, 1970; Hendrickson and Cowan, 1971). Conflicting results have been found in regenerating or recently regenerated nerves: In regenerating goldfish optic nerves the rates of both slow and fast axonal transport increase and more material is transported (Grafstein and Murray, 1969). In mammalian peripheral nerve, however, the rates either decrease slightly or remain unchanged (Ochs et al., 1960; Carlsson et al., 1971; Kreutzberg and Schubert, 1971).

13. Rapid axonal transport may be bidirectional. When nerves are crushed, materials accumulate distal to the crush as well as proximal to it. Many authors have interpreted this as evidence that there is axonal transport towards as well as away from the cell body (Lubińska et al., 1963; Kerkut et al., 1967; Lasek, 1967; Zelena, 1968; Lubińska and Niemierko, 1971; Kristensson and Olsson, 1971; Bray et al., 1971b). A few authors believe that in at least some cases the distal accumulation is an artifact and is not due to axonal transport (Weiss, 1969, 1970; Banks et al., 1969). Some authors have reported the centripetal transport of labeled proteins (Bray et al., 1971b) but there is no transport of radioactivity toward the cell body when nerve endings are incubated with labeled amino acids (Schonbach and Cuénod, 1971) or noradrenaline (Geffen et al., 1969).

Axonal Transport of Protein in the Goldfish Optic System

One of the most useful experimental systems for studying axonal transport is the goldfish retino-tectal pathway (Grafstein, 1967; McEwen

and Grafstein, 1968; Grafstein and Murray, 1969; Grafstein et al., 1970; Elam et al., 1970, 1971; Forman et al., 1971; McEwen et al., 1971; Grafstein, 1971; Forman, 1971; Elam and Agranoff, 1971a, b; Rahmann, 1965, 1967, 1968, 1971; diGiamberardino, 1971). Isotopes are injected into the goldfish eye. Some of the radioactivity which is taken up by the retinal ganglion cells is axonally transported. The axons of the retinal ganglion cells leave the eye in the optic nerves (Figure 1), cross completely at the optic chiasma, and continue in the contralateral optic tract to terminate in specific layers of the optic tectum. Figure 1 shows the goldfish visual system in a diagrammatic form; Figure 2 shows a goldfish head dissected to demonstrate the visual system.

Taylor and Weiss (1965) first proposed that neuron cell bodies could be selectively supplied with isotope for axonal transport studies by using the eye as a natural "incubation chamber." This strategy has been successfully used with many species (Mouse: Taylor and Weiss, 1965; Grafstein et al., 1972b. Rabbit: Karlsson and Sjöstrand, 1968, 1971a; Hendrickson and Cowan, 1971. Pigeon: Cuénod and Schonbach, 1971; Schonbach and Cuénod, 1971. Cat: Peterson et al., 1967. Tadpole: Goldberg and Kotani, 1967. Monkey: Turbes, 1965; Hendrickson, 1969). The optic system of the goldfish is especially convenient for this type of research. The optic nerve fibers cross completely and almost all of them innervate a single structure, whereas in most mammals the analysis is more complex because some fibers remain uncrossed and because the optic fibers innervate both the lateral geniculate and superior colliculus. The goldfish skull is soft, simplifying dissection and intracranial injection. The tectum is a flat sheet of tissue overlying a large ventricle, and it can therefore be easily removed from the brain and freed of other neural structures. A considerable amount of information is available about the anatomy (Leghissa, 1955; Schwassman and Kruger, 1967; Roth, 1969; Ito, 1971) and physiology (Jacobson and Gaze, 1963; Sutterlin and Prosser, 1970; Kaneko, 1970) of the visual system, and about protein synthesis in goldfish brain (Brink et al., 1966; Lim and Agranoff, 1969; Lim et al., 1970). When the optic fibers are cut, the

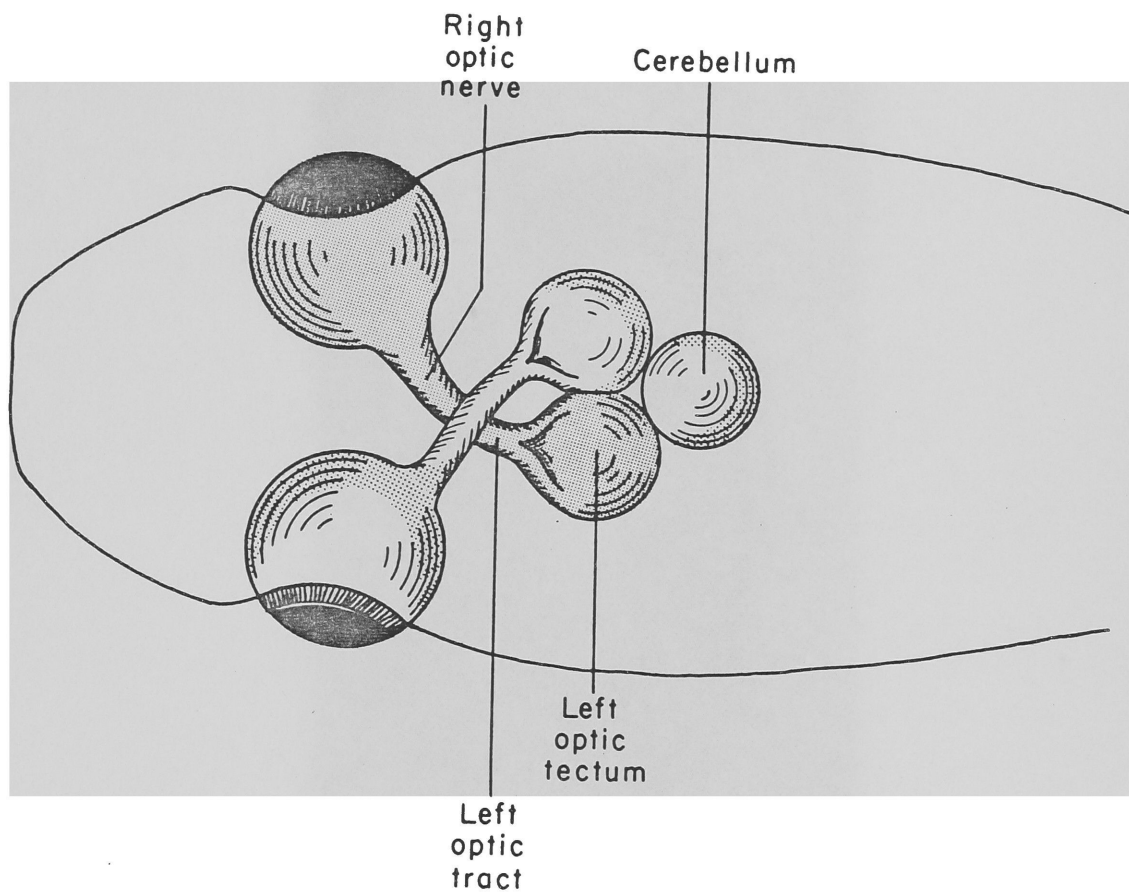


Figure 1. Diagram of the goldfish optic system.

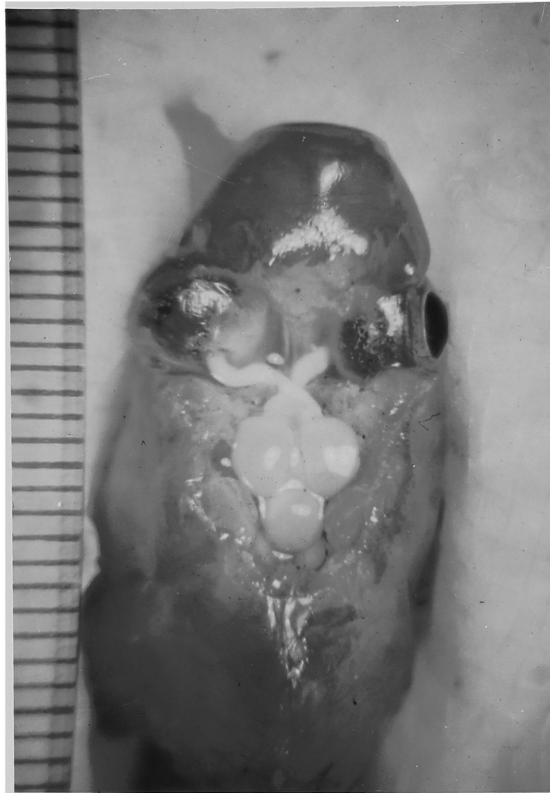


Figure 2. Photograph of the goldfish optic system. The floor of the skull and the forebrain have been dissected away to expose the optic nerves and optic tracts. Dissection and photograph by Dr. Bernice Grafstein. One division on the scale = 1 mm.

goldfish has the amazing ability to regenerate its optic fibers and reestablish normal retinotopic connections (Attardi and Sperry, 1963; Jacobson and Gaze, 1965; Gaze and Sharma, 1970). Axonal transport can thus be studied in normal as well as regenerating axons (Grafstein and Murray, 1969; Grafstein, 1971).

One difficulty in working with this system is that some of the injected isotope leaves the eye in the general circulation. Some of the radioactivity which escapes reaches the brain, and can be incorporated in the tecta. Therefore, radioactivity measured in the tecta may include not only transported radioactivity, but also a "background" of locally incorporated radioactivity. One can control for this possibility by injecting the radioisotope into only one eye. Both tecta will acquire an equal amount of background labeling, but only the tectum contralateral to the injection will contain transported radioactivity. The transported radioactivity is therefore calculated as the difference between the concentration of radioactivity in the two tecta. An example of this type of calculation is demonstrated in Figure 3. Pond fish were injected in the right eye with 4.15 μ Ci of [4,5-³H]leucine (6.0 Ci/mMole), and tecta were processed by Method 2 (see "Materials and General Methods"). Figure 3 shows the radioactivity in protein in the left and right tecta; the transported radioactivity is calculated as left minus right. The time course shown in Figure 3 of the arrival of transported radioactive protein in the tectum is similar to ones which have been published (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a). It differs from previous studies in that large pond fish were used. In this same experiment TCA-soluble radioactivity was also measured. There was an equal amount of TCA-soluble radioactivity in each tectum, and therefore, there was no transported of TCA-soluble radioactivity. Figure 4 shows the more sophisticated experiment from which Figure 3 was taken. As well as an injection of [³H]leucine in the right eye, the fish were also injected in the left eye with 0.27 μ Ci of [¹⁴C]leucine (316 mCi/mMole), and radioactive protein was examined in the optic tracts (processed by Method 2) as well as the tectum. In both the tracts and tecta,

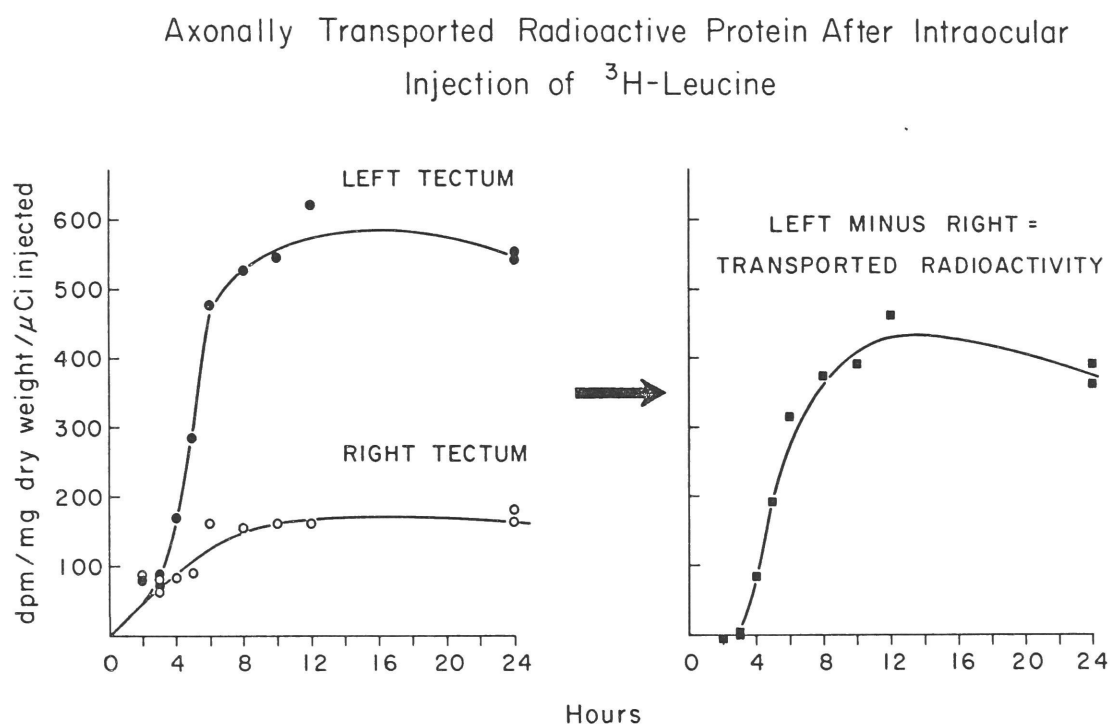


Figure 3, Axonal transport of labeled protein after an injection of ^3H -leucine into the right eye of pond goldfish. To calculate the transported radioactivity, the background radioactivity in the right tectum is subtracted from the concentration of radioactivity in the left tectum, which includes both transported and background label. See text for experimental methods. This time course was run at 20.5°C .

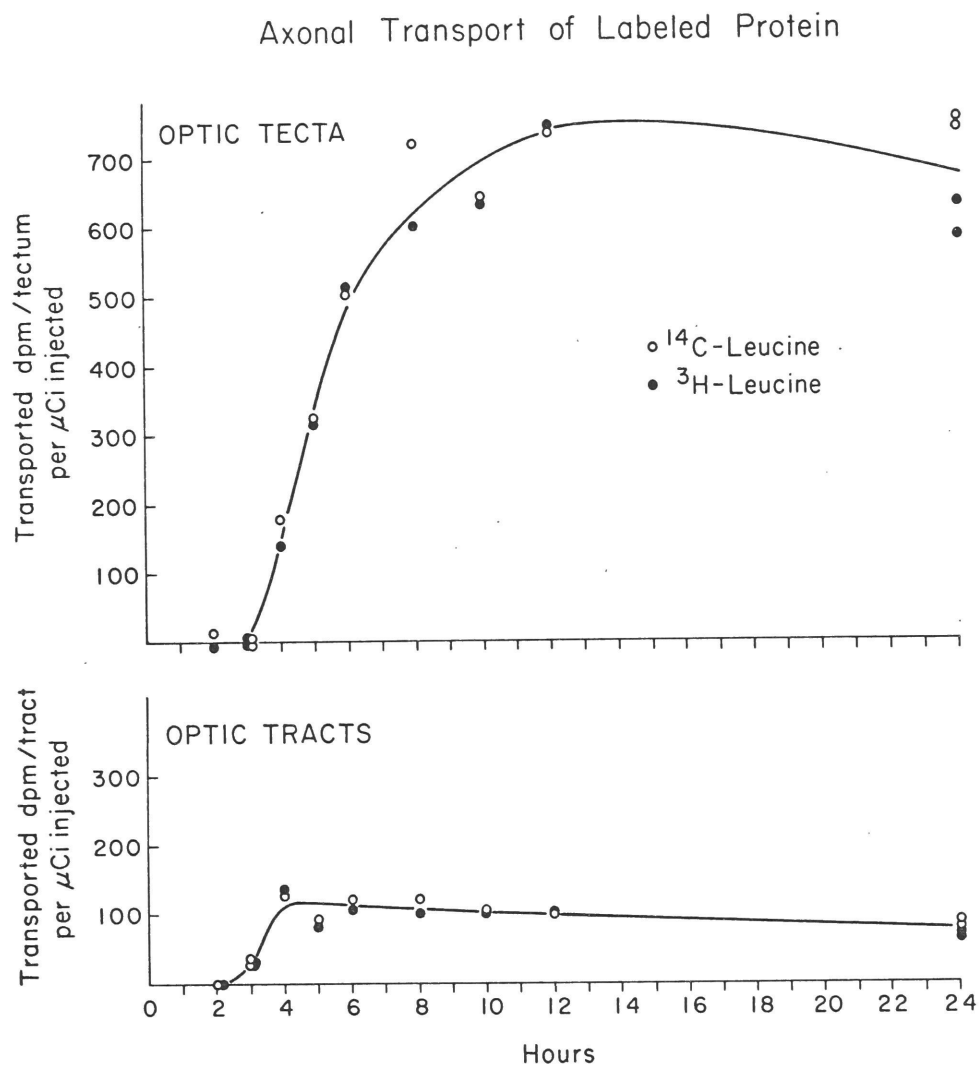


Figure 4. Arrival of axonally transported labeled protein in optic tracts and tecta after injection of [^3H]leucine into the right eye and [^{14}C]leucine into the left eye. The total transported DPM per tract and DPM per tectum has been calculated by multiplying the DPM/mg dry weight by the weights. The average weight per tectum was 1.62 ± 0.03 mg, and the average weight per tract was 0.189 ± 0.008 mg. Since lipids were not extracted, these weights include the weight of lipids and bound TCA.

transported ^3H was calculated as left minus right, while transported ^{14}C was calculated as right minus left. There is good agreement between the results obtained with the two isotopes on the opposite sides of the same fish. Transported radioactivity can also be calculated by subtraction in the optic nerves, where the transported radioactivity is on the same side as the injection. However, in the nerves there is an additional problem of radioactivity which diffuses out of the eyeball along the optic nerve, labeling the Schwann cells of the nerve immediately behind the eye (Taylor and Weiss, 1965).

There are some other disadvantages in working with goldfish. The nerves and tracts are short and the tectum is small, so for some types of chemical studies, tissue from many fish must be pooled. As would be expected in a poikilotherm, the rates of protein synthesis (Das and Prosser, 1967), axonal transport (Elam and Agranoff, 1971a; Grafstein *et al.*, 1972a), and all other metabolic processes are temperature dependent; the temperature must be controlled. Genetically homogeneous fish are not available, and the size of goldfish which are available is variable. Many parameters of goldfish metabolism are subject to seasonal variations, temperature acclimation, and unexplained differences between different shipments of fish. I have therefore tried to include internal controls whenever possible (for instance, the use of ^{14}C -amino acids when studying the axonal transport of glycoproteins labeled with ^3H -sugars).

When a labeled amino acid like [^3H]leucine is injected into the goldfish eye, at least 75% of the injected dose leaves the eye, mainly via the circulation, within 90 minutes (McEwen and Grafstein, 1968). Much of the radioactivity which remains in the eye is incorporated into cells other than the retinal ganglion cells. For instance, from 1% ([^3H]leucine, McEwen and Grafstein, 1968) to 15% ([^3H]proline, Elam and Agranoff, 1971b) of the injected dose is incorporated into the proteins of the retina, but the retinal ganglion cells are only one of the many types of cells in the retina. At most, only 0.14% ([^3H]proline, Elam and Agranoff, 1971b; see also Section I C) of the

injected dose appears in the tectum as rapidly transported protein. Amino acids differ greatly in the percentage of the injected radioactivity which is incorporated into transported protein. Only half as much [^3H]leucine is incorporated into transported protein as [^3H]proline, and with some amino acids even less is incorporated (Elam and Agranoff, 1971a). Amino acids also differ markedly in the amount of background labeling which they produce. While an intraocular injection of labeled leucine produces about an equal amount of transported and background label in protein in the contralateral tectum, the background labeling produced by proline and asparagine are so low as to be negligible (Elam and Agranoff, 1971a). Proline is thus an especially favorable precursor for studies of the axonal transport of protein.

The incorporation of [^3H]leucine into transported protein is complete by 3 hours after the injection (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a; diGiamberardino, 1971). Neither proline nor leucine label lipids. With both of these amino acids, more than 90% of the transported radioactivity can be shown by acid hydrolysis and chromatography to be unchanged amino acid incorporated in protein (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a).

Rapidly transported protein begins to enter the goldfish optic nerve about 1 1/2 to 2 hours after an injection of labeled amino acid. This delay represents both the time required to synthesize the transported protein, and the axonal transport along the several millimeters of retinal ganglion cell axon inside the eye. About 3 to 4 hours after the injection, transported protein begins to arrive in the tecta (see Figures 3 and 4). The rate at which the rapidly transported protein is moving can be calculated to be at least 40 mm/day (McEwen and Grafstein, 1968), and is believed to be in the range of 70 to 100 mm/day (Elam and Agranoff, 1971a. See Experimental Section I C). There is some indication that there may be two rapidly transported subcomponents (Grafstein and Murray, 1969; Elam and Agranoff, 1971a, b; Grafstein et al., 1972a). The arrival of the rapidly transported protein is

complete about 8 to 12 hours after the injection (Figures 3 and 4). The rapidly transported protein then turns over very slowly, with a half-life of 50 to 100 days (Grafstein et al., 1971; Elam and Agranoff, 1971b). This pattern of rapid arrival and very slow turnover suggests that only a very small fraction of the total nerve ending protein is replaced each day by rapid axonal transport. Elam and Agranoff (1971a) have estimated this daily replacement by rapid transport at about 0.5% to 1% of the total nerve fiber protein. The rates of rapid axonal transport and of protein turnover are markedly temperature dependent (Elam and Agranoff, 1971a, b; Grafstein et al., 1972a); the above data are for fish kept at 18-21°C.

Slowly transported protein begins to enter the optic nerve about 1 to 2 days after the isotope injection, and is maximal in the nerve at about 1 week. Changes in the distribution of radioactivity in the nerve with time are consistent with the movement of a wave of labeled material moving at a rate of about 0.4 mm/day (Grafstein, 1967; Grafstein and Murray, 1969). The slow component does not begin to arrive in the tectum until more than 2 weeks after the injection, and is maximal at about 3 weeks, after which it declines very slowly with a half-life of 5 weeks or more (Grafstein, 1967; Grafstein and Murray, 1969; Grafstein et al., 1972a). The slow component contains 2 to 3 times as much radioactivity as the fast component (Grafstein, 1967; McEwen and Grafstein, 1968). Its rate seems to be relatively temperature insensitive (Grafstein et al., 1972a). Both the slow and fast components are found only in the layers of the tectum which contain the optic nerve fibers and endings. The fast component labels mainly the layer containing the synaptic endings, while the slow one seems to be localized in the layer containing the axons of the optic fibers (Grafstein, 1967; McEwen and Grafstein, 1968), which suggests that different materials move in the fast and slow components. The difference in composition is also evident in the observation that the fast component is at least 75% particle-bound protein, while the slow component contains about equal quantities of soluble and particulate materials (McEwen and Grafstein, 1968). The

rapidly transported protein is tightly bound to subcellular particles, and is not significantly solubilized by low concentrations of Triton X-100, moderate concentrations of urea, or high concentrations of salts (McEwen and Grafstein, 1968; Elam and Agranoff, 1971b; Elam et al., 1971). In subcellular fractionation experiments, the rapidly transported protein has a diffuse distribution, with some enrichment in synaptosomal and microsomal fractions (Elam and Agranoff, 1971a, b; Elam et al., 1971; D. Forman, unpublished observations; see Section III C). Both the rapid and slow components contain a large number of different proteins. Tubulin can be identified as a major constituent of the slowly transported proteins (McEwen et al., 1971).

AXONAL TRANSPORT AND GLYCOPROTEINS

The axonal transport of proteins labeled with radioactive amino acids has been studied extensively. However, there has been very little examination of whether the prosthetic groups of conjugated proteins are added in the neuron cell body and then axonally transported. Alternatively, prosthetic groups might be added in the axons and endings to transported polypeptide acceptors. One important group of conjugated proteins are the glycoproteins. The subcellular location in which carbohydrates are incorporated into glycoproteins in other types of cells has been an active area of research in recent years; in the neuron this question is posed in an extreme form. Since the main sites of sugar incorporation in other cells are the endoplasmic reticulum and Golgi apparatus, it would be reasonable to suspect that sugars are incorporated into some glycoproteins in the cell body and then axonally transported. However, some authors have proposed that a major site of the incorporation of sugars into glycoproteins is the nerve endings (Den and Kaufman, 1968; Den et al., 1970; Festoff et al., 1971; Barondes, 1968b; Roseman, 1970). In this thesis I examined the question of whether the carbohydrate moieties of glycoproteins are incorporated in the neuron cell body and then axonally transported.

Glycoproteins are proteins to which carbohydrates are linked covalently. This large and varied group of proteins includes almost all secretory proteins, such as blood plasma proteins, antibodies, collagen, mucous secretions, polypeptide hormones, etc., and also includes lysosomal enzymes and many cell surface proteins. There are several recent books and extensive review articles about glycoproteins (Gottschalk, 1966; Jeanloz and Balazs, 1966 [except Volume 1A]; Rossi and Stoll, 1968; Spiro, 1969, 1970; Ginsburg and Neufeld, 1969; Winzler, 1970; Marshall and Neuberger, 1970; Balazs, 1970). Reviews with special relevance to this thesis are those concentrating on glycoproteins of brain tissue (Schmitt and Samson, 1969; Brunngraber, 1969a, 1970; Barondes, 1970) and on the glycoproteins of the plasma membrane and cell surface (Cook, 1968;

Ginsburg and Kobata, 1971; Winzler, 1970).

Glycolipids are lipids which contain carbohydrate moieties. The nervous system is rich in glycosphingolipids such as the gangliosides, cerebrosides, and sulfatides. Several recent reviews of glycoproteins have discussed glycolipids as well (Schmitt and Samson, 1969; Ginsburg and Neufeld, 1969; Roseman, 1968; Ginsburg and Kobata, 1971; Winzler, 1970). This is justified by the similarity in the oligosaccharide chains of glycoproteins and glycosphingolipids, their mode of biosynthesis, and the proposed physiological functions of these two types of substances. Indeed, in the case of some blood group substances, glycoproteins and glycolipids have identical oligosaccharide chains which are apparently synthesized by the same enzyme system (Watkins, 1967; Hakamori, 1970; Ginsburg and Kobata, 1971). Both glycoproteins and gangliosides were studied in the experiments reported in this thesis.

Acid mucopolysaccharides are a special class of glycoproteins in which the sugar moieties consist of very long chains of alternating amino sugars (which are usually sulfated) and uronic acids. Some examples are hyaluronic acid (which consists of alternating residues of N-acetylglucosamine and glucuronic acid) chondroitin 4- and 6-sulphates (where sulfated N-acetylgalactosamine and glucuronic acid alternate), and dermatan sulfate (where the units are sulfated N-acetylgalactosamine and L-iduronic acid). Because of the many negative charges on the sulfate groups and on the carboxyl groups of the uronic acids, mucopolysaccharides behave as polyanions. Their chemical and physical properties are sufficiently distinctive so that they are usually considered separately from other glycoproteins. There are several recent reviews of the biology and chemistry of mucopolysaccharides (Brimacombe and Weber, 1964; Quintarelli, 1968; Balazs, 1970).

The Structure of Glycoproteins

Although a very large number of sugars occur in nature, only seven sugars are used as the building blocks of glycoproteins and

glycosphingolipids in vertebrates: galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, fucose, and the sialic acids. They occur only in the D-configuration, except for fucose which occurs only as the L-isomer. The sugars are always in their pyranose form. Other sugars occur only in special cases: the uronic acids of the mucopolysaccharides, xylose in the linkage of mucopolysaccharide to protein, and a few reports of minute amounts of other sugars such as rhamnose (Brunngraber, 1969a). In some glycoproteins and glycolipids the sugar moiety consists of a single monosaccharide, but usually there are several sugars linked to each other by glycosidic bonds to form oligosaccharide chains. Although only a few kinds of sugars are used to form these chains, an enormous number of different oligosaccharide chains are possible. This is because the structure of the oligosaccharide chain is determined not only by the sequence and number of sugars, but also by the configurations at the anomeric carbon atoms (α or β), by the position of attachment of neighboring sugars (e.g., $1 \rightarrow 4$), by branching, and in some cases by addition of other groups (e.g. sulphate). Glycoproteins also vary in the number and kind of oligosaccharides per polypeptide, and in the positions of attachment of the oligosaccharides to the polypeptide.

In spite of their complexity, some generalizations can be made which fit all the glycoproteins which have been analyzed so far: the oligosaccharides of most glycoproteins are linked to the polypeptide by either an amide bond between N-acetylglucosamine and asparagine, or by a glycosidic bond between N-acetylgalactosamine and the hydroxyl of serine or threonine. Only two other kinds of linkages occur in special cases: collagen and some related proteins have a glycosidic bond between galactose and the hydroxyl of hydroxylysine or hydroxyproline, and mucopolysaccharides are linked by a bond between xylose and serine. Fucose and the sialic acids are found only in terminal positions at the nonreducing ends of oligosaccharide chains. Glucose, which is relatively rare, is never terminal (except when it occurs as a lone monosaccharide) (Ginsburg and Neufeld, 1969).

Glycoproteins and Mucopolysaccharides in the Nervous System

Mammalian brain proteins contain about 1.2 mg of protein-bound carbohydrate per 100 mg of protein (Brunngraber, 1970). About 85-90% of these glycoproteins are particulate (Brunngraber, 1969a). It is likely that a large fraction of the total glycoprotein and acid mucopolysaccharide protein in the brain is associated with the extra-cellular surface of the plasma membranes. This conclusion is supported by cell fractionation studies (Brunngraber *et al.*, 1967; Dekirmenjian and Brunngraber, 1969; Dekirmenjian *et al.*, 1969). The surface of most cells is rich in a polyanionic material which can be stained by several histochemical methods, and which is believed to be glycoprotein and mucopolysaccharide (reviewed by Martínez-Palomo, 1970). The inter-cellular regions of nervous tissue are rich in these stainable materials, which are especially concentrated at the synapses (Pease, 1966; Rambourg and Leblond, 1967; Bondareff, 1967; Tani and Amentani, 1971).

Little is known about the detailed chemistry of nervous system glycoproteins. Nervous system oligosaccharides are linked to protein by the same linkages as in other tissues (R. Margolis, personal communication). Nervous system glycoproteins are unusual in their high sulfate content; it has been estimated that 40% of the oligosaccharides of glycoproteins in the brain (mucopolysaccharides excluded) are sulfated (Margolis and Margolis, 1970). The level of acid mucopolysaccharides in the nervous system is low. They account for about 0.3% of the lipid-free dry weight of brain tissue (Table I of Margolis, 1969). There was some controversy as to whether there is any acid mucopolysaccharide in the brain that is not in non-neural tissues such as the blood vessels, choroid plexus, etc. However, because of the extremely low collagen content of the brain, most workers in this field now believe that connective tissues cannot account for the mucopolysaccharide found in the brain, which must therefore be associated with neurons or glia (Margolis, 1969). Elam *et al.* (1970) have demonstrated that when $^{35}\text{SO}_4$ is injected into the goldfish eye, labeled sulfated mucopolysaccharide

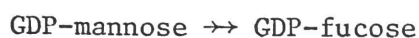
proteins (and also sulfated glycoproteins of other types) are rapidly axonally transported to the tectum.

There have been many speculations about the physiological function of the glycoproteins of the nervous system. Important roles for glycoproteins have been suggested in such functions as memory, interneuronal recognition, and synaptic transmission (Bogoch, 1968; Schmitt and Samson, 1969; Brunngraber, 1969b; Barondes, 1970). However, there is as yet almost no experimental evidence bearing directly on the role of glycoproteins in these neural activities.

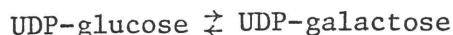
The Biosynthesis of Glycoproteins

Glycoproteins and glycolipids are synthesized by the orderly sequential transfer of monosaccharide residues from sugar nucleotides to appropriate acceptors by enzymes called glycosyltransferases. The acceptors can be the polypeptide or lipid backbone of a glycoprotein or glycolipid, or their incomplete oligosaccharide chains. Sugar nucleotides consist of sugars joined by a glycosidic ester bond to the terminal phosphate of a nucleoside-5'-diphosphate (except the CMP-sialic acids, which are nucleoside-5'-monophosphates). The chemistry of sugar nucleotides has been reviewed (Ginsburg, 1964) and nucleotide derivatives of amino sugars are discussed by Strominger (1969). Free monosaccharides are never directly incorporated into glycoproteins or glycolipids; it is only from sugar nucleotides that sugar residues are transferred to oligosaccharides.

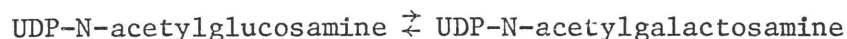
Sugar nucleotides are also intermediates in the synthesis of some of the monosaccharide components. All of the sugars found in glycoproteins can ultimately be synthesized from glucose. However, only glucosamine and mannose are synthesized from glucose directly as monosaccharides (as monosaccharide-6-phosphates) via fructose-6-phosphate. The other sugars used in glycoprotein biosynthesis are synthesized as nucleotide derivatives. For instance, fucose is synthesized in the reaction:



Galactose arises from the reaction:

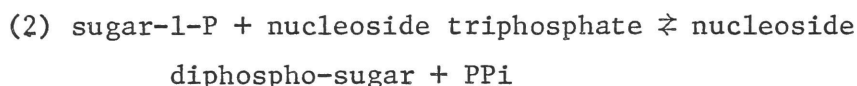
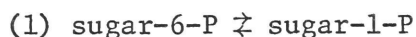


The reaction for the synthesis of N-acetylglactosamine is similar:



The synthesis of the sialic acids also proceeds via UDP-N-acetylglucosamine.

UDP-glucose, UDP-N-acetylglucosamine, and GDP-mannose are synthesized by the reactions:



Note that reaction 2 is reversible, and in fact, the enzymes which catalyze it are called pyrophosphorylases. However, inside cells ubiquitous pyrophosphatases hydrolyze the inorganic pyrophosphate, driving the formation of the sugar nucleotides to completion. An exception is the reaction which activates the sialic acids such as N-acetylneuraminic acid (NANA):



This reaction is irreversible, and the enzyme which catalyzes it is called CMP-NANA synthetase (Roseman, 1962).

Inside the cell, sugar nucleotides apparently control their own synthesis by end-product inhibition. In the rat liver, UDP-N-acetylglucosamine has been shown to inhibit the synthesis of glucosamine-6-P from fructose-6-P (see Figure 5) while CMP-NANA inhibits the synthesis of N-acetylmannosamine from UDP-N-acetylglucosamine (see Figure 7) (Kornfeld et al., 1964). GDP-fucose inhibits its own synthesis from GDP-mannose (Kornfeld and Ginsburg, 1966; Kaufman and Ginsburg, 1968). In each case, the end-product sugar nucleotide inhibits the irreversible reaction which is the first unique step in the synthesis of the sugar nucleotide from glucose. This control mechanism has implications for

experiments with exogenous labeled precursors. Each of the labeled compounds which I injected (glucosamine, fucose, and N-acetylmannosamine) enters the path of glycoprotein biosynthesis after the reaction which is inhibited by end-product inhibition. Thus, the injected precursors may preferentially saturate the pools of sugar nucleotides by shutting off the synthesis of unlabeled sugar nucleotides from glucose. This may help to produce labeling of high specific activity. On the other hand, since the exogenous sugars enter after the normal point of control, unusually large amounts of labeled sugar nucleotides may accumulate (Kaufman and Ginsburg, 1968), perhaps affecting other aspects of the metabolism of the cell (e.g. Lloyd and Kemp, 1971). Other factors also enter into the control of sugar nucleotide synthesis. For instance, the end-product inhibition of glucosamine-6-P synthetase by UDP-N-acetylglucosamine can be widely modified by the action of other substances such as AMP, UTP, and glucose-6-P (Winterburn and Phelps, 1970).

In vertebrates only one nucleotide base is used per sugar. Galactose, glucose, N-acetylglucosamine, and N-acetylgalactosamine are activated as UDP-sugars, mannose and fucose occur as GDP-sugars, while the activated form of the sialic acids is CMP-sialic acid. This contrasts with the situation in plants and bacteria, where several bases may be used for one sugar; glucose can occur as UDP, ADP, GDP, CDP, and dTDP derivatives, each processed by different enzymes. Thus, plants and bacteria can control the biosynthesis of complex heterosaccharides by separation of pathways, but animals apparently do not use this mechanism. In animals, the sequence of sugars in an oligosaccharide chain seems to be controlled mainly by the specificities of the glycosyltransferases. The optimal substrate for each glycosyltransferase is a particular sugar nucleotide and the incomplete protein-linked oligosaccharide chain produced by the previous glycosyltransferase reaction. A series of these enzymes acting sequentially produces the complex oligosaccharide chains of each glycoprotein and glycolipid. For instance, for the synthesis of the disialoganglioside G_{D1a} from lactosyl ceramide, six enzymes have been isolated from embryonic chicken brain, each of which has the

required specificity to add one sugar to the growing chain (Kaufman, et al., 1967, 1968; Basu et al., 1968; Roseman, 1968). Specific series of enzymes have also been demonstrated which act in concert to assemble the oligosaccharide chains of specific glycoproteins. Roseman has proposed that multienzyme systems which act in sequence to produce oligosaccharide chains be called multiglycosyltransferase systems (Roseman, 1968, 1970).

This mechanism of oligosaccharide synthesis provides a probable explanation for the "microheterogeneity" which is characteristic of glycoproteins. When oligosaccharides from individual glycoproteins are isolated, they usually contain some anomalous material. This material may have small amounts of inappropriate sugars incorporated, or have incomplete sequences of sugars. While other explanations are possible (such as degradation during isolation, genetic variation of individuals contributing to a pooled sample, etc.) it is widely believed that this heterogeneity is not artifactual, but is due instead to "errors" caused by the lack of absolute specificity of the glycosyltransferases (Spiro, 1969). There have been some reports suggesting the presence of a lipid intermediate in glycoprotein biosynthesis. The role of these lipids is not understood (Caccam et al., 1969; Tetas et al., 1970; Zatz and Barondes, 1969; Molnar et al., 1971).

All of the sugars of glycoproteins can ultimately be synthesized from glucose. However, labeled glucose would be an inefficient and unspecific precursor for labeling axonally transported glycoproteins because it can enter so many metabolic pathways. Three radioactive precursors were used in my experiments: D-glucosamine, L-fucose, and N-acetyl-D-mannosamine. The metabolism of these compounds will be discussed in greater detail.

Metabolism of glucosamine. The metabolism of glucosamine and its derivatives, which is summarized in Figure 5, has been reviewed by Davidson (1966). Normally, glucosamine is synthesized by glutamine-D-fructose-6-phosphate transaminase, in the reaction:

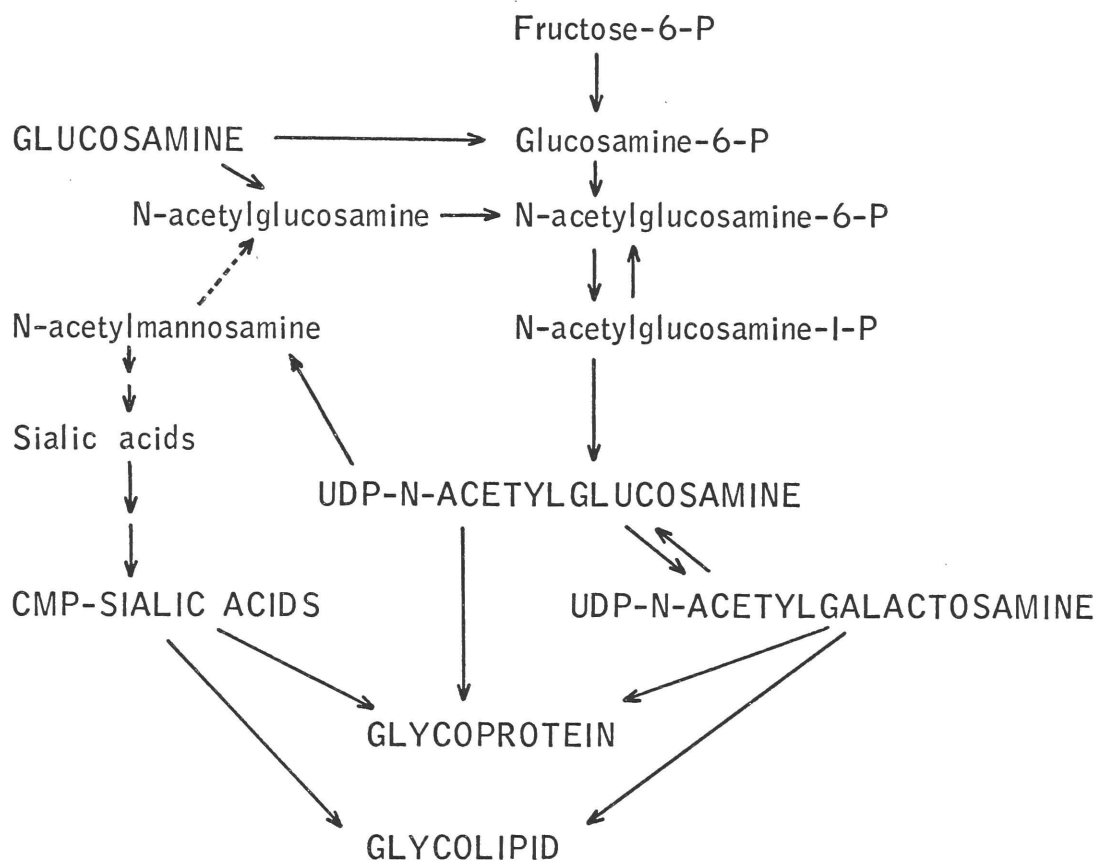
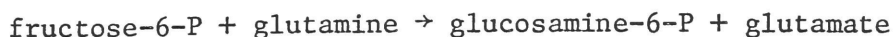
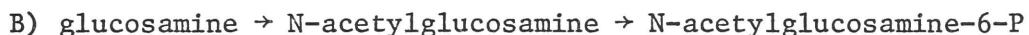
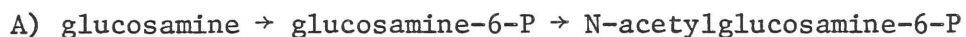


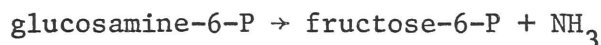
Figure 5. The metabolism of D-glucosamine and its derivatives.
Adapted from Roseman (1962), Molnar et al. (1964), and Davidson (1966).



This reaction is irreversible, and glutamine is the only known source of $-\text{NH}_2$. A different enzyme, glucosamine-6-P deaminase, catalyzes the reverse reaction. Glucosamine-6-P is rapidly acetylated, with the acetyl group coming from acetyl CoA. The N-acetylated derivatives are the physiological forms of the amino sugars; non-acetylated glucosamine occurs only briefly as an intermediate. Although the main physiological source of glucosamine is synthesis from glucose, pathways also exist for the conversion of any free glucosamine which becomes available to the cell to N-acetylglucosamine-6-P. McGarrahan and Maley (1962) studied the metabolism of exogenous glucosamine in the rat liver. They found two pathways for the conversion of glucosamine to N-acetylglucosamine-6-P:



The first, and rate limiting, reactions of these paths both have K_m 's of the same magnitude, and hence the metabolism might proceed equally along both of these paths. However, physiological concentrations of glucose strongly inhibit reaction A, so that at least in the liver, B is the predominant pathway. This may help to explain why most experimenters have found that very little experimentally administered glucosamine is converted to hexoses: the pathway for conversion of glucosamine to hexose is:



N-acetylglucosamine (and N-acetylglucosamine-6-P) can be deacetylated, but the deacetylation usually proceeds slowly in vivo. Thus, exogenous glucosamine which is metabolized in pathway B bypasses glucosamine-6-P, the intermediate for conversion to hexose. After an injection of labeled glucosamine, most of the acid-soluble radioactivity in the rat liver has been found to be a mixture of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine. There are also significant amounts of labeled N-acetylglucosamine and N-acetylglucosamine-6-P, while other acid-soluble

intermediates are found in only very small amounts (McGarrahan and Maley, 1962; Molnar et al., 1964; Kornfeld et al., 1964). The proportion of label which finds its way to N-acetylgalactosamine varies in different systems.

Pattabiram and Bachawat (1964) have examined the regional distribution in the brain of four enzymes involved in glucosamine metabolism. They found that UDP-N-acetylglucosamine pyrophosphorylase, which is necessary for glycoprotein synthesis, is concentrated in grey matter. The degradative enzymes UDP-N-acetylglucosamine phosphatase and glucosamine-6-P deaminase were more concentrated in white matter, as was N-acetylglucosamine kinase.

Metabolism of fucose. Most of the fucose found in animal glycoproteins is apparently synthesized from GDP-mannose via several enzymatic steps which yield GDP-fucose (Foster and Ginsburg, 1961). However, GDP-fucose can also be synthesized from exogenous fucose by the pathway:

1. L-fucose + ATP \rightarrow β -L-fucose-1-P + ADP
2. β -L-fucose-1-P + GTP \rightleftharpoons GDP-L-fucose + PPi

(Bekesi and Winzler, 1967; Ishihara and Heath, 1968). This pathway presumably functions in vivo to salvage fucose available from digestion or glycoprotein catabolism. These pathways are summarized in Figure 6.

Unlike glucosamine, fucose is not converted to any other glycoprotein component; incorporated radioactivity is confined solely to fucose residues (Coffey et al., 1967; Bekesi and Winzler, 1967; Kaufman and Ginsburg, 1968; Bosmann et al., 1969; Bocci and Winzler, 1969; Zatz and Barondes, 1970; Quarles and Brady, 1971; Margolis and Margolis, 1971). Acid mucopolysaccharides contain very little or no fucose (Brimacombe and Weber, 1964; Marshall and Neuberger, 1970), and although fucolipids exist, (e.g. Hakamori, 1970) measureable amounts have not been detected in the brain (Brunngraber, 1969a). In all glycoproteins which have been analyzed so far, fucose is found only at the distal, non-reducing ends of the oligosaccharide chains (Neufeld and Ginsburg, 1969). A

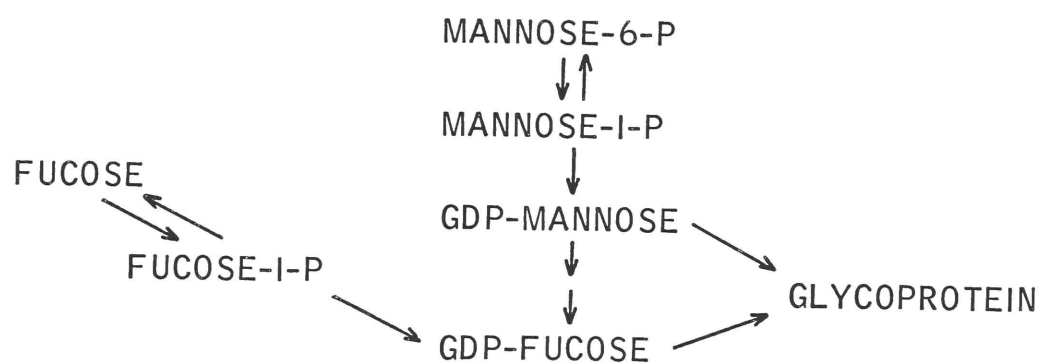


Figure 6. The metabolism of L-fucose.

catabolic pathway which converts fucose to fuconate has been reported (Schachter et al., 1969).

Metabolism of N-acetylmannosamine and the sialic acids. The sialic acids are a group of 9-carbon sugar acids, which are derivatives of neuraminic acid, a compound which does not occur naturally in an unsubstituted form. N-acetylneuraminic acid (NANA) is the predominant sialic acid in most systems (see Figure 8). N-glycolylneuraminic acid (NGNA) and a variety of O,N-diacetylneuraminic acids occur in some organisms. 8-O,N-diacetylneuraminic acid occurs in goldfish brain gangliosides (Ishizuka et al., 1970; Seiter, 1970). The metabolism of NANA has been studied much more extensively than that of the other sialic acids. The biosynthesis of NANA, as elucidated mainly by Roseman and his collaborators (reviewed by Roseman, 1962) is shown in Figure 7.

The synthesis of sialic acid begins with the synthesis of N-acetyl-D-mannosamine. In animals N-acetylmannosamine is derived from UDP-glucosamine. (Bacteria can synthesize N-acetylmannosamine-6-P from N-acetylglucosamine-6-P directly. An enzyme has been found in hog kidney which can reversibly interconvert N-acetylmannosamine and N-acetylglucosamine. However, this enzyme is believed to function only in N-acetylmannosamine catabolism.) N-acetylmannosamine appears to occur only as an intermediate in sialic acid synthesis (and hence, radioactive N-acetylmannosamine is a highly favorable precursor for labeling sialic acids). It forms NANA by an aldol condensation with pyruvic acid. The structural relationship between N-acetylmannosamine and NANA is illustrated in Figure 8. Although the reaction illustrated in Figure 8, which is mediated by the enzyme NANA-aldolase, has been useful for the synthesis of NANA in the laboratory, its physiological function is the breakdown of sialic acids. The synthesis of sialic acids, at least in animals, proceeds via phosphorylated intermediates (Figure 7) and is mediated by the enzyme NANA-9-P synthetase. The NANA-9-P is then dephosphorylated before reacting with CTP to form CMP-NANA. The synthesis of the other sialic acids has not yet been fully elucidated,

METABOLISM OF N-ACETYLMANNOSAMINE AND SIALIC ACIDS IN ANIMALS

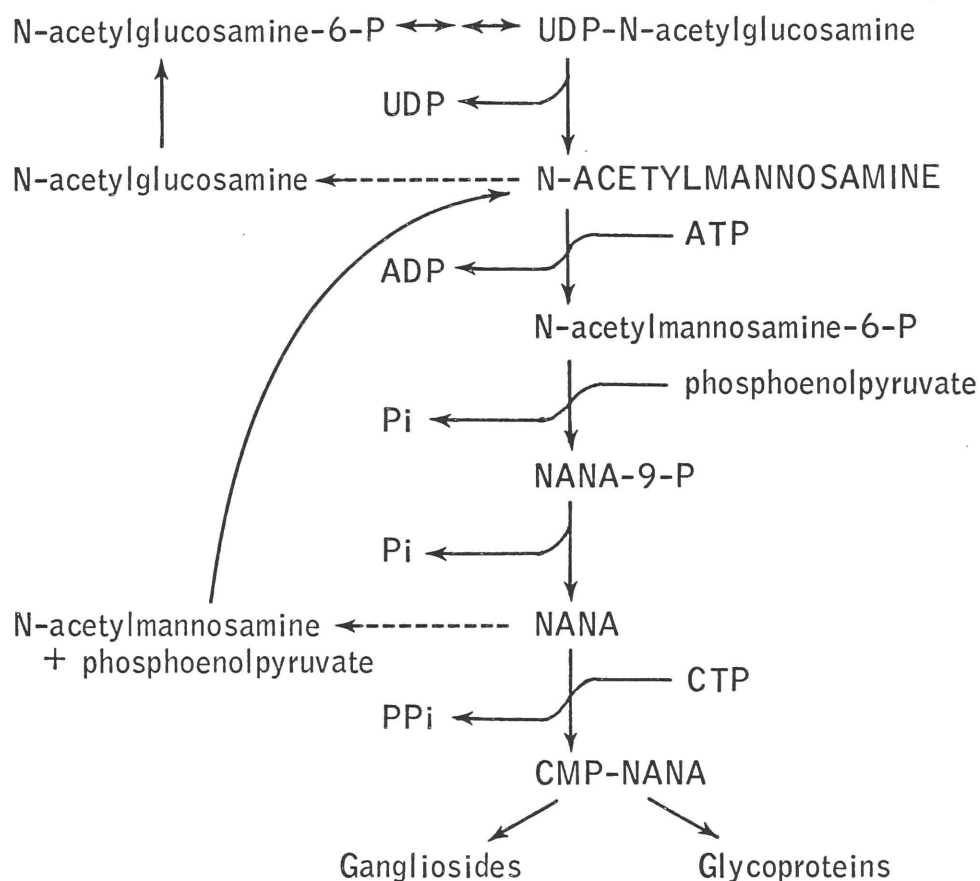


Figure 7. The metabolism of N-acetyl-D-mannosamine and the sialic acids. Adapted from Roseman (1962). Synthetic pathways are shown by solid lines; degradative reactions are shown by dotted lines.

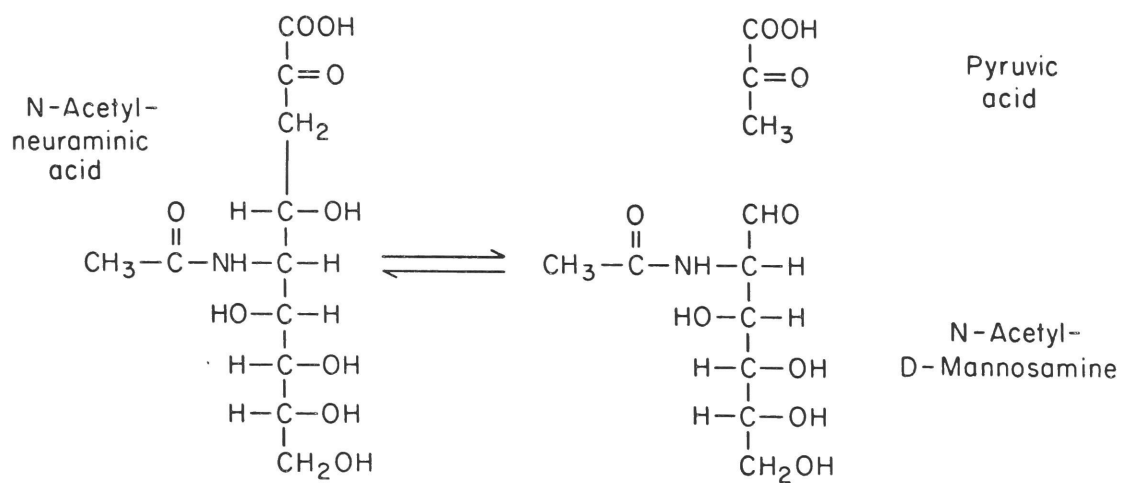


Figure 8. The reaction mediated by NANA-aldolase, illustrating the structural relationship between N-acetylmannosamine and NANA.

but is believed to follow pathways similar to the path of NANA synthesis, often involving the same enzymes.

The sialic acids, like fucose, occupy only terminal positions in the oligosaccharide chains of glycoproteins. The negative charges on the sialic acids have a large role in determining the physical and chemical properties of glycoproteins and gangliosides. They produce the negative surface charge of most cells, serve as receptors for some viruses, control the hepatic uptake of glycoproteins in the blood, and may have other important physiological functions (Cook, 1968; Morell *et al.*, 1971; Pricer and Ashwell, 1971). Sialic acids are cleaved from oligosaccharides by enzymes called neuraminidases. In addition to lysosomal neuraminidases, the brain apparently contains neuraminidases which are localized in synaptic membranes (Schengrund and Rosenberg, 1970).

The Site of Glycoprotein Biosynthesis

The subcellular site at which sugars are incorporated into glycoproteins is of special interest in this thesis. In most cells the main sites of carbohydrate incorporation are the Golgi apparatus and the endoplasmic reticulum. Evidence for this localization is provided by autoradiography (Peterson and Leblond, 1964; Neutra and Leblond, 1966a, b; Droz, 1966, 1967b; Whur *et al.*, 1969; Bennett, 1970; Bennett and Leblond, 1970, 1971; Zagury *et al.*, 1970; Haddad *et al.*, 1971), histochemistry (Rambourg *et al.*, 1969), and cell fractionation (Lawford and Schachter, 1966; Bosmann *et al.*, 1969; Schacter *et al.*, 1970; Schenkein and Uhr, 1970). The evidence suggests that the most proximal sugars are added inside the rough endoplasmic reticulum, and the more distal sugars in the Golgi (Molnar *et al.*, 1969; Rambourg and Droz, 1969; Whur *et al.*, 1969; Zagury *et al.*, 1970; Redman and Cherian, 1971). The most proximal sugars may be added while the peptide is still growing on the ribosome, since there are some reports that puromycin releases incomplete peptides from ribosomes of the rough endoplasmic reticulum which contain incorporated carbohydrate (Lawford and Schachter, 1966; Molnar and Sy, 1967; Hallinan *et al.*, 1968; Schenkein and Uhr, 1970). Fucose, a purely

terminal sugar, is added only in the Golgi region (Bennett and Leblond, 1970; Haddad et al., 1971; C.P. Leblond, personal communication). There is also evidence that mitochondria can incorporate sugars into glycoproteins and into as yet unidentified glycolipids (Bosmann and Martin, 1969; Bosmann and Hemsworth, 1970; Bosmann, 1971).

Because of their unusual geometry and special functions, it is conceivable that neurons might have special mechanisms for glycoprotein synthesis. Several authors have proposed that in neurons a major site of the incorporation of sugars into glycoproteins and glycolipids is the nerve endings. Most of the evidence for this view is from the presence of glycosyltransferases in synaptosomal fractions (Den and Kaufman, 1968; Den et al., 1970; Roseman, 1970; Festoff et al., 1971; Bosmann and Hemsworth, 1970; Brandt and Jourdian, 1971). Additional evidence comes from the rapidity of labeling in vivo of soluble proteins from a synaptosomal fraction (Barondes, 1968) and the insensitivity of that labeling to protein synthesis inhibition (Barondes and Dutton, 1969). However, there is also evidence that neurons, like other cells, incorporate sugars into glycoproteins in the Golgi apparatus and endoplasmic reticulum (Droz, 1967; Rambourg and Droz, 1969; Zatz, 1970; Zatz and Barondes, 1971b; C.P. Leblond, personal communication). In the latter case, one would expect that glycoproteins synthesized in the perikaryon may be axonally transported to the axons and endings. However, until recently there has been no experimental evidence that glycoproteins are axonally transported. The experiments described in this thesis were undertaken to provide experimental evidence of such transport, and to analyze the properties of the axonally transported glycoproteins.

MATERIALS AND GENERAL METHODS

Goldfish

Goldfish (Carrasius auratus) from a local dealer were kept in 20 gallon community tanks at room temperature (19° - 23°C) and fed daily ("Shrimp-el-ettes" or "Pool Fish Food," Longlife Fish Food Products, Harrison, N.Y.). Two sizes of goldfish were used: small fish 4-7 cm long (measured from front tip to base of tail), and large, pond-raised goldfish 9-13 cm long ("pond fish"). They were transferred to 1 gallon glass tanks during experiments. The water was tap water treated with "Aqua-D-Chlor" (Longlife Fish Food Products) or distilled water. Although distilled water might be expected to be an osmotic stress, there was never any sign of distress or illness, in keeping with the findings of others that several transfers through distilled water does not significantly deplete the goldfish of salts (S. Copans, personal communication).

Isotopes

From Schwarz BioResearch, Inc., Orangeburg, N.Y.: [^{14}C]L-proline, 260 mCi/mMole; [^{14}C]L-leucine, 312 mCi/mMole; [4,5- ^3H]L-leucine, 6.0 Ci/mMole.

From New England Nuclear, Boston, Mass.: [U- ^{14}C]L-proline, 209 mCi/mMole; [1- ^{14}C]D-glucosamine hydrochloride, 52 mCi/mMole; [6- ^3H]D-glucosamine hydrochloride, 1.15, 1.3, or 3.6 Ci/mMole; [G- ^3H]L-fucose, 4.3 Ci/mMole; N-[^3H]acetyl-D-mannosamine, 1.02 Ci/mMole.

Injections and Dissections

Isotopes were dissolved in goldfish saline (0.92% NaCl - Levine and Mussalam, 1964) and injected into the eye with a 10 μL Hamilton microsyringe as described by McEwen and Grafstein (1968). The needle was inserted into the vitreous humor at the border of the retina, and was prevented from entering more than 1-2 mm by a polyethylene sleeve. The injections rarely caused any bleeding in the eye, and the fish gave

no indication of impaired vision. The volume of the injections for the small fish was 2 μ L, while the pond fish, which have larger eyes, received 5 μ L. The amount of radioactivity injected was measured directly in each experiment by counting in Brays scintillator (Bray, 1960) and the measured (rather than the expected) values were used in calculations of DPM/ μ Ci injected. The aquarium water was changed 1-2 hours after the isotope injection.

The fish were killed by decapitation, and the brain was exposed and dissected under a dissecting microscope. When the optic tracts were studied they were removed first, cutting them as close to the chiasma as possible. The part of the brain from the optic chiasma to the back of the cerebellum was then removed from the skull. The tecta were easily peeled away as a single flat sheet of tissue. Attached structures which are not part of the tectum (namely, the remainder of the optic tract, the torus longitudinalis, and some underlying white matter, as well as any adhering blood clots) were removed. Tecta and nerves were blotted on absorbent paper before TCA precipitation, but this could not be done with the optic tracts.

Processing of Tissues for Liquid Scintillation Counting. In most of my experiments, macromolecules were precipitated by immersing freshly dissected whole or homogenized tissues in cold 10% trichloroacetic acid (TCA) at 0^o-4^oC. Since glucosamine and N-acetylmannosamine would be expected to label glycolipids, particularly gangliosides (Burton et al., 1963; Suzuki, 1967), in experiments with these isotopes it was necessary to remove lipids from the precipitated macromolecules. Polar lipid solvents are required to extract gangliosides from tissues (Suzuki, 1965). In most experiments the lipid solvent was chloroform-methanol (C-M), 1:1, a mixture which efficiently extracts gangliosides (R. Ledeen, personal communication). After TCA precipitation, the tissues were blotted with absorbent paper, and extracted overnight in C-M at room temperature with shaking. The tissues were then blotted, dried overnight on a glass surface, and solubilized in Soluene-100 (Packard Instrument Co., Downers Grove, Illinois) for liquid scintillation

counting. Before solubilization, dried tecta were weighed on a Sartorius microbalance; the dried tecta are hard, and are easy to handle with forceps. Four protocols were used for processing the tissues:

Method 1. Individual tecta. Single tecta were immersed overnight in 2.0 ml cold 10% TCA. Lipids were extracted with 2.0 ml C-M, 1:1. After drying, the tecta were weighed individually and solubilized in 0.5-1.0 ml Soluene-100 for scintillation counting.

Method 2. Pooled tecta from 3 fish. Left or right tecta from three fish were kept overnight in 6.0 ml (2.0 ml per tectum) of cold 10% TCA. Lipids were extracted with 6.0 ml C-M, 1:1. The three dried tecta were weighed as a group, and counted together after solubilization in Soluene-100. The advantage of this method was greater speed and accuracy in weighing the tecta, at the price of a loss of information about the variability between fish. In a few experiments optic tracts were processed by Method 2.

Method 3. Optic nerves and tracts. Individual optic nerves or tracts were submerged overnight in 0.5 ml cold 10% TCA. Lipids were extracted in 1.0 ml of C-M, 1:1. The dry tissue was solubilized in 0.5 ml Soluene-100. In some experiments the lengths of the freshly dissected nerves and tracts were measured under a dissecting microscope before TCA precipitation.

Method 4. Homogenates. Tissues were homogenized in distilled water or 0.32 M sucrose with small Dounce homogenizers (Kontes Glass Co., Vineland, New Jersey). In some experiments centrifugal pellets were suspended in distilled water with Pasteur pipettes. The samples (homogenates, suspended centrifugal pellets, or centrifugal supernatants) were brought to a final volume of 5.0 ml containing 10% TCA by the addition of water and 50% TCA. With very small samples smaller volumes were used. After 1 hour the TCA precipitates were sedimented in a tabletop centrifuge. They were resuspended in the same volume of 10% TCA and resedimented. The samples were kept at 0°-4°C at all times. In some experiments the lipids in the pellets were extracted

with C-M, 1:1 or ethanol-diethyl ether, 1:4. For protein measurements, precipitates were dissolved in 1.0 N NaOH and protein determined by the method of Lowry et al. (1951). For liquid scintillation counting precipitates were dissolved in Soluene-100, or samples dissolved in 1.0 N NaOH were dried on cotton or filter paper and combusted in the Packard Model 305 Sample Oxidizer.

Measurement of Radioactivity

TCA precipitates. Dried TCA precipitates were dissolved overnight in Soluene-100 at room temperature. Pieces of whole tissue were dissolved inside glass scintillation vials with shaking. Centrifugal pellets were dissolved inside the glass centrifuge tubes which were capped with Parafilm, and were then transferred to the scintillation vials in scintillation fluid. The scintillation fluid contained 5 g PPO and 0.25 g dimethyl-POPOP per liter of toluene. It was necessary to use scintillation vials with polyethylene-lined caps, since the Soluene attacked the backings of foil-lined caps. Before counting it was necessary to wait at least 12 hours for chemiluminescence to decay. In a few experiments dried TCA precipitates were wrapped in filter paper and combusted in the Packard Model 305 Sample Oxidizer.

Chloroform-methanol extracts. C-M extracts were air-dried and dissolved in the same toluene-based scintillation cocktail. It was necessary to add 0.2 ml of Soluene-100 in order to recover all the counts.

Aqueous solutions. Radioactivity in TCA supernatants and other aqueous solutions was counted in Bray's dioxane-based scintillator (Bray, 1960). In some cases, 0.5 ml of the sample was counted directly in 15 ml of Bray's solution. In most experiments, larger samples of TCA supernatants, usually 2.0 ml, were extracted three times with diethyl ether to remove the TCA, evaporated to dryness, and redissolved in 0.5 ml of H₂O before scintillation counting. Representative samples of the ether extracts were tested for each isotope studied; no radioactivity was ever detected in the ether phase.

Liquid Scintillation Counting. Samples were counted in a Packard Model 3375 Tri-Carb Liquid Scintillation Spectrometer equipped with Automatic External Standardization, which was used to measure efficiency. I confirmed the manufacturer's claim that various types of quenching (color, chemical, and dilution) fit the same calibration curve of efficiency as a function of AES Ratio. The stability of the calibration curves was checked periodically with internal standards. In double-label experiments, DPM were calculated using a computer program written by Dr. Brian Poole of the Rockefeller University (Packard Program Library No. 09672).

Control Experiments

In most of my experiments I precipitated the macromolecules in brain tissue with cold 10% TCA, extracted the lipids with lipid solvents, and measured the radioactivity in the TCA supernatant, lipid extract, and residual TCA-precipitate. It is assumed that all small molecules other than lipids are extracted by the TCA, that the radioactivity in the lipid extract is in lipid (or proteolipid), and that the radioactivity in the residual precipitate is covalently incorporated in macromolecules. This assumption would be invalid if any small radioactive molecules bound noncovalently with the macromolecules and were not removed completely by the 10% TCA. I performed some experiments to rule out this possible artifact.

Solutions of radioactive sugars in 10% TCA were prepared with [^3H]L-fucose (0.11 $\mu\text{Ci/ml}$), [$6\text{-}^3\text{H}$]D-glucosamine (0.064 $\mu\text{Ci/ml}$), and N-[^3H]acetyl-D-mannosamine (0.19 $\mu\text{Ci/ml}$). These solutions were used to process unlabeled goldfish brain tissue by Methods 1 and 4. Unlabeled whole optic tecta were fixed overnight in 2.0 ml radioactive 10% TCA at 4°C and were then processed for scintillation counting by Method 1, with or without an additional wash in 2.0 ml unlabeled 10% TCA. For Method 4, 0.5 ml aliquots of tissue homogenates of unlabeled goldfish whole brain in distilled water (5% w/v) were precipitated with 4.0 ml of the labeled 10% TCA at 4°C. The precipitates were washed with either 1 or 2

washes of 4.0 ml 10% TCA, and processed by Method 4 (including extraction with 2.0 ml of C-M, 1:1).

The amount of radioactivity which remained in the extra TCA washes, chloroform-methanol extracts, and residual precipitates is shown in Table III. No more than 0.2% of the radioactivity in the supernatants remained in these fractions. This amount is small enough to be accounted for by the labeled 10% TCA wetting the tissue; there is no evidence for specific adsorption or retention. Most of the residual radioactivity was washed out in the chloroform-methanol if there was no extra wash with 10% TCA. Less than 0.02% of the original radioactivity was found in the original precipitate even if there was no extra washing with unlabeled 10% TCA. In additional experiments, tecta labeled by transported radioactivity from [^3H]glucosamine were washed in a second volume of 10% TCA or C-M. The amount of radioactivity appearing in the washes was too low to measure reliably, but never exceeded 3% of the amount found in the first volume of 10% TCA or of C-M. In most of my experiments the level of TCA-soluble radioactivity was low enough so that "extra" washing seemed unnecessary. Accordingly, whole tecta were precipitated in a single volume of 10% TCA and TCA-precipitates of tissue homogenates were washed once. Therefore, the C-M extracts are contaminated by a small amount of TCA-soluble radioactivity. The experiment described here does not rule out the possibility that some specific metabolites of the precursors might form a tighter complex with the tissue and not be washed out by the 10% TCA.

Unlike most macromolecules, some glycoproteins are not precipitated by TCA, but these can be precipitated by phosphotungstic acid (PTA) (e.g. Taylor *et al.*, 1967). I examined tecta labeled with transported radioactivity from [^3H]glucosamine to see whether they contain labeled TCA-soluble glycoproteins. I was unable to precipitate additional radioactivity from TCA supernatants by the addition of PTA, even when unlabeled albumin was added as a carrier. Furthermore, equal amounts of radioactivity were precipitated from homogenates by 10% TCA

Table III. Control for Adsorption of Precursor to Unlabeled Tissue:
Mixing of Unlabeled Brain Tissue with Radioactive Precursors
in 10% TCA

| | $[^3\text{H}]$ Fucose | | $[^3\text{H}]$ Glucosamine | | $[^3\text{H}]$ N-Acetylmannosamine | |
|---------------------------|--|--------|----------------------------|-------|------------------------------------|--------|
| Total DPM added | 1.0×10^6 | | 5.6×10^5 | | 1.7×10^6 | |
| | <u>Per cent of total DPM recovered in:</u> | | | | | |
| <u>Whole Tecta</u> | A | B | A | B | A | B |
| Second TCA wash | - | 0.17 | - | 0.17 | - | 0.11 |
| Chloroform-methanol | 0.07 | 0.001 | 0.06 | 0.001 | 0.10 | 0.0004 |
| Precipitate | 0.003 | 0.0001 | 0.003 | 0 | 0.0008 | 0.0001 |
| <u>Homogenized Tissue</u> | C | D | C | D | C | D |
| Third TCA extraction | - | 0.12 | - | 0.11 | - | 0.12 |
| Chloroform-methanol | 0.06 | 0.01 | 0.07 | 0.009 | 0.07 | 0.007 |
| Precipitate | 0.004 | 0.002 | 0.02 | 0.005 | 0.007 | 0.001 |

- A. No extraction with unlabeled 10% TCA
 B. Extraction with 2.0 ml unlabeled 10% TCA
 C. Washed once with unlabeled 10% TCA
 D. Washed twice with unlabeled 10% TCA

with and without 0.5% PTA. I therefore concluded that measureable amounts of labeled TCA-soluble glycoproteins were not present in my experiments, and used 10% TCA as a precipitant without the addition of any PTA.

I A. TIME COURSE OF THE AXONAL TRANSPORT OF MATERIALS LABELED BY RADIOACTIVITY FROM [³H]GLUCOSAMINE

The first experiments which demonstrated the rapid axonal transport of labeled glycoproteins were performed by Dr. Bernice Grafstein of the Cornell University Medical College and Dr. Bruce McEwen of the Rockefeller University, who injected [6-³H]D-glucosamine into goldfish eyes and examined the transported radioactivity in the tecta. Grafstein sacrificed fish 1, 7, 22, 34, and 82 days after injection and examined the TCA-soluble and TCA-precipitable radioactivity. Her results are shown in Figure 9. For comparison, Figure 9A shows the results of the similar experiment with [³H]leucine. With leucine, rapidly transported protein is present 1 day after the injection, and nearly the same level is found a week later. Twenty-two days after the injection the larger component of slowly transported protein has arrived, and it also turns over slowly. There is no transported TCA-soluble component. [³H]glucosamine also labels transported TCA-precipitable radioactivity 1 day after the injection (Figure 9B), hence this material has been transported at a rapid rate. However, 22 days after the glucosamine injection, there is no large additional wave of slowly transported material as is found with amino acids. Thus it appears that glucosamine labels little or no slowly transported material. Another difference between the two isotopes is that glucosamine does produce some TCA-soluble transported radioactivity, which is present 1 day after the injection.

McEwen examined the chemical properties of the transported materials labeled with radioactivity from [³H]glucosamine. He separated the particulate and soluble macromolecules by centrifugation, and extracted lipids from the macromolecules. McEwen found that lipid solvents extract about one-quarter of the transported radioactivity and that most of the transported macromolecular radioactivity is particulate (Table IV). It is especially interesting that even 23 days after the injection only a very small amount of the transported radioactivity is in soluble macromolecules. When a labeled amino acid is used as the

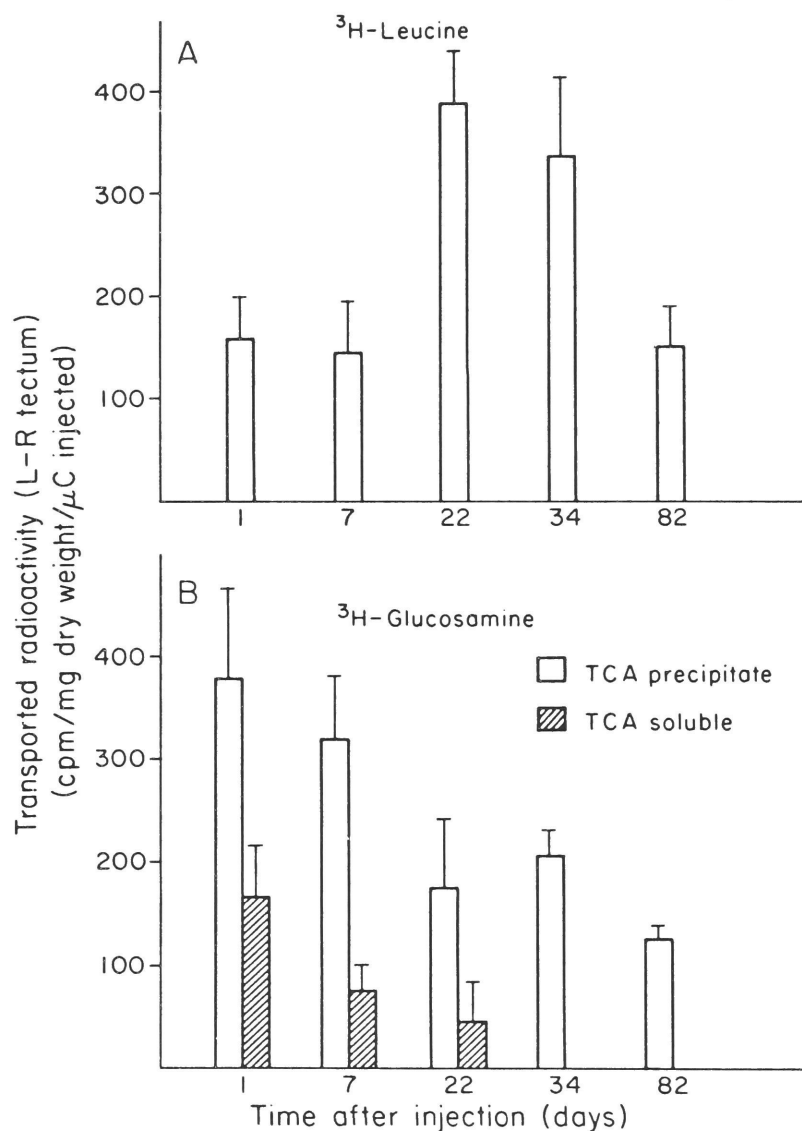


Figure 9. Comparison of transported radioactivity after injections of [^3H]leucine and [^3H]glucosamine. Fish were injected in the right eye with 4 μCi of [$4,5\text{-}^3\text{H}$]leucine (5.0 Ci/mMole) or 2 μCi of [$6\text{-}^3\text{H}$]D-glucosamine (1160 mCi/mMole). Tecta were processed by Method 1. The tecta were not extracted with C-M after TCA precipitation, so the TCA-insoluble fraction includes lipid-extractable radioactivity. Standard errors of the mean are represented by lines above the bars ($N = 5$). TCA-soluble radioactivity from glucosamine was not measured at 34 or 82 days. (Data of Dr. Bernice Grafstein. From Forman *et al.*, 1971).

Table IV. Distribution of Radioactivity in Optic Tecta after Intraocular Injection of 2 μ Ci of [3 H]Glucosamine

| <i>1 day</i> | | | <i>7 days</i> | | | <i>21 days</i> | | | |
|-----------------------------------|----------|--|-------------------------------|----------|--|-------------------------------|----------|--|-----------|
| <i>DPM per tectum</i> | <i>%</i> | <i>Concen- tration of radio- activity*</i> | <i>DPM per tectum</i> | <i>%</i> | <i>Concen- tration of radio- activity*</i> | <i>DPM per tectum</i> | <i>%</i> | <i>Concen- tration of radio- activity*</i> | |
| A. Transported (L-R tectum) | | | | | | | | | |
| Partic. | | | | | | | | | |
| macromol. | 1710 | 42 | 8.2±1.4** | 1014 | 49 | 5.9 ± 1.1 | 667 | 43 | 3.6 ± 0.4 |
| Lipid extract. | 976 | 24 | 5.1±1.0** | 593 | 28 | 3.8 ± 0.8 | 511 | 33 | 3.5 ± 0.8 |
| Sol. macromol. | 112 | 3 | 0.9±0.2*** | 80 | 4 | 0.9 ± 0.1 | 51 | 3 | 0.5 ± 0.1 |
| TCA-sol. | 1243 | 31 | 8.0±2.1*** | 394 | 19 | 4.5 ± 0.6 | 320 | 21 | 2.6 ± 0.3 |
| B. Local incorporation (R tectum) | | | | | | | | | |
| Partic. | | | | | | | | | |
| macromol. | 1992 | 36 | 9.4±1.4 | 720 | 46 | 4.8 ± 0.7 | 891 | 40 | 4.2 ± 0.3 |
| Lipid extract. | 667 | 12 | 3.1±0.5 | 318 | 20 | 1.9 ± 0.3 | 686 | 31 | 3.0 ± 0.4 |
| Sol. macromol. | 577 | 11 | 3.7±1.0 | 172 | 11 | 1.8 ± 0.2 | 190 | 9 | 1.5 ± 0.2 |
| TCA-sol | 2278 | 41 | 16.5±1.9 | 355 | 23 | 3.7 ± 0.5 | 437 | 20 | 3.8 ± 0.5 |

* Mean \pm S.D.

** DPM per μ g particulate protein

*** DPM per μ g soluble protein

Data of Dr. Bruce McEwen. (From Forman et al., 1971).

precursor in this type of experiment there is a large amount of transported radioactivity in soluble protein 23 days (but not 1 or 7 days) after the injection, due to the arrival of the slowly transported proteins which are largely soluble (McEwen and Grafstein, 1968). The particulate nature of the transported macromolecules measured 23 days after glucosamine injection is compatible with the possibility that glucosamine labels only the rapid component of axonal transport.

I examined the arrival of transported radioactivity in the tecta in detail during the first 24 hours after [^3H]glucosamine injection, in order to compare its time course with transported proteins labeled with a radioactive amino acid, and also to compare the three classes of transported materials labeled by glucosamine (TCA-soluble, macromolecular, and C-M extractable).

Methods

Small goldfish were injected in the right eye with a mixture of [$6\text{-}^3\text{H}$]D-glucosamine (1160 mCi/mMole) and [^{14}C]L-leucine (312 mCi/mole). They were kept in small tanks at $21 \pm 2^\circ\text{C}$ and sacrificed at different times after injection. Tecta were immersed in cold 10% TCA containing 0.1 M glucosamine and were processed for scintillation counting by Methods 1 or 2. It was later found that the unlabeled glucosamine in the TCA was unnecessary; identical distributions are obtained when the glucosamine is omitted. Some of the details of the experimental procedures were changed in the different experiments. For instance, different ratios of chloroform to methanol were used to extract lipids. The differences in procedure are listed in Table V. Although the procedures differed slightly, when the results were expressed as DPM/mg dry weight/ μCi injected they agreed well and could be combined into a single time-course.

Results

The arrival of transported radioactivity in the tectum is shown in Figure 10. The ^{14}C shows the pattern which is typical when amino

Table V. Differences in Procedure in Time-Course Experiments with
[³H]Glucosamine

| | Experiment | | | | |
|--------------------------------|------------|------|-----------------|------|-----------------|
| | 1 | 2 | 3 | 4 | 5 |
| Number of fish per time point | 6 | 8 | 6 | 9 | 6 |
| Volume of injection (μl) | 5 | 5 | 3 | 2 | 3 |
| μCi ¹⁴ C injected* | 0.10 | 0.10 | 0.072 | 0.10 | 0 |
| μCi ³ H injected* | 2.4 | 2.4 | 1.4 | 0.81 | 1.84 |
| Chloroform:methanol | 3:1 | 3:1 | 2:1 then 1:2 | 1:1 | 2:1 then 1:2 |
| Average weight per tectum (mg) | 0.45 | 0.34 | 0.46 | 0.55 | 0.40 |

Comments

1. Experiments 1, 2, and 3 were designed to examine the time-course of the arrival of radioactivity in the tectum. Experiment 4 is the control groups from the experiment which studied the effect of acetoxycycloheximide (Section II), and contributes the 21-hour time points. Experiment 5 examined only ³H-radioactivity at 24 hours.
2. In Experiments 1, 2, and 5 no Soluene-100 was used in counting the C-M extracts.
3. In Experiments 3 and 4 the tecta were processed in groups of three (Method 2). In the other experiments the tecta were examined individually (Method 1).

* The amounts of radioactivity injected in each experiment were measured directly by counting in Bray's scintillator.

acids are used as precursors (Figure 10A). Transported labeled protein begins to arrive about 3-3 1/2 hours after the injection. Then there is a period of rapid arrival of labeled protein which is over at about 9 hours, when a plateau is reached. Very little C-M extractable (Figure 10B) and no TCA-soluble (Figure 10A) radioactivity is transported. [³H]glucosamine labels transported TCA-precipitable and C-M extractable materials (Figure 10C and D) which arrive in the tectum with a time-course which resembles the arrival of the rapidly transported proteins. However, some ³H continues to arrive after 9 hours. The TCA-soluble radioactivity shows a similar pattern, although it seems to lag behind the other components.

For comparison, the pattern of labeling in the right tectum, which receives only blood-borne radioactivity, is shown in Figure 11. The pattern is similar for both isotopes: TCA-soluble radioactivity arrives first, and some of it is incorporated into TCA-precipitable and (to a lesser extent) C-M extractable materials. The per cent of transported and background radioactivity in the three chemical classes 1 day after an intraocular injection of [³H]glucosamine is given in Table VI. As would be expected, the more polar C-M mixtures extract more radioactivity from the TCA-precipitate. Nevertheless, the proportion of transported label in the three fractions in different experiments was roughly the same.

In Experiment 2 the distribution of radioactivity was also examined 7 days after the injection. During this period the amount of ¹⁴C radioactivity in protein was unchanged (Table VII). In a week the transported TCA-soluble radioactivity from [³H]glucosamine had declined to about one-third of its level at 24 hours. The small decline in the level of TCA-precipitable radioactivity and the small increase in the amount of C-M extractable radioactivity are not statistically significant (at the $p < 0.5$ level, t-test).

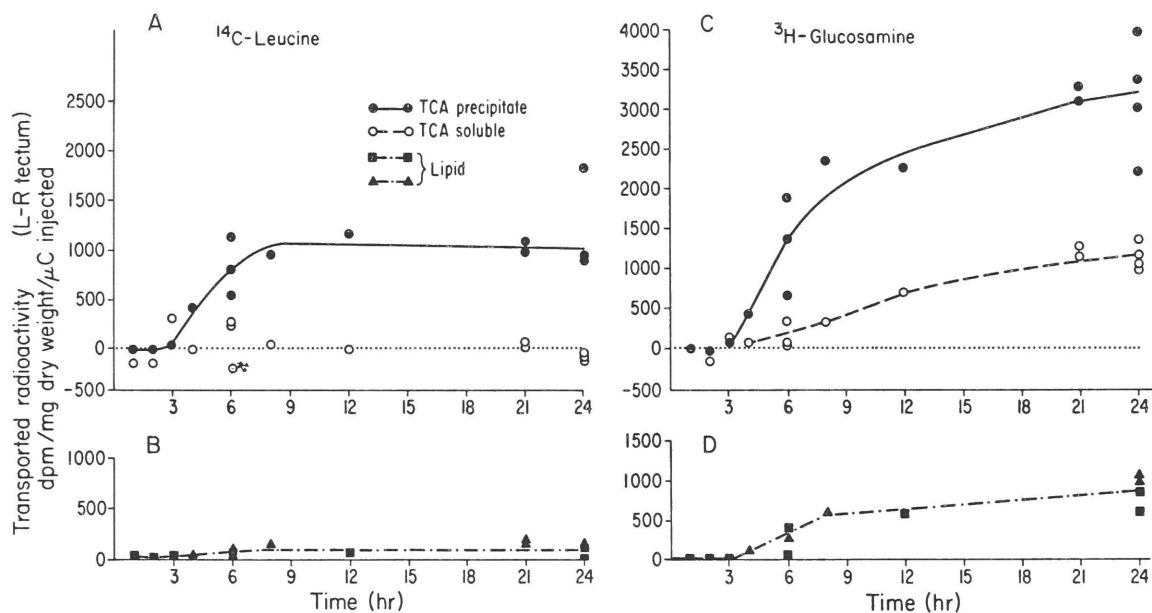


Figure 10. Time-course of appearance of transported radioactivity. Each data point is an average of between 5 and 9 fish. "Lipid" refers to the chloroform-methanol extract. Squares represent radioactivity extracted by C-M, 3:1, and triangles represent radioactivity extracted by C-M, 1:1, or by C-M, 2:1 followed by C-M, 1:2. Two anomalously high values in the lipid fraction have been omitted from D (1972 and 2039 DPM/mg dry weight/ μ Ci injected, at 21 hours).

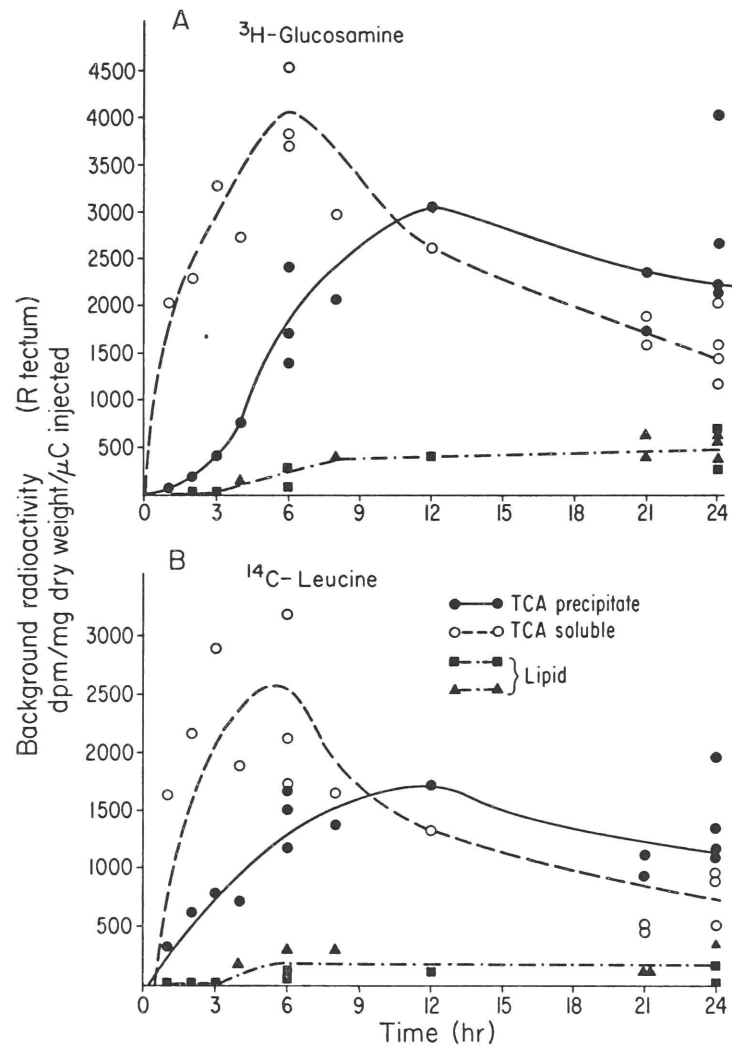


Figure 11. Time-course of appearance of radioactivity in the right tectum, reflecting the background labeling. Symbols same as in Figure 10.

Table VI. Distribution of Radioactivity one day After Injection of
[³H]Glucosamine

| | <i>Lipid extr. me- dium</i> | <i>No. of fish</i> | <i>Transported radioactivity (L-R tectum)</i> | | | | <i>Local incorporation (R tectum)</i> | | | |
|-----------------------|---|----------------------------|---|------------------|---------------|--|---|------------------|------------|---------------------------------------|
| | | | <i>% S*</i> | <i>% P**</i> | <i>% L***</i> | <i>% $\frac{L^{\S}}{P + L}$</i> | <i>% S</i> | <i>% P</i> | <i>% L</i> | <i>% $\frac{L}{P + L}$</i> |
| A. Whole tecta | 3:1 ^a | 13 | 22 | 65 | 13 | 16 | 33 | 59 | 8 | 11 |
| | 2:1 ^b | 12 | 22 | 59 | 19 | 20 | 34 | 56 | 10 | 16 |
| | 1:1 ^c | 21 | 19 | 52 | 29 | 28 | 40 | 46 | 14 | 23 |
| B. Homogenized tissue | 3:1 ^a | 8 | 31 | 45 ^{§§} | 24 | 35 | 41 | 47 ^{§§} | 12 | 21 |

* TCA-soluble.

** Solid matter after precipitation with TCA and extraction with lipid solvents.

*** Extracted with lipid solvents.

§ Proportion of lipid in TCA precipitate.

§§ Includes particulate fraction after lipid extraction plus TCA-precipitable portion of soluble fraction.

^a 3:1 chloroform-methanol.

^b 2:1 chloroform-methanol followed by 1:2.

^c 1:1 chloroform-methanol.

Values from homogenized tecta (B) are data from Dr. Bruce McEwen (see Table IV).

Table VII. Comparison of Transported Radioactivity 1 and 7 Days after Injection

| | 1 Day | 7 Days |
|---|----------------|----------------|
| <u>[³H]Glucosamine</u> (transported DPM/mg dry weight per μ Ci injected) | | |
| TCA-precipitate | 3399 \pm 531 | 2861 \pm 419 |
| Chloroform-methanol extract | 828 \pm 87 | 1144 \pm 192 |
| TCA-soluble | 1395 \pm 327 | 444 \pm 63 |
| <u>[¹⁴C]Leucine</u> (transported DPM/mg dry weight per μ Ci injected) | | |
| TCA-precipitate | 1953 \pm 228 | 1904 \pm 278 |
| Chloroform-methanol extract | 142 \pm 19 | 291 \pm 52 |
| TCA-soluble | -42 \pm 115 | -69 \pm 132 |

Discussion

The time-course of the arrival of ^{14}C -labeled proteins in the tectum (Figure 10A) agrees closely with the time-course found in previous experiments with labeled amino acids (McEwen and Grafstein, 1968; Elam and Agranoff, 1971a). The similarity of the time-courses of arrival of macromolecules labeled with ^3H from glucosamine and with $[^{14}\text{C}]$ leucine suggests that both isotopes were incorporated in the retina in about the same time period, and then transported to the tectum at the same rate. This rate is about 70-100 mm/day (Elam and Agranoff, 1971a; also see Experimental Section IC). The shape of curves like those in Figure 10 reflect not only the rate of axonal transport, but also the lifetime of the precursor pools, the time required to synthesize the transported products, the rate of turnover of the transported products in the tectum, etc. Two precursors might label materials which are transported in the axon at the same speed, and yet produce different patterns of arrival in the tectum. For instance, this is the case when transported macromolecules labeled with $[^3\text{H}]$ fucose and with labeled amino acids are compared (see Experimental Sections I C and II C). The converse, that precursors labeling materials with different rates of transport would produce similar patterns of arrival in the tectum, seems rather unlikely. Elam *et al.* (1970) based their conclusion that sulfated mucopolysaccharide proteins move at the same rate as rapidly transported proteins on the similarity of their patterns of arrival in the tectum. They have recently strengthened that conclusion by showing that the time-courses are similar at two different temperatures (Elam and Agranoff, 1971b). The time-course of the arrival of sulfated mucopolysaccharide proteins is thus also similar to Figure 10.

The three classes of transported materials labeled by $[^3\text{H}]$ glucosamine all begin to arrive in the tectum at about the same time, and have roughly similar patterns of arrival. One might expect that if the TCA-soluble material is a precursor for the local synthesis of the TCA-precipitable and C-M extractable materials in the tectum, it might arrive before the other components. An example of this sort of temporal

relationship between TCA-soluble precursors and their products is seen in the labeling of the right tecta (Figure 11). However, the transported TCA-soluble component does not precede the other components. If anything, it seems to lag behind.

Since glucosamine would be expected to label gangliosides (Burton et al., 1963; Suzuki, 1967), it was necessary to extract gangliosides from the TCA-precipitates. Gangliosides are polar lipids and require polar lipid solvents for complete extraction (Suzuki, 1965). Goldfish brain gangliosides are even more polar than mammalian brain gangliosides (Ishizuka et al., 1970; Avrova, 1971). However, the lipid solvents necessary for ganglioside extraction, such as C-M, 1:1 or 1:2 would be expected to solubilize some protein as well, especially after TCA treatment. Thus, the radioactivity observed in the C-M extract may be in ganglioside or glycoprotein (or both). The increased extraction of radioactivity into the more polar C-M mixtures is consistent with either possibility. In these experiments it was decided to accept the loss of some glycoprotein in the C-M in order to remove all labeled ganglioside from the bulk of the glycoproteins, which remain in the TCA precipitate. A phospholipid which contains glucosamine has been isolated from brain tissue (Tesoriere et al., 1970); some of the labeled C-M extractable radioactivity may be in this type of phospholipid.

As well as labeling gangliosides and conventional glycoproteins, label from glucosamine is also likely to be incorporated into sulfated mucopolysaccharide proteins, which are rapidly transported in the goldfish visual system (Elam et al., 1970). Labeling with glucosamine is complex since radioactivity from glucosamine is expected to be incorporated as three sugars (N-acetylglucosamine, N-acetylgalactosamine, and sialic acid). Glucosamine also produces a high level of background labeling, which is an experimental inconvenience. For these reasons, it seemed worthwhile to examine other labeled sugars, such as [³H]L-fucose, to see if they might be more useful as precursors for labeling glycoproteins.

I B. ABSENCE OF [^3H]FUCOSE LABELING OF SLOWLY TRANSPORTED PROTEIN IN OPTIC NERVES

There are several reasons to expect [^3H]L-fucose to be a useful precursor for labeling axonally transported glycoproteins. Fucose is unusually metabolically stable and is incorporated into glycoproteins only as fucose (Zatz and Barondes, 1970; Quarles and Brady, 1970; Margolis and Margolis, 1971). Mucopolysaccharides contain little or no fucose (Marshall and Neuberger, 1970). There are no measureable amounts of fucolipids in the nervous system (Brunngraber, 1970) and labeled fucose is not incorporated into brain lipids (Zatz and Barondes, 1970; Margolis and Margolis, 1971). Furthermore, fucose, when it occurs, is found only in the terminal positions of oligosaccharide chains (Ginsburg and Neufeld, 1969). Therefore, when glycoproteins are labeled by radioactive fucose, it is likely that the synthesis of the oligosaccharide chains is complete.

Grafstein was the first to use [^3H]L-fucose, injected into the goldfish eye, to label transported glycoproteins. Her results are shown in Figure 12. Fucose, like glucosamine, labels transported glycoprotein which is found in the tectum 24 hours after injection. There is no indication in Figure 12 of any slow component arriving 23 days after the injection. There is a small but measureable transported TCA-soluble component. The background labeling produced by [^3H]L-fucose is remarkably low. Like proline (Elam and Agranoff, 1971a), the low background makes fucose an especially favorable precursor for studies of axonal transport in the goldfish optic system.

One of the most striking differences between the labeling of transported macromolecules with sugars and with amino acids is that sugars do not seem to label the slow component of axonal transport. Since the slow component is prominent in optic nerves, I examined optic nerves for evidence of slowly transported glycoproteins labeled with [^3H]fucose.

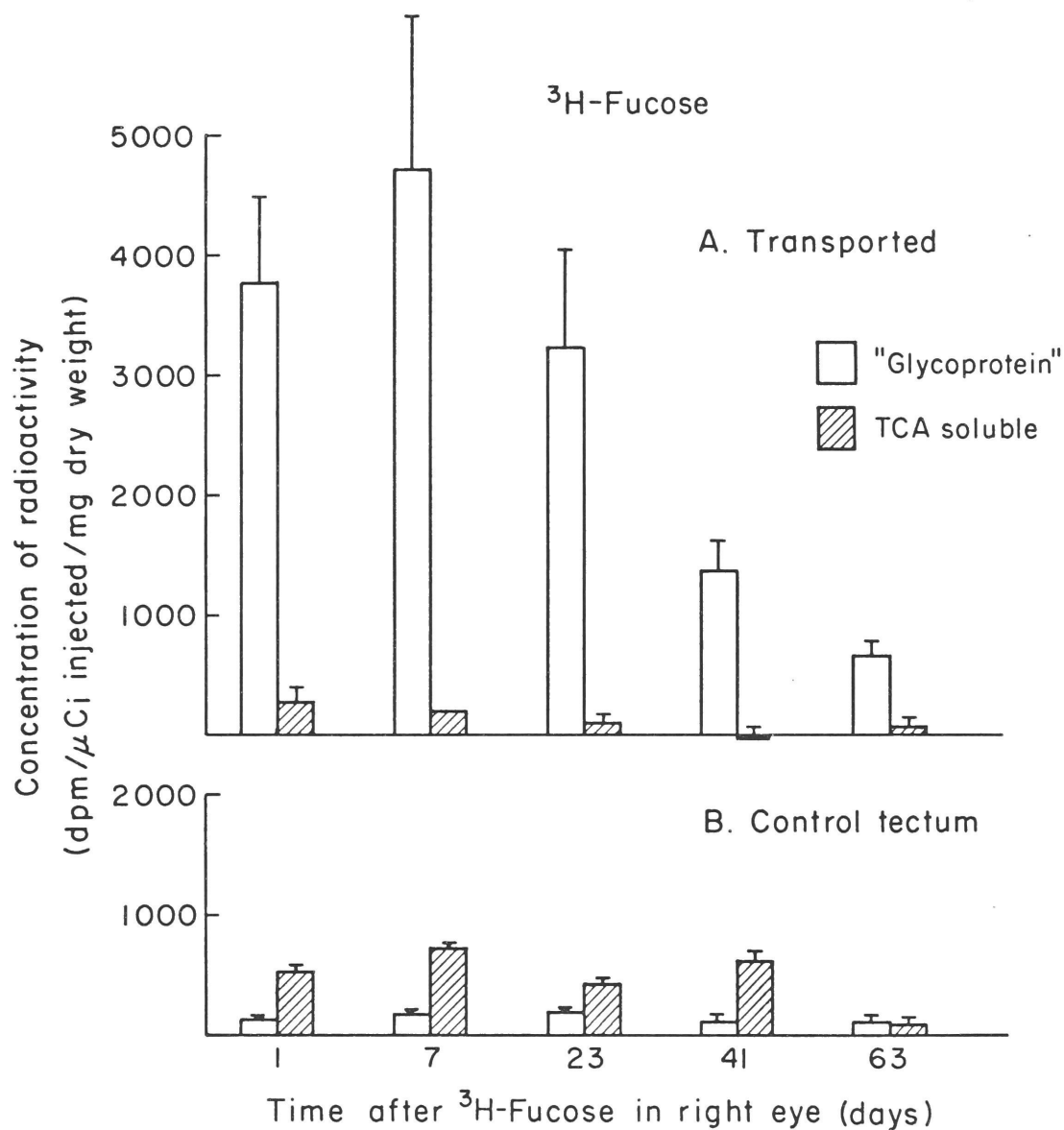


Figure 12. Radioactivity in optic tecta after an intraocular injection of [³H]L-fucose. The fish were injected with 1.2 μ Ci (4.3 Ci/mMole) in the right eye. Tecta were processed by Method 1. "Glycoprotein" refers to TCA-precipitable radioactivity. Lines above the bars are standard errors of the mean (N = 5 except at 63 days, where N = 4). Data of Dr. Bernice Grafstein. From McEwen et al. (1971).

Methods

The fish in this experiment were the same ones used by Grafstein to study [^3H]fucose-labeled, transported glycoproteins in the tecta (see Figure 12). The fish were injected in the right eye with 1.2 μC of [^3H]fucose (4.3 Ci/mMole), and sacrificed 1, 7, 23, 41, or 63 days later. After removing the tecta, Grafstein fixed the heads in Bouin's fixative, and later transferred them through several washes of 70% ethanol. I removed the right optic nerves and measured their lengths. Most of the radioactivity in these nerves was solubilized in 0.5 ml of Soluene-100 and counted in a Toluene scintillator. However, an insoluble residue remained. The small amount of radioactivity in the residue was recovered by combustion in the Packard Model 305 Sample Oxidizer, and included in the calculation of DPM per optic nerve.

Results

The acid-insoluble radioactivity in the right nerves is shown in Figure 13. There is no increase in radioactivity between 1 and 7 days; indeed, the radioactivity in the nerves is almost the same from 1 to 41 days. Thus, transported fucosyl glycoproteins in the optic nerve must turn over slowly. The radioactivity which is measured in this experiment appears to be mainly in transported radioactivity. The background labeling as measured in left nerves was extremely low. However, some of the radioactivity may be in Schwann cells labeled by precursor which has diffused out of the eye along the nerve (Taylor and Weiss, 1965).

Discussion

After an intraocular injection of [^3H]leucine, a wave of slowly transported labeled protein, which first enters the nerve about 2 days after the injection, can be found in the nerve for the next 3 weeks as it moves to the tecta at a rate of about 0.4 mm/day (Grafstein, 1967; McEwen and Grafstein, 1968; Grafstein and Murray, 1969). Seven days after the injection there is about 2.5 to 3 times as much labeled protein in the nerve as there is at 12 hours (calculated from Figure 2

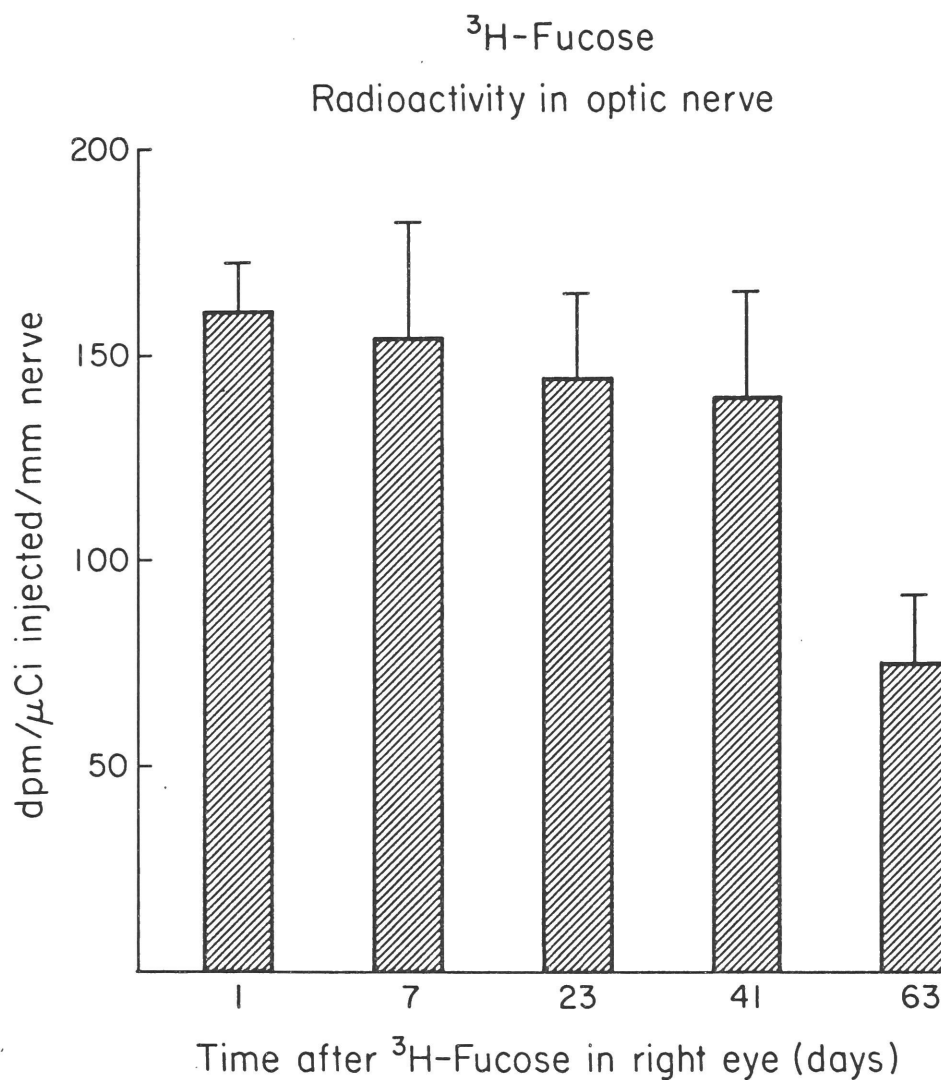


Figure 13. Acid-insoluble radioactivity in right optic nerve after an intraocular injection of [^3H]fucose. Nerves from the same fish as Figure 12. The average length of the nerves in this experiment was 3.03 ± 0.05 mm.

of Grafstein and Murray, 1969). After [^3H]fucose injection, there is no comparable increase in radioactivity between 1 day and 1 week which could be due to a slow component. Thus, at the level of resolution of Figures 12 and 13, there is no evidence in the optic tracts or tecta for slowly transported glycoproteins labeled by [^3H]fucose. Since the same is true when transported radioactivity from [^3H]glucosamine is examined in tecta (Figure 9) and tracts (B. Grafstein, unpublished observation), it appears that glycoproteins are not transported in the slow component of axonal transport.

I C. TIME COURSE OF THE AXONAL TRANSPORT OF GLYCOPROTEINS Labeled WITH [³H]FUCOSE

[³H]fucose labels rapidly transported glycoproteins and TCA-soluble materials. As in the case of glucosamine, it was of interest to compare the early time course of the transport of glycoproteins labeled by [³H]fucose with the transport of proteins labeled by radioactive amino acids. In this experiment I used large pond goldfish and was able to measure transported radioactivity in the optic nerves and tracts as well as in the tecta.

Methods

Forty-seven pond goldfish received injections in the right eye of 5 μ L containing 2.8 μ Ci of [³H]L-fucose (4.3 Ci/mMole) and 0.73 μ Ci of [U-¹⁴C]L-proline (209 mCi/mMole). The time course was run in two parts. In the first, fish were sacrificed 1, 2, 3, 4, 5, 6, 8, 10, or 24 hours after injection; in the second, they were killed at 8, 12, 15, 18, 21, or 24 hours. The results of the two experiments were compatible and have been combined. In the combined time course there are three fish per time point, with the following exceptions: 6 fish at 8 hours, 4 fish at 18 hours, and 7 fish at 24 hours. The temperature was $21 \pm 1^{\circ}\text{C}$.

The lengths of the freshly dissected nerves and tracts were measured under a dissecting microscope, and then processed individually for scintillation counting by Method 3. Tecta were processed by Method 1. Since neither fucose nor proline label a significant amount of lipid, the tissues were not extracted with C-M before drying. The size of the fish and their optic systems is given in Table VIII.

Results

Figure 14 shows the transported radioactivity found in the TCA precipitates of the optic nerve, optic tract, and tectum during the first 24 hours after injection. The overall pattern of labeling is similar in each of these tissues: after a delay, ¹⁴C-labeled proteins increase

Table VIII. Physical Measurements of Pond Goldfish and their Optic Systems

| | |
|--|--------------------|
| Length of fish (measured from front tip to base of tail) | |
| Average | 10.7 ± 0.14 cm |
| Extremes (1 fish each) | 8.5 , 14.0 cm |
| Average weight of dried tecta* | |
| | 2.05 ± 0.03 mg |
| Average length of optic nerve | |
| Fresh | 4.6 ± 0.1 mm |
| After fixation in 10% TCA† | 4.4 ± 0.1 mm |
| Average length of optic tract§ | 2.9 ± 0.1 mm |

Values are means \pm standard error of the mean (N = 47).

* Includes weight of bound TCA.

† Second group only (N = 20). Calculations of DPM/mm were based on fresh length.

§ The measured length of the optic tract was different for the two groups of fish (2.6 ± 0.1 mm and 3.1 ± 0.1 mm for the first and second groups respectively). It was difficult to measure the length of the tracts accurately. Therefore, radioactivity in the tracts has been calculated as DPM/tract without any correction for the length of the tracts

increase rapidly for about 3 to 4 hours, after which the amount of labeled protein remains about the same. Glycoproteins labeled with [^3H]fucose begin to appear at the same time as the ^{14}C -labeled proteins, but continue to arrive throughout the period studied. However, the timing of these patterns is different in each part of the optic system; as one would expect for radioactive materials being transported proximodistally, the label appears first in the optic nerve, then in the optic tract, and last in the tectum. The differences in the times of the first arrival of transported radioactivity is shown more clearly in Figure 15. For both isotopes, labeled proteins first begin to arrive in the optic nerve 1 1/2 to 2 hours after the injection, in the optic tract at about 3 1/2 hours, and in the tectum at 4 to 4 1/2 hours. Using these times and the measured dimensions of the optic system, one can calculate that this earliest labeled protein is moving at a rate of about 70-80 mm/day (Figure 16). Other methods of estimating the rate give similar results (Table IX). For instance, when the labeling period is sufficiently short to produce a rapid rise to a plateau, as is the case with labeled amino acids, a reasonable landmark is the inflection point of the rising phase. The inflection point is the period of the most rapid arrival of radioactivity, and hence corresponds to the arrival of the peak of a wave of transported material. The point at which the radioactivity is one-half its maximum value has also been used in calculating the rate of transport (McEwen and Grafstein, 1968). The half-maximal point is close to the inflection point, and is easier to locate objectively. Both of these methods give estimates of the rate of transport of ^{14}C -labeled proteins in the range of 70-85 mm/day (Table IX). Since the [^3H]fucose curve does not reach a plateau, only the time of first arrival of radioactivity can be used to calculate the rate of transport of [^3H]fucosyl glycoproteins.

Similar rates can also be calculated from Figure 10A, in which proteins labeled with [^{14}C]leucine move through the 6 mm of small goldfish optic fibers to first reach the tectum about 3 1/2 hours after injection. One must assume that proteins labeled with radioactive amino

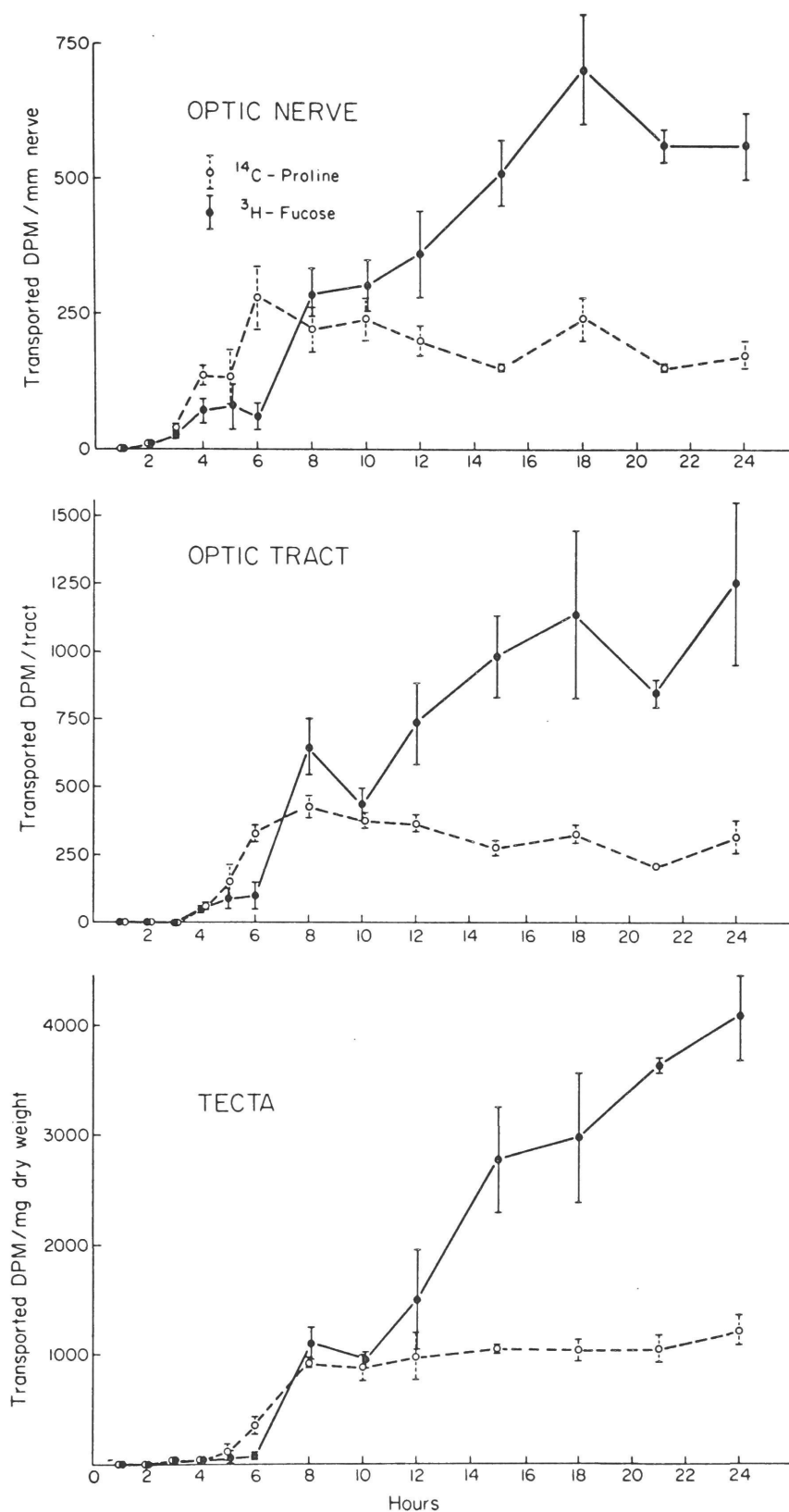


Figure 14. Time course of transported TCA-precipitable radioactivity in optic nerve, optic tract, and optic tectum. Bars show the standard error of the mean. Transported radioactivity is calculated as L - R for the optic tract and tectata, and as R- L for the optic nerve.

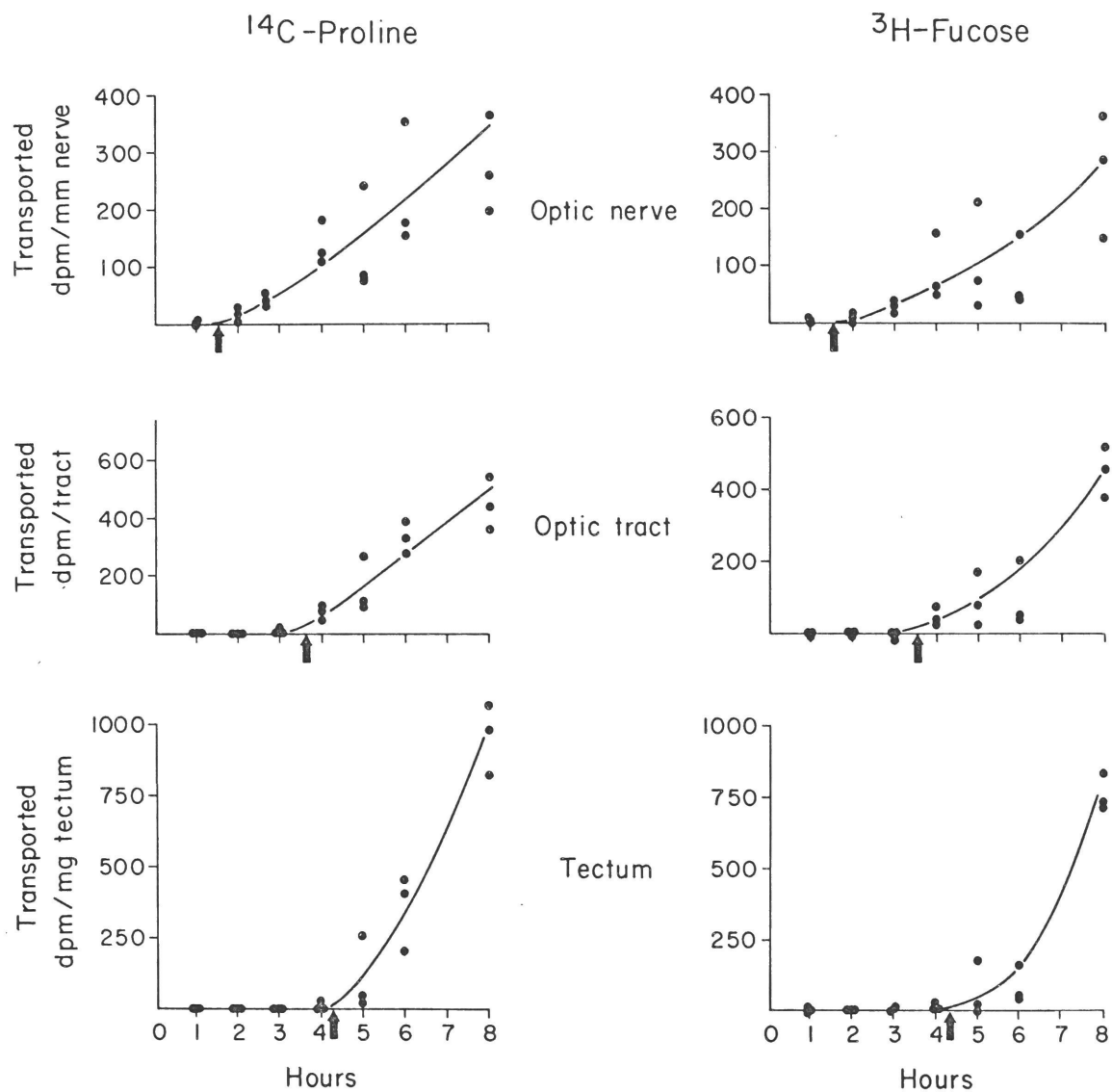
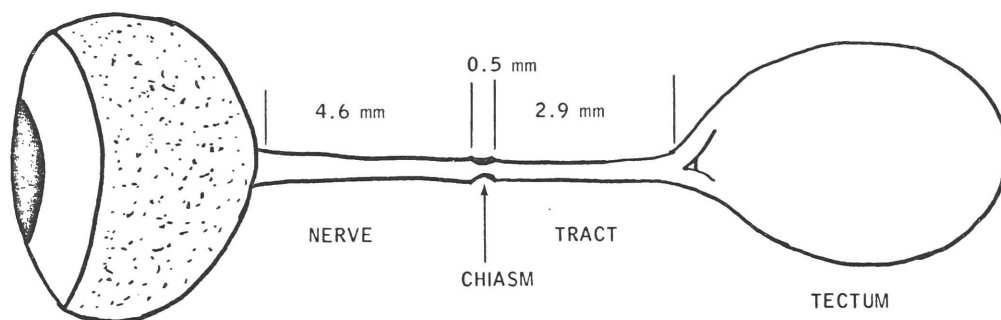


Figure 15. Arrival in the optic nerve, optic tract, and tectum of radioactive proteins labeled with [^{14}C]proline and [^3H]fucose. Includes all early data points from first series of fish.



RATE OF AXONAL TRANSPORT IN GOLDFISH OPTIC SYSTEM

| | DISTANCE | TIME | RATE |
|----------------|-----------|-----------|---------------|
| | <u>mm</u> | <u>hr</u> | <u>mm/day</u> |
| Nerve → Tectum | 8.0 | 2.5 | 77 |
| Nerve → Tract | 5.1 | 1.5 | 82 |
| Tract → Tectum | 2.9 | 1.0 | 70 |

Figure 16. An example of the calculation of the rate of rapid axonal transport, based on the first arrival of radioactive macromolecules in the parts of the optic system. The same calculation applies to both proteins labeled with [^{14}C]proline and glycoproteins labeled with [^3H]fucose. The distances are measured between proximal ends of each structure. This illustration is diagrammatic, and is not drawn to scale; the measured dimensions are indicated by the numbers above the drawing.

Table IX. Calculation of the Rate of Axonal Transport

| Time after Injection (hr) | | | Rate of Transport (mm/day) | | |
|--|-------|--------|----------------------------|------------------|-------------------|
| Nerve | Tract | Tectum | Nerve → Tectum | Nerve → Tract | Tract → Tectum |
| 1. <u>Earliest transported radioactivity</u> | | | | | |
| 1.5 | 3.5 | 4.0 | 77 | 82 | 70 |
| 2. <u>Earliest transported radioactivity</u> | | | | | |
| 1.5 | 3.5 | 4.5 | 64 | 61 | 70 |
| 3. <u>Half-maximal point</u> | | | | | |
| 4.0 | 5.4 | 6.5 | 76 | 73 | 81 |
| 4. <u>Half-maximal point</u> | | | | | |
| 4.2 | 5.4 | 6.5 | 83 | 85 | 81 |
| 5. <u>Inflection point</u> | | | | | |
| 4.0 | 5.4 | 6.5 | 76 | 73 | 81 |

The left-hand columns show the time after isotope injection when the specified portion of the labeling pattern (earliest transported radioactivity, half-maximal point, or inflection point) is measured in the nerve, tract, and tectum. The rate of transport is calculated from these times, using the dimensions shown in Figure 16. In calculations based on the arrival of the earliest transported radioactivity (1 and 2), distances are measured between the most proximal borders of the structures, as shown in Figure 16. In calculations based on the half-maximal or inflection point (3, 4, and 5), distances are measured between the midpoints of the structures. The average length of a pond-fish tectum along its long axis is 4.5 mm. The distances between midpoints are thus: nerve → tectum, 7.95 mm; nerve → tract, 4.25 mm; and tract → tectum, 3.7 mm.

Comments

1. Earliest transported radioactivity ($[^3\text{H}]$ fucose and $[^{14}\text{C}]$ proline): This calculation is illustrated in Figure 16.
2. Earliest transported radioactivity: Same as 1, but assumes that radioactivity first arrives in the tectum 4.5 hr after the injection. Estimate 1 is probably correct; although the amount was too small to be evident in Figures 14 and 15, a measurable amount of transported labeled protein was found in all tecta at 4 hr.
3. Half-maximal points of the $[^{14}\text{C}]$ proline data in Figure 14.
4. Half-maximal points of smooth curves fitted by eye to all of the $[^{14}\text{C}]$ proline data points which were averaged in Figure 14.
5. Inflection points of the smooth curves fitted to the $[^{14}\text{C}]$ proline data.

acids first begin to enter the optic nerve 1 1/2 to 2 hours after the injection; although this has never been measured with [^{14}C]leucine, it is demonstrated here in pond goldfish (Figure 14), and by Elam and Agranoff (1971a) in small goldfish, with labeled proline. The rate of transport is then 72-96 mm/day. The transported macromolecules labeled by radioactivity from [^3H]glucosamine must also move at this rate (see Discussion, Section I A).

Transported radioactivity in Figures 14 and 15 has been calculated as in previous chapters by subtracting the background concentration of radioactivity measured in the contralateral tissues. However, this was hardly necessary because the background labeling produced by both proline and fucose is extremely low. This is illustrated in Table X, which shows the distribution of the transported and background TCA-precipitable radioactivity at 8 and 24 hours. In Table X, the results are expressed as total DPM per nerve, tract, or tectum, in order to compare the relative amounts of radioactivity in these tissues. Only 0.23% of the ^{14}C -proline injected into the eye appears in transported protein, 0.16% of which is in the tectum. This is very close to the results of Elam and Agranoff (1971b) who found that 0.14% of the ^3H -proline injected into the eyes of small goldfish appeared in transported protein in the tectum 24 hours later. The efficiency of incorporation of [^3H]fucose is similar to that of proline: 24 hours after the injection, 0.20% of the injected radioactivity has been transported, 0.14% of which is in the tectum.

By summing the total amount of transported radioactivity in the optic nerve, tract, and tectum, we can calculate the total amount of transported radioactivity in the extraocular portion of the retinal ganglion cell axons. The pattern of export of transported radioactivity into the optic fibers is different for [^{14}C]proline (Figure 17) and [^3H]fucose (Figure 18). The transport of protein labeled with [^{14}C]proline stops 8 hours after the injection, while the transport of [^3H]fucose-labeled materials continues throughout the period studied. Yet, although the time course of the export of radioactivity is different for

Table X. Distribution of Radioactivity in Goldfish Optic Fibers After Intraocular Injection of [^{14}C]Proline and [^3H]Fucose

| | [^{14}C]Proline | | [^3H]Fucose | |
|---------------------------------|----------------------------|-------------------|------------------------|-------------------|
| | Transported DPM | Background DPM | Transported DPM | Background DPM |
| <u>8 hours after injection</u> | | | | |
| Nerve | 1026 \pm 165 | 11 \pm 2 | 1399 \pm 121 | 14 \pm 2 |
| Tract | 427 \pm 38 | 3 \pm 1 | 648 \pm 106 | 8 \pm 2 |
| Tectum | 1921 \pm 76 | 42 \pm 12 | 2216 \pm 343 | 58 \pm 15 |
| <u>24 hours after injection</u> | | | | |
| Nerve | 800 \pm 124 | 12 \pm 3 | 2594 \pm 294 | 30 \pm 4 |
| Tract | 317 \pm 59 | 8 \pm 1 | 1261 \pm 267 | 13 \pm 3 |
| Tectum | 2530 \pm 283 | 58 \pm 8 | 8391 \pm 753 | 104 \pm 12 |

Values are the average total DPM per optic nerve, optic tract, or tectum, \pm standard error of the mean.

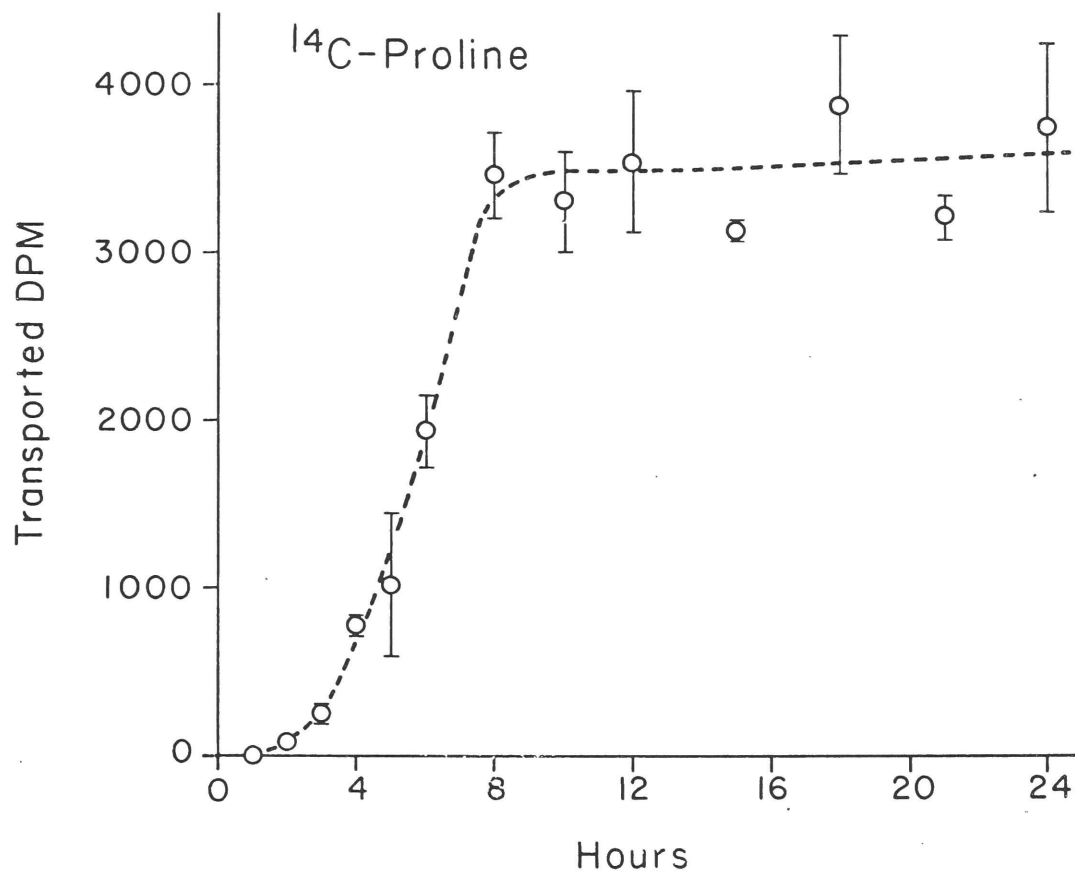


Figure 17. Total transported radioactivity in the optic pathway (optic nerve + optic tract + tectum) in protein labeled with [^{14}C]proline.

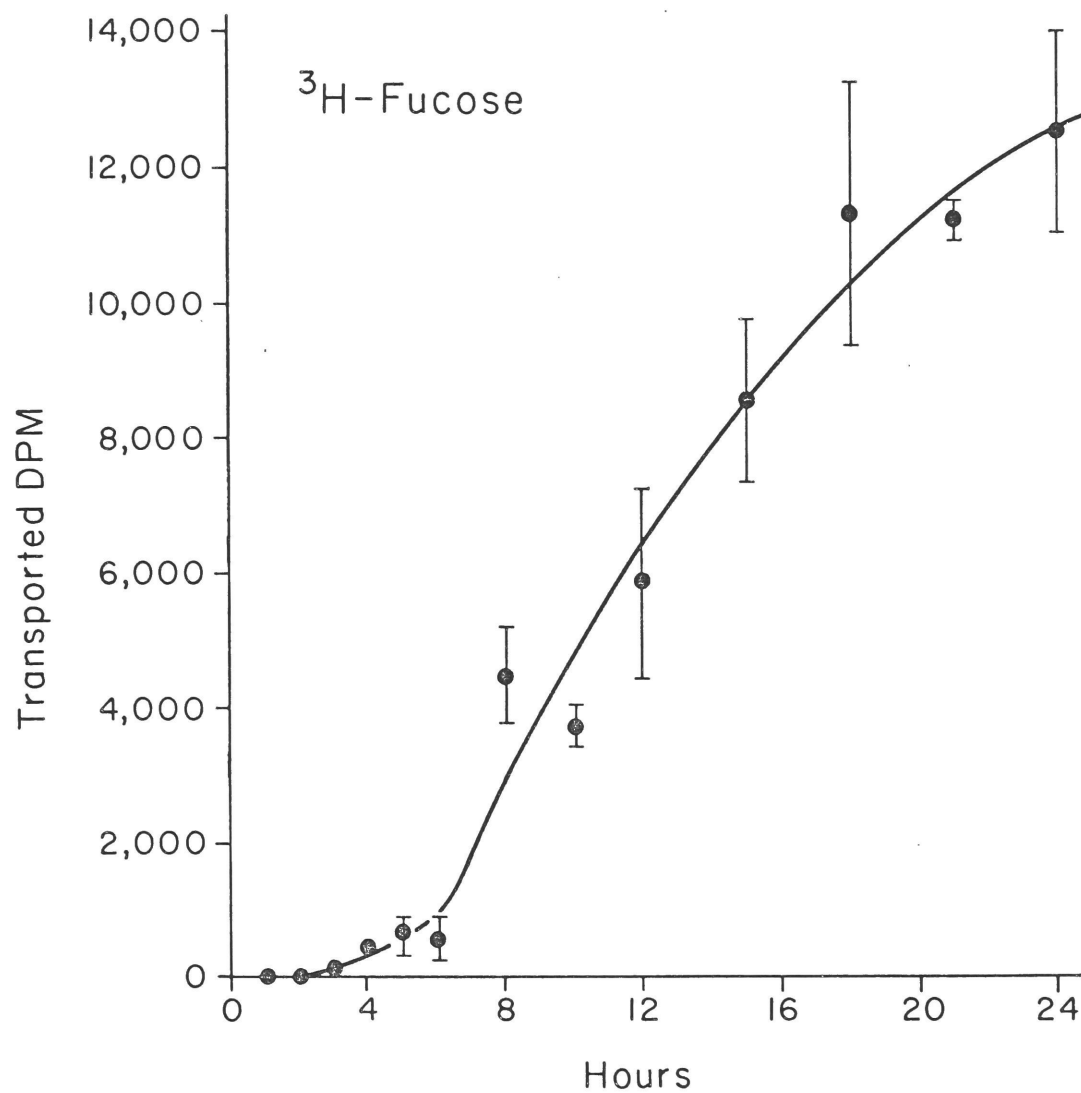


Figure 18. Total transported radioactivity in the optic pathway in glycoprotein labeled with [^3H]fucose.

the two isotopes, the per cent of the total transported radioactivity found in each part of the optic fibers is similar at all times (Figure 19).

The transported TCA-soluble radioactivity in the tecta is shown in Figure 20. As demonstrated previously, the ^{14}C radioactivity from proline is negligible. The TCA-soluble ^3H component is very small compared to the incorporated radioactivity, and it begins to arrive in the tectum later than the transported glycoprotein. The measurements of TCA-soluble radioactivity in the nerves and tracts were less satisfactory. In both nerves and tracts the measurements were highly variable, perhaps due to the difficulty of adequately blotting these tissues before TCA precipitation. When transported radioactivity in the tracts was calculated as the left-right difference, there were some anomalously high and some negative values. The TCA-soluble radioactivity in the right nerves was high at all times, apparently due to seepage of radioactivity from the eyeball. Some recent studies show that significant amounts of ^3H from $[^3\text{H}]\text{L-fucose}$ may appear in brain tissue in tritiated water (S. Bondy, personal communication; R. Margolis, personal communication. See Section III A). Since the TCA supernatants of the nerves and tracts were counted directly, I do not know how much of the TCA-soluble radioactivity measured in the nerves and tracts is in tritiated water. The TCA-soluble radioactivity measured in the tecta does not include tritiated water, since the samples were evaporated to dryness during processing for scintillation counting. Nevertheless, the measurements of TCA-soluble radioactivity in the nerves and tracts do not show any wave of transported TCA-soluble material which could serve as the precursor for the local synthesis of the glycoprotein component.

Discussion

$[^3\text{H}]\text{fucose}$ is a more convenient precursor than $[^3\text{H}]\text{glucosamine}$ for studying the axonal transport of glycoproteins, because fucose produces much lower background labeling and does not label lipids. However, the basic findings with the two isotopes are the same: 1) The

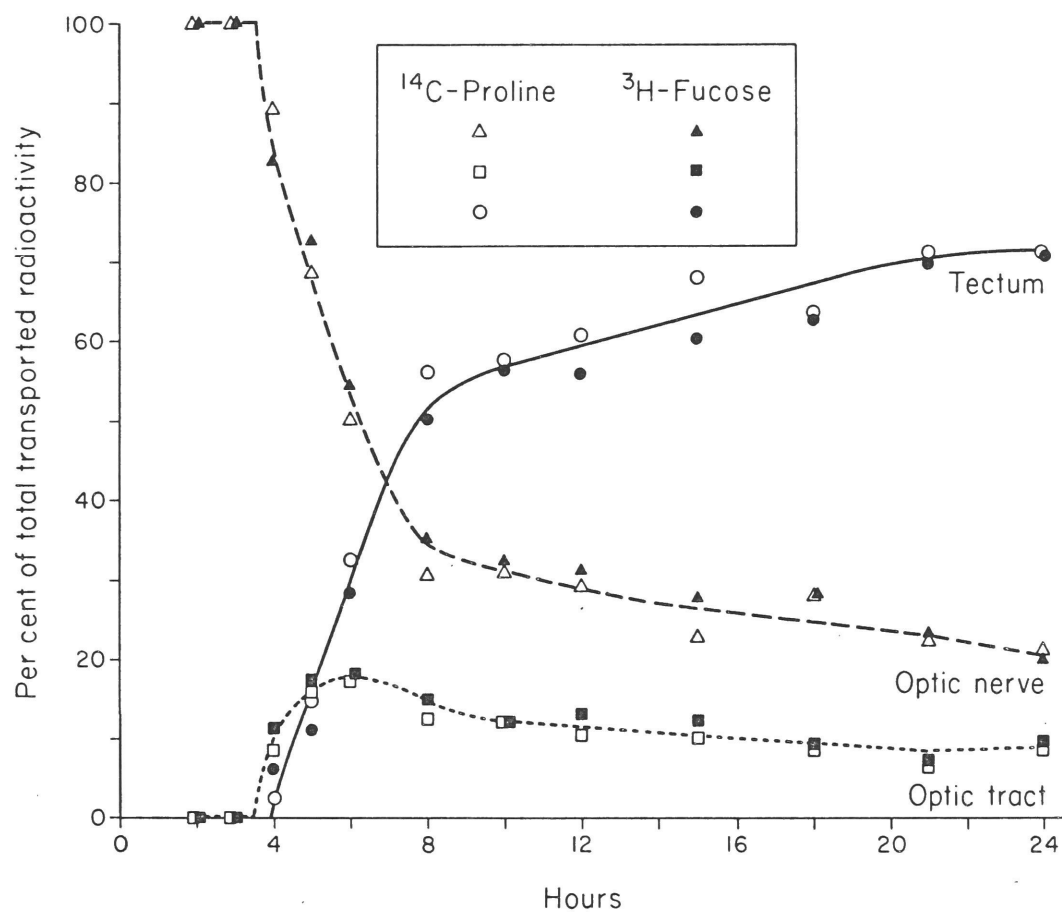


Figure 19. Per cent of total transported radioactivity in the optic nerve, optic tract, and optic tectum. The percentages were calculated for the individual fish and then averaged. The ordinate shows the time after the injection of the isotope mixture.

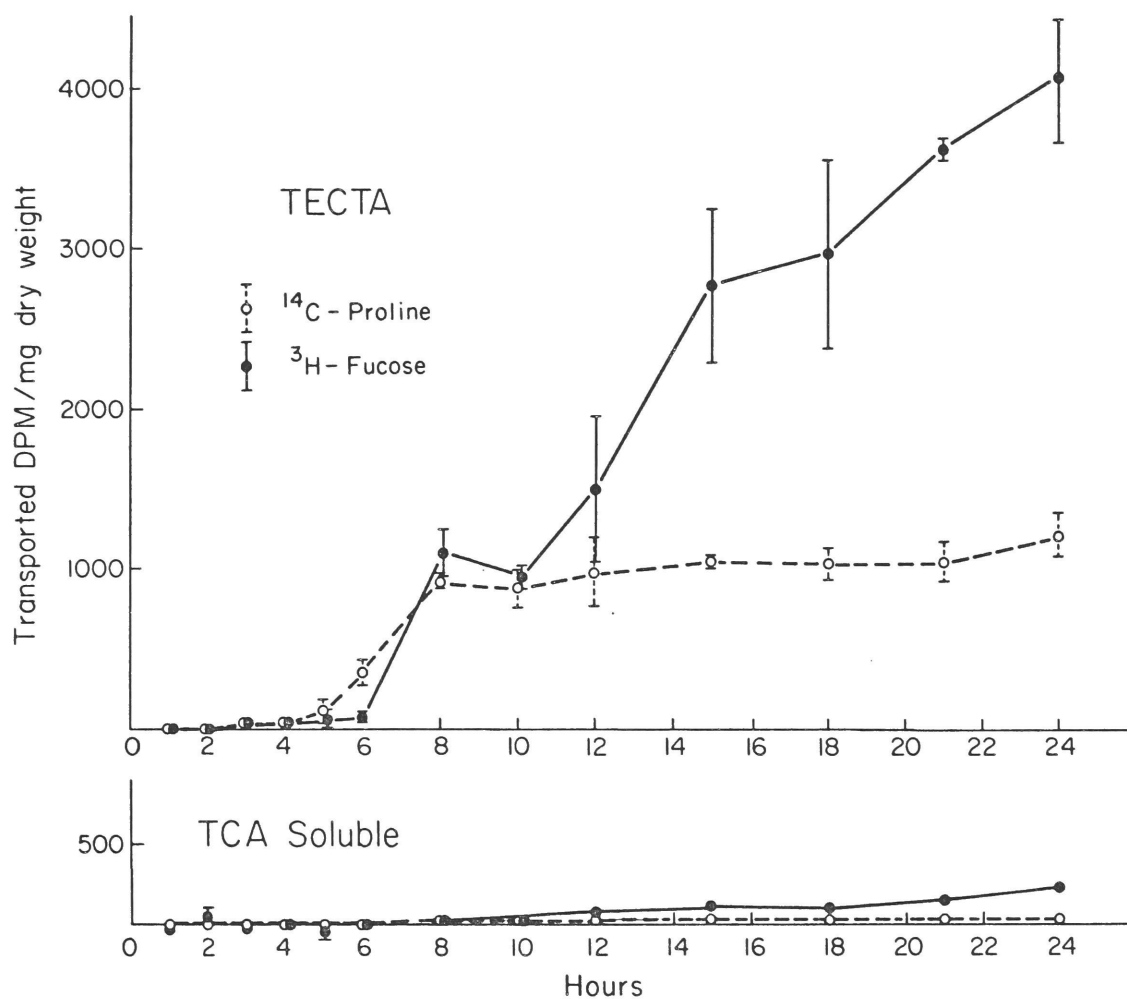


Figure 20. Comparison of transported TCA-precipitable and transported TCA-soluble radioactivity in the tecta. The TCA-precipitable radioactivity (upper curve) is the same as in Figure 14. Since the TCA-soluble material was dried and redissolved before scintillation counting, it includes only non-volatile TCA-soluble materials.

sugars label transported macromolecules which move at the same rate as rapidly transported proteins. 2) There is a small transported TCA-soluble component which lags slightly behind the transported macromolecules. 3) There is no detectable slow component. A result which was seen with [^3H]glucosamine (Figure 10), but which is much more striking with [^3H]fucose, is that the amount of [^3H]labeled-glycoprotein in the tecta continues to increase after the amino-acid-labeled protein reaches a plateau (Figures 14, 17, and 18). The reason for this difference between the [^3H]fucose and [^{14}C]proline patterns is apparently that [^3H]fucose continues to be incorporated into transported macromolecules for a much longer time after injection than [^{14}C]proline. Evidence for this explanation is presented in Section II C.

Several other workers have recently reported axonal transport of glycoproteins labeled with [^3H]fucose (Zatz and Barondes, 1971; Bondy and Morelos, 1971; Bondy and Madsen, 1971; Bondy, 1971). Karlsson and Sjöstrand, using the rabbit optic system, have confirmed most of the results reported here, including a rapid transport of fucosyl glycoproteins, an incorporation period in the retina which is longer than the interval in which amino acids are incorporated, and the apparent absence of a slow component (Karlsson and Sjöstrand, 1971c).

It is difficult to measure the rate of rapid axonal transport in goldfish optic fibers with precision. The axons are short and run for a varying distance inside the eyeball. The intra-retinal segment can be as much as 4 mm, a significant fraction of the total length of the axon. Therefore, if a pulse of material were to leave each retinal ganglion cell at the same moment and be transported at the same rate, the narrow pulse would be dispersed in the nerve as a broader band. Also, in my experiments the rather long interval of 1 hour between time points precludes a very precise estimate. Nevertheless, the several methods of calculating the rate from my data gave consistent estimates which agree with the rate of 70-100 mm/day which has been reported by Elam and Agranoff (1971a). The most accurate way to measure the rate of transport

would be to follow the progress of a sharp peak or advancing front of radioactivity by examining the distribution of radioactivity along the length of the nerve and tract at each time point (e.g., Ochs et al., 1969). This more sophisticated method also provides an estimate of 70 mm/day in the goldfish optic nerve (I. McQuarrie, personal communication). It must be emphasized that the rate of rapid transport is temperature sensitive (Elam and Agranoff, 1971a; Ochs and Smith, 1971a; Grafstein et al., 1972a). The rates reported here were determined at 19-21°C. Since the Q_{10} of the transport process is at least 2.0 (Ochs and Smith, 1971a; Grafstein et al., 1972a) the rates of rapid transport in goldfish are comparable with the faster rates measured in mammals (Introduction, Table II). When the effect of temperature is taken into account, rates of 70-100 mm/day in the goldfish at 20.5°C are equivalent to rates of 220-480 mm/day at 37°C. (The lower limit of 220 mm/day was calculated for a rate of transport of 70 mm/day and a Q_{10} of 2.0 [Ochs and Smith, 1971a]. The upper limit of 480 mm/day is based on a rate of 100 mm/day and a Q_{10} of 2.6 [Grafstein et al., 1972a]).

Transported radioactivity which is measured in an optic nerve or tract can include both radioactivity which is being rapidly transported to the tectum, and radioactivity which has been incorporated into the nerve or tract and has become stationary. If most or all of the transported radioactivity were in materials which are destined for the tectum (for instance, synaptic vesicles), one might expect to see a wave of transported radioactivity pass through the nerve and tracts, appearing in a time-course as a rise and fall in the level of radioactivity. Instead, the shape of the pattern of labeling in the nerve and tract resembles the pattern in the tecta, and there is no evidence of a sharp peak (Figure 14; also see Figure 4). There are several reasons why such discrete wave of radioactivity, if it exists, may not be evident in my data. As has been discussed above, the lengths of the intraretinal segments of the retinal ganglion cells vary, which would tend to spread out a sharp peak. Also, a rapidly transported particle moving 75 mm/day would spend about 1 1/2 hours in the optic nerve, and only 56 minutes in

the optic tract. Thus, both the time intervals and the lengths of the segments of axons sampled in this study may be too large to resolve a sharp peak of materials moving at this speed. There is evidence that when shorter time intervals or more detailed spatial distributions of radioactivity are examined, peaks of rapidly transported radioactivity can be seen (Elam and Agranoff, 1971a; I. McQuarrie, personal communication). Since [^3H]fucose is apparently incorporated into transported radioactivity continuously throughout the period which was studied, one would not expect to see a distinct peak of radioactivity. There is also no information available about the movement of individual transported molecules or particles. If they move in a spectrum of rapid rates instead of a single rate, or if their movement is discontinuous, with occasional stops, then there would be no sharp wave.

Another factor which influences the pattern of labeling is that a significant amount of transported radioactivity remains in the nerve and tract. This can be seen clearly in the [^{14}C]proline data: soon after the transport of [^{14}C]proline-labeled protein from the eye ceases (Figure 17), the amount of radioactivity in the nerve and tract (as well as in the tectum) reaches a nearly constant level (Figure 14) and the distribution radioactivity between the nerve, tract, and tectum does not change much thereafter (Figure 19). This pattern suggests that the radioactivity which is found in the nerve and tract after 8 hours is not in transit to the tectum, but rather has become part of the nerve or tract. After 8 hours there is a very slow decline in the proportion of total transported radioactivity in the optic nerve and tract, and a slight increase in the proportion in the tectum (Figure 19). This may indicate that some [^{14}C]proline-labeled proteins continue to be transported from the nerve and tract to the tectum, although this pattern would also be produced if some proteins in the nerve and tract turn over more rapidly than proteins in the tectum. Nevertheless, this change is small; the data suggests that most of the transported ^{14}C radioactivity in the nerve and tract after 8 hours is no longer moving.

With [^3H]fucose, labeled transported glycoproteins continue to be transported throughout the first 24 hours after injection (Figure 18). Yet the distribution of radioactivity between the nerve, tract, and tectum is nearly the same as the distribution of radioactivity in ^{14}C -proline-labeled proteins (Figure 19). The probable reason for the similarity of the distribution of the two isotopes, despite the difference in their time course, is that at any given moment (and particularly at late times) the amount of [^3H]fucosyl glycoprotein which is in transit in the axons is small compared to the amount which has been incorporated into them. Thus, the distribution of total [^3H]fucose radioactivity is determined mainly by the stationary radioactivity. The very small differences between the ^{14}C and ^3H patterns in Figure 19 are in the direction one would expect if ^3H but not ^{14}C is being transported after 8 hours (namely, more ^3H than ^{14}C in the nerve and tract, and less ^3H than ^{14}C in the tectum). Note that the continuous incorporation of labeled material into the nerve and tract is another factor which would reduce the sharpness of a wave of labeled material moving through them. As in the tectum, the amount of radioactivity measured in a nerve or tract may reflect the accumulation of radioactivity which had arrived earlier more than the presence of radioactivity in transit through the axon at the time of sampling.

I D. N-ACETYLMANNOSAMINE LABELS AXONALLY TRANSPORTED MATERIALS

N-acetyl-D-mannosamine is an intermediate in the synthesis of the sialic acids (Roseman, 1962). Since the only known role of N-acetylmannosamine is as a precursor of sialic acids, it should be useful for labeling glycoproteins and gangliosides. I injected tritiated N-acetylmannosamine into goldfish eyes to see whether it would label axonally transported materials.

Methods

Small goldfish were injected in the right eye with 1.1 μ Ci of N-[3 H-acetyl]acetyl-D-mannosamine (1020 mCi/mMole, New England Nuclear) in 2 μ L of saline, and maintained in small tanks at 20°C until sacrifice. The isotope was found to be radiochemically pure by thin-layer chromatography (although the detection reagent revealed an unknown non-radioactive carbohydrate, which is probably unlabeled D-mannosamine). Fish were sacrificed 9 hours, or 1, 7, or 23 days after the injection. Tecta were processed in groups of 3 by Method 2. The number of groups of fish examined were: 9 hours - 2, 24 hours - 3, 7 days - 3, 23 days - 2 groups of 3 and a third group with 2 fish.

Results

After an intraocular injection of [3 H]N-acetylmannosamine, transported radioactivity appears in the tectum (Figure 21A). The pattern of labeling differs from that produced by [3 H]glucosamine. More transported radioactivity is found in the chloroform-methanol (C-M) extract than in the residual macromolecules. At 9 hours and 1 day after injection, about two-thirds of the incorporated transported radioactivity is in the C-M extract, and this proportion increases to three-fourths of the total at 7 and 23 days. On the other hand, in the control tectum the amounts of radioactivity in the C-M extract and in the residual macromolecules are about the same (Figure 21B). The amount of transported radioactivity in both glycoproteins and C-M extractable

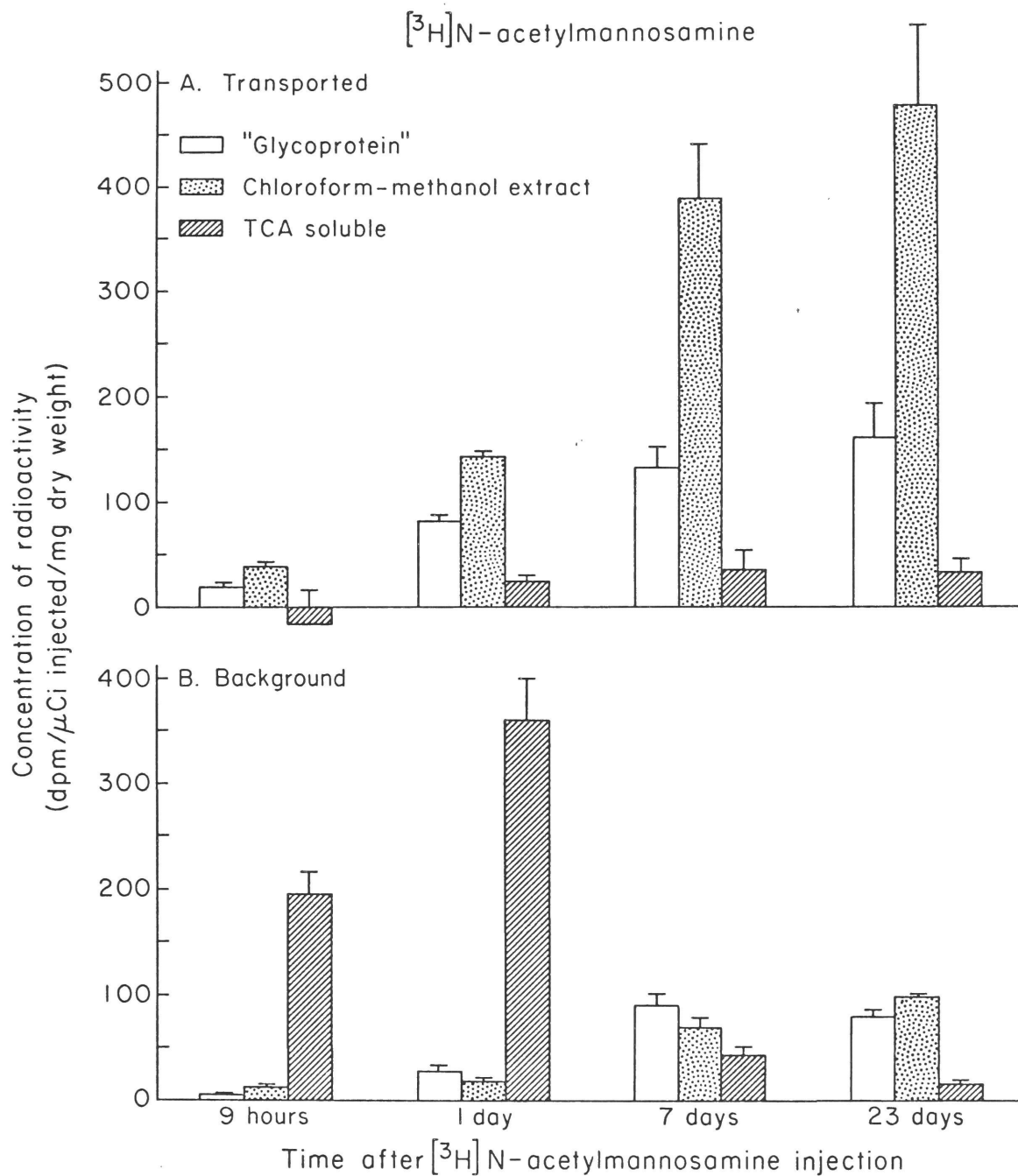


Figure 21. Radioactivity in the tectum after an intraocular injection of $[^3\text{H}]$ N-acetyl-D-mannosamine. Lines above the bars show standard error of the mean.

materials increases between 9 and 24 hours, and 7 days later there has been a further large increase. The further increases between 7 and 23 days are not statistically significant (t-test, $p < 0.05$ level). There is also some transported TCA-soluble radioactivity, although none was found at 9 hours. It is difficult to measure transported TCA-soluble radioactivity reliably at 9 and 24 hours because of the high background labeling (Figure 21B). On the other hand, the background labeling of C-M extractable material is especially low. At 24 hours there is nearly 8 times more transported than background radioactivity in the C-M extract.

Axonally transported radioactivity from N-acetylmannosamine was also examined in large pond goldfish. They were injected with 12.7 μCi , sacrificed 24 hours later, and the tecta processed by Method 1; these fish are from the same group that was used for the isolation of labeled transported gangliosides (Section IV). The distribution of radioactivity in the tecta (Table XI) agrees with the results measured in the small fish. Most of the transported radioactivity is in the C-M extract. Background incorporation into both glycoprotein and lipid is small compared to the transported component, but the background TCA-soluble radioactivity is very high. There might be a tiny transported TCA-soluble component, but it is too small to measure reliably. The incorporation per injected μCi is much lower than in the small fish; it is likely that the dose of isotope injected into the large fish was more than can be efficiently incorporated.

Discussion

More transported radioactivity from tritiated N-acetylmannosamine is found in C-M extractable materials than in macromolecules. This pattern is consistent with our belief that the label has been incorporated as sialic acid, since in the brain there is more sialic acid in gangliosides than in glycoprotein. For instance, in rat brain 68% of the NANA is C-M extractable (Brunngraber, 1969). My results also agree with other isotopic labeling studies. Quarles and Brady (1971) found

Table XI. Radioactivity in Pond Fish Tecta 1 Day after Injection of
[³H]N-Acetylmannosamine

| | Transported L-R Tectum DPM/mg dry wt | % | Background R Tectum DPM/mg dry wt | % |
|------------------------------------|--|----|---|----|
| TCA-precipitate | 346 ± 49 | 30 | 51 ± 11 | 5 |
| Chloroform- methanol extract | 790 ± 110 | 68 | 29 ± 7 | 3 |
| TCA-soluble | 26 ± 49 | 2 | 924 ± 97 | 92 |

that after an intracerebral injection of [^3H]N-acetylmannosamine in rats, 56% of the incorporated radioactivity is in ganglioside, with the remainder in glycoprotein. Labeled NANA should enter the same pathways as radioactive N-acetylmannosamine; DeVries and Barondes (1971) found that 8 hours after an intracerebral injection of [^{14}C]NANA into mouse brain, 55% of the incorporated radioactivity was in ganglioside. [^{14}C]NANA was incorporated into ganglioside and glycoprotein less efficiently than labeled glucosamine. [^3H]N-acetylmannosamine is also an inefficient precursor compared to [^3H]glucosamine for labeling transported materials in the goldfish optic system, yielding only 10%-20% as much transported radioactivity per μCi injected in small goldfish. It is not clear whether this lower incorporation is due to lesser uptake of the precursor by retinal ganglion cells, an effect of unlabeled mannosamine, or other reasons. Nevertheless, the high proportion of transported radioactivity in the C-M extract and the low background labeling make [^3H]N-acetylmannosamine a favorable precursor for examining the question of whether gangliosides are axonally transported, and it was therefore used in the experiment described in Section IV.

The transported radioactivity found in the tecta 9 and 24 hours after the injection must have been transported at a rapid rate. However, the large increase between 9 and 24 hours, and between 24 hours and 7 days raises the question of whether the N-acetylmannosamine labels any materials which are transported more slowly than the rapidly transported materials previously studied. An alternative explanation could be that N-acetylmannosamine labels materials which are rapidly transported, but that the labeling period is very long, as is the case with fucose (see Sections I C and II C). If this is the case, N-acetylmannosamine incorporation in the retinal ganglion cells must continue for more than 24 hours. Further experiments will be necessary to decide between these alternatives. However, it is clear that N-acetylmannosamine labels at least some materials which are transported rapidly.

II. STUDIES WITH ACETOXYCYCLOHEXIMIDE (AXM)

Protein synthesis inhibitors have been useful tools for studying the axonal transport of labeled proteins. The axonal transport process itself is independent of protein synthesis, and continues normally in nervous tissue in which protein synthesis has been inhibited by the drugs puromycin or acetoxycycloheximide. Therefore, these inhibitors can be applied locally to different parts of the neuron to determine where labeled proteins were originally synthesized (Peterson et al., 1967; Ochs et al., 1967; McEwen and Grafstein, 1968; Sjöstrand and Karlsson, 1969; Ochs et al., 1970).

There are no specific inhibitors of glycosyltransferases. However, the incorporation of sugars into glycoproteins can be stopped by protein synthesis inhibitors. The mechanism of action is indirect: stopping protein synthesis prevents the synthesis of the polypeptide acceptors to which sugars are transferred. When the inhibitor is first added, there may initially be some sugar incorporation into acceptors which were synthesized before the inhibitor was added. When an inhibitor is added to cells containing glycoproteins in all stages of completion, incorporation of proximal sugars (sugars nearest to the polypeptide chain) is inhibited first, while incorporation of the terminal sugars ceases last. This sequence is evidence for the view that sugars are transferred sequentially to glycoproteins after the polypeptide backbone has been synthesized (Spiro and Spiro, 1966; Herscovics, 1969).

The protein synthesis inhibitor selected for my studies is acetoxycycloheximide (AXM) (Ennis and Lubin, 1964; Sisler and Siegel, 1967). In most cells AXM acts only on cytoplasmic ribosomes (Sisler and Siegel, 1967; Beattie et al., 1967; Loeb and Hubby, 1968), but in neurons it apparently also inhibits mitochondrial protein synthesis (Goldberg, 1971). Its action is long-lasting. In goldfish brain a 0.2 μ g intracranial dose maintains near-maximal inhibition of protein synthesis for at least 8 hours (Lim et al., 1970). AXM does not inhibit

the fucosyl transferase of rat brain; it inhibits fucose incorporation into glycoprotein, but only by preventing the synthesis of peptide acceptors (Zatz and Barondes, 1971a, b).

We would predict that if axonally transported glycoproteins are synthesized in the retinal ganglion cell perikaryon, inhibition of protein synthesis in the retina would prevent the later appearance of labeled transported glycoproteins in the tectum.

Methods

Small fish were injected in the right eye with a mixture of a ^{14}C -amino acid and a ^3H -sugar in 2 μL of saline. The ^{14}C -amino acid was included to measure the amount of protein synthesis inhibition produced by the AXM. At various times before or after the isotope injection, fish in the AXM groups were injected in the same eye with 0.05 μg of AXM in 1 μL of saline. This intraocular dose of AXM strongly inhibits retinal protein synthesis, but the amount of AXM which escapes from the eye is too small to cause detectable protein synthesis inhibition in the brain (McEwen and Grafstein, 1968; confirmed here). Control fish were injected at the same time with 1 μL of saline. About 24 hours later the fish were sacrificed, and the tecta processed by Method 2. There were three groups of three fish for each time point. The AXM was a gift from Dr. T. J. McBride of the J. L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Maywood, N.J., and was produced there with the support of National Institutes of Health Contract No. PH 43-64-50.

A. EFFECT OF INTRAOCULAR AXM ON THE TRANSPORT
OF RADIOACTIVITY FROM [^3H]GLUCOSAMINE

To determine the effect of intraocular AXM on the transported materials labeled by [^3H]glucosamine, fish were injected with a mixture containing 0.81 μCi of [$6\text{-}^3\text{H}$]D-glucosamine (1.3 Ci/mMole) and 0.10 μCi of [^{14}C]L-leucine (312 mCi/mMole). AXM or saline was injected either 2 hours before or 60 seconds after (= "with") the isotope. The fish were sacrificed 21 hours after the isotope injection.

Figure 1 shows the effect of the intraocular AXM on transported (Figure 22A) and background (Figure 22B) radioactivity. AXM given at either time virtually abolishes the appearance in the tectum of transported, ^{14}C -labeled protein. It also abolishes the much smaller amount of C-M extractable ^{14}C radioactivity. Since radioactive leucine does not label transported lipids (McEwen and Grafstein, 1968), the transported radioactivity in the C-M extract must be in protein, perhaps proteolipid. There also seems to be a tiny TCA-soluble component which is abolished by the AXM; if this effect is real, it is consistent with the hypothesis that the TCA-soluble radioactivity arises from the breakdown of transported labeled protein.

AXM also prevents the arrival in the tectum of almost all of the transported macromolecules labeled by [^3H]glucosamine (Figure 22A). Injections 2 hours before or 1 minute after the isotope were equally effective, producing 94% and 92% inhibition, respectively. This suggests that the incorporation of amino sugars must be quite closely coupled with protein synthesis. If there were a large pool of preformed polypeptide acceptors for the glucosamine derivatives, one would expect to see more incorporation.

The transported radioactivity in the C-M extract is as strongly inhibited by the AXM as the macromolecular component. The transported TCA-soluble component is reduced by the AXM, but not as much as the other components. From 45% to 65% of the transported TCA-soluble radioactivity which would appear in the absence of the drug still

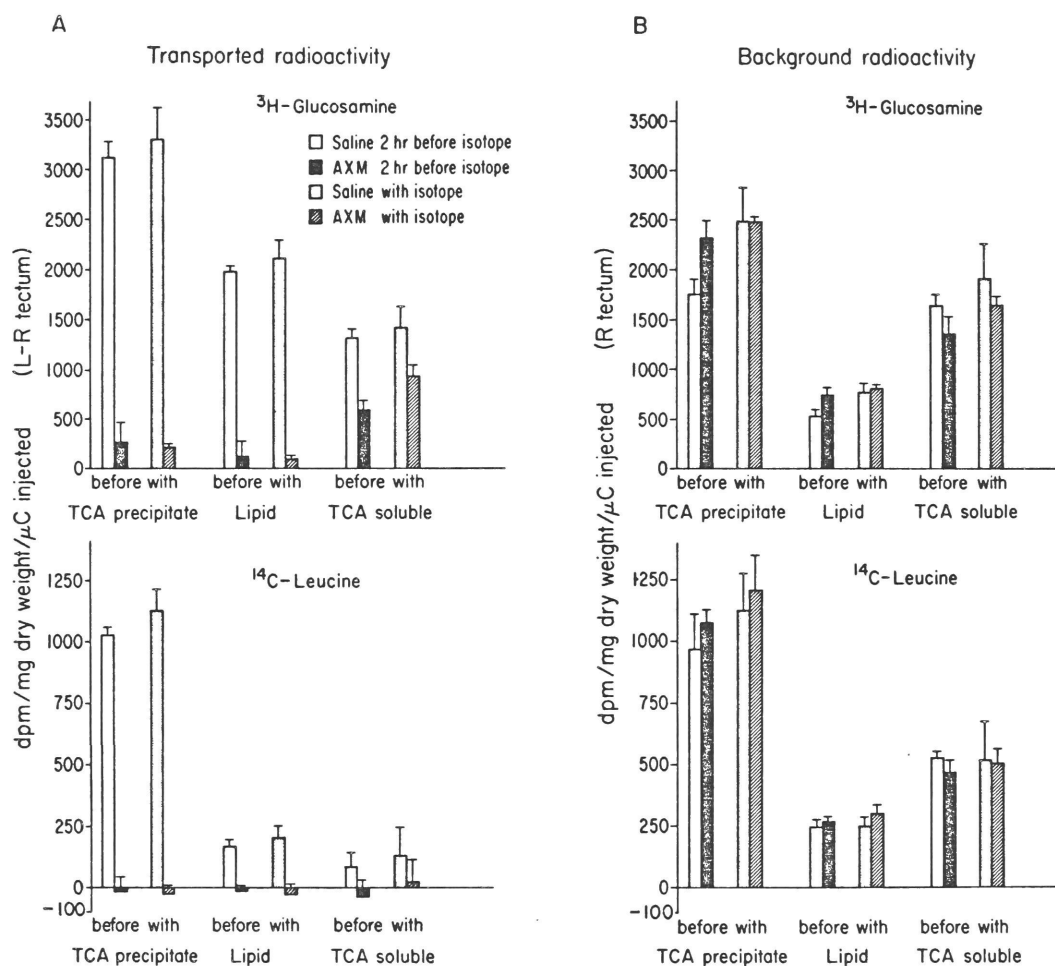


Figure 22. Effect of AXM on transported radioactivity from [^3H]glucosamine. Lines above the bars are standard errors of the mean (N = 3). "Lipid" = C-M extract.

appears after AXM.

Figure 22B shows that although retinal protein synthesis has been severely inhibited by the AXM, the dose used did not effect protein synthesis in the tecta. There was no significant difference in either the ^{14}C or ^3H background labeling between the experimental and control groups in any of the fractions (t test, $p < 0.5$ level).

B. EFFECT OF INTRAOCULAR AXM ON THE TRANSPORT OF
RADIOACTIVITY FROM [³H]FUCOSE

The isotope injection used to study the effect of AXM on the transport of glycoprotein labeled with [³H]fucose contained 1.34 μ Ci [³H]L-fucose (4.3 Ci/mMole) and 0.15 μ Ci [¹⁴C]L-proline (260 mCi/mMole). AXM or saline was injected 1 minute after the isotope injection. The fish were sacrificed 24 hours after the injection.

The results are shown in Table XII. Fucose incorporation is significantly inhibited by AXM, but not as much as the incorporation of proline. The C-M extractable components are small, and inhibited to the same extent as the protein fraction; presumably the labeled material in the C-M extract is also protein. As in the case of glucosamine, the TCA-soluble component is inhibited to a much lesser degree. In this experiment the control and AXM TCA-soluble groups are not significantly different at the $p < 0.05$ level (t test). In the following experiment (Section C) there was some inhibition.

Table XII. Effect of Acetoxycycloheximide (AXM) on Transported Radio-Activity from [^3H]Fucose

| | Transported DPM/mg dry weight/ μ Ci injected | | |
|-------------------------------------|---|---------------|--------------|
| | Control | AXM | % Inhibition |
| <u>[3H]Fucose</u> | | | |
| TCA-precipitate | 5141 \pm 363 | 916 \pm 50 | 82 |
| Chloroform- methanol extract | 234 \pm 10 | 46 \pm 10 | 80 |
| TCA-soluble | 613 \pm 43 | 540 \pm 50 | 12 |
| <u>[14C]Proline</u> | | | |
| TCA-precipitate | 6773 \pm 48 | 180 \pm 5 | 98 |
| Chloroform- methanol extract | 740 \pm 53 | 19 \pm 10 | 98 |
| TCA-soluble | 31 \pm 38 | -0.8 \pm 14 | -- |

C. EVIDENCE FOR A LONG-LIVED [^3H]FUCOSE POOL IN THE RETINA

When a mixture of [^3H]fucose and a ^{14}C -amino acid is injected into the goldfish eye, transported labeled glycoproteins continue to arrive in the tectum for hours after the arrival of amino-acid-labeled proteins has stopped (Section I C, Figure 14). This difference in pattern could be due to a difference in the length of time during which the precursors are available for incorporation. The incorporation of amino acids is complete within a few hours after injection (McEwen and Grafstein, 1968), but [^3H]fucose may be incorporated for a longer time. An alternative possibility is that the incorporation period for fucose is similar to that of the amino acids, but the mechanism of transport of the fucosyl glycoproteins could be slower or in some way fundamentally different, producing a different pattern of labeling in the tectum. We can use AXM to distinguish between these alternatives. If the incorporation period of fucose is the same as that of the amino acids, AXM given after this period is over (e.g. 4 or 6 hours after the isotope injection) should have no effect on the amount of [^3H]fucosyl glycoproteins transported to the tectum. However, if the incorporation period is longer, then the transported glycoprotein component would be inhibited by late AXM injections.

Fish were given an intraocular injection containing 1.0 μCi of [^3H]L-fucose (4.3 Ci/mMole) and 0.21 μCi of [^{14}C]L-proline (209 mCi/mMole). AXM injections were given 2 hours before, 60 seconds after (= "with") or 2, 4, or 6 hours after the isotope. In this experiment the control group did not receive saline injections. The fish were all sacrificed 24 hours after the isotope injection. Tecta were processed by Method 2, but the lipid extraction was omitted.

AXM given with or 2 hours before the isotope prevents the arrival in the tectum of transported proteins labeled with [^{14}C]proline (Figure 23A). When the AXM is given 2 hours after the isotope, a substantial amount of labeled protein is transported. However, a small amount of incorporation of [^{14}C]proline into transported protein appears to continue

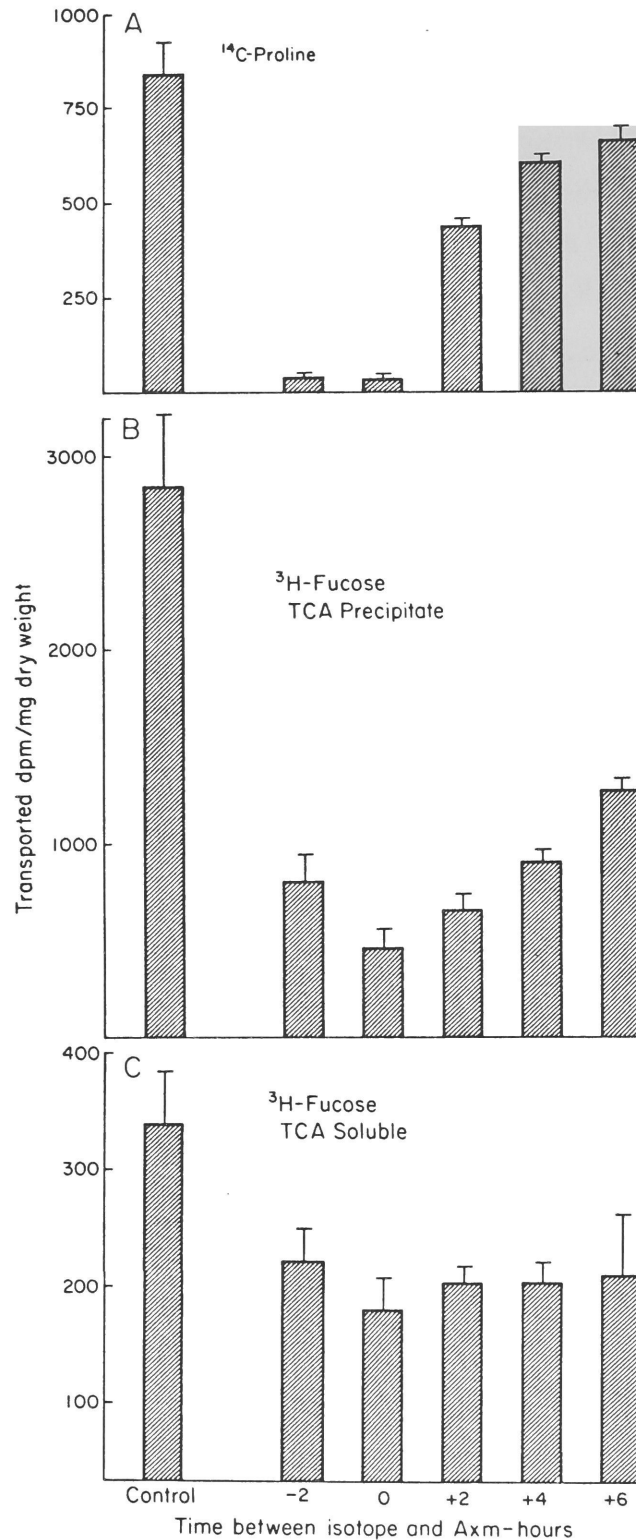


Figure 23. Effect of AXM given at different times on the arrival of transported radioactivity in the tectum. A. Transported proteins labeled with [^{14}C]proline. B. Transported glycoproteins labeled with [^3H]fucose. C. Transported TCA-soluble radioactivity from [^3H]fucose. Lines above the bars show the standard error of the mean (N = 3).

even 6 hours after the isotope injection, although the incorporation is almost complete by that time. Thus, proline is available for protein synthesis longer than leucine, which is incorporated within 2 to 3 hours after the isotope injection (McEwen and Grafstein, 1968). This finding is consistent with the observation that proteins containing [^{14}C]proline continue to leave the eye for a period of about 6 hours (Figure 17).

AXM injected with or 2 hours before the isotope greatly decreases the amount of transported glycoprotein labeled with [^3H]fucose, although the inhibition is less complete than is found with labeled amino acids (Figure 23B). On the other hand, AXM injections given at later times have a greater effect on fucose labeling than on amino acid labeling of transported protein. An injection of AXM given 6 hours after the isotope still produces 55% inhibition. Thus, more than half of the transported glycoprotein is synthesized later than 6 hours after the isotope injection. Note that since the incorporation period for fucose is so long, some of the transported labeled glycoprotein found when AXM was given with or 2 hours before the isotope may have been synthesized after the inhibition had begun to wear off. This may account for the puzzling observation that more labeled glycoprotein is transported when the AXM is injected 2 hours before the isotope (thus presumably depleting any pool of preformed polypeptide acceptors before the isotope is injected) than when the AXM is injected with the isotope (when one would expect some incorporation into newly finished polypeptides). Karlsson and Sjöstrand (1971c) have also concluded that fucose produces a long-lived precursor pool; they base their view on measurements of long-lasting TCA-soluble radioactivity in the rabbit retina after intraocular injection of [^3H]fucose.

Figure 23C shows the transported TCA-soluble ^3H radioactivity. In this experiment there was more inhibition when AXM was given with the isotope (50% inhibition) than in the preceding experiment (Section B, 12% inhibition). As in the previous experiments with labeled glucosamine and fucose, AXM inhibited the transported TCA-soluble radioactivity much less than the transported glycoprotein component.

The effect of AXM on the TCA-soluble material does not appear to be markedly time-dependent.

Discussion

When protein synthesis is inhibited in the retina, axonally transported glycoproteins fail to appear in the tectum even though tectal glycoprotein synthesis is unimpaired. Since protein synthesis inhibitors do not interfere with the rapid axonal transport mechanism (McEwen and Grafstein, 1968; Ochs and Johnson, 1969; Sjöstrand and Karlsson, 1969; Ochs *et al.*, 1970), the inhibitors must achieve their effect by preventing the synthesis of the transported glycoproteins. Thus, the results of the AXM experiments support the hypothesis that the oligosaccharide moieties (as well as the polypeptide portions) of the glycoproteins studied here are assembled in the retinal ganglion cell perikaryon and then axonally transported. This belief is strengthened by autoradiographic evidence that in neurons, as in other cells, the main sites of the incorporation of sugars into glycoproteins are the endoplasmic reticulum and Golgi apparatus in the cell body (Droz, 1967; Rambourg and Droz, 1969; C. P. Leblond, personal communication). The results presented here do not exclude the possibility that sugars are also incorporated into some types of glycoproteins in the axons and endings. The intraocular injection method selectively demonstrates axonally transported materials, and would give no indication of purely local synthesis if the source of the precursors is also local. It is worth noting, however, that since fucose is found only in the terminal positions of oligosaccharides (Ginsburg and Neufeld, 1969), at least some glycoprotein chains must be completed in the retina. The incorporation of labeled sulfate into axonally transported mucopolysaccharide proteins in the goldfish visual system is also inhibited by a small intraocular dose of AXM (Elam and Agranoff, 1971b).

Barondes and his co-workers have extensively studied the biosynthesis of glycoprotein in mouse brain, with special emphasis on the labeling of a synaptosomal fraction (which contains fragments from

nerve endings). AXM inhibits the incorporation in vivo of labeled glucosamine (Barondes and Dutton, 1969), fucose (Zatz and Barondes, 1970), and NANA (DeVries and Barondes, 1971) into most subcellular fractions. However, the incorporation of radioactivity from glucosamine into soluble proteins from the synaptosomal fraction occurs very rapidly and is uniquely insensitive to AXM. This was interpreted as evidence that some carbohydrate is locally incorporated into glycoproteins in the nerve endings (Barondes, 1968b; Barondes and Dutton, 1969). On the other hand, the incorporation of fucose (Zatz and Barondes, 1971) and NANA (DeVries and Barondes, 1971) into all synaptosomal subfractions, and also the incorporation of radioactivity from glucosamine into particulates from synaptosomes (Zatz and Barondes, 1971), is delayed in comparison to the incorporation in other subcellular fractions, and is inhibited by AXM. These observations suggested that many nerve ending glycoproteins are synthesized in the cell body, and then rapidly transported to the nerve endings. That conclusion is strengthened by the finding that fucosyltransferases are concentrated in microsomal fractions (which include fragments from the endoplasmic reticulum and Golgi apparatus). There is relatively little fucosyltransferase activity in synaptosomes and other fractions (Zatz, 1970; Zatz and Barondes, 1971b). As in our experiments, Barondes and his co-workers demonstrated that glycoproteins are axonally transported rapidly (Zatz and Barondes, 1971a), but found no evidence for any slow transport of glycoproteins (Barondes, 1968b; Zatz and Barondes, 1970).

The transported TCA-soluble radioactivity is not reduced by AXM as much as the other components. The decrease in the TCA-soluble component which does occur with AXM suggests that some of the transported TCA-soluble material arises from the breakdown of the other components. The observation that the transported TCA-soluble pool does not increase with AXM is evidence that the TCA-soluble material is not serving as a precursor for the local synthesis of the other components in the axons and endings.

The transported radioactivity from glucosamine in the C-M extract was as strongly inhibited by AXM as the macromolecular component. This might seem to suggest that the labeled material in the C-M is glycoprotein instead of lipid. The presence of radioactivity from [^{14}C]leucine and [^{14}C]proline in lipid extracts, as well as direct protein analysis, shows that C-M does extract some protein. However, several authors have reported that the incorporation of sugars into gangliosides in brain is inhibited by protein synthesis inhibitors (Kanfer and Richards, 1967; Barondes and Dutton, 1969; DeVries and Barondes, 1971; Shah and Peterson, 1971. McCluer and Agranoff, 1972 report negative results). The effect is peculiar to gangliosides; the synthesis of some other glycosphingolipids is not stopped when protein synthesis is inhibited (Kanfer and Richards, 1967; Benjamins et al., 1971). Therefore, the transported C-M extractable radioactivity from glucosamine which is inhibited by AXM might be either ganglioside or glycoprotein. The question of whether gangliosides are axonally transported is considered in Section IV.

III A. STUDIES OF THE FATE OF INTRAOCULARLY INJECTED [^3H]L-FUCOSE

When labeled fucose is injected into animals, the only radioactivity which is incorporated into macromolecules is in fucosyl residues of glycoproteins (Coffey *et al.*, 1967; Bekesi and Winzler, 1967; Zatz and Barondes, 1970; Quarles and Brady, 1970; Margolis and Margolis, 1971). To confirm that this is also the case in the goldfish optic system, I subjected TCA precipitates of tecta containing transported radioactivity from [^3H]fucose to acid hydrolysis, and used thin-layer chromatography to show that the radioactivity which was released by hydrolysis was in free fucose. In the course of these experiments I also made some observations on the nature of the TCA-soluble radioactivity, and on the fate of the injected radioactivity which does not appear in the retino-tectal pathway. In a few experiments, radioactivity from [^3H]glucosamine was also examined.

Methods

Preparation of macromolecules and acid hydrolysis. Five pond goldfish per experiment were injected in both eyes with 5.0 μCi of [^3H]L-fucose (4.3 Ci/mMole) and sacrificed 24 hours later. In these experiments both left and right tecta contained transported radioactivity; background labeling was ignored. Five tecta were homogenized in 2.0 ml of ice-cold distilled water and the homogenate was brought to a volume of 5.0 ml containing 10% TCA by the addition of water and 50% TCA. After an hour, the precipitate was separated from the supernatant by centrifugation and washed with an additional 5.0 ml of 10% TCA. The precipitate was then subjected to a series of lipid extractions: it was treated twice with C-M, 3:1, twice with 95% ethanol, and once with diethyl ether (5.0 ml of solvent per extraction), and then air dried. The powdered residue was subjected to hydrolysis in 0.5 ml of acid in a Teflon capped vial under nitrogen in a Temp-Blok (Labline Instruments, Inc., Melrose Park, Illinois). Three conditions were tried: 1) 0.1 N

HCl at 80°C for 3 hours, 2) 1.0 N HCl at 100°C for 24 hours, and 3) 0.02 N HCl in the presence of a Dowex 50 resin (Ag 50 W-X2, BioRad Laboratories, see below) for 3 hours at 100°C. The hydrolysis mixture was then dried under a stream of nitrogen. The mixture was redissolved and dried several times to remove the last traces of HCl.

Removal of ions. Since fucose is a neutral sugar it can be separated from charged molecules by passage through Dowex ion exchange resins (Spiro, 1966). The advantage of this procedure is that it removes ions which interfere with thin-layer chromatography, and also removes the unhydrolyzed protein. The cation exchange resin used was AG 50W-X2, 200-400 mesh (BioRad Laboratories, Richmond, California), washed with HCl and used as a 40% slurry in 0.02 N HCl. This resin was also used for some acid hydrolyses (i.e., the Dowex 50 resin in condition 3, Table XIV). The anion exchanger was AG 1-X8, formate form, 200-400 mesh (BioRad), washed with distilled water and used as a 50% slurry in distilled water. Small columns were made by pipetting 0.4 ml of slurry into Pasteur pipettes plugged with glass wool, and washing with distilled water. The hydrolyzate, including the suspended protein residue, was applied to coupled columns of AG 50 and AG 1 in 1.0 ml of distilled water, and washed with 3.0 ml of distilled water. A sample of the effluent (the "neutral sugar fraction" which contains the free fucose) was taken for scintillation counting and the rest was evaporated to dryness under nitrogen at 50°C. The resins and protein residue were recovered, dried, and incubated in 1.0 ml of Soluene-100 to solubilize radioactivity for scintillation counting. In some experiments radioactivity on the columns was eluted with 2N HCl (the AG 50 column) or concentrated formic acid (the AG 1 column) and counted separately.

Cellulose thin-layer chromatography. Materials were chromatographed on Eastman Cellulose Chromagram Sheets #6064 in an Eastman Chromagram Developing Apparatus. Three chromatographic systems were used: A) ethyl acetate - pyridine - acetic acid - water, 5:5:1:3, developed twice (Klenck et al., 1970). B) n-butanol - pyridine - 0.1 N HCl, 5:3:2, developed once. C) n-butanol - ethanol - water, 10:1:2,

developed twice (Spiro, 1966). The hydrolyzate was dissolved in a volume of water and applied to the cellulose sheets as a streak 1.5 cm wide. Samples of appropriate sugars (2-5 μ g) were run in separate lanes. These lanes were removed from the sheet and the position of the standards was visualized by a AgNO_3 -NaOH method as modified by Dr. H.-D. Klenck: the chromatograms are sprayed with a reagent containing 0.5 ml saturated AgNO_3 in 100 ml of acetone, with enough water added to redissolve the white precipitate which forms (about 3.0 ml). After this dries, the plates are sprayed with methanolic NaOH (2 gm NaOH dissolved in a minimum of water, per 100 ml of methanol). Sugars (and some other substances) appear as brown spots. After waiting 5-10 minutes for full color development, the chromatograms can be sprayed with 5% sodium thiosulphate in 50% methanol to intensify and preserve the spots.

Radioactivity in the chromatographed hydrolyzates was determined by liquid scintillation counting. Strips of the Chromogram sheet (0.25-1.0 mm wide) were soaked for a few minutes with 0.5 ml Soluene-100 in plastic vials, and then 10 ml of the standard toluene scintillation fluid was added. Before putting the vials in the counter they were placed for a minute in an ultrasonic bath (Heat Systems Electronics, Inc., Plainview, L.I., N.Y.) to dislodge any cellulose still on the plastic backing. In pilot experiments, the Soluene and sonication steps were found to be necessary for reliable recovery of counts. A sample of hydrolyzate applied to a cellulose strip without chromatography was used to measure the recovery of radioactivity.

TCA-soluble radioactivity. The TCA-supernatants were extracted three times with diethyl ether to remove the TCA. To determine the amount of tritiated water and non-volatile radioactivity, one of two duplicate aliquots was evaporated to dryness and redissolved in water, and then the samples were counted in Brays scintillator. The remainder was evaporated to dryness and studied by thin-layer chromatography. In some experiments the samples were passed through ion exchange resins (see "Removal of Ions"), and in some experiments the samples were heated with HCl to hydrolyze acid-labile compounds.

Measurement of radioactivity in fish tank. Three 1.0 ml samples were removed from a tank of measured volume at various times after [^3H]fucose injection. One sample was counted directly in Bray's scintillator, and the second was evaporated to dryness and redissolved in 1.0 ml of distilled water before counting in Bray's scintillator. The third sample was evaporated to dryness, incubated with 0.5 ml Soluene-100, and counted in a toluene scintillator.

Measurement of radioactivity in structures of the eye. Whole eyeballs which had been injected with [^3H]fucose were immersed overnight in 10 ml cold 10% TCA, and then transferred to cold 70% alcohol. The retina, lens, and other tissues of the eye were dissected out and either dissolved in Soluene-100 or wrapped in filter paper and combusted in the Packard Model 305 sample oxidizer.

Results

Appearance of radioactivity in the fish tank. The appearance of radioactivity in the fish tank is shown in Figure 24. Although the radioactivity present 15 minutes after the injection is probably due to the leakage of [^3H]fucose from the injection site, the gradual, nearly linear increase thereafter suggests that radioactivity is being excreted by the fish. Most of this radioactivity is volatile, and hence is presumably tritiated water. The measurements of non-volatile radioactivity in Bray's scintillator and in a toluene scintillator after Soluene solubilization gave closely similar results (hence the low level of the non-volatile radioactivity is not an artifact due to the precipitation of labeled protein in the Bray's solution). By 24 hours after the injection, 54% of the injected radioactivity is in the water of the tank, and 80-90% of that radioactivity is in tritiated water.

Fate of injected radioactivity. Table XIII shows the radioactivity found 24 hours after injection in some structures of interest. About one-third of the injected dose is not accounted for in Table XIII. Presumably that radioactivity is present elsewhere in the body of the goldfish.

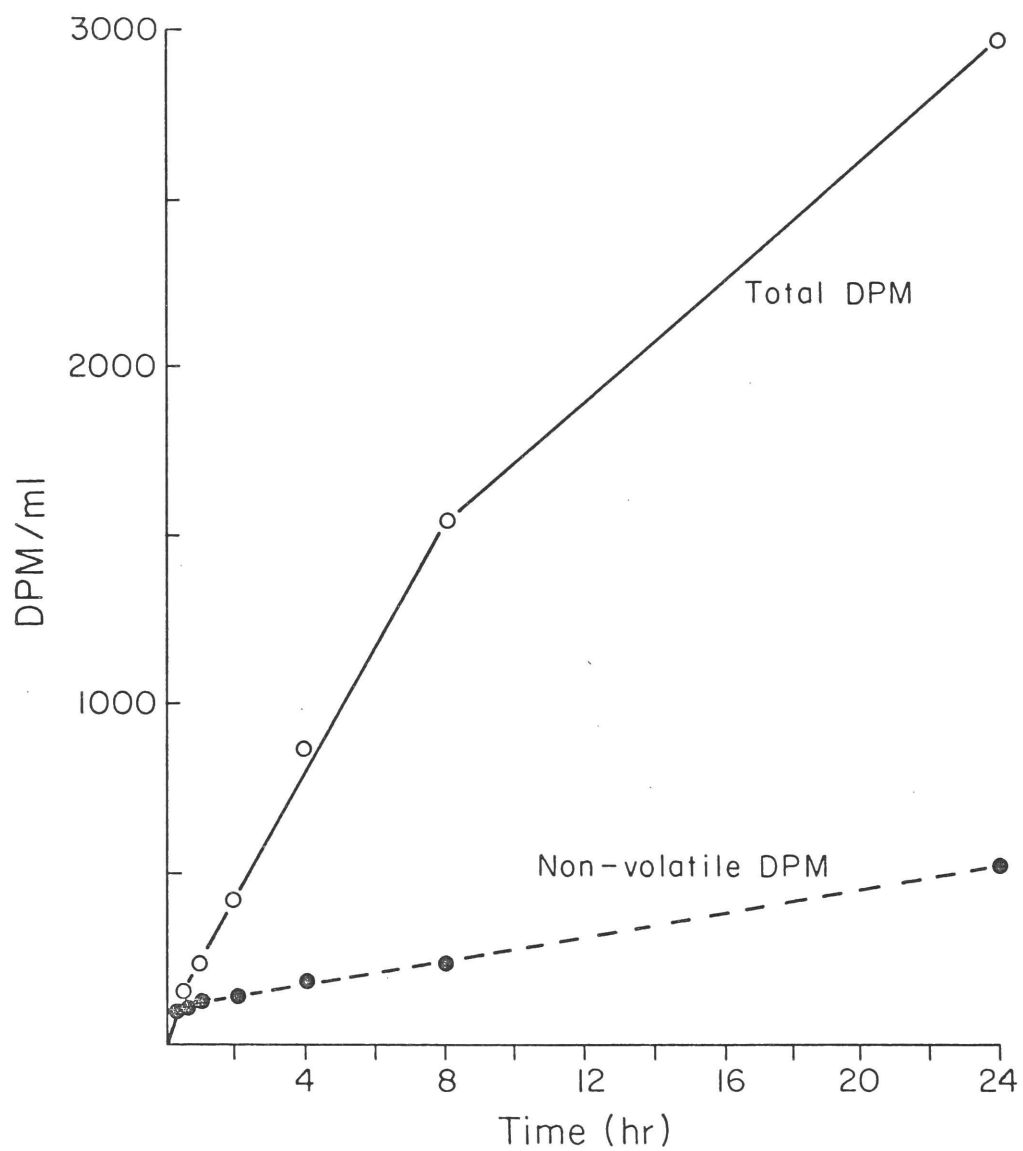


Figure 24. Appearance of radioactivity in fish tank water after an intraocular injection of [^3H]L-fucose.

Table XIII. Fate of Injected Radioactivity 24 Hours after Intraocular
Injection of [^3H]Fucose

| | % of Total Injected Radioactivity | |
|----------------------------|-----------------------------------|--------------|
| Tank water | 54 | |
| As tritiated water | | 45-49 |
| Non-volatile | | 5-9 |
| TCA-soluble, in eye | 4 | (varies 1-8) |
| Incorporated in eye | 7.5 | |
| In retina | | 6.9 |
| In other structures of eye | | 0.6 |
| Transported protein | 0.2 | |
| Nerve, TCA precipitate | | 0.04 |
| Tract, TCA precipitate | | 0.02 |
| Tecta, TCA precipitate | | 0.14 |
| Tecta, TCA-soluble | | 0.007 |
| accounted for: | 66% | |

Acid hydrolysis and thin-layer chromatography of TCA-precipitable radioactivity. The amount of radioactivity which was released from TCA precipitates by acid hydrolysis and recovered in the neutral sugar fraction is shown in Table XIV. Relatively mild hydrolysis conditions (1, and especially 3) were sufficient to release most of the radioactivity. This is typical of fucose, which is usually more easily hydrolyzed from oligosaccharides than some of the more proximal sugars (Spiro, 1966). Nearly all of the radioactivity could be released with more vigorous conditions (2). The values in Table XIV are for radioactivity which was not recovered remained in the protein residue; very little was adsorbed by the ion exchange resins.

When the materials in the neutral sugar fraction were separated by cellulose thin-layer chromatography, most of the radioactivity appeared in a single peak which moved with the mobility of L-fucose (Table XV). The results from a typical chromatographic separation are shown in Figure 25. Taking into account the radioactivity lost during hydrolysis or on the ion-exchange resins (Table XIV), at least 80%, and at best (98% X 94% =) 92% of the total radioactivity in the TCA-precipitate was recovered as free [³H]fucose.

The analysis of the incorporated radioactivity from [³H]glucosamine is more difficult, since optimal hydrolysis conditions must be determined for each sugar. If the conditions are too mild the sugars are not removed from the glycoprotein; if the conditions are too strong the sugars are destroyed. The conditions which are needed to remove all the hexosamine almost inevitably destroy all of the sialic acids. The deacetylation of N-acetylhexosamines during acid hydrolysis must also be taken into consideration (Spiro, 1966; Graham et al., 1970). I did not study the nature of the radioactivity from glucosamine thoroughly. In several experiments, I hydrolyzed lipid-free TCA precipitates from tecta containing radioactivity from glucosamine in 0.02 N HCl in the presence of Dowex-50 (H⁺ form) at 100°C for 24 hours. From 70% to 95% of the radioactivity was released from the precipitate. When the

Table XIV. Acid Hydrolysis

| Hydrolysis Conditions | % of Radioactivity in TCA Precipitate Recovered in Neutral Sugar Fraction after Acid Hydrolysis |
|---------------------------------------|--|
| 1) 0.1 N HCl, 80°C, 3 hr | 91 |
| 2) 1 N HCl, 100°C, 24 hr | 98 |
| 3) 0.02 N HCl + Dowex 50, 100°C, 3 hr | 88 |

Table XV. Identification of Radioactivity in Neutral Sugar Fraction
from Acid Hydrolyzate by Cellulose Thin-Layer Chromatography

| Chromatographic System | Hydrolysis Conditions (see Table XIV) | % of Recovered CPM Moving with [3 H]Fucose | % Recovery of CPM Applied to Chromatogram |
|------------------------|--|---|---|
| A | 1 | 91 | 104 |
| | 2 | 94 | - |
| | 3 | 80 | - |
| B | 1 | 93 | 101 |
| C | 1 | 90 | 81 |

Chromatographic Systems

- A. Ethyl acetate - pyridine - acetic acid - water, 5:5:1:3
- B. n-Butanol - pyridine - 0.1 N HCl, 5:3:2
- C. n-Butanol - ethanol - water, 10:1:2

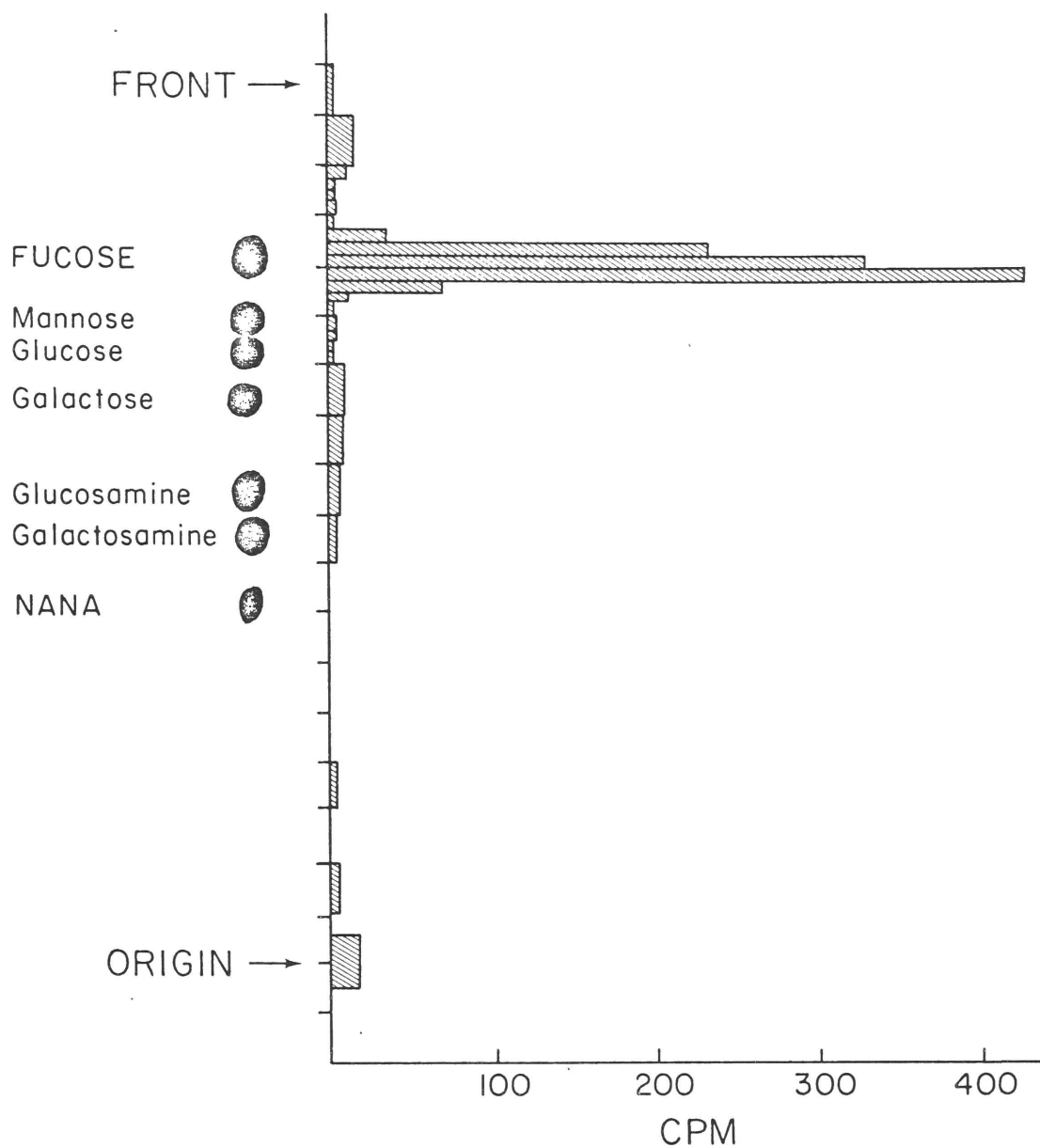


Figure 25. Recovery of transported TCA-precipitable radioactivity as free [^3H]fucose after acid hydrolysis and cellulose thin-layer chromatography. Hydrolysis conditions 1, chromatographic system A (see Tables XIV and XV). Spots are tracings of standard sugars. Distance along chromatogram is divided into centimeters. Bars show CPM in strip of indicated position and width.

hydrolyzate was passed through coupled Dowex columns a variable amount, usually about 70%, was retained by the Dowex-50; this could all be eluted with 2 N HCl. About 70% of the eluted radioactivity moved with the mobility of the hexosamines in chromatographic system A. Almost all of the radioactivity moved with glucosamine, but glucosamine and galactosamine were not sufficiently separated to allow calculation of the amount of galactosamine. Most of the radioactivity which passed through both Dowex columns moved with the mobility of the N-acetylhexosamines, and after treatment at 100° for 3 hours in 1.0 N HCl this material also moved with the mobility of the hexosamines. Again most of the radioactivity was glucosamine. Overall, I was able to recover at least 50% of the incorporated radioactivity as hexosamine, mainly as glucosamine. I did not determine the amount of radioactivity in sialic acid. Although these experiments are not definitive, they suggest that at least half of the transported TCA-precipitable radioactivity from [³H]glucosamine is incorporated as N-acetylhexosamine, almost all of which is N-acetylglucosamine.

TCA-soluble radioactivity. About 10% to 20% of the TCA-soluble radioactivity in the tectum 24 hours after injection was volatile, and is probably tritiated water. (Note that TCA-soluble radioactivity discussed elsewhere in this thesis refers only to non-volatile radioactivity, except where noted.) A variable amount of the non-volatile radioactivity moved with the mobility of free fucose; this ranged from 23% to 62% in different experiments. There was also usually a peak at the origin and radioactivity spread from the origin to the fucose peak. Much of the unidentified radioactivity could be adsorbed by a Dowex 1 column. There is some indication that some of the radioactivity which did not move with fucose is in fucose derivatives, perhaps GDP-fucose or fucose-1-P. The amount of radioactivity in the fucose peak was increased by heating, acid hydrolysis, or aging. In one experiment, when the TCA-soluble radioactivity which had been adsorbed by the Dowex 1 column (and hence was not free fucose) was eluted with concentrated formic acid, 90% of the eluted radioactivity moved with the mobility of

free fucose. Presumably, this material was converted to free fucose by the formic acid treatment.

The results of some experiments on the TCA-soluble radioactivity from labeled glucosamine were similar: a variable amount of the TCA-soluble radioactivity, usually 20 to 30%, passed through Dowex columns and moved in chromatographic system A with the mobility of the N-acetylhexosamines, mostly N-acetylglucosamine. Evidence that this material was N-acetylhexosamine was provided by heating in 1.0 N HCl for 2 1/2 hours at 100°C to de-acetylate it; it then moved with the mobility of the hexosamines, mainly glucosamine. There were also several other peaks of radioactivity, some of which were adsorbed by the Dowex columns. With aging or heating, the size of these peaks declined and the size of the N-acetylhexosamine peaks increased. By acid hydrolysis, 40%-60% the radioactivity in the other peaks could be converted to materials which moved with the mobility of N-acetylhexosamine or hexosamine. These results suggest that much of the TCA-soluble radioactivity from [³H]glucosamine is N-acetylglucosamine or its metabolites, perhaps UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, and N-acetyl-glucosamine-6-P (see Figure 5). In these experiments I used both [³H]glucosamine and [¹⁴C]glucosamine with a symmetrical double-labeling experimental design (see Section III C), and thus was able to compare transported and background radioactivity. Transported and background TCA-soluble radioactivity from glucosamine had similar patterns at all stages of the analysis.

Discussion

The main finding of this section is that when [³H]L-fucose is injected into the goldfish eye, the transported TCA-precipitable radioactivity is almost entirely in fucosyl residues of glycoproteins; the fucose is not converted to any other incorporated sugar. This agrees with the results of other studies of the metabolism of L-fucose in the brain (Zatz and Barondes, 1970; Quarles and Brady, 1971; Margolis and Margolis, 1971; Karlsson and Sjöstrand, 1971c) and in other animal

tissues (Coffey et al., 1967; Bekesi and Winzler, 1967; Bosmann et al., 1969). The large amount of tritiated water which appeared in the tank could be due to metabolism or ^3H exchange in the goldfish (S. Bondy, unpublished results; R. Margolis, unpublished results), or to metabolism by enteric or other bacteria (Bocci and Winzler, 1969). In any event, when the labeled L-fucose is converted to other substances, little or no radioactivity finds its way into transported glycoproteins. In a single experiment in which D-fucose was tried instead of L-fucose ([6- ^3H]D-fucose, Calatomic, 200 mCi/mMole), a small amount of TCA-precipitable radioactivity was transported. This radioactivity could be released by acid hydrolysis, and it then migrated with a mobility which was quite different from that of D- or L-fucose.

The non-volatile TCA-soluble radioactivity apparently included some free fucose and other labeled materials. The evidence suggest that some of the other materials are unstable derivatives of fucose. Since GDP-fucose is an unstable compound which is especially sensitive to acid hydrolysis (Ishihara and Heath, 1968), it is a likely candidate. Since my experiments were not designed to minimize GDP-fucose breakdown, it is impossible to estimate how much of the radioactivity recovered as free fucose may have originally been in GDP-fucose. I did not pursue these experiments far enough to rigorously confirm the presence of GDP-fucose. The results with the TCA soluble radioactivity from glucosamine were similar; most of the radioactivity was apparently in N-acetylhexosamines and unstable derivatives of the N-acetylhexosamines. Although these studies are incomplete, none of the data are inconsistent with the results of previous studies of the metabolism of L-fucose (Foster and Ginsburg, 1961; Bekesi and Winzler, 1967; Ishihara and Heath, 1967) and D-glucosamine (McGarrahan and Maley, 1962; Molnar et al., 1964; Kornfeld et al., 1964; Davidson, 1966) in animal tissues.

III B. [³H]FUCOSE LABELS MAINLY PARTICULATE PROTEINS

A simple way to distinguish between slowly and rapidly transported proteins labeled with radioactive amino acids is to homogenize the tissues containing the transported proteins and use ultracentrifugation to separate the proteins which are bound to sedimentable subcellular particles ("particulate" proteins) from the proteins which remain in the supernatant ("soluble" proteins). Rapidly transported proteins are mainly particulate; a large proportion of the slowly transported proteins are soluble (McEwen and Grafstein, 1968; Bray and Austin, 1969; Ochs et al., 1969; Sjöstrand and Karlsson, 1969; Sjöstrand, 1970; Cuénod and Schonbach, 1971). McEwen showed that the rapidly transported glycoproteins labeled with radioactivity from [³H]glucosamine are also mainly particulate (Table IV). I examined transported glycoproteins labeled with [³H]fucose; they too are mainly particulate.

Methods

Four pond fish per experiment were injected with 4.1-4.4 μ Ci of [³H]L-fucose (4.3 Ci/mMole, New England Nuclear) in the right eye and sacrificed 24 hours or 23 days later. Pooled left or right tecta or optic tracts were vigorously homogenized in 2.0 ml ice-cold distilled water with a Dounce homogenizer, and spun at 101,000 X g_{av} for 75 minutes in a Spinco Model 40 rotor in the Spinco Model L Ultracentrifuge. The supernatants were removed with a Pasteur pipette. The soluble proteins in the supernatant and the particulate macromolecules in the centrifugal pellet were precipitated with TCA and processed by Method 4. The TCA precipitates were dissolved in 1.0 N NaOH. Protein was determined by the method of Lowry et al. (1951), and radioactivity was measured using the Packard Model 305 Oxidizer. In some cases lipids were extracted with 2 X 2.0 ml of C-M, 1:1, and analyzed separately for protein and radioactivity.

Results

Table XVI shows the distribution of protein in the optic tracts and tecta. About 34% of the protein in the tracts, and 44% of the protein in the tecta is liberated as soluble protein. This distribution of protein is essentially the same as was found by McEwen (McEwen and Grafstein, 1968; also confirmed in the experiment shown in Table IV). However, in both the optic tracts (Table XVII A) and optic tecta (Table XVIII A) the proportion of the total protein-bound transported radioactivity which appears in the soluble fraction at 24 hours is very low, less than 10% in all cases. This is true both 1 and 23 days after the injection. The concentration of radioactivity in soluble protein is much lower than in particulate protein in both tracts (Table XVII B) and tecta (Table XVIII B). Background incorporation of [^3H]fucose is low, and usually includes a greater proportion of soluble protein than the transported component.

Discussion

The finding that most of the transported glycoprotein labeled with [^3H]fucose is particulate is not surprising, since most of the glycoprotein of the nervous system is particulate (Brunngraber, 1969a; 1970). The finding is interesting, however, in relation to the evidence that glycoproteins are transported only in the rapid component of axonal transport. In a variety of neurons, rapidly transported proteins labeled with radioactive amino acids are mainly particulate, while a large fraction of the total protein of nervous tissue, and also a large fraction of the slowly transported protein, is soluble. Transported glycoproteins labeled by [^3H]fucose, by radioactivity from [^3H]glucosamine (Table IV), and by $^{35}\text{SO}_4$ (Elam and Agranoff, 1971b) are mainly particulate. It is especially interesting that transported glycoproteins labeled with [^3H]fucose are mainly particulate even 23 days after the injection (as is also true of transported glycoproteins labeled by [^3H]glucosamine - Table IV). If some of the slowly transported soluble proteins are glycoproteins which contain fucose, their presence might

Table XVI. Protein and Per Cent Soluble Protein in Optic Tracts and Tecta

| | <u>Tecta</u> | <u>Tracts</u> |
|---|----------------|---------------|
| Total Protein per Tectum or per Tract (μ g)* | 640 \pm 50 | 82 \pm 10 |
| % Soluble* | 44.1 \pm 1.5 | 34 \pm 2 |

*Mean \pm standard error of the mean (N = 4).

Table XVII. TCA-Precipitable Radioactivity in Optic Tracts after
Intraocular Injection of [^3H]Fucose

A. Distribution of radioactivity (DPM/mg total protein per μCi injected)

| | <u>Transported</u> | <u>%*</u> | <u>Background</u> |
|---------------------|--------------------|-----------|-------------------|
| 1 DAY | | | |
| Particulate protein | 2865 | 92 | 212 |
| Soluble protein | 243 | 8 | 31 |
| 23 DAYS | | | |
| Particulate protein | 3913 | 95 | 62 |
| Soluble protein | 188 | 5 | 50 |

B. Concentration of Radioactivity (DPM/mg protein in fraction per μCi injected)

| | <u>Transported</u> | <u>Background</u> |
|---------------------|--------------------|-------------------|
| 1 DAY | | |
| Particulate protein | 4540 | 355 |
| Soluble protein | 735 | 88 |
| 23 DAYS | | |
| Particulate protein | 5922 | 177 |
| Soluble protein | 511 | 164 |

*Per cent of transported TCA-precipitable radioactivity.

Each value is the average of two experiments. Because the 1 day and 23 day experiments were performed on different shipments of fish of slightly different size, the amount of labeling in the two groups is not exactly comparable. Hence, the apparent increase in the radioactivity in transported particulate protein between 1 and 23 days may not be significant.

Table XVIII. TCA-Precipitable Radioactivity in Optic Tecta after
Intraocular Injection of [^3H]Fucose

| A. <u>Distribution of radioactivity</u> (DPM/mg total protein per μCi injected) | | | |
|---|--------------------|-----------|-------------------|
| | <u>Transported</u> | <u>%*</u> | <u>Background</u> |
| 1 DAY | | | |
| Particulate protein | 3147 | 94 | 51 |
| Soluble protein | 208 | 6 | 39 |
| 23 DAYS | | | |
| Particulate protein | 3293 | 97 | 49 |
| Soluble protein | 94 | 3 | 14 |
| B. <u>Concentration of radioactivity</u> (DPM/mg protein in fraction per μCi injected) | | | |
| | <u>Transported</u> | | <u>Background</u> |
| 1 DAY | | | |
| Particulate protein | 5752 | | 86 |
| Soluble protein | 493 | | 70 |
| 23 DAYS | | | |
| Particulate protein | 5971 | | 90 |
| Soluble protein | 212 | | 57 |

*Per cent of transported TCA-precipitable radioactivity.

Each value is the average of two experiments.

have been detected as an increase between 1 and 23 days in the proportion of transported soluble glycoprotein. The absence of any increase is compatible with the possibility that glycoproteins are transported only rapidly. The particulate nature of rapidly transported proteins suggests that they are components of membraneous organelles. Present methods do not shed any light as to whether the small amount of rapidly transported soluble proteins are transported as free soluble proteins, are inside organelles which are lysed by homogenization in distilled water, or form some other association with particulate protein which is disrupted by water treatment.

Zatz and Barondes (1971a) recently reported that glycoproteins labeled with [³H]fucose are rapidly transported in mouse brain. Their evidence is based on the dynamics of the labeling of glycoproteins in a synaptosomal fraction (which contains fragments of nerve endings). Although they also presented evidence for the axonal transport of particulate glycoproteins, they placed special emphasis on the axonal transport of soluble proteins obtained from the synaptosomal fraction by lysis with distilled water. Zatz and Barondes emphasized the soluble proteins because they are less contaminated with proteins from sources other than nerve endings than are the particulates from the synaptosomal fraction (Barondes, 1968b). However, the soluble proteins of the synaptosomal fraction constitute only 14.6% of the total synaptosomal protein (Table III of Barondes, 1968). One can calculate that most of the radioactivity in their synaptosomal fraction is particulate (Table XIXA). However, one cannot calculate exactly how much of this radioactivity is transported and how much is due to local incorporation or contamination from sources other than nerve endings. Since Figures 2 and 3 of their paper (Zatz and Barondes, 1971a) suggest that most of the rapidly transported glycoprotein in the synaptosomal fraction arrives in the nerve endings between 2 and 4 hours after the intracerebral injection, the radioactivity which appears in the synaptosomes between 2 and 4 hours is probably mostly transported rather than locally synthesized. But even if one looks only at the increase in labeling in the synaptosomal subfractions that occurs

Table XIX. Calculation of the Amount of Particulate and Soluble Protein-bound Radioactivity in Synaptosomal Fractions from Mouse Brain after Intracerebral Injection of [^3H]Fucose

| | Protein* | Whole† Synaptosomal Protein | Per cent of Total Radioactivity |
|----------------------------------|----------|-----------------------------------|---------------------------------------|
| | cpm/mg | cpm/mg | |
| A. <u>4 Hours after Isotope</u> | | | |
| Soluble | 1235 | 180 | 8 |
| Particulate | 2472 | 2111 | 92 |
| B. <u>4 Hours minus 2 Hours§</u> | | | |
| Soluble | 967 | 141 | 12 |
| Particulate | 1240 | 1059 | 88 |

*From Zatz and Barondes (1971a), Table I. Results expressed as cpm in soluble protein/mg soluble protein, and cpm in particulate protein/mg particulate protein.

†Results expressed as cpm/mg total synaptosomal protein. Based on assumption that 14.6% of protein of synaptosomal fraction is soluble (Barondes, 1968b).

§See text for rationale of calculation B.

between 2 and 4 hours (Table XIX B), one still finds that most of the labeled glycoprotein is particulate. Thus, although Zatz and Barondes (1971) emphasized soluble proteins for technical reasons, their data is not inconsistent with the bulk of the rapidly transported glycoproteins being particulate.

The particulate nature of rapidly transported glycoproteins labeled with [³H]fucose has been confirmed in the rabbit optic system (Karlsson and Sjöstrand, 1971c).

III C. EXAMINATION OF THE SUBCELLULAR DISTRIBUTION OF
TRANSPORTED RADIOACTIVITY FROM [^3H]GLUCOSAMINE
WITH A SYMMETRICAL DOUBLE-LABELING METHOD

In order to understand the function of axonally transported glycoproteins, it will be necessary to know their subcellular location. Cell fractionation techniques are available for determining the subcellular location of materials in the brain (Whittaker, 1965; de Robertis, 1967) which can be applied to fish brain (Whittaker and Greengard, 1971). I undertook to determine the subcellular location of the axonally transported macromolecules labeled with radioactivity from glucosamine. The results of some differential centrifugation experiments from that series are presented below. Although these experiments represent only an initial step toward determining the subcellular location of axonally transported glycoproteins and glycolipids, they demonstrate a general method which can be used to study axonally transported materials labeled with precursors such as glucosamine which have a significant amount of background labeling.

In simple experiments with precursors with significant background labeling, it is easy to reliably measure the amount of transported radioactivity as the difference between the tecta. In experiments with many steps, the possibility increases that uncontrollable experimental factors such as unequal recoveries could introduce differences between samples from the left and right tecta, leading to invalid estimates of transported radioactivity. This problem is most acute in continuous systems such as density gradients, gel electrophoresis, etc. where the collection of perfectly corresponding fractions cannot be assured. One approach to the calculation of transported radioactivity in the presence of background labeling is to use symmetrical double-labeling. In the experiments described below I injected [^3H]glucosamine into the right eye, and [^{14}C]glucosamine into the left eye of the same fish. Transported ^3H is then calculated as left minus right, while transported ^{14}C is calculated as right minus left. If the results from both isotopes

agree closely, one can have confidence that spurious difference have not been introduced, and that the calculations of transport are valid. An example of symmetrical double-labeling is shown in Figure 4 in the Introduction.

Methods

Six pond goldfish per experiment received an injection of [6-³H]D-glucosamine (1.16 Ci/mMole) in the right eye and [1-¹⁴C]D-glucosamine (52 mCi/mMole) in the left eye 24 hours before sacrifice. The amounts injected varied in different experiments. In one experiment (Table XX) whole tecta and tracts were processed by Method 2.

In the cell fractionation experiments, I used a modification of the standard "four fractions" differential centrifugation procedure (Whittaker, 1965). Pooled tecta or tracts from the left or right sides were gently homogenized in 1.4 ml of 0.32 M sucrose using a Dounce homogenizer (Kontes Glass Co., Vineland, New Jersey). After samples were taken for scintillation counting and protein determination, 1.0 ml of the homogenate was centrifuged in a Lourdes Model A-2 "Betafuge" at $1100 \times g_{av}$ for 10 minutes. The pellet was resuspended in 1.0 ml 0.32 M sucrose and spun again at $1100 \times g_{av}$ to give a pellet which is designated the "N" fraction. The combined supernatants were spun at $16,000 \times g_{av}$ for 20 minutes to yield a pellet ("P₂") and a supernatant which was in turn sedimented at $101,000 \times g_{av}$ for 120 minutes in a Spinco Model 40 rotor in a Spinco Model L Ultracentrifuge. The final pellet is designated the "M" fraction, and the supernatant is the "S" fraction. The temperature was 0-4°C throughout the fractionation. The pellets were suspended in distilled water, and macromolecules in samples of the fractions were precipitated with 10% TCA containing 1 mM D-glucosamine. Duplicate samples were dissolved in Soluene-100 for scintillation counting or in 0.5 N NaOH for protein analysis by the method of Lowry et al. (1951). The TCA supernatants were extracted three times with diethyl ether to remove the TCA, concentrated by evaporation, and counted in Bray's scintillator (Bray, 1960). Efficiency of counting was measured with the

Packard Automatic External Standard, and ^3H and ^{14}C disintegrations per minute were calculated using a computer program written by Dr. Brian Poole of the Rockefeller University (Packard Program Library No. 09672). Note that in the cell fractionation experiments, the lipid was not removed from the TCA precipitate, which therefore includes labeled lipids as well as macromolecules.

Results

Table XX shows the radioactivity in whole tecta and tracts which were immersed in TCA and processed by Method 2. The distribution of transported TCA-precipitable, C-M extractable, and TCA-soluble radioactivity in pond fish tecta is similar to the distribution found in small goldfish (Tables IV and VI). In the tracts a greater percentage than in the tecta of the total transported radioactivity is TCA-soluble (which is also the case with $[^3\text{H}]$ fucose: see Tables XVII and XVIII). Table XX shows fairly good agreement in the distribution of ^3H and ^{14}C .

The results of the differential centrifugation experiments are presented in Figure 26 and Table XXI. In order to compare the results obtained with $[^3\text{H}]$ and $[^{14}\text{C}]$ glucosamine, the radioactivities in Figure 26 have been converted to relative values by dividing the concentration of radioactivity in each fraction by the concentration in the homogenate. In the tecta, the three particulate fractions (N, P_2 , and M) have about the same concentration of transported TCA-precipitable radioactivity as the homogenate (Figure 26). There is some enrichment of the label in the M fraction. However, the greatest proportion of transported radioactivity is found in the fraction with the most protein, the P_2 fraction (Table XXI). The concentration of transported radioactivity in the soluble macromolecules (S fraction) is very low, and represents only a few per cent of the total transported radioactivity. On the other hand, the concentration of the transported TCA-soluble radioactivity from the tecta is high in the S fraction, and about two-thirds of the transported acid-soluble material is found in this fraction. The particulate fractions all have lower concentrations of transported TCA-soluble radioactivity than the homogenate.

Table XX. Distribution of Radioactivity in Optic Tracts and Tecta

| | ³ H | | | | ¹⁴ C | | | |
|---|------------------------|----|----------------------|----|------------------------|----|----------------------|----|
| | Transported | | Background | | Transported | | Background | |
| | Radioactivity (L-R) | | Radioactivity (R) | | Radioactivity (R-L) | | Radioactivity (L) | |
| | dpm/mg | % | dpm/mg | % | dpm/mg | % | dpm/mg | % |
| Tecta—data from intact tecta | | | | | | | | |
| P ^a | 1638 | 59 | 722 | 44 | 489 | 64 | 334 | 44 |
| L ^b | 682 | 25 | 206 | 13 | 186 | 24 | 83 | 11 |
| S ^c | 446 | 16 | 694 | 43 | 89 | 12 | 338 | 45 |
| Tecta—data from homogenized tecta | | | | | | | | |
| P + L ^d | — | 83 | — | 62 | — | 87 | — | 61 |
| S | — | 17 | — | 38 | — | 13 | — | 39 |
| Optic Tracts—data from intact tracts | | | | | | | | |
| P | 1782 | 33 | 467 | 21 | 594 | 27 | 152 | 27 |
| L | 952 | 18 | 96 | 4 | 252 | 11 | 30 | 5 |
| S | 2592 | 49 | 1628 | 74 | 1364 | 62 | 392 | 68 |
| Optic Tracts—data from homogenized tracts | | | | | | | | |
| P + L | — | 50 | — | 31 | — | 41 | — | 42 |
| S | — | 50 | — | 69 | — | 59 | — | 58 |

^a = TCA precipitate after lipid extraction

^b = Lipid (chloroform-methanol extract)

^c = TCA-soluble

^d = TCA precipitate, lipids not extracted

Six large pond fish received 1 μ Ci of [6-³H]D-glucosamine in the right eye and 0.4 μ Ci of [1-¹⁴C]D-glucosamine in the left eye. They were sacrificed 24 hours later, and the optic tracts and tecta were processed by Method 2. Values for homogenized tracts and tecta are from the homogenates of the cell fractionation experiments. Since different amounts of isotope were injected in each cell fractionation experiment, only the average per cent distribution of radioactivity is presented.

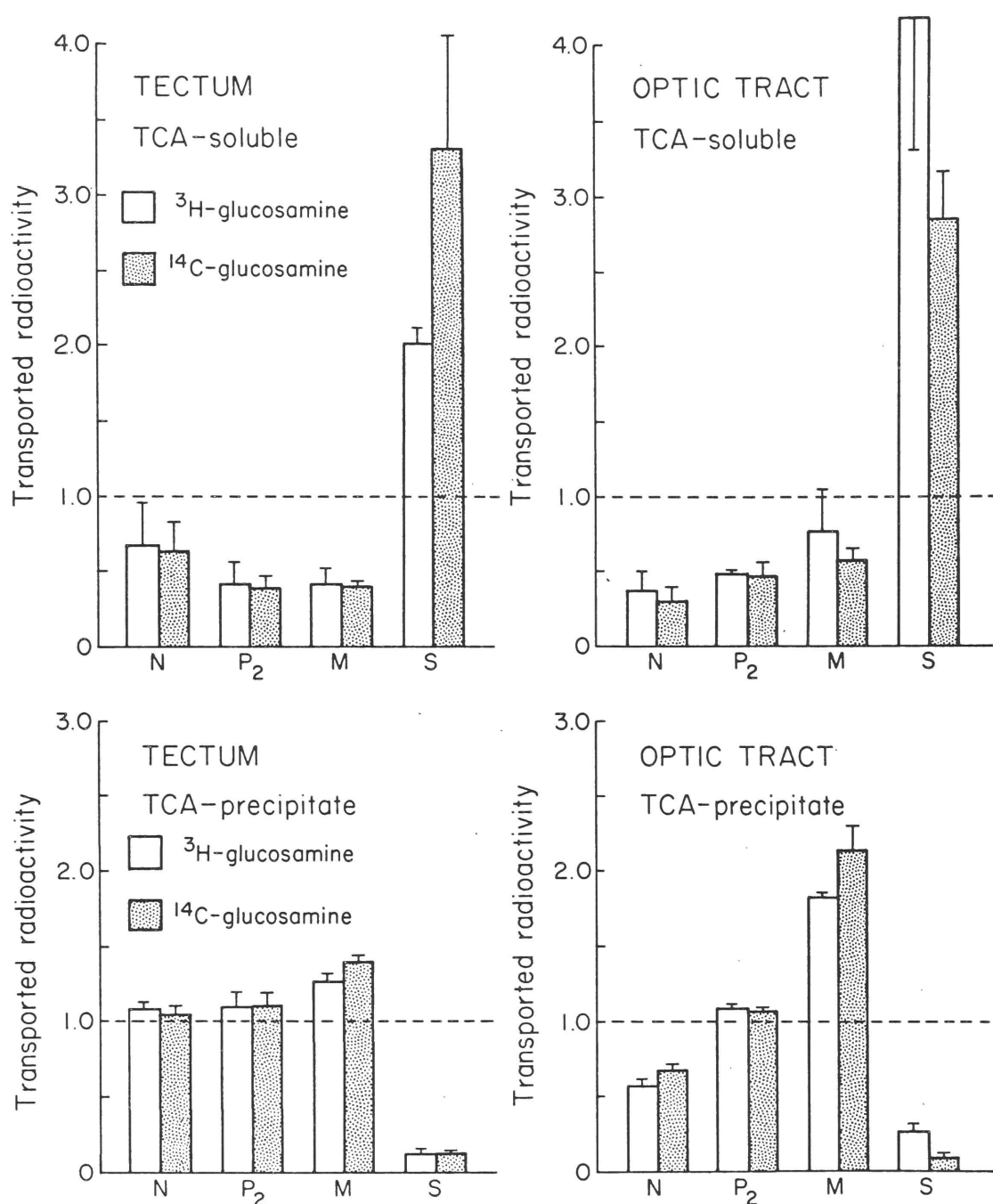


Figure 26. Subcellular distribution of TCA-precipitable and TCA-soluble transported radioactivity in the optic tracts and tecta 24 hours after the injection of radioactive glucosamine. The concentration of transported radioactivity (DPM/mg protein) in each fraction has been divided by the concentration of transported radioactivity in the homogenate. N: "crude nuclear" fraction, P₂: "crude mitochondrial" fraction (mitochondria, synaptosomes, myelin fragments), M: microsomes, S: supernatant. Each value is the average of three experiments. Lines above the bars show standard error of the mean. Lipids were not extracted from the TCA precipitates.

Table XXI. Distribution of Recovered Radioactivity and Protein in Subcellular Fractions

| Per cent recovered in fraction | N (%) | P ₂ (%) | M (%) | S (%) |
|--|--------|--------------------|----------|---------|
| <i>Tecta</i> | | | | |
| Protein | 17 ± 1 | 40 ± 0.4 | 20 ± 0.4 | 23 ± 1 |
| Transported TCA-precipitable radioactivity | 19 ± 1 | 50 ± 1 | 28 ± 2 | 3 ± 0.2 |
| Background TCA-precipitable radioactivity | 13 ± 1 | 42 ± 2 | 31 ± 1 | 13 ± 1 |
| Transported TCA-soluble radioactivity | 11 ± 2 | 17 ± 2 | 9 ± 2 | 64 ± 3 |
| Background TCA-soluble radioactivity | 9 ± 2 | 25 ± 2 | 15 ± 1 | 50 ± 3 |
| <i>Optic Tracts</i> | | | | |
| Protein | 24 ± 2 | 30 ± 1 | 22 ± 1 | 24 ± 1 |
| Transported TCA-precipitable radioactivity | 15 ± 1 | 34 ± 2 | 47 ± 2 | 5 ± 1 |
| Background TCA-precipitable radioactivity | 14 ± 2 | 21 ± 3 | 26 ± 3 | 37 ± 3 |
| Transported TCA-soluble radioactivity | 7 ± 1 | 16 ± 1 | 11 ± 1 | 68 ± 1 |
| Background TCA-soluble radioactivity | 16 ± 3 | 17 ± 2 | 18 ± 2 | 54 ± 4 |

D-Glucosamine-6-³H was injected into the right eye, and D-glucosamine-1-¹⁴C was injected into the left eye 24 h before sacrifice. The values for ³H and ¹⁴C did not differ significantly, and they have been averaged together. "Background" radioactivity refers to radioactivity on the right side for ³H and on the left side for ¹⁴C. N: "crude nuclear" fraction, P₂: "crude mitochondrial" fraction (mitochondria, synaptosomes, myelin fragments), M: microsomes, S: supernatant. Values are the averages of three experiments ± standard errors. Average recoveries of protein and radioactivities range from 92–101%. The amount of protein in the 1.0 ml of tectum homogenate which was fractionated averaged 3.25 ± 0.06 mg, while in the case of the tracts the average was 0.38 ± 0.06 mg

A similar pattern is found in the tracts (Figure 26). TCA-soluble transported radioactivity in the tracts is found mainly in the S fraction, where this radioactivity has been considerably enriched. As in the tecta, the S fraction contains little TCA-precipitable transported radioactivity. However, in the tracts there is greater enrichment than in the tecta of transported incorporated radioactivity in the M fraction, which contains almost half of the transported TCA-precipitable radioactivity but less than a quarter of the total protein (Table XXI).

Discussion

These experiments demonstrate that symmetrical double-labeling is a feasible approach for measuring axonally transported radioactivity in the presence of background labeling. The agreement between the results obtained with [^3H]- and [^{14}C]glucosamine provides the necessary internal control against the possibility that differences between the left and right samples arising during the fractionation have produced invalid calculations of transported radioactivity. In most of the data presented here the numerical values obtained with [^3H]- and [^{14}C]glucosamine are very close. In the cases where the numbers diverge (e.g. the TCA-soluble radioactivity in the optic tracts) the two isotopes still illustrate the same quantitative relationships between the various chemical and subcellular fractions. In general, when ^3H and ^{14}C label the same molecule in different positions, as is the case here ([6- ^3H]glucosamine, [1- ^{14}C]glucosamine), one can expect similar results with both isotopes when the precursor molecule is incorporated into macromolecules with little alteration, but the results could be quite different if the precursor is extensively metabolized. The agreement between the results for the two isotopes is consistent with the hypothesis that the label has been incorporated into macromolecules (and glycolipids) as N-acetylhexosamine or sialic acid. If a large fraction of the injected glucosamine has been converted to glucose and then to its metabolites, more divergent results would be expected.

Symmetrical double-labeling has the obvious advantage of producing twice as much data per experiment as experiments with one isotope. This benefit is partly offset by the added difficulties in counting and calculation of radioactivity. Symmetrical double-labeling could also be used to compare transported and background labeling in the same tissue when calculation of the amount of transported radioactivity is either unnecessary or impossible. For instance, one could use this technique with density gradients, chromatography, etc., to determine whether the patterns of transported and background labeling are similar or distinctly different without quantifying the amount of transported radioactivity. While symmetrical double-labeling can be used to study transported radioactivity in the presence of significant background radioactivity, it would clearly be preferable, when possible, to use precursors like proline and fucose which produce a background so low that it can be ignored.

The cell fractionation experiments confirm the particulate nature of the transported TCA-precipitable radioactivity and the low level of labeling in soluble macromolecules in the tecta (Table IV), and extend those observations to the optic tracts. The subcellular location of the transported macromolecules cannot be determined without biochemical and morphological characterization of the fractions, and additional experiments. The enrichment of transported TCA-precipitable radioactivity in the M fraction of the optic tracts is reminiscent of the enrichment of rapidly transported proteins in microsomal fractions from nerves which has been observed by several authors (Ochs *et al.*, 1967; Kidwai and Ochs, 1969; Sjöstrand and Karlsson, 1969; D. Forman, unpublished results). The composition of microsomal fractions from nerves is not known, but they are expected to contain fragments of plasma membrane, "neuroendoplasmic reticulum," and synaptic vesicles, but not mitochondria or large myelin fragments.

Elam and Agranoff have studied the subcellular distribution of rapidly transported macromolecules with ^3H -proline and $^{35}\text{SO}_4$ in the

goldfish optic tectum (Elam and Agranoff, 1971a and b; Elam et al., 1971). They followed the procedure of Whittaker (see Whittaker, 1965) which involves differential centrifugation followed by subfractionation of the P_2 fraction on discontinuous density gradients. Their differential fractionation procedure was similar to the one I used, and produced a similar distribution of radioactivity. The distribution of radioactivity from both isotopes (Figure 7 of Elam and Agranoff, 1971b) was similar to the distribution of the TCA-precipitable radioactivity in the tectum in Figure 26. An important finding was that the distribution of [^{14}C]proline- and [^{35}S]sulfate-labeled macromolecules closely paralleled each other, suggesting that both isotopes are localized in the same subcellular particles. Purified nuclei from the N fraction, and purified ribosomes from the M fraction had very low specific activities (Elam and Agranoff, 1971a). The distribution of transported radioactivity was essentially the same from 5 to 11 hours after injection.

Elam and Agranoff presented some biochemical and morphological information about their fractions. The radioactivity does not correlate especially well with a mitochondrial marker, which was nearly absent from the M fraction. Electron micrographs of their fractions show profiles which resemble synaptosomes in all the particulate fractions, including the mitochondrial, N, and M fractions (Elam et al., 1971). Thus, the wide distribution of radioactivity is probably due to the presence of fragments from the nerve endings in all the particulate fractions. In studies of the subcellular distribution of rapidly transported proteins in the rabbit lateral geniculate (Sjöstrand and Karlsson, 1968; Karlsson and Sjöstrand, 1971a, c) and pigeon optic tectum (Cuénod and Schonbach, 1971), the labeled protein was also distributed among many fractions and no conclusion could be drawn about its subcellular location except the generalization that it is mainly particulate.

IV. AXONAL TRANSPORT OF GANGLIOSIDES

In the experiments presented in Experimental Sections I A., I D., and II A., TCA precipitates were extracted with lipid solvents to remove glycolipids from the glycoproteins. The presence of transported radioactivity in the C-M extracts suggested that there may be some axonal transport of glycolipids. However, the radioactive materials in the C-M extracts were not characterized chemically. It seemed likely that some of this material was ganglioside, since glucosamine and N-acetylmannosamine are good precursors for labeling gangliosides (Burton *et al.*, 1963; Suzuki, 1967; Barondes, 1968b; Yogeewaran *et al.*, 1970; Quarles and Brady, 1971). The experiment described in this section examined the question of whether labeled gangliosides are axonally transported after an intraocular injection of tritiated N-acetylmannosamine. The experiment was performed in the laboratory of Dr. Robert Ledeen of the Albert Einstein College of Medicine. The guidance and help of Dr. Ledeen and of Dr. Robert Yu is gratefully acknowledged.

Gangliosides. Brain tissue is rich in glycolipid, most of which is glycosphingolipid. Glycosphingolipids contain sugars which are glycosidically linked to the C₁ primary hydroxyl of ceramide, which consists of sphingosine or a closely related long-chain amino alcohol (long chain base) with a fatty acid attached to the amino group of the sphingosine by an amide bond. Gangliosides are glycosphingolipids which contain sialic acids. The sialic acid is usually NANA, but N-glycolylneuraminic acid and O-acetylated sialic acids also occur in some species. In brain gangliosides the sialic acid residues are attached to the same ceramide-tetrasaccharide backbone: galactosyl- β (1 \rightarrow 3)-N-acetylgalactosaminyl- β -(1 \rightarrow 4)-galactosyl- β -(1 \rightarrow 4)-glucosyl-(1 \rightarrow 1)-ceramide. Gangliosides are also found in other tissues, but the gangliosides of other tissues usually have oligosaccharide structures different from the brain gangliosides (Ledeen *et al.*, 1968; Handa and Burton, 1969; Puro *et al.*, 1969; McCluer, 1970; Ledeen, 1970; Wiegandt, 1970, 1971). Gangliosides are found in a higher concentration in the brain than in

other tissues, and account for 6% of the total mammalian grey matter lipids by weight (Svennerholm, 1970). Brain gangliosides are found mainly in neurons (Derry and Wolfe, 1967; Mårtensson, 1969) where they are localized in the neuronal plasma membranes (Eichberg *et al.*, 1964; Brunngraber *et al.*, 1967; Lapetina *et al.*, 1967; Spence and Wolfe, 1967; Wiegandt, 1967; Trotter and Burton, 1969; Dekirmenjian and Brunngraber, 1969; Dekirmenjian *et al.*, 1969). On the other hand, cerebrosides and sulfatides are localized mainly in the glia (Mårtensson, 1969; Balasubramanian and Bachawat, 1970; Wiegandt, 1971). Reviews are available covering the chemistry of gangliosides (Ledeen, 1966; McCluer, 1968; Mårtensson, 1969; Klenk, 1969; Wiegandt, 1971) and other aspects of brain gangliosides (Schmitt and Samson, 1969; Svennerholm, 1965, 1970).

The negative charges of the sialic acids make the physical properties of the gangliosides different from most other lipids, and the isolation procedures for gangliosides are designed around these properties. Gangliosides require polar lipid solvents to extract them from tissue, such as chloroform-methanol mixtures containing 50-60% methanol (Suzuki, 1965). In the Folch partitioning procedure (Folch *et al.*, 1957), gangliosides partition into the aqueous upper phase while most other lipids remain in the lower, hydrophobic phase. Gangliosides remain in solution in large micelles, so that dialysis can be used to separate the gangliosides from small molecules which also partition into the Folch upper phase. Additional purification steps were added in our procedure to remove any remaining radioactive contaminants.

The gangliosides of fish brain have been examined recently (Ishizuka *et al.*, 1970; Yu and Ledeen, 1970; Seiter, 1970; McCluer and Agranoff, 1971; Avrova, 1971; Avrova and Zabelinskii, 1971). The ganglioside concentrations in fish brain which have been reported are shown in Table XXII; these levels are only 1/3 to 1/5 of the concentrations found in mammalian brains. The ceramide portions of fish brain gangliosides contain a smaller proportion of stearic acid and more palmitic and monoenoic acids than mammals (Avrova and Zabelinskii, 1971). They also have a somewhat different distribution of long chain

Table XXII. Concentration of Ganglioside Sialic Acid in Fish Brain

| Tissue | Micromoles sialic acid per gram wet weight | | | Method | Reference |
|--|---|------------------|---------------------|--------|--------------------------------------|
| | Folch u.p. | Dialyzed u.p. | Purified further | | |
| *1 Goldfish tectum | 0.96 | 0.97 | 0.70 | GLC | This thesis |
| 2 Goldfish whole brain | 0.76 ± 0.03 | 0.75 | | GLC | Yu and Ledeen, 1970 |
| †3 Goldfish tectum | 1.04 | | 0.65 | GLC | Pilot study for this thesis, 1969 |
| Goldfish whole brain | 0.91 | | 0.58 | | |
| 4 Carp and other fish, whole brain | | 0.82 | | Th | Ishizuka <u>et al.</u> , 1970 |
| §5 Carp whole brain | | 1.17 ± 0.03 | | R | Avrova, 1971 |
| 6 Goldfish whole brain | | | 0.90 | R | McCluer and Agranoff, 1972 |

Analytical Methods

GLC: Gas-liquid chromatography (Yu and Ledeen, J. Lipid Res., 11:506).

Th: Thiobarbituric acid (Warren, J. Biol. Chem., 234: 1971).

R: Resorcinol (Svennerholm, Biochim. Biophys. Acta, 24: 604).

Comments

* Right tectum values only

† This pilot study was undertaken with Dr. Ledeen in 1969. The dialyzed Folch upper phases were purified by base treatment and DEAE Sephadex chromatography. These values are not as reliable as 1 and 2.

§ This value is probably too high. It is close to the result obtained by Yu and Ledeen (1970) using the resorcinol method, which they demonstrated to be too high because of false chromogens. Also, Avrova notes the presence of a water-soluble contaminant which is not ganglioside, and which accounts for 5-10% of the sialic acid in the dialyzed Folch u.p. The contaminant is probably soluble glycoprotein.

bases. Although 4-sphingenine ($C_{18:1}$) is the major long chain base of both mammalian and fish brain gangliosides, mammals have much more eicosa-4-sphingenine ($C_{20:1}$) (Avrova and Zabelinskii, 1971). The sialic acids in fish brain gangliosides are apparently attached to the same ceramide-tetrasaccharide backbone as mammalian brain gangliosides, but fish differ in two important respects. In fish polysialogangliosides predominate. They apparently have no monosialoganglioside, and only a small amount of disialogangliosides (the predominant species in mammalian brain). Instead, goldfish brain gangliosides are mainly tri-, tetra-, and pentasialogangliosides, with the tetrasialogangliosides predominating (Ishizuka *et al.*, 1970; McCluer and Agranoff, 1972; Avrova, 1971). This fits into an evolutionary trend in which the more primitive vertebrate classes have a lower concentration of brain gangliosides, and more polysialogangliosides (Avrova, 1971). Fish brain gangliosides also contain an unusual sialic acid, 8-0,N-diacetylneuraminic acid (Ishizuka *et al.*, 1970; Sieter, 1970). This gives them unusual thin-layer chromatographic patterns. However, the 8-0 acetyl bond is very labile to alkaline hydrolysis. Base treatment, as was used here, converts the 8-0,N-diacetylneuraminic acid to NANA.

Methods

Injections and Dissections

Large pond goldfish were injected in the right eye with 12.3 μ Ci of N-[3 H]acetyl-D-mannosamine (1020 mCi/mMole), and sacrificed 24 hours later. Pooled left tecta and pooled right tecta from 43 fish were frozen in liquid nitrogen, and used to isolate samples of gangliosides from the left ("L") and right ("R") sides. Tecta from five additional fish were processed by Method 1. The distribution of radioactivity in those fish is presented in Table XI (Section I D.).

Isolation of Gangliosides

The procedure used to isolate the gangliosides is summarized in Table XXIII. The details are as follows:

Table XXIII. Procedure for Isolating Radiochemically Pure Gangliosides

| Step | Purpose |
|--|--|
| 1. Extract lipids in C-M, 1:1, followed by C-M, 1:2 | |
| 2. Folch partitioning *I | Removes most other lipids |
| 3. Enzyme treatment | Hydrolyzes labeled sugar nucleotides |
| 4. Dialysis, with EDTA *II | Removes small molecules, including products of enzyme treatment |
| 5. DEAE Sephadex column | Removes protein and neutral lipids |
| 6. Base treatment | Saponifies residual phospholipid |
| 7. Dialysis *III | Removes ions from preceding steps |
| 8. Unisil column *IV | Removes remaining protein and sulfatide |

* Samples for analysis of sialic acid and radioactivity were taken at the points indicated by the Roman numerals, and will be called:

- I. Folch upper phase
- II. Dialyzed Folch upper phase
- III. Upper phase before Unisil
- IV. Purified ganglioside

Extraction of gangliosides. The wet tecta were weighed in tared glass-stoppered centrifuge tubes, and were then extracted in 30 volumes (11 ml) of C-M, 1:1 by magnetic stirring at room temperature for one hour. The protein was sedimented by centrifugation. The supernatants were filtered through medium porosity sintered glass funnels, and the residues were re-extracted with 11 ml of C-M, 1:2 (Suzuki, 1965). The combined supernatants from each side were dried under nitrogen. The weights of the wet tissue, dry extracted tissue, and lipid extracts are given in Table XXIV.

Folch partitioning. The lipids were dissolved in 10 ml of C-M, 2:1, and partitioned by the method of Folch et al. (1957) with 2.0 ml of physiological saline. The lower phases were repartitioned once with saline-methanol, 1:1, added to make a total volume of 12 ml, and the surfaces were washed with some more saline-methanol. The pooled upper phases were dried under nitrogen.

Phosphodiesterase treatment and dialysis. Kanfer and Brady (1967) reported that sugar nucleotides are retained by dialysis bags. They can be removed by enzyme treatment (Kanfer and Brady, 1967; Suzuki and Chen, 1967). The upper phases were incubated at 37° for 2 hours with 1.0 Unit of phosphodiesterase from Crotalus adamanteus venom, Type II (Sigma Chemical Co.) and 0.25 Units (0.01 mg) of E. coli alkaline phosphatase (Sigma Chemical Co.) in 1.0 ml 0.1 M tris-glycine buffer, pH 8.4, containing 0.03 M MgCl₂. After the incubation, 10 ml of chloroform-methanol, 1:1, was added to precipitate the enzymes, and centrifuged. The supernatants were filtered through sintered glass funnels and evaporated nearly to dryness. They were then redissolved in 0.05 M EDTA, dialyzed for 2 days against distilled water, and lyophilized.

DEAE Sephadex Fractionation. Yu and Ledeen (1971) have found that chromatography on DEAE Sephadex and Unisil columns is necessary to remove all traces of glycoprotein and glycopeptide from gangliosides in the dialyzed Folch upper phases. In both procedures, gangliosides and proteins are adsorbed to the columns, and then the gangliosides are

Table XXIV. Weights of Tecta for Isolation of Gangliosides

| | Left | Right |
|---|-------|-------|
| Total wet weight (g) | 0.366 | 0.368 |
| Wet weight per tectum (mg) | 8.5 | 8.6 |
| Total dry weight after lipid extraction (mg) | 39.4 | 38.4 |
| Dry weight per tectum (mg) | 0.92 | 0.89 |
| Total weight of lipid extract before Folch partitioning (mg) | 37.3 | 37.2 |

selectively eluted. For each ganglioside sample, 1 gm of DEAE Sephadex A-25 (Pharmacia Fine Chemicals, Inc.) was washed 10 times with methanol-chloroform-0.8 M sodium acetate, 60:30:8 ["60:30:8(Ac)"] and then three times with methanol-chloroform-water, 60:30:8 ["60:30:8(W)"]. The slurry was used to pack a column (height 5-7 mm) which was washed with an additional 200 ml of 60:30:8(W). The lyophilized gangliosides were applied to the column dissolved in 25 ml of 60:30:8(W), and washed with an additional 75 ml. No radioactivity appeared in the fractions which passed through the columns. The gangliosides were eluted with 100 ml of 60:30:8(Ac). The eluate was flash evaporated and then lyophilized to dryness.

Base Treatment and Dialysis. Remaining phospholipids can be removed by base treatment. The upper phases were incubated for 2 hours at 40°C in 0.2 M NaOH in methanol. Most of the ethanol was evaporated with a stream of N₂ and the residues were incubated briefly at 40°C in distilled water to hydrolyze methyl esters, and then dialyzed for 2 days against distilled water. Note that the base treatment converts the 8-0,N-diacetylneuraminic acid to NANA.

Unisil Fractionation. Besides the DEAE Sephadex, a second column was necessary to remove all of the protein. We used columns made of 1.0 gm of Unisil (Clarkson Chemical Co., Williamsport, Pa.; 200-325 mesh) washed with chloroform. The lyophilized samples were dissolved in C-M, 1:1, and applied to the column in 40 ml C-M, 85:15. They were washed with an additional 110 ml of C-M, 85:15. No radioactivity was found in this fraction. The purified gangliosides were eluted with 150 ml of C-M, 2:3.

Analysis of Sialic Acid

Sialic acid was measured by the gas-liquid chromatographic method of Yu and Ledeen (1970) using a beef brain ganglioside calibration curve. As well as being one of the most sensitive methods available, this method has the special advantage in this study of being insensitive to false chromogens in goldfish ganglioside fractions which interfere with

sialic acid analysis by other methods (see Table VI of Yu and Ledeen, 1970).

Measurement of Radioactivity

Aliquots of liquid samples were evaporated on pieces of filter paper, and combusted in the Packard Model 305 Sample Oxidizer for liquid scintillation counting. Solid samples were also combusted after being wrapped in filter paper and being compressed with a pellet press (Parr Instrument Co., Moline, Illinois). The combustion method made it possible to show that the radioactivity which was bound to the dialysis bags, and also the radioactivity which was lost in the pellet of precipitated enzyme protein, was negligible.

Results

The results of the sialic acid analyses are shown in Table XXV. The amount of ganglioside sialic acid found in the goldfish tecta is similar to values which others have measured in fish brain (see Table XXII). Some sialic acid was lost from the L sample between the analysis of the undialyzed and dialyzed Folch upper phase; the reason for this loss is unknown. Since loss does not usually occur at this step (Yu and Ledeen, 1970), the values for the right tectum are probably more representative of the recovery one can expect with this method. About 20-30% of the sialic acid present in the dialyzed upper phase was lost during the purification procedure. While some of this represents loss of ganglioside during transfers, it is very likely, on the basis of previous experience with these methods, that most of the loss is due to the removal of glycoprotein and glycopeptides present in the upper phase (R. Ledeen, personal communication).

The distribution of radioactivity is shown in Table XXVI. Only a small amount of radioactivity in the C-M extract, less than 2% of the total, remained in the Folch lower phase. However, almost all of the radioactivity in the right tectum sample, and half of the radioactivity in the left tectum sample, was lost when the enzyme-treated upper phase

Table XXV. Sialic Acid

| Fraction | Corrected total micrograms of NANA | |
|---|------------------------------------|----------|
| | Left | Right |
| Folch upper phase | 106 | 109 |
| Dialyzed Folch upper phase | 85 | 110 |
| Purified ganglioside | 66 | 79 |
| <u>Recoveries</u> | <u>%</u> | <u>%</u> |
| % of Folch upper phase recovered as purified ganglioside | 62 | 72 |
| % of dialyzed Folch upper phase recovered as purified ganglioside | 78 | 72 |

The amount of lipid-bound sialic acid has been expressed as micrograms of NANA. The amount of 8-O,N-diacetylneuraminic acid is unknown, since it was all converted to NANA by base treatment. Goldfish brain gangliosides contain only 0.4% N-glycolylneuraminic acid, which was not measured here (Yu and Ledeen, 1970). The total amount of NANA has been corrected to the total amount which would have been found if no aliquots had been removed for sialic acid analysis and scintillation counting.

Table XXVI. Distribution of Radioactivity

| Fraction | Corrected Total DPM | |
|--|---------------------|----------|
| | Left | Right |
| Folch upper phase | 48,147 | 22,034 |
| Folch lower phase | 649 | 331 |
| Insoluble residue | 16,824 | 3,495 |
| Dialyzed Folch upper phase | 23,362 | 263 |
| Purified ganglioside | 18,292 | 214 |
| <u>Recoveries</u> | <u>%</u> | <u>%</u> |
| % of DPM in Folch upper phase recovered after dialysis | 48 | 1.1 |
| % of DPM in dialyzed Folch upper phase recovered in purified ganglioside | 78 | 81 |

The measured total DPM have been corrected to the value which would have been found if aliquots had not been removed for sialic acid analysis and scintillation counting in previous steps.

The radioactivity in the residue includes both radioactivity in glycoprotein, and in adsorbed small molecules. When the residue was washed with cold TCA, some radioactivity appeared in the TCA. The measured radioactivity in the left tectum residue decreased from 427 DPM/mg dry weight, to 413. The radioactivity in the right tectum decreased from 91 DPM/mg dry weight, to 49.

was dialyzed. This is consistent with the high level of TCA-soluble background radioactivity found 24 hours after a [^3H]N-acetylmannosamine injection (see Section I D., Figure 21 and Table XI). Most of the difference between L and R was not lost during the dialysis (Tables XXVI and XXVII), as one would expect from the small size of the transported TCA-soluble component produced by [^3H]N-acetylmannosamine (Figure 21 and Table XI). The recovery of radioactivity through the remaining steps of the purification is about the same as the recovery of sialic acid. (The level of radioactivity in ganglioside on the R side is so low that it amounts to only a few counts per minute per aliquot. Hence, the recovery of radioactivity on the R side cannot be calculated with precision.)

Using the measurements of radioactivity and sialic acid, one can calculate the amount of transported radioactivity as the difference in the DPM per μg NANA (Table XXVII). There is a large left-right difference, showing that labeled gangliosides have been axonally transported. The specific activity of the transported material in the Folch upper phase remains about the same as the gangliosides are purified. The background labeling is extremely low, amounting to only about 1% of the amount transported.

It is interesting to compare the distribution of radioactivity found with TCA precipitation and C-M extraction (Method 1) and the distribution found during the isolation of gangliosides. This is done in Table XXVIII, in which the distribution of radioactivity from the ganglioside isolation has been calculated as DPM/mg dry weight, and is compared with the results in Section I D., Table XI. There are some discrepancies as to the exact concentration of radioactivity in some fractions (viz., between the transported C-M extract of Method 1 and the transported non-dialyzable lipids from the ganglioside extraction, and between the background TCA-soluble radioactivity from Method 1 and the background dialyzable radioactivity from the ganglioside extraction). On the whole, however, the patterns of radioactivity shown in Table XXVIII are similar in both the amount and distribution of radioactivity. This strengthens our belief, based on solubility properties, that when

Table XXVII. Concentration of Radioactivity

| Fraction | DPM/ μ g NANA | | |
|----------------------------|-------------------|-------|-------------------|
| | Left | Right | Transported (L-R) |
| Folch upper phase | 453 | 203 | 250 |
| Dialyzed Folch upper phase | 276 | 2.4 | 274 |
| Upper phase before Unisil | 266 | 2.6 | 263 |
| Purified ganglioside | 277 | 2.7 | 274 |

Table XXVIII. Comparison of Radioactivity from Ganglioside Isolation with Radioactivity from TCA-Precipitation, Chloroform-Methanol Extraction after Intraocular Injection of [^3H]N-Acetylmannosamine

| TCA Precipitation, Chloroform-Methanol Extraction (Method I)* | | | Ganglioside Isolation | | |
|---|----------------------|-----------|----------------------------------|----------------------|-----------|
| <u>Fraction</u> | <u>DPM/mg dry wt</u> | <u>%†</u> | <u>Fraction</u> | <u>DPM/mg dry wt</u> | <u>%†</u> |
| A. TRANSPORTED | | | | | |
| C-M extract | 790 (± 110) | 68 | Dial. Folch u.p. + Folch l.p. | 593 | 60 |
| Protein | 346 (± 49) | 30 | Washed residue | 364 | 37 |
| TCA-soluble | 26 | 2 | <u>Dialyzable§</u> | 34 | 3 |
| | | | Purified ganglioside | 458 | - |
| B. BACKGROUND | | | | | |
| C-M extract | 29 (± 7) | 3 | Dial. Folch u.p. + Folch l.p. | 16 | 2 |
| Protein | 51 (± 11) | 5 | Washed residue | 49 | 7 |
| TCA-soluble | 924 (± 97) | 92 | <u>Dialyzable§</u> | 609 | 90 |
| | | | Purified ganglioside | 6 | - |

* Data from Section I D., Table XI.

† % of total transported or background DPM.

§ Includes TCA-soluble radioactivity from residue.

tecta are processed by Method 1 after [^3H]N-acetylmannosamine injections, as in Section I D., the radioactivity in the TCA-precipitate is in glycoprotein, the TCA-soluble radioactivity consists of molecules small enough to be dialyzable, and the transported radioactivity in the C-M extract is mainly ganglioside (contaminated with some glycoprotein and a tiny amount of labeled lower phase lipids).

Discussion

The results presented in Table XXVII, which shows a large left-right difference in the radioactivity in highly purified gangliosides, strongly suggests that when [^3H]N-acetylmannosamine is injected into the goldfish eye some of the label is incorporated into gangliosides which are synthesized in the retina and then axonally transported. This finding has several interesting implications: 1) Many laboratories are currently investigating the metabolism of gangliosides (Suzuki, 1967; Harzer *et al.*, 1969; Quarles and Brady, 1971; DeVries and Barondes, 1971). The results of these labeling studies, especially regional studies, should be re-evaluated to take into account the contribution of movements of labeled gangliosides as well as synthesis in the observed patterns of radioactivity. 2) Several authors have reported that the incorporation of labeled sugars into gangliosides is inhibited when protein synthesis is inhibited (Kanfer and Richards, 1967; Barondes and Dutton, 1959; Shah and Peterson, 1971; DeVries and Barondes, 1971). This phenomenon is unique to gangliosides; the incorporation of sugars into other glycosphingolipids is independent of protein synthesis (Kanfer and Richards, 1967; Benjamins *et al.*, 1971; McCluer and Agranoff, 1971). If the synthesis of gangliosides is closely linked to the synthesis of the proteins with which they are axonally transported, it would explain the effects of the protein synthesis inhibitors on ganglioside synthesis. The observation that AXM inhibits the transported C-M extractable material labeled with radioactivity from [^3H]glucosamine (Section II A., Figure 22) is consistent with this hypothesis (although that material has not yet been proved to be ganglioside).¹ One unexplained contradiction,

¹See footnote on pp. 151-152.

however, is the results of McCluer and Agranoff (1971) who confirmed the results of Kanfer and Richards (1967) on rats, but were unable to demonstrate an inhibition of [3 H]galactose incorporation into ganglioside in goldfish brain when synthesis was inhibited with puromycin. 3) Many laboratories have concluded, on the basis of subcellular fractionation studies, that gangliosides are localized in the neuron plasma membrane (Eichberg *et al.*, 1964; Brunngraber *et al.*, 1967; Lapetina *et al.*, 1967; Spence and Wolfe, 1967; Wiegandt, 1967; Trotter and Burton, 1969; Dekirmenjian and Brunngraber, 1969; Dekirmenjian *et al.*, 1969). Gangliosides are apparently not present in mitochondria or synaptic vesicles. Thus, the rapid axonal transport of gangliosides represents the transport of a plasma membrane "marker."

4) The amount of gangliosides in nerves is very low (Svennerholm, 1970; Wiegandt, 1971), and much of the nerve ganglioside is probably located in the plasma membrane of the axons (Dekirmenjian *et al.*, 1967). However, some ganglioside has been measured in membranes from intra-axonal sources (R. Lasek, personal communication; G. DeVries, personal communication). This intra-axonal ganglioside may represent ganglioside which is being axonally transported for incorporation into plasma membrane further down the axon.

5) Several authors have demonstrated the presence in synaptosomal fractions of glycosyltransferases which incorporate sugars into gangliosides (and glycoproteins). They therefore conclude that the main site of synthesis of the oligosaccharide chains of gangliosides (and glycoproteins) is the nerve ending (Den and Kaufman, 1968; Roseman, 1970; Festoff *et al.*, 1971). In the brief accounts available to date it is impossible to evaluate the purity of their fractions. Nevertheless, it is quite possible that gangliosides are synthesized both in the cell body for transport and also locally in the nerve endings. If this is the case, it will be important to determine the relative quantitative contributions and the functional significance of gangliosides synthesized in each location. So far there is no satisfactory experimental method for comparing synthesis in the two locations. It is possible that the

axonal transport of ganglioside is not physiologically important. An example of this sort is provided by the axonal transport of labeled noradrenaline (Geffin and Rush, 1968; Livett et al., 1968). When radioactive noradrenaline is supplied selectively to neuron cell bodies, it is taken up by the amine storage granules and axonally transported with them to the nerve endings. However, the quantitative contribution of the noradrenaline carried to the endings in the granules is very small compared to local synthesis and local uptake of exogenous noradrenaline. Similarly, gangliosides might be transported in vesicles which are plasma membrane precursors, and yet most axonal and nerve ending gangliosides could be locally synthesized. On the other hand, axonal transport may be the main source of gangliosides in axons and endings. This is a fertile question for further investigation.

GENERAL DISCUSSION

In the studies presented in this thesis, labeled sugars were injected into the goldfish eye to serve as precursors for materials which are axonally transported in the optic nerve. The main results obtained with radioactive glucosamine and fucose agreed closely. Labeled N-acetyl-mannosamine was studied less extensively, but also gave similar results: The sugars label glycoproteins which are rapidly transported to the tectum at the same rate as other rapidly transported proteins, about 70-80 mm/day. There was no evidence for any slow transport of glycoprotein. Most of the transported glycoproteins are tightly bound to sedimentable subcellular particles.

The observation that transported glycoproteins appear in the optic nerve fibers after an injection of precursor into the eye is presumptive evidence that the carbohydrates are incorporated into the glycoproteins in the cell body of the retinal ganglion cells and then transported. The detailed time course of labeling is consistent with that hypothesis. Further evidence for a retinal site of synthesis comes from the experiments with AXM. When AXM is injected into the eye in a small dose which inhibits protein synthesis in the retina but does not effect protein synthesis in the brain, the amount of transported glycoprotein which appears in the tectum is greatly reduced. Since AXM does not interfere with the mechanism of axonal transport (McEwen and Grafstein, 1968; Ochs et al., 1970), the drug must be acting by preventing the synthesis of the glycoproteins in the retina.

The occurrence of transported TCA-soluble radioactivity raised the possibility that small molecules may be axonally transported, and then incorporated into macromolecules in the axons and endings. Such glycoproteins would then appear to have been axonally transported. This seems unlikely, since the TCA-soluble component contains much less radioactivity than the transported macromolecules at all times. Furthermore, instead of preceding the transported glycoproteins as one might expect of a precursor, the TCA-soluble materials seem to lag behind them.

The experiments with AXM also provide evidence that the transported TCA-soluble material is not a precursor of the glycoproteins: When the synthesis of transported glycoprotein is inhibited with AXM, one would expect that the pool of transported TCA-soluble material would increase if it is a precursor of the glycoprotein. Instead, it decreases somewhat. Thus, the evidence suggests that the transported glycoproteins are not products of local synthesis from transported precursor, but rather that the sugars are incorporated in the retina before transport. This conclusion is in accord with autoradiographic evidence that in most cells, including neurons, carbohydrates are incorporated into glycoprotein in the endoplasmic reticulum and Golgi apparatus. The demonstration that glycoproteins are axonally transported does not preclude the possibility that additional carbohydrates are incorporated into glycoprotein in the axons and endings, using precursors from local sources. To date it has not been possible to measure the relative contributions of axonal transport and local synthesis to the metabolism of glycoproteins in axons and nerve endings. Local incorporation of carbohydrate into glycoprotein might involve the activity of glycosyltransferases in the axons and nerve endings (Den and Kaufman, 1968; Den *et al.*, 1970; Festoff *et al.*, 1971), synthesis by synaptic mitochondria (Bosmann and Martin, 1969; Bosmann and Hemsworth, 1970; Bosmann, 1971), or the glycosylation of cell surface glycoproteins by extracellular glycosyltransferases which are free in the extracellular fluids (Den *et al.*, 1970) or on the surface of other cells (Roth *et al.*, 1971).

As well as labeling glycoproteins, glucosamine and N-acetylmannosamine also label transported materials which are soluble in lipid solvents. The C-M extractable material labeled by N-acetylmannosamine was identified as ganglioside.¹ This represents the first demonstration that these

¹Since this thesis was accepted, Ledeen and I have obtained additional evidence that gangliosides are axonally transported. Goldfish were injected in the right eye with [³H]glucosamine and in the left eye with [¹⁴C]glucosamine (see Section III C) and the tecta were removed 24 hours later. Gangliosides in the tecta were isolated by the methods described in Section IV. Almost all of the transported C-M extractable radioactivity

glycolipids, which occur in especially high concentrations in the brain, are axonally transported. As in the case of the glycoproteins, the relative roles of the axonal transport and the local synthesis of gangliosides remain to be determined.

It is interesting to speculate on the subcellular location of the transported glycoproteins, since knowledge of their location will probably be necessary for an understanding of their physiological function. Synaptic vesicles are believed to be rapidly transported (at least in the case of catecholamine storage granules [Dahlström, 1971]), but the available evidence suggests that synaptic vesicles contain little glycoprotein (Dekirmenjian and Brunngraber, 1969; Dekirmenjian et al., 1969). (A report that synaptic vesicles are rich in acid mucopolysaccharides [Vos et al., 1969] cannot yet be accepted, since the "synaptic vesicle" fraction in that study appears to have been heavily contaminated with plasma membrane fragments.) The transported glycoproteins may be in lysosomes, since lysosomal hydrolases are glycoproteins (Goldstone and Koenig, 1970). Almost nothing is known about the axonal transport of lysosomes. Mitochondria may carry the transported glycoprotein, since mitochondria contain glycoprotein and can even synthesize them (Bosmann and Martin, 1969; Bosmann and Hemsworth, 1970; Bosmann, 1971). However, the enrichment of the transported proteins in microsomal subcellular fractions (see Section III C) and evidence that mitochondria are transported slowly (Barondes, 1966; Weiss and Pillai, 1965) both argue against mitochondria being a major carrier of the rapidly transported glycoproteins studied in the goldfish optic system.

Footnote 1 cont.

was found in the purified gangliosides. The gangliosides were separated by thin-layer chromatography on silica gel G using 2 solvent systems: propanol-H₂O, 70:25 (developed once), and chloroform-methanol-2.5 N NH₄OH (developed 3 times). In all cases, 95% or more of the radioactivity co-migrated with the gangliosides. This was shown both by scraping the individual bands and scintillation counting, and by autoradiography of the thin-layer plates (¹⁴C label only). The radioactivity in each band was roughly proportional to the resorcinol staining intensity (an indicator of sialic acid content). More than 80% of the total radioactivity was found in 2 major bands which are probably tri- and tetrasialogangliosides. The patterns of radioactivity in the transported and locally synthesized gangliosides labeled by glucosamine were similar.

In histochemical studies, the most prominent location of glycoproteins in the brain is at the plasma membrane, where they form the cell surface and are especially concentrated at synapses (Pease, 1966; Rambourg and Leblond, 1967; Bondareff, 1967; Martínez-Palomo, 1970). It thus seems likely that much of the transported glycoprotein is cell surface material. The transport of gangliosides is consistent with this hypothesis, since gangliosides are also believed to be located mainly at the cell surface (Cook, 1968; Schmitt and Samson, 1969; Ginsburg and Kobata, 1971). Other plasma membrane constituents such as acetylcholinesterase and adenyl cyclase are also rapidly transported. The location of these materials while they are in transit inside the axon is not known; perhaps they are transported as vesicles of the ubiquitous but poorly understood "neuroendoplasmic reticulum," and join the plasma membrane of the axons and synapses by a process of exocytosis. Some evidence has been presented for a mechanism of this type (Kasa, 1968). A possible model for this process is seen in intestinal columnar epithelial cells, where the terminal carbohydrates of cell surface glycoproteins are added in the Golgi complex, and the glycoproteins then move in small vesicles to the cell surface (Bennett and Leblond, 1970). At the cell surface, glycoproteins undoubtedly help to determine the physical and chemical properties of the membranes and the intercellular spaces. Thus, they might have a role in physiological activities such as intercellular recognition, learning, and synaptic transmission (Bogoch, 1968; Schmitt and Samson, 1969; Brunngraber, 1969b; Barondes, 1970). The rapid axonal transport of cell surface glycoproteins may provide a mechanism by which the properties of synaptic membranes could be rapidly controlled by events in the neuron cell body.

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