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Phospholipids and cerebrosides of the normal chick sciatic nerve were extracted by Folch procedures and separated with silica gel thin-layer chromatography using various chloroform-methanol solvents. The subsequent chemical assays were expressed as a function of the developmental stages of the chick from 15 days of incubation to 7 days after hatching (28 days from onset of incubation).

A histological study of chick sciatic nerves at ages 18, 20, 22 and 25 days was conducted to identify the period of myelination. Luxol Fast Blue G stain was used to identify the myelin. Myelin is poorly identified at 18 days but notably present at 25 days.

The assays of the phospholipids, expressed as relative percent of the total phospholipid phosphorus, indicated that ethanolamine-phosphoglyceride ratio increases significantly from 17 days to 23 days from onset. Sphingomyelin ratio also increased during this time, but not as much. While the relative amount of

serinephosphoglyceride remained constant and cholinephosphoglyceride diminished.

In vivo introduction of inorganic ^{32}P was used to follow the metabolism of the phospholipids. The period of most active uptake of this substrate was also from 17 to 23 days from onset. The substrate failed to be absorbed well just prior to hatching. This was possibly due to inactivation of the allantoic circulation responsible for assimilation of the substrate prior to hatching.

In the sciatic nerve of the 16 day chick embryo there is 3 μg of cerebroside per mg of wet weight of tissue. As development takes place, this component increases to 26 μg per mg wet weight of tissue at 27 days from onset.

Cerebroside and phospholipid metabolism was followed with in vivo injection of galactose-1-^{14}C. At the earlier ages, the substrate was metabolized predominantly into non-lipid components. But from 20 to 28 days, the radioactivity was recovered increasingly from the lipid fractions of the tissue.

Proteolipid protein in the lipid extract was also determined. It was found in this tissue at all ages, but only after hatching is there a notable increase in its amount.

Implications of these lipid changes as coincident to myelination in the peripheral nerves were discussed.

Patterns of Lipid Changes with Growth of Chick Sciatic Nerve

by

Hector John Munn

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Redacted for privacy

Professor of Biochemistry-Biophysics
in charge of major

Redacted for privacy

Chairman of the Department of Chemistry

Redacted for privacy

Dean of Graduate School

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Typed by Gwendolyn Hansen for Hector John Munn

To

H L and P M

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INTRODUCTION

Embryological growth and development of an organism follows an orderly, reproducible sequence of chemical events which are less extensively described than morphological changes. The specific details through which genetic information is able to guide the course of these events also need description. This thesis describes an attempt to identify the appropriateness of a particular biological cell system, the chick sciatic nerve, for study of the order of events in development.

Differentiation and its Control

Current concepts of biological development and its control focus initially upon the deoxyribonucleic acid (DNA) of the nucleus. A full complement of nuclear material is obtained from the two gametic cells that unite at fertilization. Then under the provision of proper nutrition and environment, the development from a single cell to a mature embryo begins. The single cell divides to form two daughter cells. Subsequent divisions continue which gradually produce cell groups displaying different characteristics. These characteristics distinguish specific tissue types and organs that act together to fulfill the life requirements of the organism. The segregation during the

embryological growth period of cell type varying in biochemical repertoire as a result of release of genetic information is referred to as differentiation (Ebert, 1967). Even though the cell types have different functions or products, they are all integrated to give an organism that is independently functional and able to utilize the provisions of its surroundings for the maintenance of its life processes.

Of particular interest, however, are the mechanisms by which differentiation is controlled. Normal growth and functioning of a cell is dependent upon the proper nutrients being available at the proper time so that the cell can build products that are needed as precursors to other products of the same or adjacent cells at a later time. Mechanisms must exist to determine when the cells begin a function and later to determine when to cease or decrease that function.

This control is understood to be mediated by the nuclear DNA. According to current theory (Jacob and Monod, 1961; Liuzzi et al., 1965), the nuclear DNA embodies information in the form of a unique sequence of nucleotides. Information is released to the cell processes by means of messenger ribonucleic acid (mRNA). The mRNA controls the order in which amino acids are polymerized to form proteins. These proteins in turn may be the cell product or they may catalyze part of the cellular metabolism that yields a cellular product (Weiss and Kavanau, 1957).

However, even though cells have become differentiated as the

embryo develops, the gross nature of the DNA and the sequence of the nucleotides of the DNA are apparently conserved from cell division through cell division by means of the precise mechanism of DNA replication. The assumption would follow that DNA of every cell of a single individual is the same. There is no direct evidence for this assumption and, in fact, alternate assumptions have been proposed (Ebert, 1967). Regardless of which assumption is made concerning the intercellular constancy of the DNA, there is some mechanism by which the DNA functions in the control of differentiation. It is therefore of interest toward the understanding of chemical differentiation to seek information concerning the mechanism of release of information from DNA and the subsequent events that lead to a unique cellular product.

Choice of Living System

In a study of the control of differentation it is necessary to select an appropriate metabolic system for investigation. Some guidelines need to be suggested for this selection.

First, a limited number of cell types should be involved. A single cell type is not necessarily desirable because it is apparent from study of early embryological changes that differentiation occurs as a result of the interaction of cells in slightly different environments (Lillie, 1952; Markert, 1958; Schmitt, 1967). On the other

hand, a study of changes involving many cell types in the system would be too complex to make single events unambiguous.

The second guideline is that the cellular system should have unique cell products or reactions that can be chemically approached, identified and measured. Obviously there are many cellular functions that are ubiquitous to all cells and these would not be usefully applied to the study of differentiation. Thus, only a restricted few cells can receive attention for their utility to provide specific information about differentation.

The third test is related to the second in that it is desirable that the biochemical metabolites, if unique, also have a low turnover rate. Without a low turnover rate, a significant but small change would be soon masked by subsequent changes and become unobservable.

Fourth, for practical reasons, the cellular system should have a fairly short period during which the cell products are produced. Thus their investigation would not involve undue time for information to become available through experimental procedures.

The last guideline relates to the selection of the organisms or tissue that is studied. A major thrust of investigation of differentiation has been directed to invertebrate organisms. This is because invertebrates are thought to consist of less complex cell systems. On the other hand, the embryological information describing the vertebrates, especially avian embryology, is much more complete.

Avian embryology thus affords a firmer basis on which to add new methods or to which new information may be related.

A consideration of the many tissues and organs of the many vertebrate embryos that may meet these criteria leads to the consideration of myelin and the process of myelination in chick embryos as a system to study the mechanism of differentiation and its control.

Myelin and Neural Tissue

Myelin is a structural component of neural tissue. It is found in close association with neurons. These neurons are interconnected into a vast network that may be given the rough structural categories of central nervous system (CNS) and peripheral nervous system (PNS). The CNS consists of the brain and spinal cord which are the centers of correlative and cognitive activities. The PNS includes tissue that is either motor or sensory in function. It is composed of ganglia, nerve trunks, nerve fibers and sensory organelles that are found exterior to the spinal cord.

Each neuron consists of a cell body into which several afferent neurites or dendrites carry impulses and from which usually one efferent neurite or axon carries the impulse. The cell bodies contain the nucleus and the major portion of ribonucleic acid. Neurons with sensory function have their cell bodies in the spinal ganglia. They receive impulses through dendrites from the sensory end plates and

transmit them to the spinal cord by means of the axons that enter the dorsal root of the spinal cord. The motor nerves have their cell bodies in the spinal cord and send their impulses to muscles by means of axons that form the ventral root of the spinal cord.

Various nerve trunks consist of great bundles of motor axons and sensory dendrites may be found as the most obvious anatomical structures of the PNS. The largest of these nerve trunks in birds is the sciatic (ischiatic) nerve. It arises from the fourth through the eighth lumbo-sacral nerves and passes to the posterior and outward through the iliosciatic foramen of the pelvis, down the inner posterior aspect of the thigh to supply the hind limb. It is mostly composed of motor neurites (Bradley, 1960; McLeod et al., 1964).

In addition to neurons, the other major type of cells in the nervous system are the neuroglia cells. These cells are given names depending upon their location and form. An astrocyte is found in the CNS in association with capillaries and other neuroglia cells. It has many fine processes, hence the name astrocyte. Oligodendroglia are also found in the CNS. They are larger than astrocytes and are structurally associated with neurons. The Schwann cell is found in the PNS, but has the same structure and function as oligodendroglia. The term glia is often used as a collective, abbreviated synonym for all neuroglia (Bodian, 1967). The main cell product of glia cells is myelin. The myelin or the presence of the glia cell is necessary for

the proper functioning of the neuron (Hydén, 1959; Hydén and Pigon, 1960).

Electron micrographs of sections of peripheral nerves indicate that the myelin material is composed of a set of concentric layers surrounding the neurites. The fixative-stain for the electron micrograph that demonstrates these layers shows alternating light and dark rings. Also, the dark rings have two alternating intensities. Complete interpretation of the different rings is still being debated, but by comparison with the plasma membrane of the glial cells and by following changes during the early stages of myelination it is possible to derive several reasonable conclusions about the origin of the myelin. The plasma membrane is stained as three layers: a dark outer layer, a lighter inner layer and an unstained layer between (Finean, 1962). Initial stages of myelination show the plasma membrane forming a process extending towards the neurite membrane (Uzman, 1964). The cytoplasm is gradually excluded so that the two inner layers are juxtaposed and appear as one. The glial process now wraps or is wrapped around the neurite until two outer dark surfaces are juxtaposed and also appear as one. The wrapping process continues until several layers of plasma membrane have been formed (Herndon, 1964; Hirano, 1966). If the assumption is made that the stain is absorbed by the proteinaceous coating of the outer and inner surfaces of the plasma membrane, then the unstained layer is

thought to be lipid (Vandenheuvel, 1966).

The final multiple layered structure of the mature myelinated neurite has a large enough cross section to be resolved by the light microscope, even though the individual layers of myelin are not resolved except with electron microscopy. Although all neurons and their processes are found associated with glial cells, the extent or degree of myelination is variable. Some neurites of the CNS have only one set of glial membranes around them and are referred to as unmyelinated neurons. Generally, the neurons of the PNS are highly myelinated.

The myelin, then, may be equated with the plasma membrane as far as its origin and chemical composition is concerned. The cellular membranes are thought to consist of a lipoidal center layer that is actually the thickness of two molecules of phospholipid with the non-polar fatty acid portions of their molecules directed toward each other (Danielli and Davson, 1935; Robertson, 1959; Vandenheuvel, 1963). The result is an interdigitating double layer of molecules. These are held together principally by hydrophobic bonding (Wallach and Zahler, 1966). The polar end of the phospholipid molecules are directed away from the center to provide polar bonding sites for water and proteins. This interpretation is based largely on myelin electron micrographs.

Membranes other than myelin, such as in mitochondrial membranes, may not call for similar structural interpretation as that held by Danielli (Green and Perdue, 1966).

Chemical Analysis of Myelin

Numerous attempts have been made to obtain a chemical analysis of the myelin in order to confirm the Danielli model. These attempts vary in aspects such as: species studied, neural organ or tissue studies and biochemical purification attempted, if any. The list is great, but some of the outstanding reports should be mentioned. Clouet and Gaitonde (1956) and Gent et al. (1964) studied rat brain protein and lipid. Folch-Pi (1955) used whole mouse brain to quantitatively estimate all identifiable components. Autilio, Norton and Terry (1964) prepared myelin concentrates with sucrose gradient centrifugation. A similar procedure is used by Adams, Davison and Gregson (1963) except that ion content and pH were specifically controlled. El-Eishi (1967) studied chick spinal cord myelin histochemically and biochemically without any attempt at particulate fractionation. He shows evidence for myelination between 18 and 21 days after onset of incubation. Human brain lipid composition is the concern of O'Brien and Sampson (1965). Eichberg, Whittaker and Dawson (1964) were interested primarily in phospholipids of myelin as compared to other cellular organelles and membranes of guinea

pig brain.

The listing of studies of chemical composition of neural tissue or specifically of myelin is much more extensive. These studies mentioned give substance to the idea that myelin is composed of a relatively select group of compounds or classes of compounds. These are proteins, phospholipids, cholesterol, and cerebrosides.

Proteins are extractible in various forms. Proteolipids were first described as a distinct component of brain lipid (Folch et al., 1951) but not of lipid extracted from the peripheral nervous system (Folch et al., 1958). Trypsin resistant protein is noted in the PNS by Adams (1955) using histochemical methods. Wolfgram (1966) extracted a protein from bovine brain at pH 2 that is insoluble at pH 5 which he suggests is possibly a specific component of myelin. The major study of phospholipids of myelin has been mentioned (Eichberg et al., 1964). But it should be added that there was little indication that myelin phospholipids were different from phospholipids of other membranes except for slight quantitative variations.

However reports of cerebrosides by Lewin and Hess (1965) strongly support this class of compounds as a "quantitative histochemical referent for myelinated fibers in nervous tissue" (p. 219). Also cerebrosides are noticeably lost in controlled demyelination of chick sciatic nerve (Berry et al., 1965).

Not only is the chemical composition of myelin well reported,

but investigation of the turnover rates of these components shows a definite grouping of substitutents that have halflives in myelin of from 8 to 14 months (Eng and Smith, 1966). This conforms to the suggested guideline for choice of tissue to use in an investigation of control mechanisms.

Time of Myelination

A relatively brief period during which myelination occurs is suggested by electron micrography (Geren, 1954), histological studies (El-Eishi, 1967) and by biochemical studies (Garrigan and Chargaff, 1963). These studies show myelination to be a phenomenon that takes place just prior to hatching of the chick embryo.

A consideration of the embryological origin of the glial cells also leads to this conclusion. Neural tissue arises from the dorsal plate of the ectoderm. As this enfolds to form the neural tube, some of the cells near the developing myotomes do not become part of the neural tube but become associated into what is recognized as the neural crest and later as the spinal ganglia. Among the cells first forming the neural plate, a major portion do not become neurons, but on differentation become glial cells (Romanoff, 1960). They have been noted as early as the second day of development (Reddick, 1951). In the chick spinal cord, glial cells begin myelination on the 12th day (Bensted et al., 1957). However, as the motor neurons, for example,

begin to send neurites to the maturing muscles of the myotomes, the associated glial cells continue to multiply and to spread out along the neurites. When the neurites cease to be rapidly extending, the glial cells then begin to extend their myelin processes that wrap the neurites (Lillie, 1952). This myelination continues rapidly over a brief span of time until the proper maturation level is reached (Cravioto, 1965).

Although many sources of neural tissue are available for study, it would be appropriate to be able to compare the biochemical changes with well documented embryological growth information. The chick is the most widely investigated vertebrate. It also is conveniently cultured and assessed as to age (Hamburger and Hamilton, 1951). Chick embryos lend themselves nicely to tracer studies (Siek and Newburgh, 1965). Peterson and Murray (1955) have been successful in culturing neuroglial cell systems. This in vitro preparation of a differentiated cell system holds promise of still greater refinement for the study of differentation.

Thus the objective of this research is to describe the normal structure and lipid composition of the chick sciatic nerve and its changes with development. These normal patterns can then be related to patterns that result from stress circumstances impressed upon the system that counter the normal developmental processes. Thereby

it is hoped to eventually gain insight into the requirements and intercellular relationships of differentiation.

MATERIALS AND METHODS

Chick Sciatic Nerves

Eggs of White Rock strain (Hubbard pullet and White Mountain rooster) were obtained from Jenk's Hatchery, Tangent, Oregon and maintained at 10°C until used. The eggs were incubated at 98°F dry bulb and 86°F wet bulb in a Jamesway incubator, Model 252-B. Hatching time averaged twenty and one half days. About 90% of the eggs hatched.

In order to obtain embryos of several ages at one time from which to dissect tissue for simultaneous biochemical analysis, groups of eggs were set every day at noon for the planned number of days. The ages of the embryos on the day of dissection was not determined from the setting date but rather from the hatch date of a representative sampling that was left to incubate.

Material was also obtained from chicks at ages beyond hatching. These chicks were kept in a brooder and provided only water for the first three days. This is the period of time that the chick still receives yolk nourishment. On the fourth day, a ration of Purina chick starter mixture was provided as food.

Dissection of the sciatic nerve followed removal of the embryo from the shell and decapitation. Dorsal and leg skin was also

excised and the sciatic nerve exposed by severing the superficial muscles of the upper thigh. The nerve was severed near the spinal cord distally to the spinal ganglia. Care was taken to remove any adipose tissue and any portions of ganglia. The nerve was then severed distally to the knee joint before its ramifications became too numerous.

The dissected nerves were placed in chick Ringer's solution over ice until enough were obtained for the desired experiment. A wet weight of 100 to 150 mg of tissue was required for each determination. The number of nerves needed for a determination varied with age and ranged from approximately 24 nerves at 15 days to 8 at 28 days.

Lipid Extraction

The extraction procedure for obtaining phospholipids, proteolipids and cerebrosides was a modification of that used by Folch, Lees and Stanley (1957). The tissue samples were removed from the chick Ringer's solution, carefully blotted to remove surface liquids and then rapidly weighed on glassine paper to obtain the wet weight. The paper was weighed immediately after the removal of the tissue to obtain the tare weight. This tissue was placed into a conical, ground glass tissue grinder and hand homogenized in one ml of cold methanol (M) This homogenate was transferred to a tared, stoppered ten ml centrifuge tube containing two mls of chloroform (C).

Additional C:M:H_2O (38:19:3 by volume) was added to yield a combined volume of approximately five mls.

This mixture of homogenate and extracting solvents was kept at ice temperature for about 20 minutes and mixed occasionally with a Vortex mixer. The walls of the tubes were then washed with C:M:H_2O (38:19:3) and centrifuged in a clinical centrifuge at top speed for two minutes. The supernatant was pipetted into a 50 ml centrifuge tube. The residue was resuspended two more times in C:M:H_2O (38:19:3). Two milliliters of solvent was used each time. The first washing was for 30 minutes and the second for four hours. The combined C-M extract will be referred to as the "Folch extract."

The extent of the extraction was checked by doing further washes of the residue and assaying for lipid phosphorus in the wash. Less than one percent of the total lipid phosphorus was recovered in these washings.

The Folch extract was washed with 0.2 volumes of chick Ringer's solution. This was followed by three more washes with small amounts of pure upper phase solvent (Folch et al., 1957). The extract was centrifuged following each washing to hasten the separation of the phases. No "fluff" occurred similar to that described by Folch, et al. (1957). The washings were removed with a pipet and combined into a fraction referred to as the aqueous wash. Determinations were made on this aqueous wash after it was partially evaporated

to remove chloroform and methanol.

The washed Folch extract was evaporated nearly to dryness at 20°C and placed in a vacuum desiccator over potassium hydroxide pellets overnight at 4°C. This dryed extract was taken up in C:M (2:1). Since the solvent has a high expansion coefficient, all aliquots used for chemical determinations were made at 20°C.

For chemical measurements on the remaining residue, it was washed with 95% ethanol and 5% ice cold trichloroacetic acid (TCA) to give an acid soluble fraction (Santen and Agranoff, 1963). After dehydration with absolute ethanol and ether, the residue was placed in a vacuum desiccator until the weight achieved a constant value. This dry weight gives an approximate value of tissue size. Two milliliters of one N KOH was added to the dried residue and digested at 37°C until a clear hydrolysate was formed. This hydrolysate was then diluted to appropriate volumes with one N KOH. Aliquots were taken for determination of DNA or radioactivity.

Chemical Determinations

The Bartlett (1959) adaptation of the Fiske-Subbarow procedure was used to determine inorganic phosphorus content in the total lipid extract and in fractionated extracts from thin-layer chromatography.

Cerebrosides were determined by a modification of the methods of Hess and Lewin (1965). Aliquots of the washed Folch extract

containing 1 to 8 μg of galactose were taken and the solvent evaporated. Two milliliters of orcinol reagent (0.22 g orcinol dissolved in 100 ml of 50% H_2SO_4 and used within 12 hours) was added to each sample tube. The tubes were chilled in ice water and the orcinol reagent thoroughly mixed before heating in a boiling water bath for 25 minutes. Care was taken to insure that every tube received similar heating. The tubes were placed in ice water following heating. The color intensity was measured spectrophotometrically on a Bausch and Lomb Spectronic 600 at 425 mμ using one cm cell path versus a solvent blank. Standards containing between 0 and 8 μg of galactose were treated to the same procedure. The amount of cerebroside was assumed to be 4.6 times the amount of galactose.

Proteolipid protein in the washed Folch extract was determined by the Lowry method using the Folin phenol reagent (Lowry et al., 1951). The "Reagent B" or $CuSO_4$ in potassium tartrate was stabilized by the addition of 2.5 ml of one N NaOH per 100 ml of reagent. The protein standard was bovine serum albumen.

Thin-layer Chromatography of Lipids

Quantitative analysis of the lipids was accomplished by a modification of the procedure of Skipski, Peterson and Barclay (1964). Aliquots of washed Folch extract that contained about 5 to 7 μg of lipid phosphorus were used for a quantitative estimation of specific

phospholipids separated by thin-layer chromatography (TLC). The stationary phase was Silica gel H (Brinkmann Instruments, Westbury, New York) that was slurried in 0.001 M Na_2CO_3 before spreading at a thickness of 0.25 mm. The plates were activated at 110°C for one hour. Application of sample was conducted through a box as described by Cruess and Sequin (1965) through which a stream of nitrogen flowed to encourage solvent evaporation.

The plates were eluted using an ascending solvent system consisting of chloroform, methanol, glacial acetic acid, and water (65:25:8:4). The solvent was allowed to move to approximately one cm from the top of the plates. The elution chamber was lined with Whatman No. 1 paper and allowed to equilibrate for two hours before use. The solvent was renewed daily.

Following the evaporation of the solvent from the eluted plates, the lipid material was visualized by placing the plate in a chamber filled with iodine vapor. When the lipid was just visible, the plates were removed and the location of the components marked by scratching the silica lightly. The iodine sublimed from the plate in a few minutes.

The identification of the chromatographed lipids was made by comparison with standard lipids obtained from Pierce Chemical Company, Rockford, Illinois. However, the phospholipids were further checked by their reaction to Zinzadse spray (Dittmer and

Lester, 1964). Further, ethanolaminephosphoglyceride (EPG) and serinephosphoglyceride (SPG) were identified with ninhydrin spray; while cholinephosphoglyceride (CPG) and sphingomyelin (SPM) were identified with Dragendorf's reagent (Skidmore and Entenman, 1962). Cerebroside was identified by means of the orcinol reagent used as a spray. The cerebroside turns a bright orange.

The lipid components that were localized on the silica gel plates were then scraped off with a razor blade onto glassine paper and transferred into tubes for determination of the phosphorus content by the Bartlett method. Prior to doing a spectrophotometric measurement, the silica gel was removed by centrifugation. The amount of the phospholipid recovered was expressed as the fraction of the weight of the specific phospholipid phosphorus to the total lipid phosphorus.

The solvent system C:M:glacial acetic acid:H_2O (160:35:10:1) was used to select cerebrosides from the phospholipids and neutral lipids on TLC. After identification with iodine and removing the material from the plate, the silicic acid with the cerebroside was placed into a short glass column containing C:M (2:1). This procedure completely excluded air and permitted elution of the cerebroside from the column with about four bed volumes of solvent. Recovery of the cerebroside was greater than 60% by this method. This procedure is similar to that of Cruzner and Davison (1967).

Hydrolysis of Galactose from Cerebroside

In order to confirm the presence of galactose in the cerebroside, the cerebroside from the preparative TLC was evaporated and hydrolyzed in six N HCl according to Brand and Sperry (1941). The hydrolyzed product was deionized with Dowex 1-X8, 200-400 mesh that had been converted to the formate form. The deionized eluate was evaporated to dryness before resolution in water for further treatment.

To separate the galactose from the deionized hydrolysate, the sample was streaked on washed Whatman No. 1 filter paper and chromatographed with n-butanol:ethanol:water (10:1:2) for 96 hours (Spiro, 1960). A standard sample of galactose was spotted on the edge of the paper. Following development and drying, the region containing the standard was cut off and treated with periodic acid and benzidine to identify the galactose region (Gordon et al., 1956). The regions corresponding to the standard of the main paper were then removed. The galactose was eluted with water and assayed as previously described.

Galactose-1-^{14}C in vivo Injection

D-Galactose-1-^{14}C (New England Nuclear Corporation, Boston, Mass.) was diluted with sterile chick Ringer's solution to an activity

of ten μc per ml in a sterile flask fitted with a serum stopper. Individual injections were made with a pre-sterilized one cc tuberculin syringe with 0.1 cc calibrations. The unused diluted galactose was frozen between injection operations.

The injection of the eggs was made through an opening punched in the large end of candled eggs. The needle was inserted far enough to place the solution onto the allantoic membrane on the lower surface of the air space.

For hatched ages, the injection was made through the skin of the ventral abdominal region into the body cavity. In no case was any embryo or chick killed or deformed by causes known to be related to the radioactive label or the injection procedure.

The injection plan varied according to the objective. For the determination of the uptake with age of the chick, the injection was conducted 12 hours before the chick was sacrificed. For the determination of the accumulative uptake and for increasing the amount of radioactivity in the cerebrosides, multiple injections of one μc were given every 24 hours.

Determination of Radioactivity

Radioactivity was determined with a Packard Tri-Carb Model 3003 Liquid Scintillation Spectrophotometer. The ^{14}C was counted at gain setting of 18 and discriminator setting of 50-1000. One channel

was set with 2% gain and 50-200 discrimination for the determination of quenching by means of internal standardization.

The scintillation fluids used were Bray's (Bray, 1960) and toluene-methanol (PPO 6 g, POPOP 0.1 g, methanol 414 ml, toluene 586 ml). The toluene-methanol fluid was used initially for samples dissolved in C:M which would readily be evaporated. Bray's scintillation fluid was used exclusively in later determinations. Whenever the sample had been dissolved with KOH, 0.1 ml of formic acid was added to the one ml aliquot before adding the 15 ml of Bray's scintillation fluid.

Expression of Radioactivity Data

An aliquot of the stock injection solution was diluted with water. The radioactivity was determined and used as a measure of the actual activity injected into the eggs or chicks for each experiment. For samples on which both the radioactivity and the galactose were determined, the radioactivity was expressed as:

$$\frac{\text{Total cpm recovered/total cpm injected}}{\text{mg galactose}}$$

In those experiments in which total radioactivity was being followed, the radioactivity of a fraction was expressed as a percentage of total activity:

$$\frac{\text{Cpm in fraction} \times 100}{\text{Total radioactivity recovered}}$$

In vivo Uptake of Inorganic ^{32}P into Total Lipid

Carrier-free $H_3{}^{32}PO_4$ in water was obtained from New England Nuclear Company with a stated activity to noon on a given date. All counting data were calculated back to this hour and day by using the decay factors listed by Wang and Willis (1965). An injection solution was made that contained four μc per μl of solution by dilution with either 0. 25M potassium monohydrogen phosphate or with sterile chick Ringer's solution. An aliquot of the injection stock was diluted quantitatively and its radioactivity determined as a measure of the injected radioactivity.

Injection was conducted in the same way as for the galactose ^{14}C. That is, injection was made into the air space before hatching and subcutaneously following hatching. The required shift in mode of injection produces a discontinuity in the data.

After the lipids were extracted, an aliquot of the total extract was taken and the solvent evaporated before addition of the toluene-methanol scintillation fluid. Duplicate samples were so ordered in the scintillation counter that they could be averaged to a single mid-time and corrected from this time for the decay of the ^{32}P. No quenching correction was found necessary for this system.

The uptake of ^{32}P was expressed as follows:

$$\text{Uptake} = \frac{\%\ \text{activity recovered}}{\text{g P recovered}} = \frac{\frac{\text{cpm recovered}}{\text{cpm injected}} \times 100}{\text{g P recovered}}$$

Histological Methods

Histological preparations of the chick sciatic nerves for ages 18, 20, 22 and 25 days were made. The dissected tissue was washed in chick saline to remove any blood and immediately placed in calcium-formal fixative for 24 hours (Salthouse, 1962). Occasionally the tissue was placed in ten percent formalin until the concurrent chemical procedures could be conducted on tissue taken at the same time. Prior to staining, the calcium-formal treatment was repeated on the tissue stored in formalin. The tissue was dehydrated in a graded isopropanol series: 50%, 60%, 90% and 100% (isopropanol in water, by volume). This was followed by three baths of "Technicon Dehydrant" and two baths of "Technicon Clearing Agent" before impregnation with Paraplast (m. p. 56-57°) for two hours. The entire embedding operation was conducted with the aid of an Autotechnicon.

The embedded tissue was sectioned into ten μ sections. The staining procedure utilized the stain, Luxol Fast Blue G (Solvent Blue 34, Matheson, Coleman and Bell). The procedure is described by Salthouse (1962). A 0.1% solution of Luxol in isopropanol was used and the stain was applied by immersion for three hours at 35-40° C.

Destaining was accomplished with 0.005% lithium carbonate for one half to one minute. The Luxol stain was followed by a hematoxylin-eosin counter stain applied with the standard Harris procedure. This combination of stains is designed to yield blue-black myelin, dark red nuclei and pink connective tissue.

Photomicroscopy

Color photographs were made with Ectachrome X, ASA 25 film through an AO Spenser series LB Phasestar microscope with a 35 mm camera, achromatic objectives and base illuminator. Tissue dimensions were determined with a stage micrometer whose photograph was carried through the same enlargement operations as the photographs of the tissue.

RESULTS

Dissection and Weight Bases

In order to standardize the extraction procedures to be applicable to a given tissue size, all experiments were conducted on a total tissue mass of between 100 and 150 mg wet weight. Samples larger than this resulted in an incomplete extraction and samples less than this increased the error by losses into the solvents. All extractions were carried out in a single glass stoppered centrifuge tube to eliminate transfer losses. For younger ages a greater number of embryos were necessary. The increased number of tissue pieces with greater surface area leads to slightly exaggerated wet weight values as is shown on Table 1.

Table 1. Typical dissection data.

Day from onset	Number of nerves	Wet weight grams	*Dry weight grams	% Dry weight / Wet weight
16	20	0.1249	0.0104	8.3
18	12	0.1171	0.0101	8.6
20	10	0.0988	0.0095	9.6
21	10	0.1177	0.0116	9.8
23	8	0.1064	0.0084	7.9
24	6	0.1001	0.0094	9.4
28	6	0.1071	0.0178	11.4

*Dry weight is that of the dry, extracted tissue following ether washing. See methods.

Even though there are possible procedural errors, it was decided to use wet weight as the basis for expression of data. Dry weight basis would be more ideal except that using the Folch extraction procedures it is necessary to extract the tissue without delay. This is a common procedure by experimenters using Folch methods. Sometimes the dry weight of each extracted fraction is determined and summed for total dry weight. This procedure was tried and was unsatisfactory. The extraction procedures remove about 1% of the wet weight. This would yield a net dry weight to wet weight ratio of about 10 to 11% for embryos and chicks older than 18 days and 7 to 9% younger than 18 days.

A third basis that is often used to express tissue components is that of DNA quantity. Figure 1 shows the change of DNA per weight as a function of developmental age. DNA changes with growth are of two types or a combination of two types. If growth is represented by rapid cell division with a majority of the cells of the same overall dimensions, then the factor DNA per wet weight unit should be constant with growth. However, if growth is a matter of cells increasing in size by extension of their cell walls or by addition of mass to the cell, then growth will be represented by a decrease in the ratio, μg DNA/mg wet weight.

Figure 1 shows a decrease in the DNA/wet weight ratio prior to hatching while after hatching a constant ratio obtains. This later

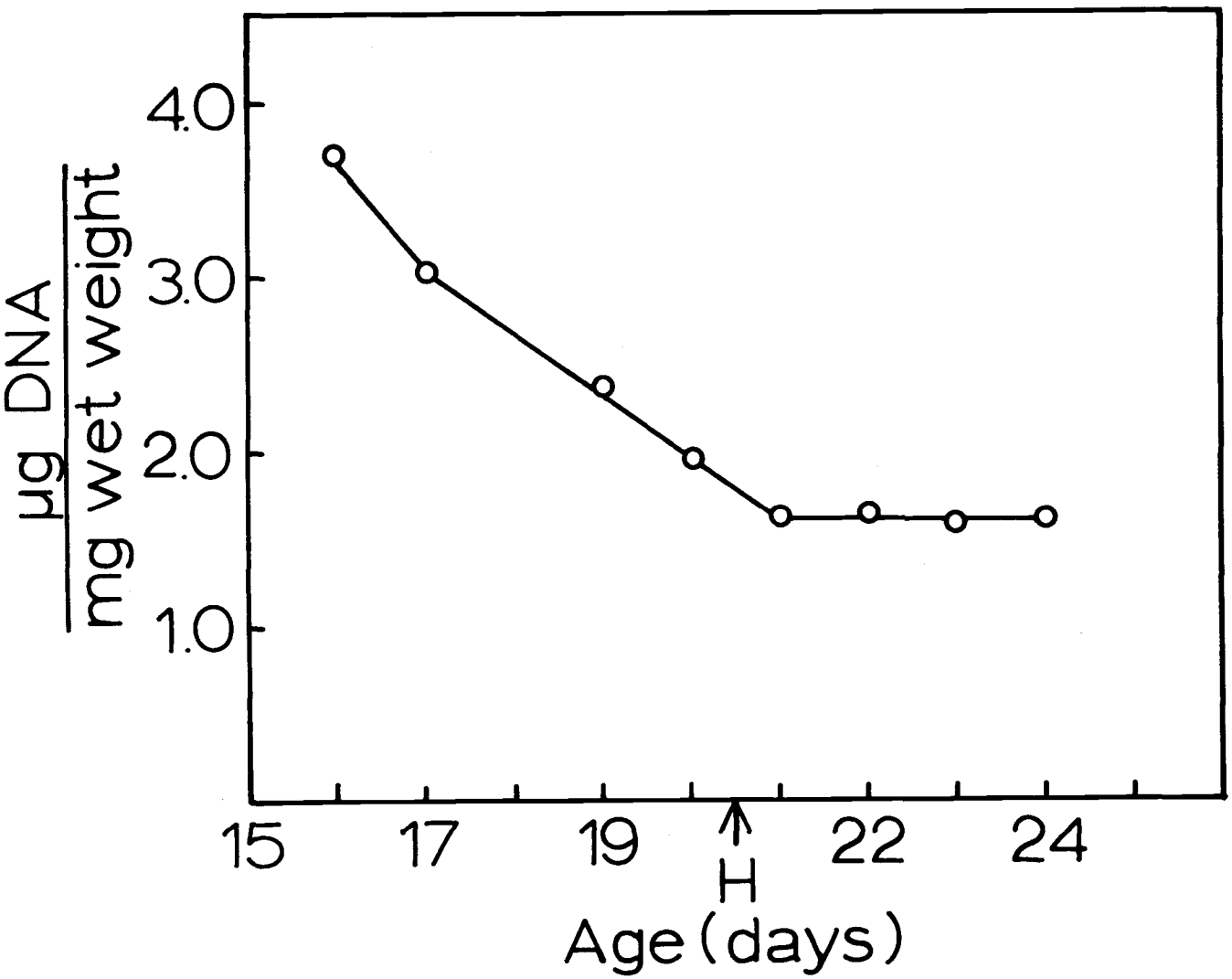

Figure 1. Change of DNA in chick sciatic nerve with development.

situation can thus result from changes that are not involving growth or are a matter of changes that involve chemical conversions of precursor material into products within the cell.

Since the ratio of DNA to wet weight varied during development, it was considered better not to express the data on the basis of DNA.

Phospholipid Phosphorus

Total inorganic phosphorus was determined in the Folch extract as described under Methods. This information is summarized in Figure 2. Curve A shows an overall pattern of change in phospholipid phosphorus up to 30 days. The rate of change from day to day is demonstrated with Curve B. There is a fairly constant rate of increase up to 17 days and between 23 and 28 days. However, a more rapid rate of increase occurs between 17 and 19 days and again between 21 and 23 days. These two periods of rapid change are interrupted just before hatching, 19-20 days, by a period of retardation.

The total lipid extract was separated into individual phospholipids by means of TLC with an acidified chloroform-methanol solvent. Figure 3 is a photograph of one plate that is representative of this separation after iodine exposure. The intensity of the iodine was not deep enough for the photographic record in all cases, but the lipid regions are marked for removal in the next step. Note the complete separation of the four dominant phospholipids by this method. The

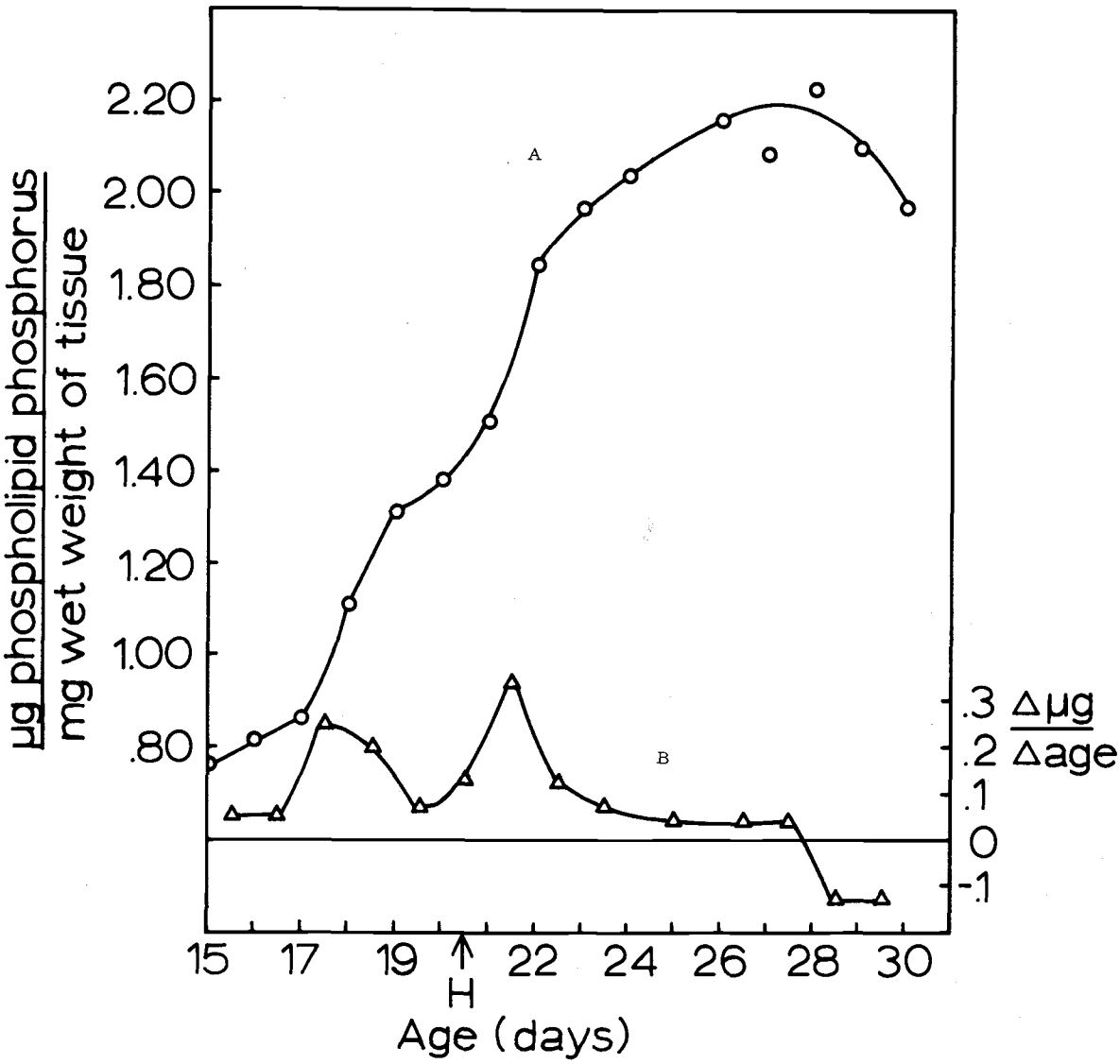

Figure 2. Change of phospholipid phosphorus of chick sciatic nerve with development.

A. Change of phospholipid phosphorus
B. Rate of change of phospholipid phosphorus

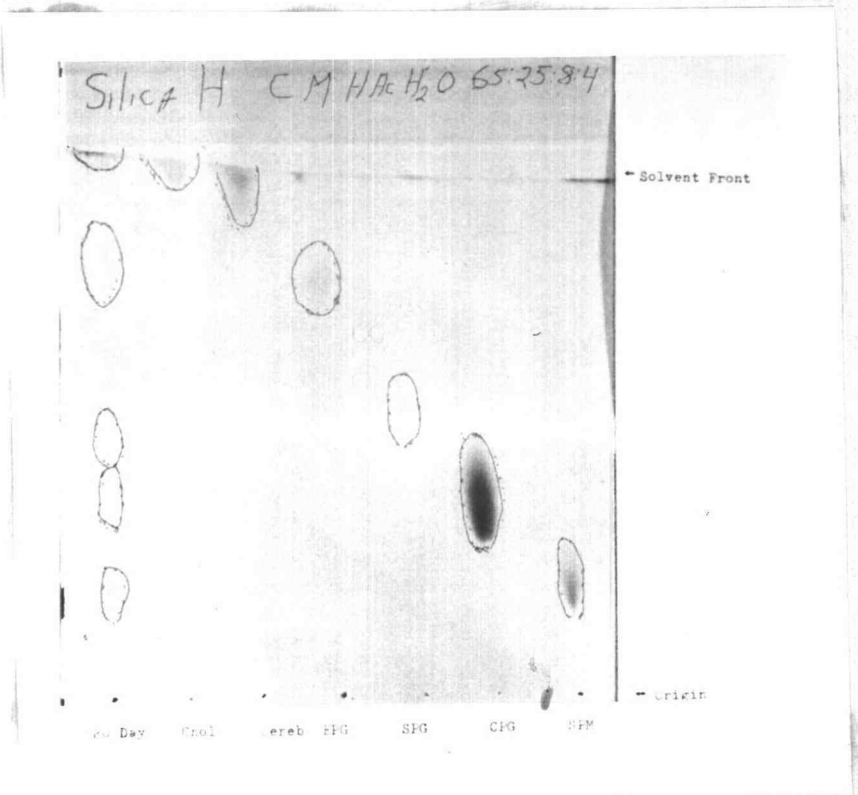

Figure 3. Thin-layer chromatogram of Folch lipid extract of sciatic nerve of a 20 day chick embryo. Stationary phase is Silica gel H slurried in 0.001 M Na_2CO_3 spread 0.25 mm thick on 20 X 20 cm glass plates. Solvent is chloroform: methanol: glacial acetic acid: water (65:25:8:4 by volume). Visualized with iodine vapors. Known compounds used for comparison include: cholesterol (Chol), cerebroside (Cereb), ethanolamine phosphoglyceride (EPG), serine phosphoglyceride (SPG), choline phosphoglyceride (CPG) and spingomyelin (SPM).

cerebrosides move with the solvent front.

The separated phospholipids were scraped from the plate and assayed for inorganic phosphorus. The recovery of the inorganic phosphorus by this method varied between 70 to 100%. In order to compare the different determinations, the amount of each lipid is expressed as the percentage of the total lipid recovered from a given determination. This information is summarized in Figure 4.

Cholinephosphoglyceride occurs in the highest relative amount in the early ages and shows a steady decline throughout the growth period. There are no periods of significant change during this decline.

Ethanolaminephosphoglyceride is next highest in quantity at 15 days. The level of EPG declines slightly but then increases for the three days prior to hatching. It then decreases slightly at hatching before increasing after hatching to reach a constant level at about four days after hatch.

Serinephosphoglyceride shows a nearly constant ratio prior to hatching with a slight rise after hatching. The separation of EPG and SPG on TLC is not always complete. Occasionally when SPG appears higher than usual, the determination of EPG will be less than usual. This source of error has lead to some license in the interpretation of the points plotted on the graph.

Finally, sphingomyelin is lowest in quantity in the early ages. This component shows a gradual increase with development that

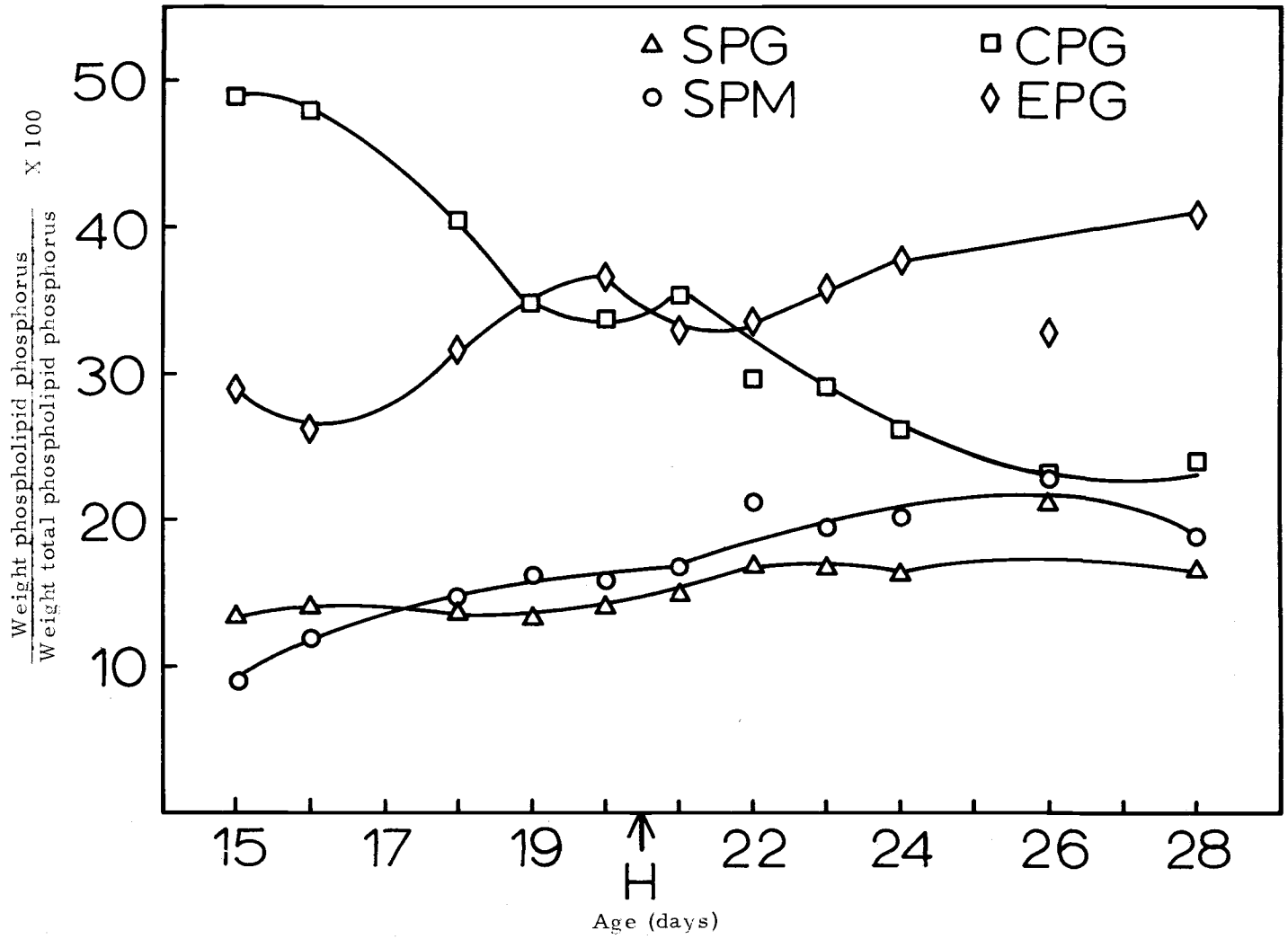

Figure 4. Changes in the relative percentage of phospholipids in chick sciatic nerve lipid extract with development.

somewhat mirrors the opposite change in the CPG. The final ratios of CPG and SPM are both in the region of 20% of the total lipid

Several attempts were made to identify the presence of inositolphosphoglyceride (IPG) in this tissue using the spot test of Feigl and Gentil (1955). The test was not positive though similar extractions of chick brain show the presence of inositol (Siek and Newburgh, 1965). The conclusions possible are that IPG is not present in chick sciatic nerve or that the quantity of IPG is too small to be demonstrated using the smaller samples employed.

Uptake of ^{32}P as a Function of Time

Eighteen day old embryos were injected with four μc of ^{32}P each and incubated for various lengths of time up to 24 hours. The embryos were then sacrificed at different times, the total lipid fraction prepared and radioactivity determined as described in Methods. Figure 5 summarizes the results.

After a brief lag period of about one hour, there is an increase in the rate of uptake. This rate continues at about the same level through 24 hours of incubation. Since the rate of change of uptake is nearly the same at 12 hours as at 24 hours after injection, the subsequent determinations were made with eggs or chicks that were injected with the label 12 hours before termination. This experiment indicates that the length of time of the incubation following injection

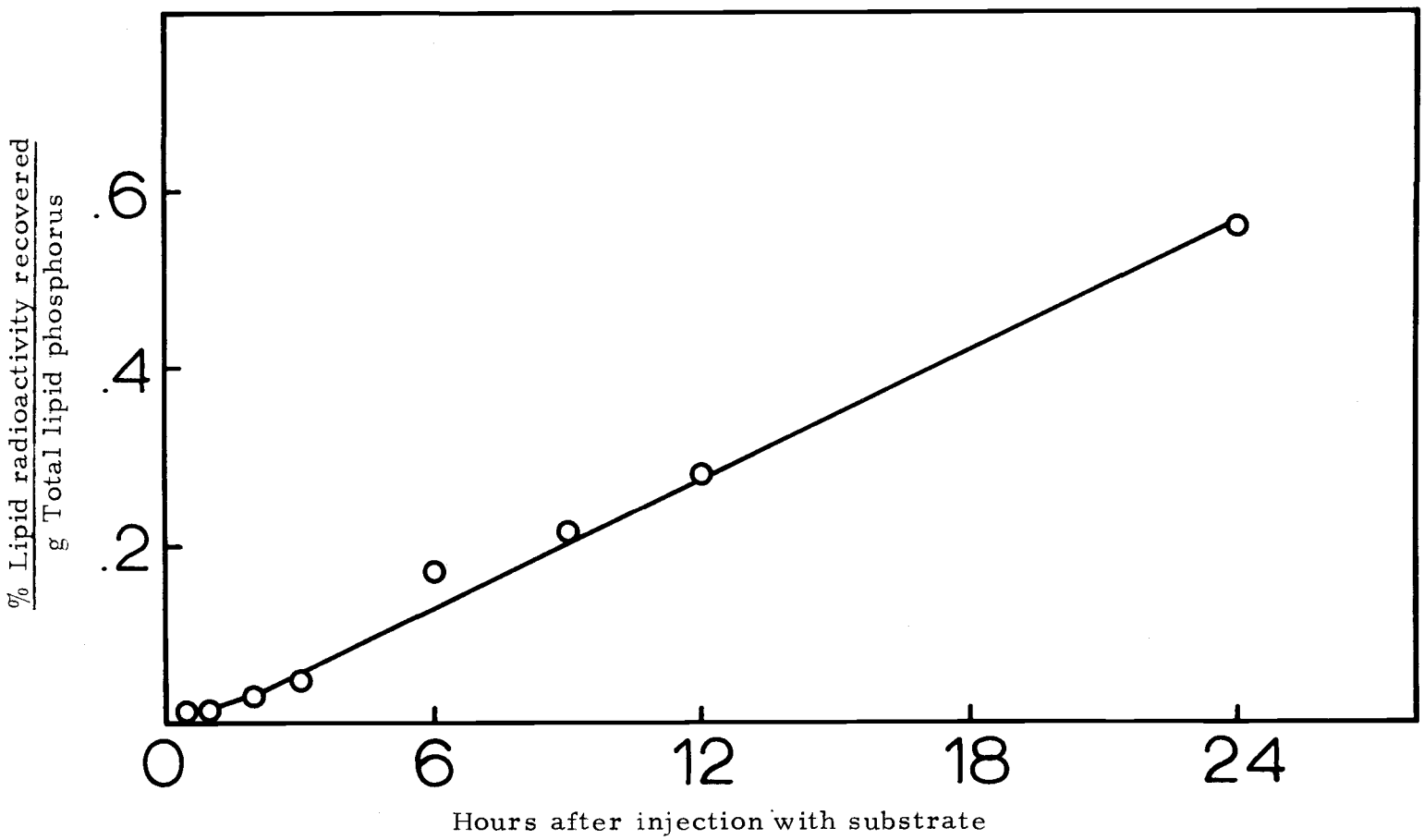

Figure 5. Uptake of ^{32}P into sciatic nerve of 18 day chick embryo as a function of post injection incubation time.

must be carefully controlled in order to avoid variation of the results.

Uptake of ^{32}P with Development

Embryos or chicks of ages from 15 to 24-1/2 days from onset were injected with four μc of ^{32}P and allowed to continue incubation or growth for 12 hours before termination. Again the total lipid was extracted and the radioactivity and phosphorus content were determined. The data on this uptake are given in Figure 6.

These data are from two series of determinations of different ages and therefore could not be numerically averaged. A graphical average is shown. The resulting pattern shows trends that are of interest. The uptake decreases from 15 to 17 days. This is followed by an increase to about 19 days. The hatching event is preceded by a slight decrease in the uptake. The change in mode of injection from an air space injection to a subcutaneous injection before and after hatch, respectively, is noted by a sizeable increase in the uptake after hatching. There is a rapid decrease from the highest level just following hatch so that a minimum is reached at about 24 days. Also, the rate of decrease after hatch is nearly the same as the rate of decrease just prior to hatch.

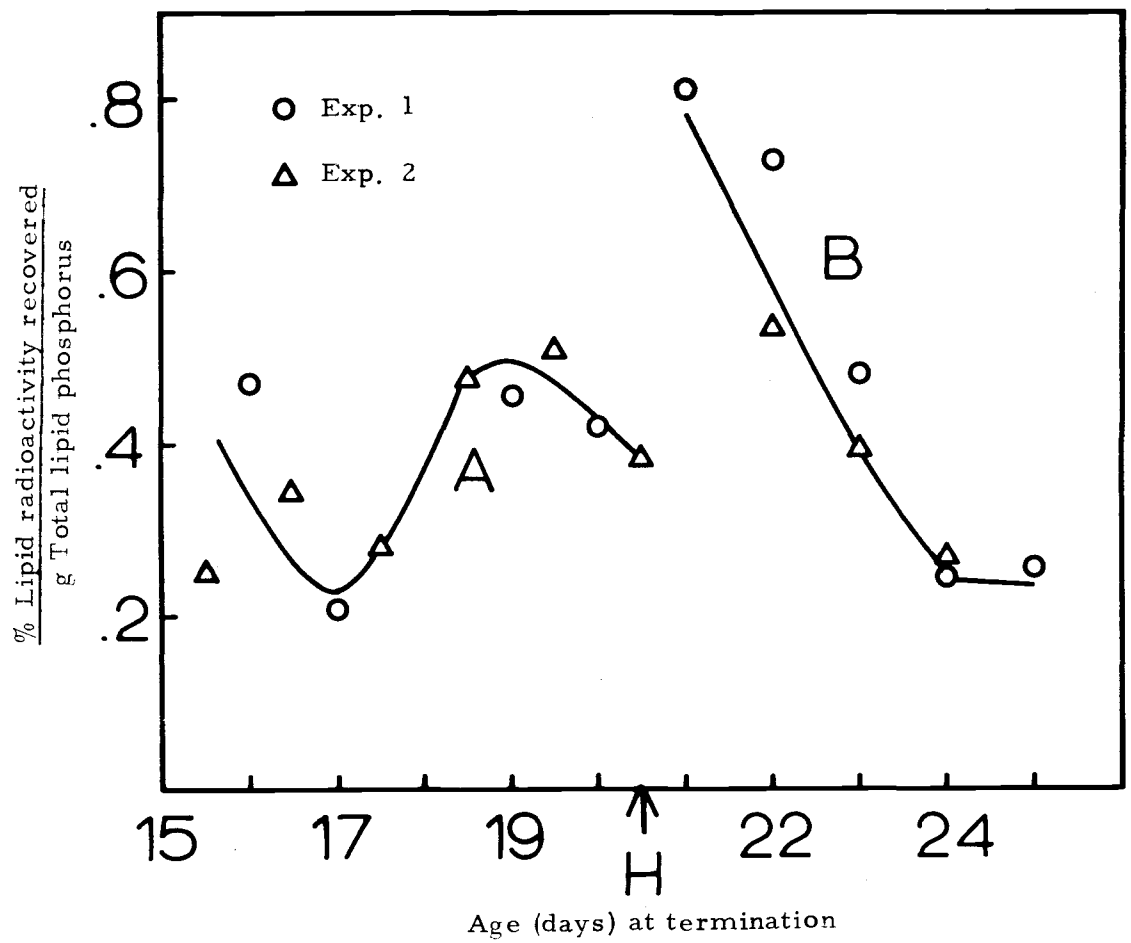

Figure 6. Uptake of P^{32} into lipids of chick sciatic nerve with development.

A: Allantoic absorption of substrate
B: Subcutaneous absorption of substrate

Changes in Proteolipid Protein

The total lipid extract was also assayed for protein content according to the Lowry method as noted in the Methods sections. Figure 7 shows the changes in μg of protein per mg of wet weight as a function of age from 15 days to 28 days of development. The pre-hatch level of protein is relatively low with a maximum at 16 days after which the level decreases prior to hatching. The event of hatching is concurrent with a rapid increase to 22 day maximum. A second minimum occurs at 24 days after which a rapid increase occurs.

Total Cerebroside

The orcinol method for determination of galactose was used to estimate the quantity of cerebroside present in the total lipid extract of the chick sciatic nerve with development. The sigmoidally shaped curve of Figure 8 gives the results of this series of tests. This is the average of four different series of determinations. Not all four cover the same portion of the development period, however. Data for ages above 25 days are from one series only.

It is of interest that this component of the lipids does not show any significant variations during development other than that which is typical of a rapid growth phenomenon. There is a slow period up to about 16 days, a regular growth stage through the hatching period,

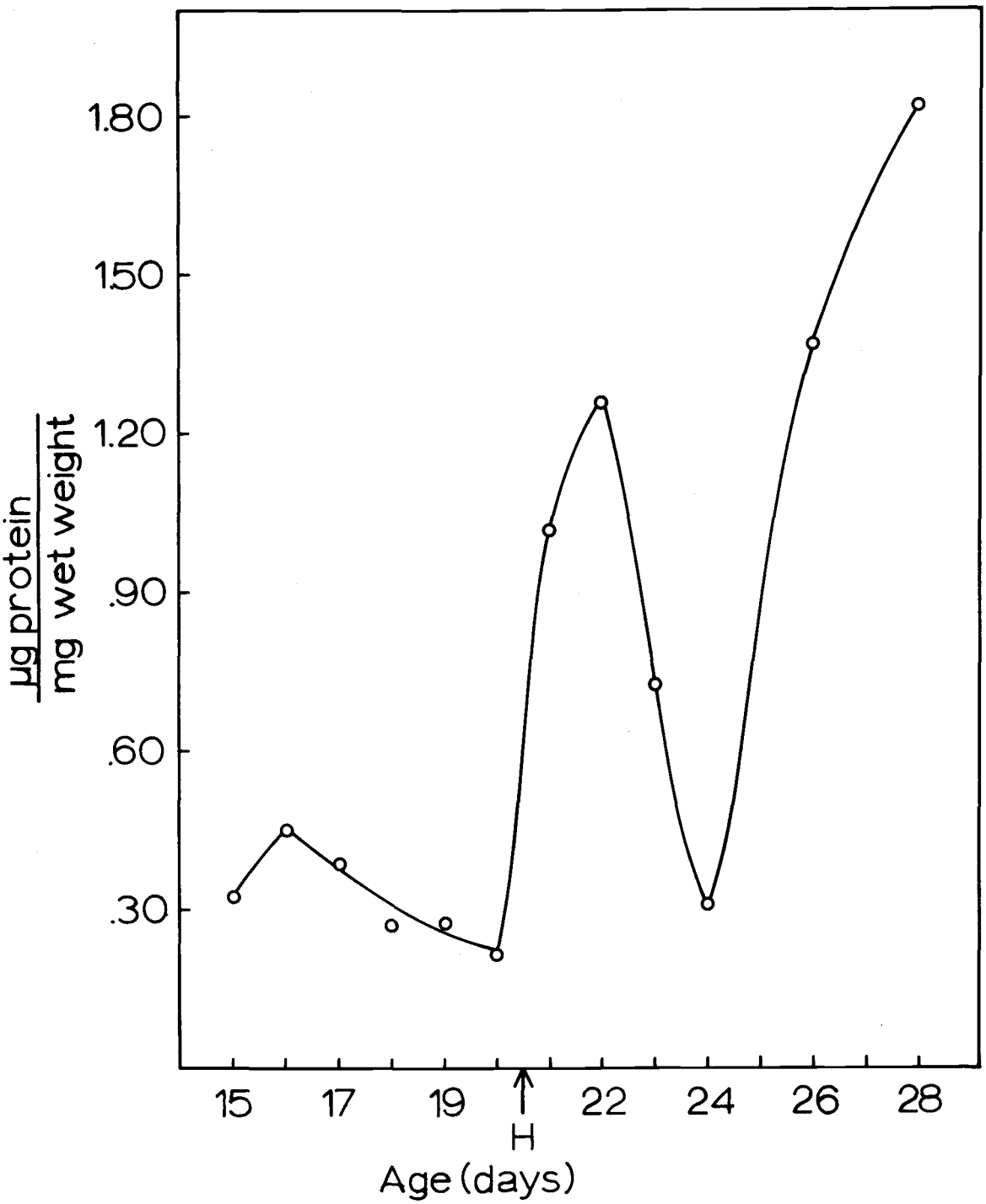

Figure 7. Change in proteolipid protein of chick sciatic nerve with development.

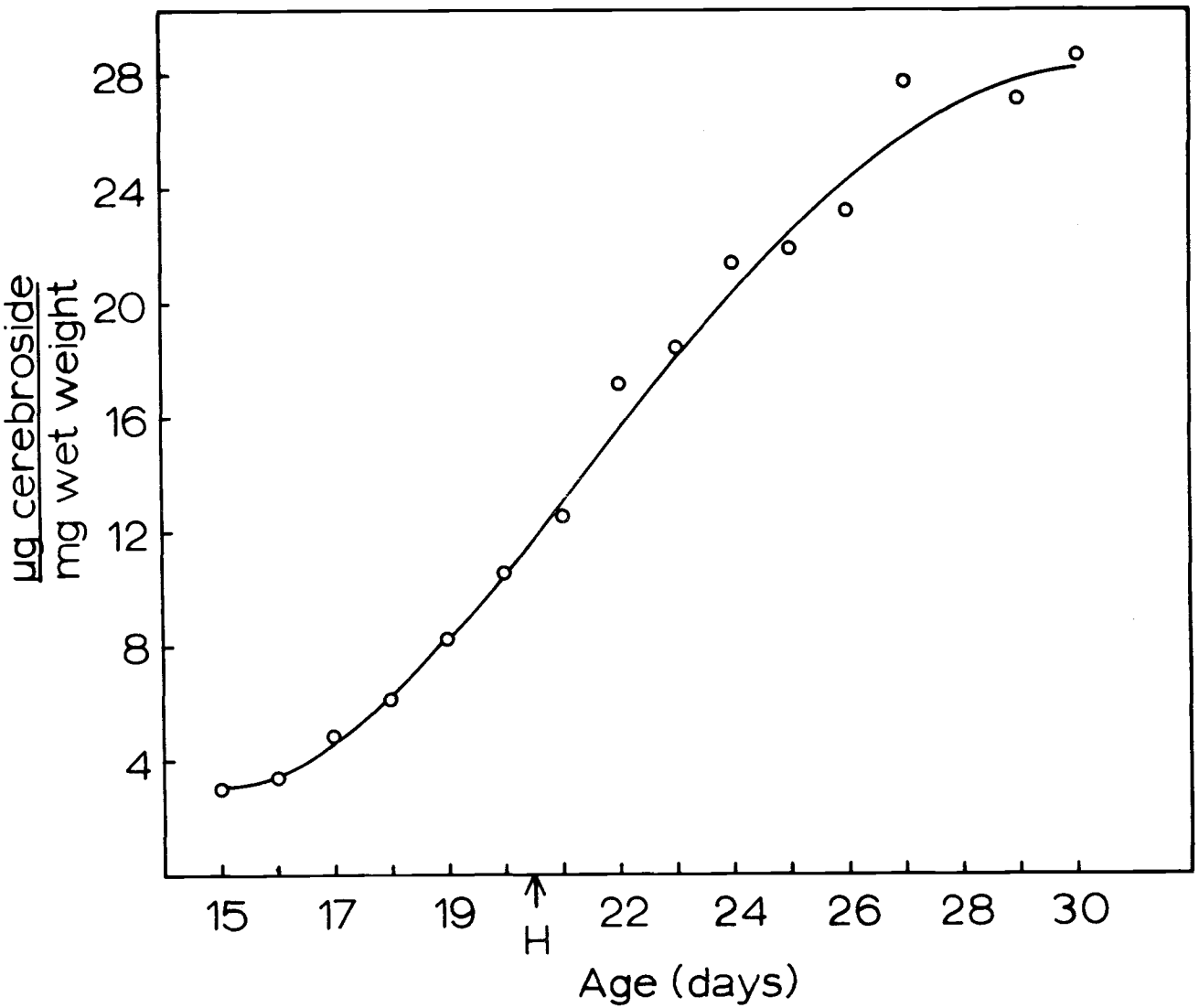

Figure 8. Change in total cerebroside content of chick sciatic nerve with development.

and a retardation at the older ages.

There are no obvious effects corresponding to the day of hatching similar to what is observed in the case of phospholipids.

Galactose-1-^{14}C Uptake Into Tissue Fractions

Table 2 shows the results for the experiments in which galactose-1-^{14}C was used as a metabolic tracer. As the Methods section described, this possible precursor of cerebroside was injected into the air space of incubated eggs or subcutaneously into chicks and allowed to be metabolized for 12 hours before dissection of the sciatic nerve and preparation of the lipid fractions. These data express the radioactivity recovered in each fraction of the separated components as a function of development. Also shown is the percentage this radioactivity represents of the total activity found in the sciatic nerve.

Even though the amount of galactose ^{14}C injected at each age was the same, the amount that could be recovered from the tissue steadily declined. This can best be accounted for by the increased size of the embryo or chick and the increased amount of dilution that takes place with growth. Since the galactose can enter the glycolysis pathway by inversion by UDP glucose epimerase, as the metabolism increases, a greater amount will be lost to this route.

Several observations can be made concerning the percentages of radioactivity recovered in the four fractions. The 5% TCA extract is

designed to remove any soluble protein. The percentage of radioactivity of this portion was nearly constant through the entire growth period. This radioactivity is of unknown source. It is possible that this low, constant level could be a function of the constant amount of solvent used that would consistently remove a given portion of the material present.

Table 2. Galactose ^{14}C uptake into chemically separated components of chick sciatic nerve with development.

Days From Onset	Quench corrected cpm					% of total cpm			
	L	F	T	R	Total	L	F	T	R
16	646	873	220	2473	4212	15.3	20.7	5.2	58.7
18	559	607	132	1725	3026	18.4	20.0	4.5	57.0
20	717	707	110	1412	2946	24.3	24.0	3.7	48.9
21	693	521	100	1332	2646	26.2	19.7	3.8	50.3
23	717	648	64	562	1991	36.0	32.5	3.2	35.4
24	589	372	53	261	1275	46.2	29.2	4.2	20.5
28	255	58	21	117	451	56.5	12.9	4.6	25.9
B	0	21	0	0	21				

Symbols: L = total lipid extract washed according to Folch method
F = aqueous wash of the total lipid extract
T = 5% TCA extract
R = residue remaining after lipid and TCA extractions

The insoluble residue includes many components. These include DNA, RNA, and insoluble protein, which is the major component. No attempt has been made to fractionate or analyze this portion. The radioactivity in this fraction has the largest percentage at day 16 where

the determinations begin. From this high level of 58.7% there is a steady decline until beyond hatching when a nearly constant level is reached.

Contrary to this latter effect, the radioactivity in the total lipid extract shows a steady increase from a level of 15% at day 16 to over half of the radioactivity toward the end of the test period. This fraction consists of phospholipids, proteolipid protein, neutral lipids and cerebrosides. At this point, the amount of activity in these various sub-components was not distinguished.

Finally, the radioactivity of the aqueous wash of the total lipid extract shows a percentage of nearly 20% prior to hatching, it is higher after hatching and then diminishes. This fraction is known to contain gangliosides. Since gangliosides also have a galactose moiety, it is possible that the added galactose is a precursor of gangliosides.

Accumulative Uptake of Galactose-1-^{14}C

The previous data raise a question if any of the radioactive materials are lost during the incubation period through metabolic replacement and if the radioactive material will build up to a greater amount if more substrate is provided. This accumulative aspect was studied by using six groups of eggs that were incubated together. Four groups were inoculated with one μc of carrier free galactose-1-^{14}C per egg. These groups represented the ages 16, 17, 18 and 19

days from onset. A fifth group of eggs was given a daily injection of one μc per egg per day on the same four days as the groups receiving only one μc. This last group received a total of four μc and should reflect the same total uptake as the sum of the uptake in the groups that were inoculated only once.

All five groups and an uninoculated group were incubated until day 20 when all of the eggs were opened, dissected and the lipid extract prepared. The radioactivity and galactose content in the total lipid extract was determined. These results are given in Table 3. The galactose is expressed in terms of total cerebroside present.

Table 3. Radioactivity and cerebroside quantity in total lipid extract from chick sciatic nerve. Variation in age at time of inoculation.

Group	Age at inoculation	Total cpm recovered / mg wet weight	μg cerebroside / mg wet weight
1	16	4.49	14.7
2	17	6.47	13.2
3	18	9.20	13.1
4	19	10.25	15.4
Total groups 1-4		30.41	
5	16-19	31.02	18.0

Uptake of Galactose-1-^{14}C into Cerebroside

There is also a question if the galactose is the component that carries the radioactivity in the lipid extract. It is useful to be able to

relate the radioactivity to the cerebroside content of the lipid extract. To do this an aliquot of the total lipid extract of the samples described in the previous paragraph were used. The embryos were all at the age of 20 days when sacrificed. The extract was fractionated by TLC, the spots located, scraped from the plate into the scintillation vials and assayed for radioactivity. Figure 9 shows the thin-layer chromatograph that gives a good separation of the cerebrosides. There are two spots that are positive to the cerebroside spray test. Each of these was counted separately and the amount of radioactivity summed. This was then expressed in terms of activity of the total cerebroside. The amount of cerebroside was taken from the determination of galactose in the total lipid extract. Thus a specific activity of the total cerebroside in the sciatic nerve extract could be expressed as the total cpm in the cerebroside per μg cerebroside. This information is given in Table 4.

Table 4. Activity in cerebroside isolated from sciatic nerve of 20 day embryos inoculated with galactose ^{14}C.

Group	Age at inoculation	cpm in cerebroside / μg cerebroside
1	16	0.338
2	17	0.489
3	18	0.664
4	19	0.725

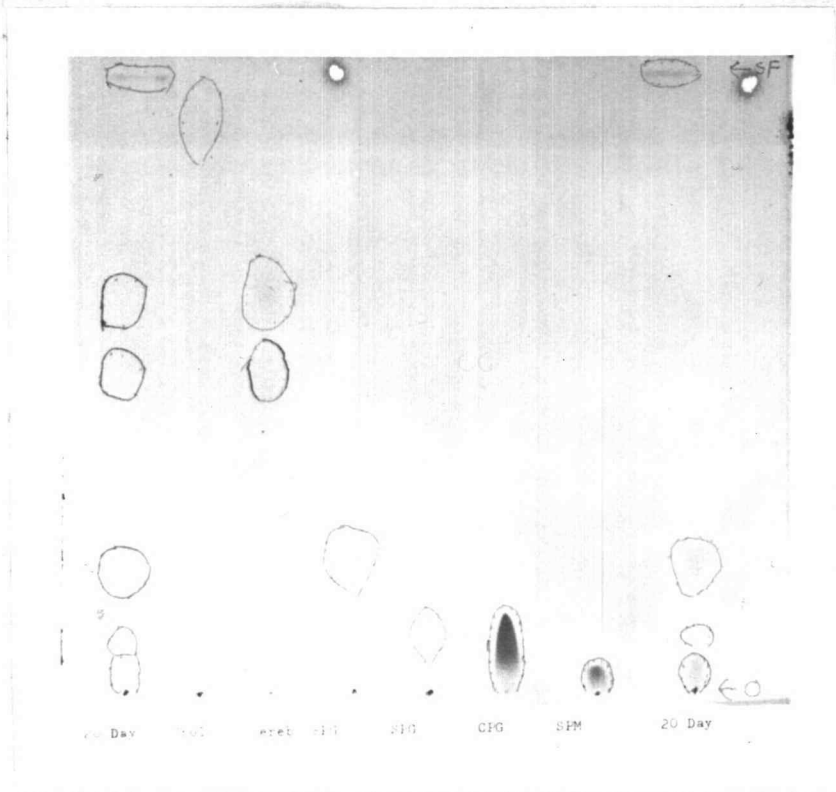

Figure 9. Thin-layer chromatogram of Folch lipid extract of sciatic nerve of a 20 day chick embryo showing separation of cerebroside. Stationary phase is Silica gel H slurried in 0.001 M Na_2CO_3, spread of 0.25 mm thick on 20 X 20 cm glass plate. Solvent is chloroform:methanol:glacial acetic acid:water (160:35:10:1 by volume). Standards and abbreviations are the same as for Figure 3.

Recovery of Galactose from Radioactive Tissue

The total lipid fraction was assayed for galactose and an aliquot fractionated by preparative TLC. The cerebroside was located by I_2, removed and eluted. This sample was assayed for galactose and radioactivity and an aliquot evaporated and hydrolyzed. Following the hydrolysis, the acid was removed by deionization. The eluted material was made up of five ml volume and aliquots assayed for galactose and radioactivity. An aliquot was streaked on paper and chromatographed. The galactose region was identified, removed and eluted. The eluate was assayed for galactose and radioactivity. These results are summarized in Table 5.

Table 5. Specific activity of products of each step in the purification of galactose from galactose-1-^{14}C labelled lipid.

Sample	Specific Activity (cpm/μg galactose)
Total lipid	8.28
Cerebroside	15.58
Hydrolyzed cerebroside	2.65
Galactose from paper chromatogram	1.24

This shows that even though there is considerable concentration of the label into the cerebroside as compared to the total lipid, the label is not maintained strictly in the galactose portion of the

cerebroside molecule. Admittedly, this experiment was of an exploratory nature and should be refined. But the trend shown here and in the next experiment are consistent.

Uptake of Radioactivity of Galactose-1-^{14}C into Phospholipids

At the same time that the radioactivity of the cerebroside is noted, it is possible to determine the degree of activity that is recoverable from the other lipid components. The same TLC plates were used as for the cerebroside assay. Reference to Figure 9 shows that this solvent system does not distinguish between CPG and SPG, but that EPG is nicely separated and SPM is adequately separated. The results for the group inoculated at 18 days and sacrificed at 20 days were typical of the relative radioactivity in the lipid types. This is given in Table 6.

Table 6. Ratios of radioactivity in TLC fractions to the total activity recovered from the plates.

Lipid type	% activity in spot of total activity recovered
Neutral lipid	21.6
Cerebroside	26.8
EPG	19.2
CPG, SPG	15.9
SPM	23.0
blank	3.5

Although over a quarter of the radioactivity is accounted for by the cerebroside fraction, it is obvious that the galactose label is highly metabolized and shunted into the glucose metabolic pathway. Thus the glyceride, fatty acid or base portions of the phospholipids could all carry the radioactivity.

Histology of the Chick Sciatic Nerve

Color photomicrographs of the histological preparations of the chick sciatic nerves from ages 18, 20, 22 and 25 days are mounted as Figures 10, 11, 12, 13, respectively.

Myelin stains a blue-black with the Luxol blue G. The nuclei stain a dark red from the counter stain while the connective tissue is a light red. Red blood cells stain red with darker red nuclei. The consistency of the staining procedure can be roughly judged by the colors of the red blood cells from preparation to preparation.

In each photomicrograph a region on the perimeter of the nerve trunk was chosen to show the relative amount of connective tissue present. In all cases there is a small amount of adipose tissue between the nerve trunks. A band of connective tissue rings each nerve trunk and extends somewhat into the nerve trunk to shape it into regions. In some cases the connective tissue is wavy around the perimeter of the nerve trunk. This indicates shrinkage due to

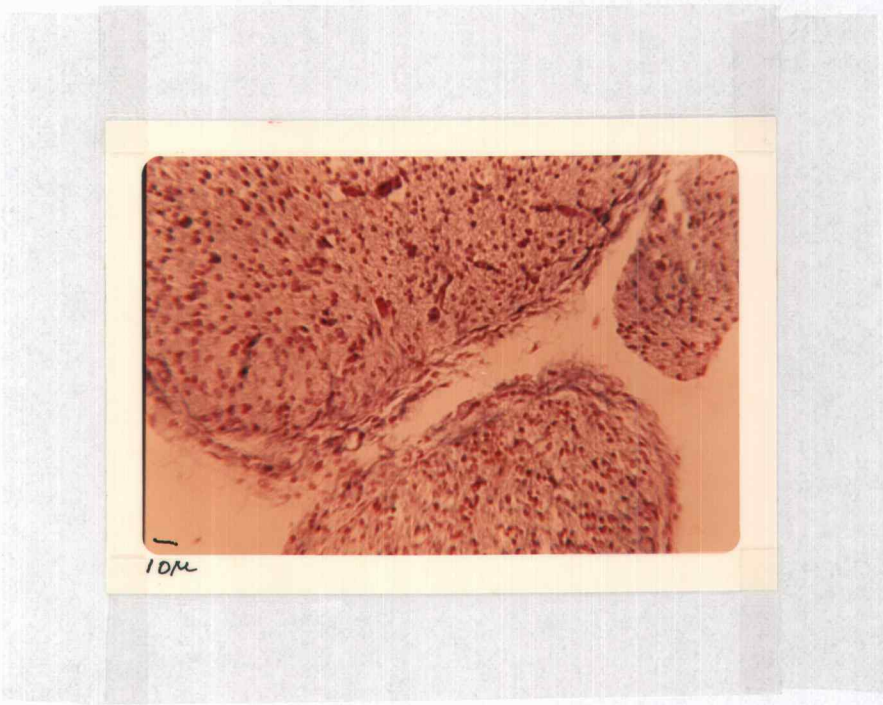

Figure 10. Cross section of 18 day chick sciatic nerve. Luxol Fast Blue G stain with hematoxylin-eosin counterstain. 300X

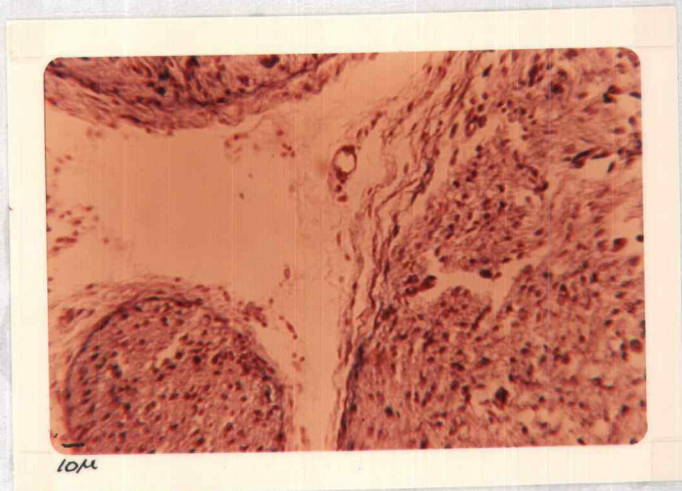

Figure 11. Cross section of 20 day chick sciatic nerve. Luxol Fast Blue G stain with hematoxylin-eosin counterstain. 300X

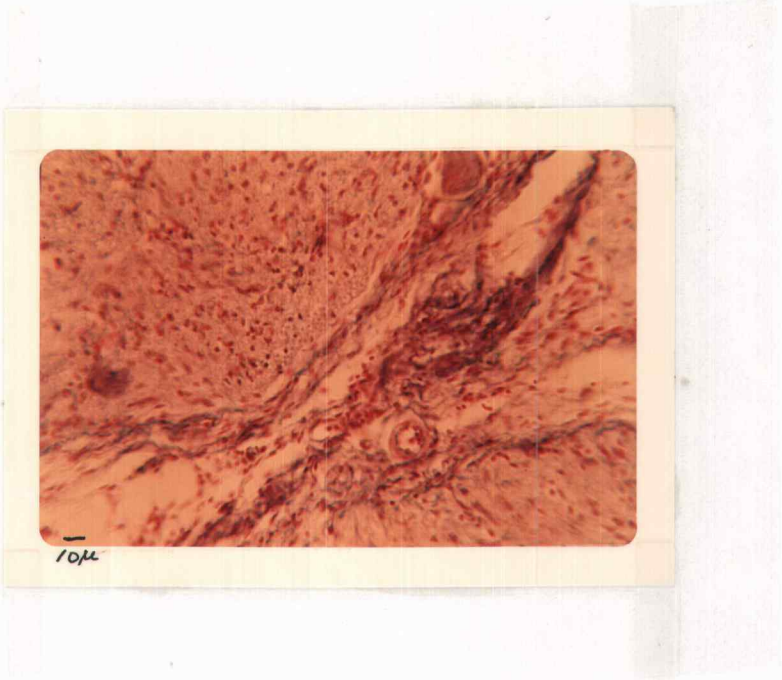

Figure 12. Cross section of 22 day chick sciatic nerve. Luxol Fast Blue G stain with hematoxylin-eosin counterstain. 300X

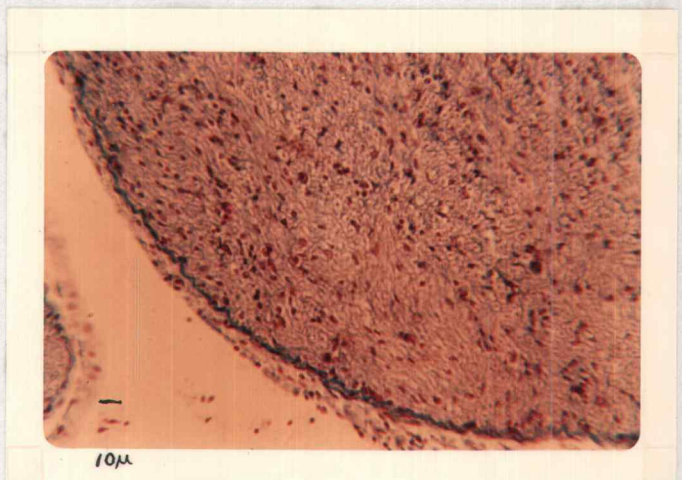

Figure 13. Cross section of 25 day chick sciatic nerve. Luxol Fast Blue G stain with hematoxylin-eosin counterstain. 300X

dehydration of the tissue. Other than the neural fibers and glial cells, some small capillaries may be also identified.

A comparison of the tissue from different ages of development shows an increase in structural definition and myelin staining properties. If the mature myelin structure is present in a good cross section, it appears as a blue ring of varied thickness and diameter with a dot in the center. This is thought to be the result of the stain adhering principally to proteinaceous material of the tissue after the lipid material has been dissolved out in the dehydration of the tissue. If the neurons are sectioned obliquely, the myelin shows a banded or fibrous appearance. In the tissue from 18 day embryos, there is little indication of myelin structure or staining. Both structure and staining properties of myelin can be identified in the tissue from 20 day embryos. Then for both the 22 day and 25 day chick nerves, there is extensive myelin staining and structural pattern demonstrated.

DISCUSSION

Previous investigations of growth patterns in lipids of the chick embryo in this laboratory have lead to the consideration of lipids of neural tissue as involved primarily in myelination (Siek and Newburgh, 1965; Bieber, Cheldelin and Newburgh, 1962). The next step chosen for this line of investigation is then to explore and describe the lipids in a specific, myelinating tissue, the chick sciatic nerve, with the view of noting a specific property through which the chemical and structural changes of myelination could be associated.

After a brief review of the problems of making chemical determinations on chick sciatic nerves, there will be an analysis of the significance of the chemical determination of the lipids studied. This will be followed by a consideration of the attempts at in vivo tracer studies using the two radioactive substrates, inorganic ^{32}P and galactose-1-^{14}C. A final comment will be made about the histological preparations made of the tissue.

The Chick Sciatic Nerve as Source Tissue

The attempt to obtain precise chemical data from biological material involves minimizing a number of problems imposed by biological variability and accounting for any non-random events encountered during the growth period. The use of the chick sciatic

nerve encounters several of these. The most outstanding of these can be noted as: defining age or growth stage, feeding and nutritional aspects, the physiological requirements of the event of hatching, genetic variability and associated tissue variability, and finally, the variety of cell types in addition to glial cells in the tissue chosen. These biological problems are matched by problems encountered in conducting the chemical analyses. The limitations imposed by these biological and chemical parameters will be discussed in turn.

The embryological time of the structural event of myelination has been thought to be between 18 days and hatching in the chick. Starting with this approximation and preliminary studies, the time span chosen for the experiments was taken between 15 day embryo and the 7 day old chick (28 continuous days). The sciatic nerve is quite small before 15 days. The analysis could have started with 12 day embryos, but this is impractical due to the great number of embryos needed to be dissected for one experiment. Following hatching, the care and feeding of the chicks effectively limits the range to 7 days after hatching. The chicks do not require food for the first three days and some will survive for two more days, but beyond this, feeding is required.

Although the age stages of embryonic chicks has been well established by the work of Hamburger and Hamilton (1951), in practical situation during the pressure of dissection, the measurement of

every chick to verify the proper stage assignment is inconvenient. Occasionally, a chick was discarded because of obviously retarded development in relation to the other embryos used in the experimental group. But for the most part, the eggs were taken randomly so that the biological variability of laying and incubation that results in a hatching variation of one to two days could be averaged out. Ages of hatched groups were more certainly established since a group could be collected just after hatching that has an age variation of only a few hours.

The event of hatching imposed a discontinuity on this study. Casual observation indicates that the development of the embryo that climaxes with hatching approaches this event with a series of special actions. The major shift of structure is in the respiration and blood circulation. Prior to hatching, blood circulates in an extra-embryonic network below the shell and allantoic membrane. About 6 to 12 hours prior to hatch, the beak breaks the shell. Presumedly at this time, the lung respiration begins to function and supersedes the extra-embryonic respiration. Also at this time, the blood is withdrawn from the last remnants of the extra-embryonic circulation so that when the chick is released from the shell, it usually does not lose blood.

Another factor at hatching seems to be of a protective nature. The newly hatched chick can endure unusual buffeting without bleeding

or bruising. For example, decapitation at this time does not result in the same degree of bleeding as before or somewhat after hatching. Thus a form of metabolic quiescence is imposed. Some of the data might support this observation.

Hatching also imposes a methodological discontinuity on the mode and site of injection of radioactive substrates before and after hatching. In this case, injection into the air space assumes that the material will be absorbed into the extra-embryonic circulation and thence be transported to the liver. Post hatch injections were made into the peritoneum. The substrate must pass into the peritoneal circulation and be carried to the liver. The effect of the absorbing membranes on the passage of the material in each case is an unknown factor. It is surprising that even greater extents of discontinuity of the result were not observed.

Genetic variation was minimized by use of a particular strain of chicken. Even then, variation could be noted in size, rate of development after hatch and amount of adipose tissue near the nerve when dissected. The adipose tissue could be removed by careful dissection. It is assumed that by utilizing the cross section of eggs as received from the hatchery, it would be possible to compare separate runs within this project.

It is to be noted that the histological preparations adequately demonstrate that the tissue presents a major component of neural and

glial cells (Figures 10 to 13). Since no neural nuclei are included in the tissue used, the nuclei are mostly those of the glia. Blood cells, connective tissue, adipose tissue and small blood vessels can be noted. Certainly lipids would be extracted from these cells as well as the glia cells. However, they represent a fairly constant factor with growth and the major cell that is undergoing change is the glial cell.

These sources of biological variability are matched by the variability of the methodology. Various studies of the chloroform-methanol extraction procedure shows it to be a consistent and complete extraction method. Assays of phosphorus, galactose, protein and DNA were done in triplicate or in quadruplicate when possible. The spectrophotometric reading of the replicate samples were generally within 5% of each other. The galactose readings could vary as much as 10% unless great care was taken to insure uniform heat development of the orcinol color.

Where scintillation counting was involved, duplicate counting vials were used and time-averaged as in the case of the ^{32}P studies. However for the TLC samples of ^{14}C, there was only one sample per determination. Duplicate determinations were then utilized. These would vary by at least five percentage points of each other.

Thus there are severe limitations imposed by the biological variation, the chemical assay methods and the procedure for determining the radioactivity. These suggest a relatively high degree of

caution is needed in the interpretation of the data. Even though there must be caution in attributing significance to changes that take effect in one day, a larger picture can be demonstrated from the smaller changes. When more than one component demonstrates a similar pattern, or when an interrelated pattern is shown, then it is felt that these can project a significant degree of useful meaning to the patterns observed.

The Chemical Characteristics of Chick Sciatic Nerve

Two major questions are being asked in this investigation. What are the normal chemical characteristics of the chick sciatic nerve as it develops? Are there changes in the development of the chemical structure of the sciatic nerve that are significant indicators with which to correlate biochemical and structural events? Most of the data relates to the first question. There are possible answers to the second question, but they are highly tentative.

DNA

Clearly only a select group of chemical components of this system have been considered. The emphasis has been directed to the lipid components, except for the DNA assay. The DNA changes seem to indicate that the cellular multiplication period occurs prior to the beginning of the investigation period at 15 days from onset (Figure 1). From this time to hatching, the tissue is primarily engaged in growth by material acquisition. The cells may be dividing some, but the

decrease in the DNA per wet weight ratio indicates that the addition of bulk to the cell is the predominant growth characteristic at this time.

However, about the time of hatching and thereafter, the growth of the cells in terms of increase in the size of a given cell ceases or slows. This shift coincides with the maturation of the organism into a free living state from the embryonic state. Greater functional demands begin to be placed on the nervous system in place of the limited demands of the shell environment.

Phospholipids

The parallel of developmental variations with hatching is also noted in the changes that occur in phospholipid quantities (Figure 2). At about 17 days the rate of change in the total phospholipid becomes quite high, but as the hatching event becomes eminent, this rate lags until after hatching. The post-hatch growth period shows only a brief continuation of the rapid rate until it appears that the process is essentially over at about 28 days. An examination of the specific phospholipid involved in the retardation prior to hatching indicates that EPG and possibly SPG are responsible (Figure 4).

The in vivo introduction of ^{32}P was utilized in the hope that an especially unique period of change might be identified thereby (Figure 6). However, the hatching event placed a pronounced discontinuity on the interpretation of these results. Perhaps the rate never drops

from the rapid rate of uptake identified at 19 days. But as the embryo prepares to hatch, the allantoic circulation fails to absorb the radioactive substrate. Under the provision of a more direct route of introduction of the substrate into the organism, there is an apparent return to rapid uptake of the substrate. But by this time the rate of change of the phospholipid is diminishing, the other organ systems are entering a rapid growth phase following hatching and the net effect is for uptake of ^{32}P into the sciatic nerve phospholipids to diminish rapidly. At this point in the analysis, it appears that it would have been useful to know which, if any, of the phospholipids was most rapidly labelled. However, this attack was not taken when it seemed apparent that the in vivo labelling studies were marred by the absorption discontinuity introduced by hatching.

Returning to a consideration of the net changes of the phospholipids during the myelination period (Figure 4), it can be observed that the greatest relative increase occurs in the EPG and SPM fractions. This increase is at the expense of the CPG, while SPG remains relatively constant. The two phospholipids, EPG and SPM, are singled out by Eng and Smith (1966) as myelin lipids. Since the CPG is relatively higher at the onset of the study period, it might then have a more predominate function in various non-myelin membranes present in the neurite and primordial glial cell.

Table 7 shows some comparative figures for the percentages of

phospholipids in various nervous tissues in addition to the embryonic tissue in this project.

Table 7. Percentages of specific phospholipid to total phospholipid from neural tissue of several sources.

Phospholipid	Source tissue			
	Human brain[a]	Guinea pig brain[b]	Adult chicken sciatic nerve[c]	28 day chick nerve[d]
EPG	40	20	37	40
SPG	14	20	14	16
CPG	32	46	49[e]	24
SPM	14	10		21
IPG		4		

[a] O'Brien and Sampson (1965).

[b] Eichberg, et al. (1964).

[c] Berry, et al. (1965).

[d] Figure 4, age 28 day.

[e] CPG and SPM not separated.

This comparison of phospholipid percentages shows that human brain and guinea pig brain both demonstrate higher CPG. Since brain contains more non-myelinated components, the CPG may be expected to be higher. The results on adult chicken sciatic nerve indicates that there is little change in the percentages following the test period used in this project.

It is of interest that Berry et al. (1965) did not report the presence of IPG in the chick sciatic nerve. It is reported in chick

brain, however (Siek and Newburgh, 1965).

Proteolipid Protein

Perhaps the chick peripheral nerve is unique with regard to proteolipid protein content (Figure 7). Lipid extraction of mammalian tissue by Folch et al. (1958) found very little proteolipid protein in the PNS and the conclusion was made that the absence of proteolipid protein was a characteristic of PNS. The chick sciatic nerve has a rather low amount of proteolipid protein prior to hatching but the amount raises rapidly for the first three days following hatch. The level briefly diminishes, then returns to the higher level for the older ages at the upper end of the study period. This proteolipid protein is not the same as the structural protein that is present in all cells. The proteolipid protein is thought to have a major role in myelin formation within the CNS.

Even though structural protein is abundant in most tissues, it was not analyzed in this project. The functional distinction between proteolipid protein and structural protein has not been defined. The two types of protein are defined on the basis of extraction procedures. The proteolipid protein is extracted along with the lipid, presumably as a lipid covered protein micell, while the structural protein is not. The proteolipid protein may be a transient form of structural protein. Though amino acid analysis seems to indicate that it is different (Folch

and Lees, 1951).

Tuqan and Adams (1961) agree with Folch and Lees (1951) by finding proteolipid primarily in the CNS of human tissue. They then identify the protein of PNS as trypsin resistant protein. Though PNS tissues from several organisms were reported to have trypsin resistant protein, the chicken peripheral nerves were not included in their list.

If the proteolipid protein found in this case in the chick sciatic nerve is related to myelination, it is strange that it is found in significant quantities only after hatching. Information is not available to know if this protein is present in adult chicken PNS. Therefore it is interesting to note the presence of proteolipid protein, but to relate it to myelination on the basis of this finding seems unwarranted.

Cerebrosides

Total cerebroside content in chick sciatic nerve is different from those components mentioned so far in that it does not reflect a noticeable variation at the time of hatching. The period of rapid increase in amount of cerebroside begins at 16 days (Figure 8). The rate reaches its point of most rapid change at day 21 then gradually decreases. The data seem to suggest a leveling out of amount on a wet weight basis, though these older ages were not as certain due to nutritional variations.

The chick sciatic nerve cerebroside can be compared to the chick brain cerebroside as reported by Garrigan and Chargaff (1963) (Table 8). If one assumes that the cerebroside is in the myelin as does Garrigan and Chargaff, then this comparison suggests that there is a greater concentration of myelinated neurites in the sciatic nerve than in the brain. The sciatic nerve would then be a more appropriate system on which to study myelination.

Table 8. Comparative amount of cerebroside in chick brain[a] and chick sciatic nerve[b] at various ages.

Age (days)	μg cerebroside per mg wet weight of tissue	
	Chick brain	Chick Sciatic Nerve
15	1.85	3.00
18	2.06	6.05
20	3.02	10.50
20.5	4.78	11.50
22.5	5.14	16.75

[a]Garrigan and Chargaff (1963).

[b]Figure 8

Uptake of ^{14}C from Galactose Substrate

The galactose-1-^{14}C uptake study was undertaken with the notion that a precursor of cerebroside could significantly label the cerebroside with respect to the other lipid moities and as such, it

could serve as an index of the presence of cerebrosides. The data demonstrates that the galactose enters the glycolysis pathway to a greater extent than simply serving as a precursor for the cerebroside. The radioactivity is distributed to all tissue components.

The data seem to show a difference in the stages of development in which the structural protein and the lipids are produced. If it can be assumed that the total insoluble residue (R) consists essentially of protein, its relative percentage of radioactivity is highest at the start of the study period. This would be the time for the essential framework of the cells to be formed. The maturing glial cells would be producing mainly cell membranes that would be largely lipid and only a minor portion of structural protein. The data show that by the time of hatching, lipid production becomes the major cell function. This is consistent with the idea that myelination is occurring at this time, but not direct evidence for the idea.

Table 3, which demonstrates accumulative factors of uptake, confirms the conclusions drawn from Figure 8 that a relatively consistent rate of increase of cerebroside is shown through the growth period.

In short, the labelling experiments fail to give much information in addition to that obtained by direct analysis of the chemical components with development.

Conclusions

The relationship of these several lipid components of the chick sciatic nerve to the process of myelination remains somewhat tentative. It would be attractive to conclude that ethanolamine-phosphoglyceride, spingomyelin and cerebrosides undergo a significant degree of increase in the tissue simultaneous with the myelination process. This time period for myelination in the chick peripheral nerves is approximately from the eighteenth day of incubation to four days after the chick hatches as judged from the histological preparations. The lipids thought to be associated with this event have a period of rapid accumulation beginning the seventeenth day of incubation and lessening at six days after hatch.

The three lipid components along with cholesterol appear to be implicated increasingly as myelin markers. No one component can be singled out as unusually significant. Expression of a ratio number between cerebroside, ethanolaminephosphoglyceride and spingomyelin to cholesterol is as yet the nearest indicator of myelination (Eng and Smith, 1966).

It may still be possible to view cerebrosides as at least somewhat predominant to phospholipids and cholesterol. It is felt that the use of a precursor that is metabolically more closely associated to the product cerebroside might still serve to be an appropriate myelin

indicator.

As said, a direct lipid-myelin association is yet to be made. Some preliminary experiments were directed to an autoradiographic identification of a lipid substrate in a histological preparation. This approach should be carried out using the electron microscope in order to obtain adequate resolution.

Perhaps this definition of the development of lipids in a specific embryonic nervous tissue will provide some understanding of the glial cell and its period of major growth. Its differentation from primordial cells has taken place long before this event however, and this investigation provides little understanding of this larger problem.

SUMMARY

Differentiation of the nervous tissue produces the glial cells (Schwann cells) that produce an extended cell membrane, myelin, that covers the neural processes of the mature neurons. This glial cell membrane is composed of a relatively limited number of lipid types along with structural protein. An understanding of the factors that bring about the elaboration of myelin at a particular stage of development is sought. To this end, a study of myelin lipids was undertaken in order to determine if there is a direct association of a chemically measureable component to the structural features characteristic of myelin.

Chick embryos and hatched chicks from the ages of 15 days to incubation to 7 days after hatch (28 days from incubation) were used as the source of sciatic nerve tissue. This tissue, without extraneous lipid and spinal ganglia, was submitted to the Folch lipid extraction procedure. The lipids were separated by means of silica gel thin-layer chromatography in which various chloroform-methanol solvents were used. These lipid assays along with concurrent assays of DNA and proteolipid protein were related to the chick developmental stages.

A comparison of the relative amounts of the specific phospholipids to the total phospholipid identified ethanolaminephosphoglyceride

as demonstrating the most significant relative increase. Sphinogomyelin ratio also improved with development; while serinephosphoglyceride ratio remained constant and the cholinephosphoglyceride ratio diminished. The ages of 17 days to 23 days could be identified as the period of most change in the phospholipids.

The amount of DNA as compared to wet tissue weight decreases in the embryonic nerve until hatch takes place. From this time on there is little change in this component. This was thought to demonstrate that the growth of the tissue is in the size of the cells prior to hatch, but shifts to growth in the number of cells following hatch.

Proteolipid protein in the lipid extract was found to be present in small but measurable amounts prior to hatch. After hatching occurs the amount of proteolipid protein increases. No significance was attached to this finding at this time.

In vivo introduction of inorganic ^{32}P and galactose-1-^{14}C were used to follow the metabolic changes in the lipids. The period of most active uptake of ^{32}P was also from 17 to 23 days. However, since injection of the substrate was made into the egg air space prior to hatching, the pre-hatching inactivation of the allantoic circulation, which absorbs the substrate, also diminished the uptake of the ^{32}P.

Galactose-1-^{14}C does not serve as a selective precursor of cerebroside. At earlier ages, the substrate predominantly entered the metabolism of the non-lipid cell components. As development

took place, the radioactivity was increasingly recovered from the lipid components.

The analysis of the amount of cerebroside in the chick sciatic nerve showed that there is three μg per mg of wet tissue weight in the 16 day embryonic nerves. By the age of 27 days this has increased to 26 μg per mg wet tissue weight. This is significantly greater than chick brain assays of cerebroside. Since the changes in amount of cerebroside were unaffected by the hatching process, it serves as a more ideal component to consider for further investigation. However, no single component of the peripheral nerve seems to be ideally associated with myelination.

A histological study of the chick sciatic nerves at ages 18, 20, 22 and 25 days using Luxol Fast Blue G as a myelin stain demonstrated little myelin at 18 days, but increasing myelin staining properties and definition of structure by 25 days.

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