STUDIES ON THE TURNOVER OF PROTEIN, GLYCOPROTEIN AND GANGLIOSIDES IN THE BRAINS OF GALACTOSE INTOXICATED CHICKS

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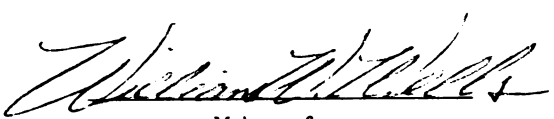

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ABSTRACT

STUDIES ON THE TURNOVER OF PROTEIN, GLYCOPROTEIN AND GANGLIOSIDES IN THE BRAINS OF GALACTOSE INTOXICATED CHICKS

By

James Carlisle Blosser

A study of the effects of feeding chicks toxic levels of D-galactose on brain amino acid levels, polyribosomal aggregation, protein turnover, glycoprotein, ganglioside and mucopolysaccharide synthesis and neural lysosomal stability was undertaken. A number of amino acids known to be associated with the tricarboxylic acid cycle were significantly altered in concentration; alanine, glutamate, and glutamine were decreased while asparate levels increased. In addition, leucine concentrations were depressed. Brain polyribosomal profiles were similar to those from controls. The ratio of monoribosomes to polyribosomes was 0.10 $\pm$ 0.01 while yields of ribosomal material were typically 0.50 mg per gram of brain. Protein synthesis rates, as judged by the *in vivo* incorporation of L-[U-^{14}C] leucine and L-[guanidino-^{14}C] arginine, were not decreased in whole brain or in the

nuclear, microsomal, mitochondrial, or soluble fractions. Protein degradation rates, as measured by the in vivo loss of L-[guanidino-^{14}C] arginine, were similar to those measured in controls. Microsomal protein had the longest half-life (60 hours) of the subcellular fractions studied. The average half-life for total protein was 36 hours.

Both [6-^{3}H] glucosamine and [6-^{3}H] mannosamine were incorporated into glycoprotein and ganglioside fractions at enhanced rates in galactose-fed chicks. The increased utilization of [^{3}H] glucosamine for glycoprotein synthesis was greatest in the microsomal fraction and typically 50 per cent greater than controls over the first 30 minutes. Free glucosamine concentrations from galactose treated animals were similar to those in controls (2.2 $\pm$ 0.1 and 1.9 $\pm$ 0.1 nmoles/gram tissue, respectively) as were levels of glycoprotein bound hexosamine. Approximately 80 per cent of the tritium was incorporated as hexosamine into the glycoprotein fraction. In similar analyses of the ganglioside fraction, neutral and amino sugar contents were unaltered from those of control chicks and radioactivity was found to be distributed between hexosamine (70%) and sialic acid (30%). [^{3}H] Mannosamine was incorporated into glycoprotein of galactose-fed chicks at three times the rate of that in control animals and radioactivity was solely in

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sialic acid. Similar results were obtained in the ganglioside fraction. Although [^{3}H] glucosamine was taken up faster by mucopolysaccharides in galactose-fed chicks than in controls, normal incorporation rates of $^{35}SO_4$ into chondroitin sulfate suggests that this subclass of acid polysaccharides are not affected. That precursors to the carbohydrate moieties are utilized at an enhanced rate while the content their bound products remain unaltered is suggestive of a faster turnover rate.

Neural lysosomes from galactose-fed chicks demonstrated decreased stability to hypoosmotic and temperature shock when compared with controls. This increased lability to osmotic shock could be duplicated by preincubation of normal lysosomes in solutions of galactose and galactitol. Further, the increased fragility induced, in vivo, by galactose feeding could be reversed by removing the diet from the chicks for 8 hours, and was accompanied by large reductions in the levels of galactose and galactitol in the brain. The free activities (non-sedimentable at 20,000 g) of both β-galactosidase and β-Nacetylhexosaminidase were elevated above those of controls, and the increase was found to be proportional to the summation of brain galactose and galactitol concentrations. Together, these data indicate that increased fragility of lysosomes is a result of the accumulation of galactose and galactitol by the

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lysosomes. The possibility of lysosomal dysfunction as a result of increased lability and release of acid hydrolases into the cytoplasm is consistent with the concept of a faster turnover rate of carbohydrate units of glycoprotein and gangliosides. Whether a similar mechanism operates in the brains of galactosemia patients contributing to their frequent irreversible brain damage is now open to further exploration.

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A THESIS

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The author wishes to thank Professor William W. Wells for his challenging ideas, constant optimistic support, and more than generous financial assistance. Although numerous other individuals could be mentioned for their roles in facilitating my growth in development of critical thought and reliable values, I would like to single out in particular Drs. Harvey H. Knull and Leslie P. Kozak for their unselfish attention, honest criticisms, and constructive discussions. The friendly and tension-free atmosphere of the laboratory created by the research associates and fellow graduate students has also been appreciated. Finally, I would like to express my deep gratitude to my parents, whose unselfish love and encouragement has allowed this venture to be undertaken.

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ABBREVIATIONS

UDPAG	uridine diphospho-N-acetyl glucosamine
CMP	cytidine monophosphate
TCA	trichloroacetic acid
UDP	uridine diphosphate
Tris	tris (hydroxymethyl) aminomethane
ATP	Adenosine triphosphate
EDTA	Ethylenedinitrilotetraacetic acid
ADP	Adenosine diphosphate
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
DPM	decompositions per minute
CPM	counts per minute

CHAPTER I

INTRODUCTION

Organization of the Thesis

For convenience, Chapters II, III, and IV have been organized in article form as found in biochemical journals. Each chapter includes an introduction, methods, results, and discussion section. A bibliography is provided at the end of each chapter. Chapter II has been published as it appears under the title, "Studies on Amino Acid Levels and Protein Metabolism in the Brains of Galactose-Intoxicated Chicks" by J. C. Blosser and W. W. Wells, Journal of Neurochemistry, 19, 69 (1972). Chapter IV, with omission of the data on plasma levels of acid hydrolases, has been accepted for publication in the Journal of Neurochemistry under the chapter title by J. C. Blosser and W. W. Wells. Abstracts for these two manuscripts are included in the text of the thesis.

Research Objectives and Rationale Of Experimental Approach

Although the neuropathological consequences of galactosemia can be largely avoided therapeutically by

restriction of dietary galactose, the underlying biochemical causes of mental retardation are unknown. Research in this area, by implementation of model systems, is designed to elucidate the altered biochemical parameters contributing to motor and sensory dysfunction and will hopefully lead to an understanding of the biochemical causes of impaired mental function in the human galactosemic. Such altered factors may also be common to a number of other known inherited mental disorders, for example the amino acidopathies, and hence contribution could be made to an understanding of their etiologies. Chicks appear to be a useful model for studying the galactose toxicity syndrome due to the low levels of the sugar nucleotide pathway enzymes in brain tissue and their high susceptibility to the neurotoxic effects of galactose.

The initial observation of depressed levels of ATP and phosphocreatine and energy charge in the brains of galactose-fed chicks (Kozak and Wells, 1969) prompted a consideration of the consequential effects on anabolic metabolism. Altered amino acid levels (Kinoshita, et al., 1965) and decreased protein synthesis (Dische, Zelmenis, and Youlous, 1957) were known to occur in the lenses of galactose-fed rats, giving further impetus to undertake a study of protein turnover and its possible rate limiting mechanisms in the brains of galactose-fed chicks.

In vivo radioactive tracer studies with leucine and arginine indicated that protein turnover in general was not affected and an investigation of protein subclasses was then begun by studying glycoprotein synthesis. [^{3}H] glucosamine was found to be incorporated at an enhanced rate into the glycoprotein, glycolipid, and mucopolysaccharide fractions. Because of the ambiguity in interpretation of tracer studies of this sort, other labeled precursors of these glycomacromolecules were used to verify the above observations. If any or all of these fractions are being synthesized at a greater rate than normal, any precursor should be utilized at a faster rate. Sodium [^{35}S] sulfate incorporation rates into the mucopolysaccharide fraction was normal, but [^{3}H] mannosamine was incorporated into glycoprotein and glycolipid as N-acetylneuraminic acid much faster than in control animals. Since levels of glucosamine, galactosamine, and N-acetyl neuramimic acid in the glycolipid and glycoprotein of whole brain were essentially unaltered, the possibility of a faster turnover rate was considered. Levels of β-N-acetylhexosaminidase and β-galactosidase, both lysosomal enzymes, were found to be elevated in post lysosomal supernatant fractions of tissue homogenates and subsequent studies demonstrated enhanced fragility of the neural lysosomes as a result of accumulation of galactose and galactitol in the brain.

These findings are consistent with the hypothesis of a faster turnover rate of glycoprotein and/or glycolipid.

Literature Review

Galactosemia is an inherited autosomal recessive disease characterized by the inability to metabolize D-galactose. A myriad of clinical manifestations of the disorder including liver disease, cataracts, and mental retardation are the result of a deficiency of galactose-1-phosphate uridyltransferase [E.C. 2.7 7.12] activity (Isselbacher, Anderson, Kurahashi and Kalckar, 1956). The purpose of this review is to briefly describe the clinical, genetic, and biochemical characteristics of the disease, compare the animal model systems used for studying the galactose toxicity syndrome, and describe the roles suggested for galactose and its metabolites in contributing to the pathological effects of galactose feeding in animals and cell cultures.

Human Hereditary Galactosemia

Symptoms.--The symptoms associated with galactosemia usually appear shortly after birth and are casually related to the ingestion of galactose in the form of lactose in the mother's milk. The most common initial symptom is a failure of the infant to thrive and is characterized by a loss of weight, refusal to eat, lethargy, and the development of diarrhea and vomiting.

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Jaundice is a common occurrence and often proceeds to cirrhosis of the liver. Cataracts and mental retardation are observable within the first two months if ingestion of milk continues. Other symptoms include amino aciduria, proteinuria, and galactosuria reflecting renal malfunction and occasionally hypoglycemia. There appears to be a wide range of variation in severity of the symptoms. In a number of cases the disease is fulminating, accompanied at times by convulsive activity, with death ensuing shortly thereafter. Galactosemia has also been diagnosed in individuals with relatively mild symptoms in early life, but who often are found to suffer from mental retardation in later life (Isselbacher, 1966).

All the above symptoms except mental retardation can be reversed if the disease is diagnosed quickly and the infant is placed on a galactose free diet. The degree of mental retardation, as judged by IQ, appears to range widely when dietary therapy is started by the first year of life. Those with normal IQ are found but usually have special learning defects and psychological problems (Sadler, Inouye and Hsia, 1969; Komrower and Lee, 1970). The highest IQ's reported as a group are those of children whose mothers restricted lactose ingestion during the child's prenatal life (Donnell, Koch and Bergren, 1969).

At present, no therapy or cure for galactosemia is available beyond dietary control. The recent demonstration of lambda phage transduction of a galactose operon into the DNA of cultured human galactosemic fibroblasts and the resultant expression of galactose-1-phosphate uridyltransferase activity in the cell, suggests the possibility of introducing the gene to cells in vivo which lack transferase activity (Merril, Geier and Petricciani, 1971). Successful introduction of the gene into, say, liver cells might allow galactosemics to thrive unaffected on normal or partially restricted diets.

Genetics.--Numerous investigations of galactose-1-phosphate uridyl transferase activity in red and white blood cells in family lives have shown that galactosemia is transmitted by an autosomal recessive gene. Heterozygotes normally have 50 per cent of normal activity (reviewed by Segal, 1971). In at least one case the genetic defect appears to be due to a point mutation. Antibody to the normal transferase enzyme was shown to be quantitatively absorbed to galactosemic erythrocyte preparations which contained no transferase activity (Tedesco and Mellman, 1971). This is consistent with the findings by Beutler (1965) of several individuals whose red blood cell enzyme levels were 50 per cent of normal but whose parents had levels 75 per cent of the

normal activity. Depending upon the location of a point mutation, the resultant structural change in the enzyme could moderately reduce enzymatic turnover rate or render the enzyme completely inactive.

Galactose Metabolism in Galactosemics.--The uridine nucleotide pathway for conversion of galactose to glucose has been largely eludicated by Leloir (1951) and is represented by Steps 1 through 3 in Figure 1. Absence of galactose-1-phosphate uridyl transferase (reaction 3) blocks the major metabolic route for galactose and other oxidative and reductive pathways become more prominent.

Galactose-1-phosphate is the initial metabolite to accumulate before the metabolic block. Elevated levels have been measured in red blood cells, liver, and kidney of galactosemics (Sidbury, 1960). However, both substrate and product inhibition of galactokinase (Figure 1, reaction 1) (Cuatrecases and Segal, 1965) as well as phosphatase activity (Figure 1, reaction 4) would tend to decrease the formation of this intermediate, a possible toxic metabolite.

The reduction of galactose by aldose reductase (Figure 1, reaction 7) to galactitol was demonstrated when the polyol was detected in urine and plasma of a galactosemic patient (Wells, Pittman and Egan, 1964). Subsequent studies (Quan-Ma, H. Wells, W. Wells, Sherman and Egan, 1966) with tissues of two infants with

galactosemia who died from the illness showed accumulation of galactitol in all tissues with highest concentrations in brain and skeletal muscle where the enzyme has highest activity. Tracer studies with D-[1-^{14}C] galactose in a galactosemic patient further demonstrated the major significance of this pathway in the absence of transferase activity (Egan and Wells, 1966). However, due to the relative high Km (15 mM) (Hayman and Kinoshita, 1965), this pathway is active only when high levels of galactose are present.

Galactose oxidation to galactonic acid by galactose dehydrogenase (Figure 1, reaction 8) followed by decarboxylation to xylulose (Figure 1, reaction 9) has been described in rat liver (Cuatrecasas and Segal, 1966). Although tracer studies with [1- or 2-^{14}C] galactose suggested an insignificant role for this pathway in normal man, similar studies in galactosemics indicate that this metabolic route may play a role in galactose metabolism (Segal and Cuatrecasas, 1968).

Another pathway for galactose metabolism which could bypass the transferase block involves UDP-galactose formation from UTP and galactose 1-phosphate by uridine diphosphate galactose pyrophosphorylase (Figure 1, reaction 6). Although this activity has been found in human liver (Isselbacher, 1957) the levels are low and do not increase with age (Abraham and Howell, 1969).

Further, it is quite probable that the enzyme is not specific for galactose-1-phosphate and is in fact UDP-glucose pyrophosphorylase (Figure 1, reaction 5) (Knop and Hansen, 1970).

Comparison of the Rat and Chick as Model Systems

In order to delineate the biochemical mechanisms underlying disruption of normal cellular processes in galactosemia, most investigations have depended on changes induced in animals, most notably the rat and chick, by feeding high levels of galactose in their diet. It must be remembered that since all the enzymes of the uridine nucleotide sugar phosphate pathway are present in these systems, the situation is not entirely analogous to a complete block at the transferase step. Success in inducing toxic symptoms depends upon overloading the enzymes of the Leloir pathway.

The major pathologic changes in rats fed 20 or 40 per cent of galactose in their diet include cataract formation, poor growth rate, and kidney damage characterized by amino aciduria (Craig and Maddock, 1953). Fetuses from pregnant rats fed high-galactose diet in addition demonstrate moderate fat infiltration of the liver (Spatz and Segal, 1965). Although brain abnormalities are not usually observed (Handler, 1947), a combined ethanol-50 per cent galactose diet will evoke

neural degeneration, liver necrosis and death presumably due to inhibition of UDP galactose-4 epimerase (Tygstrup and Keiding, 1969). Quan-Ma and Wells (1965) demonstrated galactitol accumulation in liver, muscle, brain, kidney, and intestine of rats fed high-galactose diet. When [1-^{14}C] galactose or [1-^{14}C] glucose was administered to 4-day-old rats, no difference in the excretion patterns of $^{14}CO_2$ could be seen, suggesting that the rat has a relatively good capacity for galactose oxidation via the Leloir pathway (H. Wells, Gordon and Segal, 1970). Measurements of enzyme activities of the uridine nucleotide pathway in rat liver showed transferase levels to be the highest (Segal, 1971).

The chick, on the other hand, develops severe neurotoxic symptoms including eliptiform convulsions, tremors, ataxia, and ultimately death when fed high levels of galactose (Dam, 1944; Rutter, Krichevsky, Scott and Hansen, 1953). Histological studies have revealed that the only significant lesion is in nervous tissue (Rigdon, Couch, Creger and Ferguson, 1963). Neurons, primarily in the basal ganglia, medulla, and occipital lobes were frequently found to be degenerated.

Both galactose and galactitol accumulate in tissues, particularly in brain, kidney, and muscle (H. Wells and Segal, 1969). Oxidation of [1-^{14}C] galactose to $^{14}CO_2$ occurred much slower than [1-^{14}C] glucose

oxidation and in tissue slice studies, brain and intestine demonstrated much lower galactose oxidation rates than kidney and liver (H. Wells, et al., 1970). In contrast with the rat, oxidation of [^{14}C] galactonic acid did not occur (Wells, et al., 1970). Galactose-1-phosphate is known to accumulate in nervous tissue (Kozak and Wells, 1969), suggesting that galactose-1-phosphate uridyl transferase is the rate limiting step in the pathway. However, evidence is conflicting. Transferase activity in the brains of female chicks was found to be one-half that of males and the lowest of the Leloir pathway enzymes (Mayes, Miller and Myers, 1970). Further, brain galactose-1-phosphate levels were twice those of male chicks, consistent with transferase enzyme as the rate-limiting step. However, [1- or 2-^{14}C] galactose oxidation studies with male and female chicks showed no difference in rates of $^{14}CO_2$ evolution from the whole animals (H. Wells, et al., 1970).

Due to the high susceptibility to galactose neurotoxicity, the chick has been adopted by several laboratories as a model for the effects of galactose and its metabolites on the brains of galactosemics. Recent criticism of this model has suggested that hyperosmolality and not abnormal galactose metabolism causes the toxicity syndrome (Malone, Wells and Segal, 1971). Chicks given water containing 10 per cent galactose develop osmolalities

up to 470 milliosmols. However, when chicks are given a synthetic diet containing galactose (40 per cent by weight) osmolalities of only 335 are attained with normal levels at 305 (Knull and Wells, 1972). Knull and Wells also demonstrated that intraperitoneal injection of glucose into chicks fed the synthetic diet can reverse the gross symptoms of toxicity while raising the osmolality of the blood further, casting doubt on the role of hyperosmolality as a major factor in inducing neuropathological symptoms in synthetic diet-fed animals.

Possible Roles of Galactose, Galactitol and Galactose-1-Phosphate in the Etiology of Galactose Toxicity Syndrome

Cataract Formation in the Lens.--Galactitol formation in the lens is considered to play the primary role in inducing cataracts by causing imbibition of water as a result of its poor diffusion properties (Kinoshita and Merola, 1964). In vitro experiments with rat lens have shown that cataracts can be prevented by balancing the osmolality of the incubation medium with galactitol formation in the lens. Kinoshita and coworkers (1968) were also able to block water uptake and cataract formation with an inhibitor of aldose reductase, 3,3-tetramethylene-glutaric acid, for 3 days in an incubation medium containing galactose. The first

change occurring after water imbibition is a marked reduction of glutathione (Suppel, 1966) followed by decreases in amino acid levels (Kinoshita, Merola and Hayman, 1965), glycolytic enzymes and glycolytic rate (Suppel, 1967) within 2 days. Reduction in protein synthetic rate is also known to occur (Dische, et al., 1957). Earlier attempts to implicate galactose-1-phosphate in cataract formation appear less likely as this metabolite accumulates late in the disorder. The finding that individuals with galactokinase deficiency also develop cataracts substantiates this conclusion (Gitzelmann, 1967).

Aminoaciduria.--Galactose or galactose-1-phosphate are thought to play a role in this disorder. Incubation of rat kidney cortex slices with galactose inhibits amino acid accumulation in kidney tubule cells (Thier, Fox, Rosenberg and Segal, 1964). Similar inhibition has been reported in rat intestinal mucosa incubated with galactose (Saunders and Isselbacher, 1965). The fact that patients observed so far with galactokinase deficiency do not demonstrate amino aciduria further implicates galactose-1-phosphate in the inhibition of renal amino acid transport (Gitzelmann, 1967).

Biochemical Changes in the Brain.--Kozak and Wells (1969) observed reduction in levels of ATP by 15 to

20 per cent, phosphocreatine by 30 per cent, and energy charge by 10 per cent prior to onset of convulsive activity in chicks fed a high galactose diet. Further studies with radioactive tracers demonstrated a rapid turnover of galactose-1-phosphate while relatively little galactose was being metabolized (Kozak and Wells, 1971). This combined with the discovery of a phosphatase (Figure 1, reaction 4) with a low Km for galactose-1-phosphate and a Vmax several times greater than that for galactokinase or galactose-1-phosphate uridyl transferase, led to the formulation of a novel mechanism shown below to explain the depression of ATP levels (Kozak and Wells, 1971).

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{galactokinase}} \text{Galactose-1-P} + \text{ADP}$$

$$\text{Galactose-1-P} + H_2O \xrightarrow{\text{phosphatase}} \text{Galactose} + \text{Pi}$$

$$\text{ATP} + H_2O \longrightarrow \text{ADP} + \text{Pi}$$

The result is a loss of ATP without further metabolism of galactose.

Reduced cerebral glucose levels have also been implicated as a contributor to the depressed high energy phosphate reserves in chicks. Glucose and glycogen were found to be largely depleted during the later stages of the toxicity syndrome and high energy phosphates were rapidly utilized with little lactate formation during

ischemia (Granett, Kozak, McIntyre and Wells, 1972). Further, when the debilitated animals were injected intraperitoneally with glucose, there was a rapid though temporary recovery of the animals accompanied by an increase in brain glucose levels and a return of ATP and phosphocreatine to normal concentrations (Knull and Wells, unpublished results). However, it is doubtful that reduced glucose levels initiate the lowering of high energy phosphate levels. ATP is depressed by nine hours after galactose ingestion begins, while glucose levels have altered only slightly (Granett, et al., 1972). Impairment of glucose transport into the brain by high levels of serum galactose has been suggested as a cause for low brain glucose concentrations (Granett, et al., 1972). The total amount of [^{14}C] glucose in the brain following intraperitoneal injection was considerably lower than that found in controls.

In contrast, brain glucose levels are depressed to only a small extent and ATP levels are unaffected in rats fed high galactose diets (W. Wells, et al., 1969). This is presumably due to the greater capacity of rats to metabolize galactose via the sugar nucleotide phosphate pathway. However, other abnormalities have been observed in rat nervous tissue during galactose ingestion. Gabbay and Snider (1970) found decreased nerve conduction in sciatic nerve accompanied by accumulation of galactitol

and osmotic swelling, an effect similar to that in diabetic neuropathy in which sorbitol accumulates in nerve cells. Concentrations of selective amino acids are decreased in fetal rats when mothers are injected with large amounts of galactose (Carver, 1966). A decrease in the ability of seretonin to stimulate the contraction of stomach fundi in rat was shown to be a result of a deficiency in the seretonin receptors which are thought to be glycolipid in character (Woolley and Gommi, 1964).

Galactose Toxicity in Cell Cultures and Red Blood Cells.--The growth of human galactosemic fibroblasts is inhibited by galactose and cellular degeneration and death occurs by 72 hours. Electron micrographs have exhibited dilation of the endoplasmic reticulum during the incubation period (Miller, Gordon and Bench, 1968). ATP levels have been found to be reduced in galactosemic red blood cells during incubation with galactose (Pennington and Prankerd, 1958); however, this has not been confirmed (Zipursky, et al., 1965). E. coli. with galactose-1-phosphate uridyl transferase deficiency show impaired growth rate in galactose containing media but galactokinase deficient mutants do not, suggesting galactose-1-phosphate as the toxic agent (Kurahashi K. and Wahba, 1958; Yarmolinsky, Wiesmeyer and Kalckar, 1959).

Subsequent studies by Sundararajan (1963) have shown galactose-1-phosphate interference with induction of glycerol kinase.

Sidbury (1957) has proposed that galactose-1-phosphate inhibition of phosphoglucomutase is a major cause of galactose toxicity. The intermediate has been shown to accumulate in red blood cells (Sidbury, 1960) of galactosemics. However, inhibition has been shown only *in vitro* and in the absence of cofactor glucose-1-6-diphosphate.

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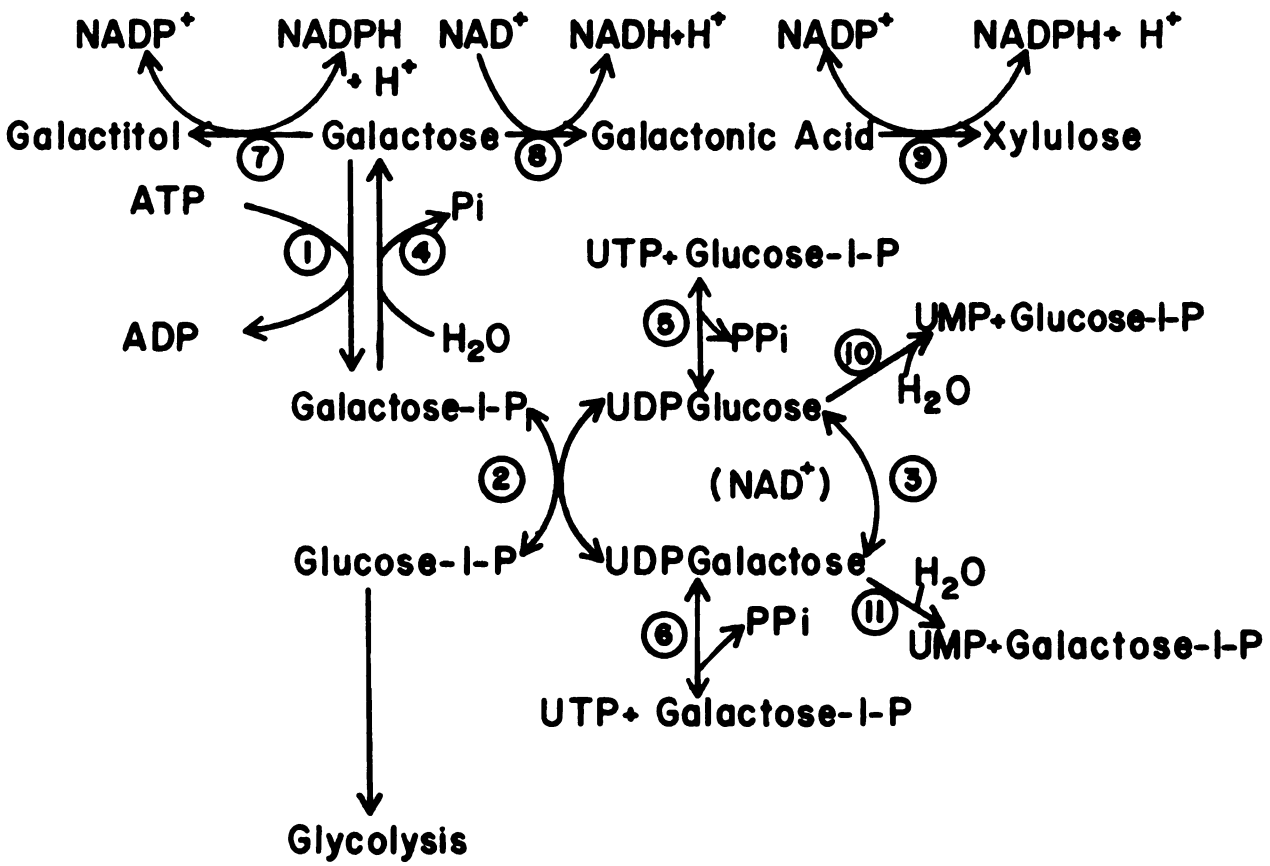
Figure 1. Major metabolic pathways of D-galactose. 1. galactokinase (E.C. 2.7.1.6); 2. galactose 1-phosphate uridyltransferase (E.C. 2.7.7.12); 3. UDP glucose 4-epimerase (E.C. 5.1.3.2); 4. galactose 1-phosphate phosphatase; 5. UDP glucose pyrophosphorylase (E.C. 2.7.7.9); 6. UDP galactose pyrophosphorylase (E.C. 2.7.7.10); 7. aldose reductase (E.C. 1.1.1.21); 8. galactose dehydrogenase (E.C. 1.1.1. 48); 9. galactonic acid dehydrogenase; 10. UDP glucose pyrophosphotase; 11. UDP galactose pyrophosphotase.


NADP+
NADPH + H+
NAD+
NADH+H+
NADP+
NADPH+ H+
Galactitol
Galactose
Galactonic Acid
Xylulose
ATP
ADP
Pi
H2O
UTP+ Glucose-1-P
PPi
UMP+Glucose-1-P
H2O
Galactose-1-P
UDPGlucose
(NAD+)
Glucose-1-P
UDPGalactose
H2O
PPi
UMP+Galactose-1-P
UTP+ Galactose-1-P
Glycolysis

CHAPTER II

STUDIES ON AMINO ACID LEVELS AND PROTEIN METABOLISM IN THE BRAINS OF GALACTOSE-INTOXICATED CHICKS

Abstract

Levels of free amino acids, profiles of polyribosomes, and rates of protein synthesis and degradation were examined in the brains of chicks fed toxic levels of galactose. The content of a number of amino acids were altered; alanine and leucine were most strikingly depressed, whereas levels of aspartate were elevated. Polyribosomal profiles were unaltered. There appeared to be no detrimental effect on protein synthesis as judged by *in vivo* incorporation of L-[U-^{14}C] leucine and L-[guanidino-^{14}C] arginine. Likewise, the half-lives of proteins, measured by the loss of L-[guanidino-^{14}C] arginine, were similar in experimental and control groups. In contrast, initial rates of incorporation of [^{3}H] glucosamine into glycoproteins were enhanced. The effect was greatest in the microsomal fraction and typically 50 per cent greater than controls. Levels of free

glucosamine and protein-bound hexosamine were essentially unaltered in the galactose-fed chicks.

Introduction

Galactose toxicity in the chick is characterized by ataxia, tremors, and severe seizures within 36-48 hours when animals are fed a diet containing 40 per cent (w/w) galactose (Dam, 1944; Rutter, Krichevsky, Scott and Hansen, 1953). Such neurological disturbances when induced by drugs, diet, or electroshock are associated with disruption of the control mechanisms for protein synthesis in the brain. Electroshock-induced seizures or high levels of phenylalanine are accompanied by disaggregation of polyribosomes in rats and mice (Vesco and Giuditta, 1968; MacInnes, McConkey and Schlesinger, 1970; Aoki and Siegel, 1970), and in studies of galactosemic fibroblasts, electron micrographs have revealed dilation of endoplasmic reticulum from polyribosomes (Miller, Gordon and Bensch, 1968). In rats aberrations in brain protein synthesis have been implicated in association with perturbation of levels of free amino acids induced by high levels of galactose (Carver, 1966) or of phynylalanine (Agrawal, Bone and Davison, 1970). Reduction in the rate of protein synthesis is accompanied by a depression of ATP levels in electroshocked brain slices (Lipmann, 1970). Furthermore, the rate dependence of histidyl tRNA synthetase in Salmonella

tymphimurium is known to be sensitive to energy charge in vitro (Brenner, Delorenzo and Ames, 1970). These latter observations are of particular interest in light of the reported depression of brain ATP, creatine phosphate, and energy charge in the galactose-intoxicated chick (Kozak and Wells, 1969).

Accordingly, the present study was undertaken to investigate the effects of galactose toxicity in the chick on polyribosomal aggregation, pool sizes of free amino acids, and resultant effects on protein synthesis and degradation in the brain. Protein turnover, in subcellular fractions, as well as in total brain, was studied in vivo with [^{14}C] leucine or [^{14}C]arginine, and one protein subclass (glycoprotein) was examined with [^{3}H]glucosamine.

Materials and Methods

Animals and Materials

Day-old Leghorn cockerels, purchased from Cobbs, Inc. (Goshen, Ind.) were housed in a brooder at 32°C. Animals were placed on a synthetic diet described by Rutter et al. (1953), with 40 per cent (w/w) of the diet replaced with D-galactose (General Biochemicals, Inc., Chagrin Falls, Ohio). L-[U-^{14}C]Leucine was obtained from either New England Nuclear (Boston, Mass.; 23·6 mCi/mmol) or Amersham Searle (Chicago, Ill.; 344 mCi/mmol;

L-[guanidino-^{14}C] arginine was from New England Nuclear (4.58 mCi/mmol) or from Amersham/Searle (25 mCi/mmol), and D-[6-^{3}H]glucosamine (3.6 Ci/mmol) was from New England Nuclear. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NADP oxidoreductase; EC 1.1.1.49) and pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40) were purchased from Boehringer Mannheim (New York, N.Y.), and hexokinase Type C-300 (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1), and lactate dehydrogenase Type III (L-lactate: NAD oxidoreductase; EC 1.1.1.27), were purchased from Sigma Chemical Co. (St. Louis, Mo.).

In Vivo Tracer Studies

Individual control and experimental chicks were injected intracerebrally with 10-20 µl of labelled precursor in 0.15 M-NaCl solutions. Animals were decapitated at appropriate times, and whole brains were removed and placed in ice or ice-cold buffer (0.01 M-tris, pH 7.0, in 0.25 M-sucrose) (see Legends to figures for details). Brains were weighed and homogenized in 5 vol. of the same buffer in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 850 g for 10 min. to obtain a crude nuclear fraction which was further purified for nuclei according to the method of Casola and Agranoff (1968). For a crude mitochondrial fraction, the 850 g

supernatant fraction was then sedimented at 1,000 g for 20 min. Microsomal and soluble protein fractions were separated by centrifuging the 11,000 g supernatant fraction at 100,000 g for 90 min.

Preparation of Samples for Counting

Protein was treated according to the method (Method A) described by Lim and Agranoff (1966) for counting. The powdered tissue was weighed on an analytical balance, dissolved in 0.5 ml of hyamine hydroxide for 3 min. at 80°C, and then mixed with 10 ml of toluene-based scintillation fluid (2,5-diphenyloxazole, 5 g; 1,4-bis-[2-(5-phenyloxazolyl)]-benzene, 0.3 g; toluene to 1 litre). Radioactivity was then counted in a Beckman c.p.m. 100 liquid scintillation spectrometer as c.p.m. and corrected to d.p.m. by means of an external standard ratio and quenching curve for either ^{14}C or ^{3}H, as required. Efficiency for full channel ^{14}C c.p.m. was 95 per cent. In the glycoprotein experiments, the method of Robinson, Molnar and Winzler (1964) was followed for isolation of the protein fraction and subsequent counting of radioactivity; the procedure was modified slightly by adding 4 per cent (w/v) Cabosil to the scintillation fluid composed of 10 g of 2,5-diphenyloxazole, 100 g of naphthalene and 1 litre of dioxane. Protein concentrations were measured by the

method described by Lowry, Rosebrough, Farr and Randall (1951) after treatment with 0.2 M-sodium hydroxide treatment for 30 min. at 90°C.

Determination of the Specific Radioactivity of Free [^{14}C] Leucine

The TCA-soluble supernatant fraction was extracted six times with diethyl ether to remove TCA and then treated with Dowex 50W-X8 [H^+] (50-100 mesh) to separate amino acids from sucrose. Samples were reduced in volume with a flash evaporator, spotted on Whatman 3 MM paper, and chromatographed overnight with butanol-acetic acid-water (4:1:5, by vol.). The area on the chromatogram corresponding to leucine, isoleucine, and phenylalanine was eluted with water and diluted to a known volume. A portion of this sample was counted in the dioxane-based scintillation fluid. Isoleucine and phenylalanine, being essential amino acids, are unlabelled and do not interfere with the radioactive determinations. Another portion was treated according to the method of Gehrke and Stalling (1967) to convert the amino acids to the corresponding N-trifluoroacetyl-n-butyl ester. The derivatives were analyzed quantitatively with proline as an internal standard on a Tabsorb column with a Hewlett-Packard Model 402 gas chromatograph.

Preparation of Tissue for Free Amino Acid Quantification

Chicks fed respective diets for 44 hours were decapitated and the brains were removed within 15 seconds and frozen in liquid N_2. Tissue was powdered at dry ice temperature and stored at -90°C until needed. Amino acids were extracted and prepared for analysis by a modification of the procedure described by Levi, Kandera and Lajtha (1967). Tissue was treated with 5 vol. of 0.6 M-$HClO_4$, centrifuged to remove the residue, and the $HClO_4$-soluble fraction was neutralized with 1.2 M-KOH; the $KClO_4$ was removed by centrifugation at 0°C. The extract was treated with 50 µmol of Na_2SO_3 and 10 µmol of cysteine per g of original tissue to oxidize glutathione and remove it from the glycine-alanine region of the chromatogram. Samples corresponding to either 4 or 40 mg of original tissue were then applied to a one-column amino acid analyzer using the gradient system of Piez and Morris (1960).

Polyribosomal Profiles

Polyribosomes were isolated from chick brain by the method described by Earl and Morgan (1968) in a homogenizing medium consisting of 0.02 M-tris (pH 7.6); 0.001 M-EDTA; 0.1 M-KCl; 0.01 M- or 0.004 M-magnesium acetate; and 0.2 M-sucrose. Polyribosome pellets could be stored at -90°C for two weeks without alteration

in polyribosomal profiles. Polyribosomal pellets isolated from pools of eight chick brains were lightly dispersed in 0.75 ml of resuspension buffer with a loose-fitting Potter-Elvehjem homogenizer, and corrected to approximately the same concentrations ($A_{260\ nm}$/mg ribosomes = 11.3). Portions were placed on 15-30 per cent (w/v) linear sucrose gradients containing the buffer already described with 4 mM-magnesium acetate, and tubes were centrifuged at 25,000 rev./min for 3.5 hours in a 25-1 SW rotor in a Beckman Model L2 centrifuge. Tubes were punctured at the bottom, and the gradient was pumped through a flow cell (0.5 mm) of a Gilford Model 2000 spectrophotometer at 260 nm.

Determination of Protein-Bound Hexosamine

Dried protein fractions were hydrolysed for 3 hours in 4 M-HCl (2 ml/mg) at 100°C. Hexosamine was determined by a modification of the Elson and Morgan method for amino sugars (Boas, 1953).

Estimation of the Specific Radioactivity of Free [^{3}H] Glucosamine

Groups of eight chicks were injected intracerebrally with 1 µCi of [^{3}H] glucosamine 44 h after being placed on control and experimental diets and after 10 or 20 min. were decapitated into liquid N_2. Brains were chipped out and powdered in a dry ice-chilled pan

and mortar and pestle at a temperature of -50°C. We have observed that values for labile intermediates obtained by this procedure are indistinguishable from those determined for brains chipped out of skulls in a walk-in freezer at -20°C. The following operations were all carried out at 4°C. Portions of the powdered tissue were homogenized in a Potter-Elvehjem homogenizer with 5 vol. of 10 per cent (w/v) TCA. The TCA-insoluble material was sedimented by centrifugation at 12,000 g for 10 minutes and the TCA was extracted from the supernatant fluid with five successive treatments with an equal volume of diethyl ether. The extract was adjusted to pH 7 and passed through a Dowex 50 X 8 (NH_4^+ form) (200-400 mesh) column (7 cm X 0.9 cm dia.) according to the method described by Thompson, Morris and Gering (1959). After washing the column with 50 ml of H_2O, the amino compounds were eluted with 60 ml of 2 M-NH_4OH. The NH_4OH was removed by lyophilization and the residue was taken to a known volume with H_2O. Essentially all of the radioactivity in the NH_4OH eluate co-chromatographed with standard glucosamine on Whatman 3 MM paper in a butanol-pyridine-H_2O (6:4:3, by vol.) solvent system.

Glucosamine was quantified by a modification of the fluorometric assays for glucose and ATP described by Lowry, Passonneau, Hasselberger and Schulz (1964). Glucosamine was converted to glucosamine 6-phosphate with

hexokinase (EC 2.7.1.1) and the ADP formed was quantified by coupling with pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27), and observing the oxidation of NADH. The assay mixture contained 0.1 M-tris HCl (pH 7.5); 4 mM-$MgCl_2$; 80 mM-KCl; 0.25 mM-ATP; 0.125 mM phospho-enol-pyruvate; 1.4 µM-NADH; 1 µl of lactate dehydrogenase; 3 µl of pyruvate kinase; 1 µl of hexokinase; and H_2O and extract to a final volume of 2.0 ml. Samples were corrected for any residual glucose contamination by the enzymic method described by Lowry et al. (1964). Glucosamine and glucosamine 6-phosphate did not intere with the glucose 6-phosphate dehydrogenase assay. In order to test the isolation procedure for glucosamine, 12.6 nmol of standard glucosamine, as judged by the assay, were added to a TCA homogenate of 2 g of brain tissue obtained as described above. Triplicate samples equal to 0.5 g of original tissue, with and without the added glucosamine, were then carried through the isolation procedure described above. Added glucosamine was determined by the fluorometric assay on samples equivalent to 0.1 g of original tissue and found to be 0.67 $\pm$ 0.06 nmol or an average recovery of 106 per cent.

Results

[^{14}C]Leucine Incorporation in Vivo into Brain Protein

A number of amino acid levels were altered in chicks fed galactose (Table 1); however, glutamine, glutamate, aspartate, leucine, and alanine appeared to be most markedly affected. The severity of decrease in the latter two (25 and 40 per cent, respectively), compounded with their already low levels, suggested possible inhibitory effects on rates of protein synthesis at the level of their respective tRNA charging enzymes.

To investigate this possibility, as well as the effect of lowered ATP levels on protein synthesis in the galactose-fed chick, incorporation of [^{14}C]leucine into protein *in vivo* was studied over a 20 minute period. As shown in Table 2, measurements based on d.p.m./mg of protein alone are deceptive. The rate of incorporation of [^{14}C]leucine is based not only on the rate of polypeptide formation but also on the concentration and, hence, the initial specific radioactivity of free leucine. In the case of the galactose-fed chick, the lower level of free leucine resulted in a higher initial specific radioactivity and was reflected in the greater incorporation of radioactivity into protein. After corrections were made for the specific radioactivity of the precursor, virtually no difference in incorporation of leucine into

protein was detected. When protein samples from this experiment were subjected to 72 hours hydrolysis at 110°C in 6 M-HCl, essentially all radioactive material in the hydrolysate cochromatographed on paper with leucine in the butanol-acetic acid-water solvent system.

Polyribosomal Profiles and Rates of Synthesis of Nascent Polypeptide Chains

Since the above experiment was based on analysis at only one time, the experiment was repeated with [U-^{14}C] leucine, measuring only the rate of synthesis of the nascent polypeptide on the polyribosomes. The incorporation of [U-^{14}C]leucine into polypeptide (d.p.m./mg ribosome) is approximately 25 per cent faster in the galactose-fed animals, (Figure 2), but if corrected for the elevated specific radioactivity of leucine, the incorporation curves would be identical.

Polyribosomes isolated in this same experiment were centrifuged on sucrose density gradients and sedimentation rates detected with a flow cell as outlined in Materials and Methods. Representative profiles from a number of runs are presented in Figure 3. The ratios of monoribosomes to polyribosomes, calculated by integrating separately the areas of the monoribosomal peak and polyribosomal peaks of three separate experiments were 0.100 ± 0.010 for control and 0.099 ± 0.012 for

galactose-fed chicks. Yields were typically 0.50 mg of ribosomes/g of brain from both control and experimental groups.

In Vivo Catabolism of Brain Protein

Studying rates of degradation of proteins is complicated in these experiments by the relatively short life of the chicks fed galactose in comparison to the long half-lives of most brain protein. ATP levels fall and galactose 1-phosphate levels rise after 9 hours of galactose feeding (Kozak and Wells, 1971), but tremors or convulsions are not seen until 30-36 hours after initiation of the diet, with death of the chicks ensuing by 48-54 hours.

To partially circumvent this difficulty, chicks were injected intracranially with L-[guanidino-^{14}C] arginine 2 hours before placement on the respective diets. Figure 4 illustrates the degradative rates of protein over the 48-hour period. The average half-life of total protein over the 2-day period was approximately 36 hours for both galactose- and control-fed chicks. Likewise, no significant difference in the decay curves of the subcellular fractions could be detected; apparent half-lives of degradation were 36 hours for the soluble fraction and 60 hours for the microsomal fraction. The biphasic character of the curve for the crude mitochondrial fraction

precluded half-life determinations; however, it did not appear to reflect a recycling of labelled arginine. The radioactivity in the TCA-soluble fraction 24 hours after injection was typically less than 500 d.p.m./g of brain, and as estimated by paper chromatography in butanol-acetic acid-water (4:1:5, by vol.), was largely [^{14}C]urea.

Biosynthesis of Glycoprotein

Advantage was taken of the previously observed equal rates of protein synthesis from [^{14}C]leucine in experimental and control animals by using both [6-^{3}H] glucosamine and L-[guanidino-^{14}C]arginine as glycoprotein precursors. The ratio of ^{3}H to ^{14}C indicated the rate of synthesis of the carbohydrate moiety of glycoprotein relative to that for total protein synthesis. Arginine was also chosen because of its unaltered levels in brains of galactose chicks (Table 1). [^{3}H] Glucosamine was incorporated at a significantly enhanced rate into microsomal and crude mitochondrial protein fractions in the galactose-fed chicks (Figure 5). The observed increase in incorporation from [^{3}H]glucosamine was most obvious in the glycoprotein-rich microsomal fraction, whether expressed on the basis of per mg of protein or relative isotope ratio (Figure 5B).

Levels of free glucosamine, as determined by the fluorometric assay, were similar (1.9 $\pm$ 0.1 nmol/g of tissue for controls, 2.2 $\pm$ 0.1 nmol/g of tissue for

galactose-fed), an observation suggesting that a precursor, specific radioactivity effect as seen with the [^{14}C]leucine studies did not exist. To further investigate this possibility , the specific radioactivities of free glucosamine were measured in a separate experiment (described in Methods). Specific radioactivities at 10 and 20 minutes after injection were 190,000 d.p.m./nmol, and 95,000 d.p.m./nmol, respectively, for controls and 30,000 d.p.m./nmol and 20,000 d.p.m./nmol for galactose-fed animals (range of error, approximately 20 per cent). These values were indicative of a faster rate of utilization of free glucosamine which was consistent with the enhanced incorporation of tritium into glycoprotein.

Preliminary measurements demonstrated that 80 per cent of the tritium in the total protein fraction at 30 minutes was associated with [^{3}H]hexosamine. Also, hexosamine levels in the total protein fraction were identical and typically 8.2 $\pm$ 0.8 and 8.5 $\pm$ 1.3 μg/mg of protein for control and galactose-fed animals, respectively.

Discussion

In general, our amino acid analyses are in good agreement with those reported by Levi et al. (1967) for hen brain. The slight variations observed may reflect the developmental stage of the animal, an effect seen in other instances (Agrawal, Davis and Himwich, 1969).

Earlier studies have shown that high levels of galactose alter the levels of brain amino acids. Carver (1966) demonstrated a decrease in concentration of a number of amino acids in fetal rat brain following injection of galactose into the mother. The alterations of free amino acid levels observed in our studies do not correlate well with those reported for fetal rat brain except for the similar decreases in alanine and leucine. A variety of factors, including mode of introduction of D-galactose (diet versus injection), duration of exposure to high levels of galactose (2 days versus 1 h), or differing susceptibility of the two species to galactose intoxication, may contribute to the dissimilarities.

It is difficult to ascribe alterations in levels of amino acids seen under galactose feeding to any one factor. The altered concentrations of amino acids associated with the glycolytic pathway and the tricarboxylic acid cycle (alanine, aspartate, glutamate) may reflect changes in metabolic flux rates of these pathways in response to increased energy requirements. Alanine levels, in particular, may be depressed as a result of a greater diversion of pyruvate into the tricarboxylic acid cycle. Restricted energy reserves may cause selective impairment of cellular membrane transport systems for different amino acids. Battistin, Grynbaum and Lajtha (1969) have demonstrated in brain slices that

the sensitivity to a decrease in the energy supply was not identical for all amino acids and varied with the type of inhibitor used to restrict energy reserves. Whether galactose directly competes with certain amino acids for transport in brain has not been adequately assessed to our knowledge, although evidence for a common carrier of galactose and selective amino acids in intestine appears to be conflicting (Munck, 1968; Saunders and Isselbacher, 1965).

The alteration in amino acid levels and the reduction of ATP content appear to have had no effect on turnover of brain proteins in chicks fed galactose for 48 hours. This conclusion was demonstrated by the identical time course for the labelling of protein in subcellular fractions with [^{14}C]arginine and [^{14}C]leucine, the [^{14}C] leucine incorporation into peptidyl tRNA, the similarity of the polyribosomal profiles, and equal half-lives of protein in the control and experimental animals. One difficulty in correlating altered levels of free amino acids with a possible regulatory role in rates of protein synthesis is the uncertainty of the effective concentration of amino acids in different cytoplasmic locations and in different cell types. Apparently, leucine and alanine levels localized at the sites of protein synthesis in the cytoplasm, mitochondria and nucleus are sufficiently higher than the K_m of their respective tRNA synthetases

and, therefore, exhibit little control function. Likewise, the lack of observed control of rates of protein synthesis by the high-energy phosphate levels may reflect unaltered levels of ATP at protein synthetic sites or insensitivity of the energy-requiring reactions in vivo to moderate reductions in the adenine energy charge.

The half-lives calculated for degradation of protein were substantially lower than those reported in adult rat brain by Piha, Cuénod and Waelsch (1966), and Lajtha (1959). Several factors, including age of animals, type of radioactive amino acid used, and time course over which the degradation rates were followed, may all contribute to the faster decay rates which we observed. That protein synthesis slows down in mammalian brain during latter stages of development has been well substantiated (Johnson and Luttges, 1966), and may be accompanied by a deceleration of degradation rates. Arias, Doyle and Schimke (1969) have examined the effect of different amino acid tracers on measured half-lives of protein in liver. The recycling of radioactivity, more substantial with other amino acids, was minimized with L-[guanidino-^{14}C]arginine. Finally, our studies were carried out over a relatively short time period (48 h) and, thus, would largely reflect the degradation rates of the proteins turning over most rapidly.

The biphasic nature of the curve for the crude mitochondrial fraction (Figure 3) may reflect the two processes of degradation of protein in mitochondria and of incorporation of labelled protein into myelin and nerve endings by axoplasmic flow--both present in this fraction. Reports by Piha et al. (1966) and Barondes (1968) suggest that both of these processes function simultaneously.

One source of error in our protein degradation experiments is the non-steady state level of protein metabolism; i.e. there is a net increase in protein with time in the growing chick, with the effect of decreasing the observed half-life. However, brain wet weight increases less than 10 per cent over the 2-day period, and because of the well-developed nature of the young chick brain, one would not expect more than parallel increases of 10 per cent in protein.

Most interesting is the accelerated rate of incorporation of [^{3}H]glucosamine into protein fractions in the galactose-intoxicated chicks. The much lower values for specific radioactivity of free glucosamine in the galactose-fed chick in comparison to those in control animals are indicative of a much faster rate of turnover of the free glucosamine pool, presumably because of increased requirements for glycoprotein and/or glycolipid biosynthesis. The increased rate of incorporation

may reflect elevated rates of synthesis of glycoproteins or only their carbohydrate moieties. Since protein-bound levels of hexosamine were equal in the two dietary groups, increased rates of synthesis would have to be accompanied by increased rates of degradation. Whether the apoprotein is being synthesized at a greater rate cannot be ascertained from present experiments. [^{14}C] Arginine was incorporated at the same or slightly faster rates into protein of each subcellular fraction studied in the galactose-fed chicks. However, these rates are an average of individual synthetic rates of all types of protein. The increased incorporation of tritium into glycoprotein could also be explained by either an alteration in the regulatory control of the enzymes converting glucosamine into other carbohydrate precursors of glycoproteins or an elevation of the specific radioactivity of the nucleotide N-acetyl hexosamine intermediates as a consequence of depression of their concentrations. These questions, together with possible parallel effects in the glycolipids, are subjects of further investigation in our laboratory.

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TABLE 1. Analysis of Free Amino Acids and Ammonia in the Brains of Chicks Fed Control or Galactose-Containing Diets.

Compounds	Control	Galactose
	(μmol/g wet wt.)	
Aspartate	2.310 ± 0.071	3.127 ± 0.091*
Glutamate	7.642 ± 0.148	6.912 ± 0.123‡
Glycine	1.059 ± 0.111	1.008 ± 0.010
Alanine	0.273 ± 0.003	0.172 ± 0.005†
Valine[1]	0.064 ± 0.004	0.058 ± 0.004
Methionine	0.052 ± 0.005	0.050 ± 0.002
Isoleucine	0.0197 ± 0.002	0.0181 ± 0.0017
Leucine	0.051 ± 0.001	0.038 ± 0.003‡
Tyrosine	0.042 ± 0.002	0.034 ± 0.003§
Phenylalanine[1]	0.061 ± 0.003	0.071 ± 0.005
γ-Aminobutyrate	2.010 ± 0.088	1.759 ± 0.063
Lysine	0.553 ± 0.023	0.471 ± 0.011§
Histidine	0.222 ± 0.004	0.194 ± 0.009§
Tryptophan	0.021 ± 0.002	0.020 ± 0.005
Arginine	0.138 ± 0.003	0.133 ± 0.005
Ammonia[2]	0.526 ± 0.103	0.630 ± 0.087
Glutamate[2]	8.000 ± 0.117	6.085 ± 0.388†
Glutamine[3]	5.913 ± 0.107	5.130 ± 0.062*

*$P < 0.01$; †$P < 0.005$; ‡$P < 0.025$; §$P < 0.05$; [1]valine chromatographs with galactosamine and phenylalanine with β-alanine; [2]Determined fluorometrically (Folbergrova, Passonneau, Lowry and Schulz, 1969); [3]Determined fluorometrically as glutamate by difference before and after hydrolysis for 2 h at 100°C with 2 M-H_2SO_4.

Each value represents an average (s.d.) of three separate determinations on a pool of 12 brains from each experimental group. Serine, threonine, glutamine, and asparagine were not quantified by the amino acid analyser because of overlap.

TABLE 2.--Incorporation of L-[U-^{14}C]Leucine into Brain Subcellular Fractions.

Fraction	Diet	d.p.m./mg of Protein	d.p.m./mg of Protein: d.p.m./µµmole of free Leucine
Supernatant	Control	2720 ± 130	6200 ± 250
	Galactose	3500 ± 380	7030 ± 600
Microsomes	Control	4930 ± 800	13,000 ± 1250
	Galactose	6750 ± 350	13,250 ± 400
Crude Mitochondrial	Control	4760 ± 630	12,610 ± 900
	Galactose	6370 ± 450	12,800 ± 600
Nuclear	Control	2120 ± 300	5625 ± 400
	Galactose	2450 ± 450	5000 ± 750

Male, day-old chicks (eight/group) were fed the respective diets for 44 h. They were injected intracranially with 20 µl (1 µCi) of L-[U-^{14}C]leucine (2 µCi/µg) and killed 20 minutes later. Each value represents an average of determinations from two pools of four brains (± range).

Figure 2. Incorporation of L-[U-^{14}C]leucine into peptidyl tRNA on the polyribosomes. Control and galactose-fed chicks fed their respective diets for 44 h were injected intracranially with 0.5 μCi of L-[U-^{14}C]leucine. Animals were killed at times indicated in the Figure (eight per experimental group per time period) and polyribosomes were isolated as described in the text section on Methods. After diluting the polyribosomes to equal concentrations, triplicate aliquots were prepared for counting of radioactivity. Ribosomes and protein were precipitated with 10% (w/v) TCA at 0°C for 30 minutes, centrifuged, and then heated at 90°C for 20 minutes with 10% (w/v) TCA. The precipitate was collected on a glass filter (Whatman GF/C, 2.4 cm), washed with 5% (w/v) TCA, dried, and counted with the glass filter in toluene-based scintillation fluid. Standard deviations are denoted by vertical bars.

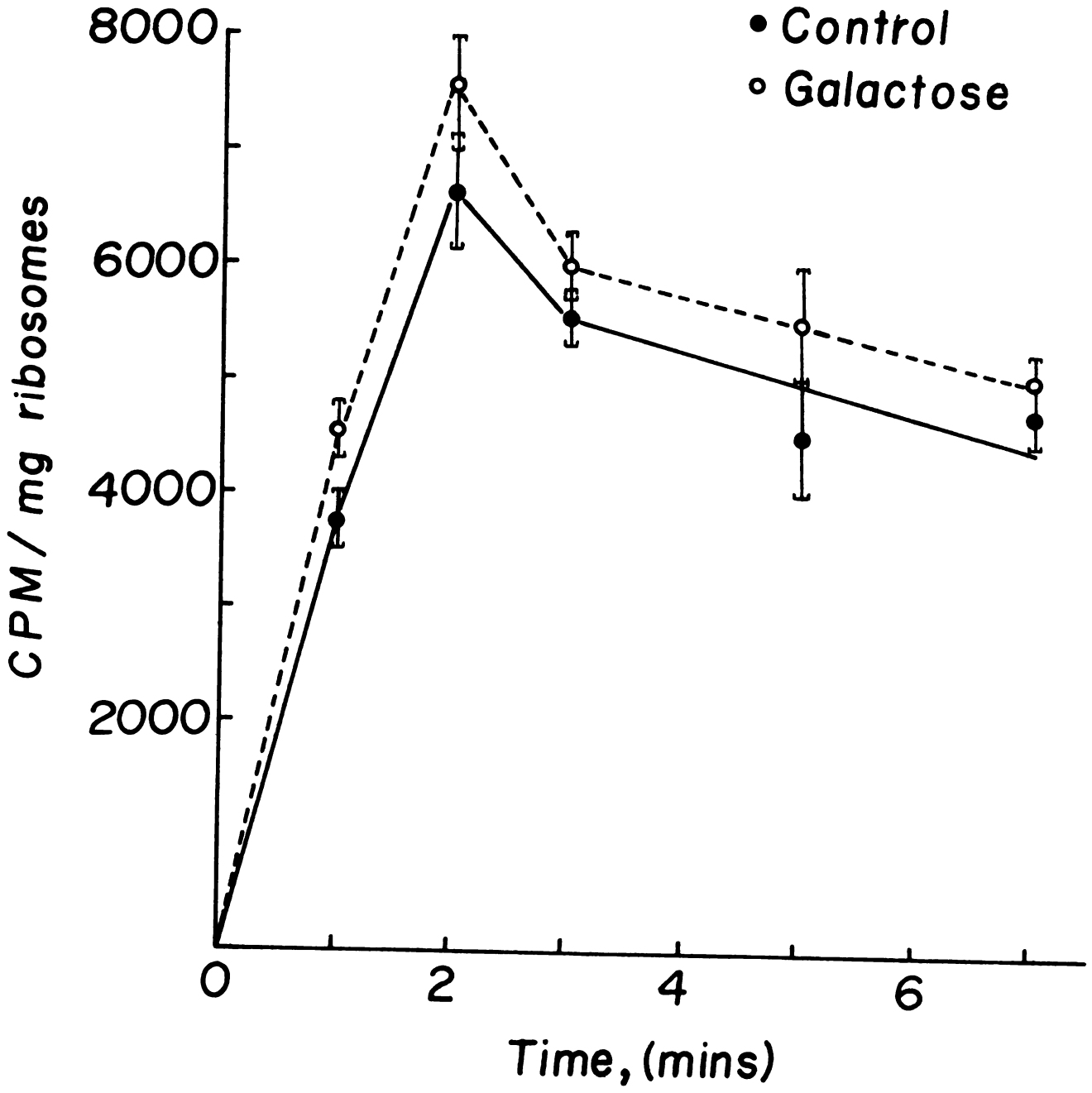

8000
6000
4000
2000
CPM / mg ribosomes
• Control
° Galactose
0
2
4
6
Time, (mins)

Figure 3. Polyribosomal profiles from two typical experiments are shown. For experimental details, see Methods section.

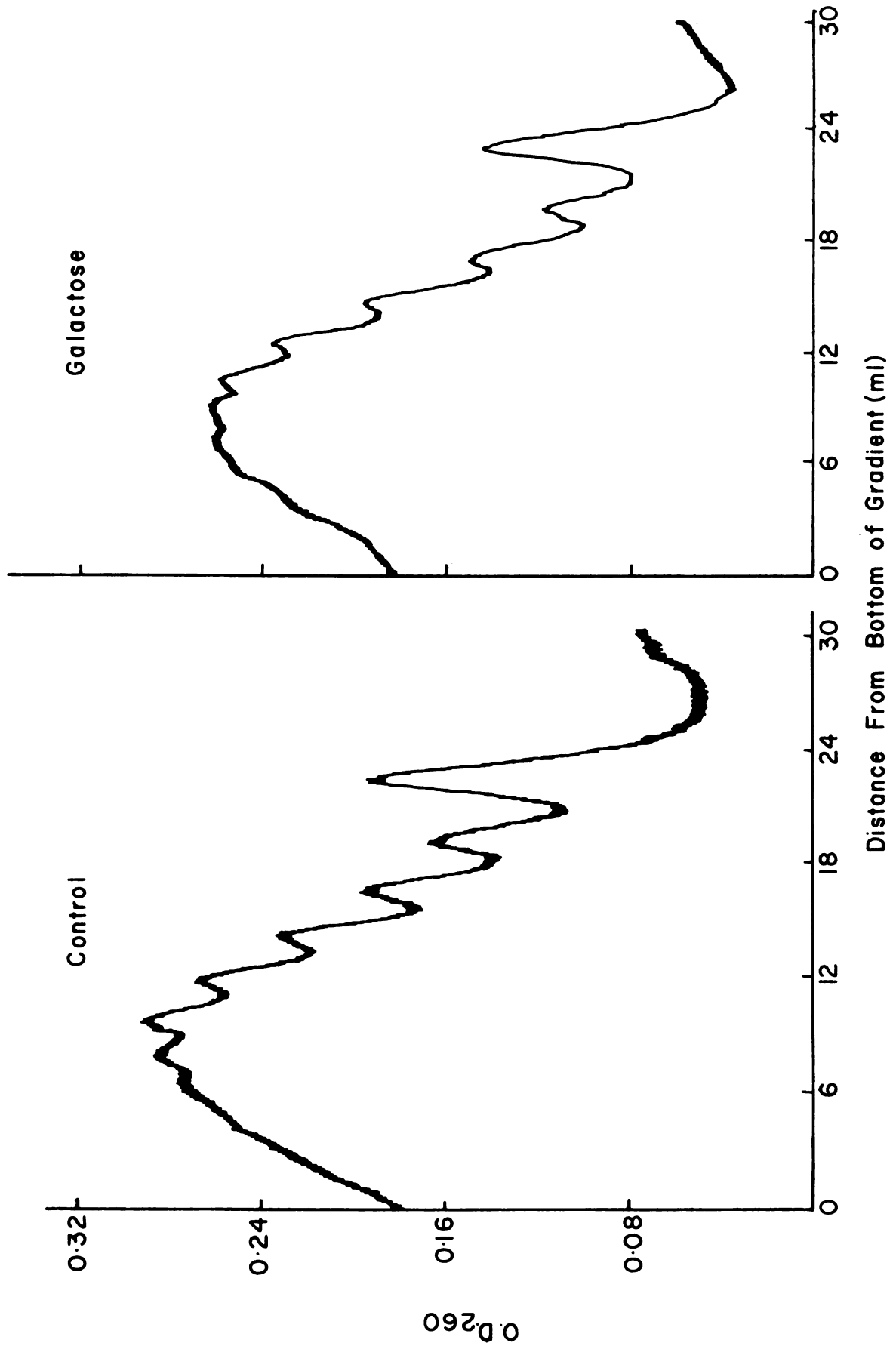

Galactose
Control
O.D260
0·32
0·24
0·16
0·08
0
6
12
18
24
30
Distance From Bottom of Gradient (ml)

Figure 4. *In vivo* rates of protein degradation as illustrated with L-[guanidino-^{14}C] arginine.

Day-old chicks were injected intracranially with 0.5 μCi of L-[guanidino-^{14}C]arginine and placed on the corresponding diets 2 hours later. Twelve chicks from each experimental group were killed at times indicated in the Figure. Triplicate determinations were made on each pool as outlined in Methods. For total protein, five 1 ml samples were taken from the crude homogenate and prepared for counting of radioactivity by Method C of Lim and Agranoff (1969). Standard deviations rarely exceeded 10 per cent for multiple determinations. Control, ●———●; Galactose, O-----O.

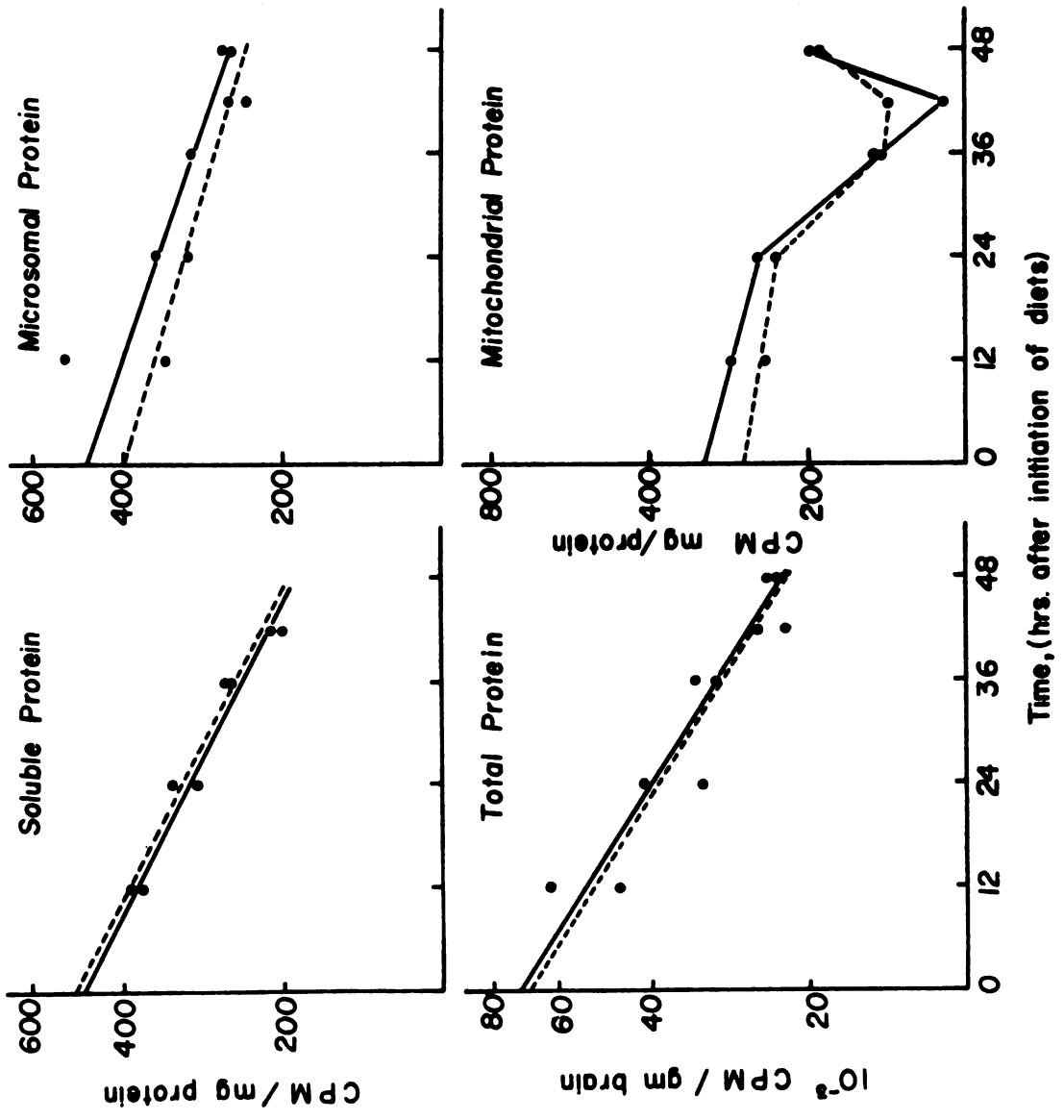
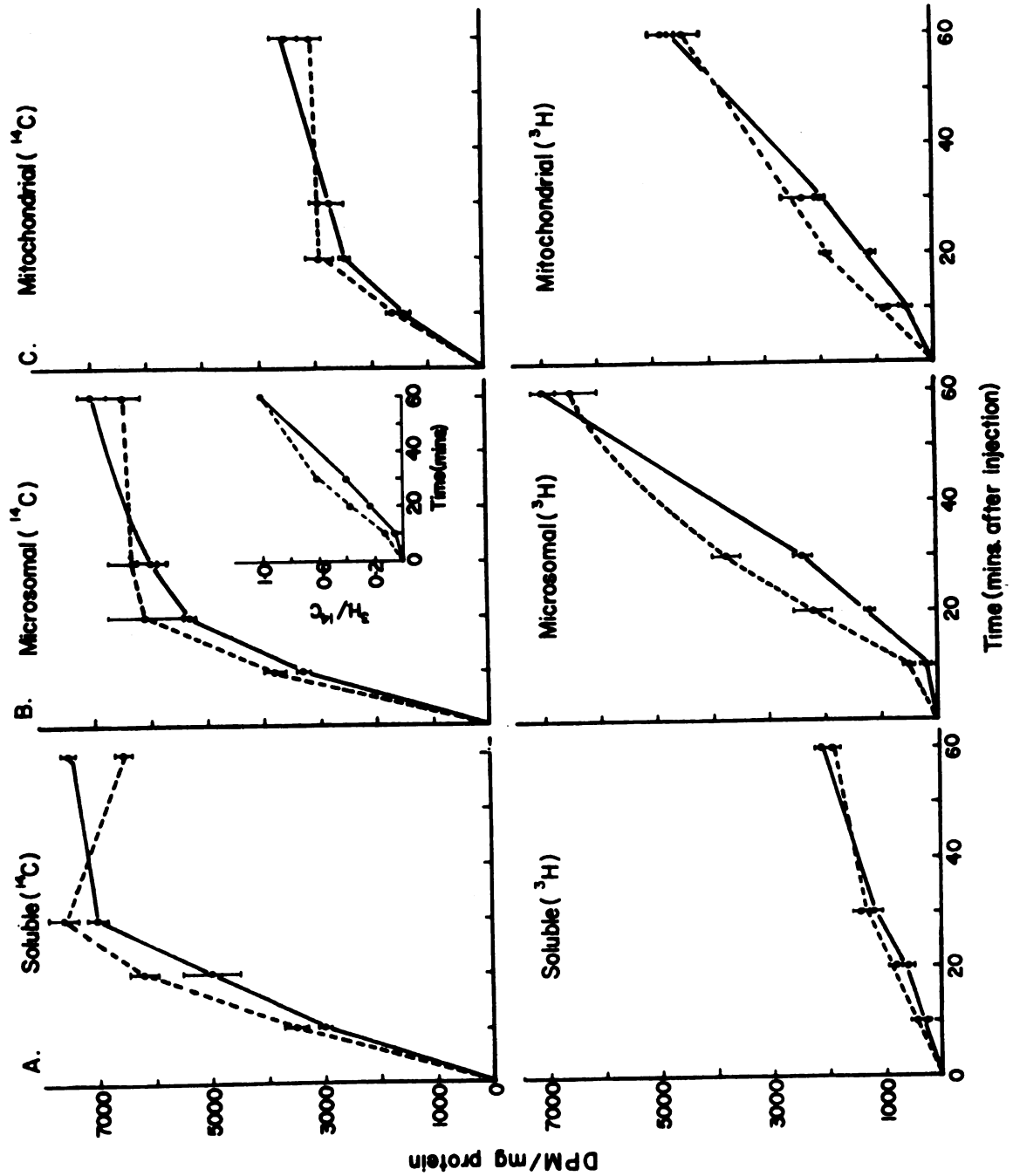

Microsomal Protein
Mitochondrial Protein
Soluble Protein
Total Protein
CPM / mg protein
CPM mg/protein
10⁻³ CPM / gm brain
Time, (hrs. after initiation of diets)
600
400
200
800
60
40
20
0
12
24
36
48

Figure 5. Incorporation of D-[6-^{3}H]glucosamine and L-[guanidino-^{14}C]arginine into the different subcellular protein fractions of galactose- and control-fed chicks.

Chicks were injected intracranially with 1 μCi each of the respective isotopes 44 h after initiation of diets and killed (12 per experimental group for each time period) at times indicated in the Figure. Identical results were obtained in a second experiment. A, B, and C represent incorporation of radioactivity into the protein fraction of the soluble, microsomal and crude mitochondrial fractions, respectively. The inset in B refers to the $^{3}H/^{14}C$ ratio at each time period in the corresponding subcellular fraction.

Control, ●——●; Galactose, O----O. Standard deviations are indicated by vertical bars.


A.
Soluble (^{14}C)
B.
Microsomal (^{14}C)
C.
Mitochondrial (^{14}C)
Soluble (^{3}H)
Microsomal (^{3}H)
Mitochondrial (^{3}H)
DPM/mg protein
Time (mins. after injection)
Time (mins)
$^{3}H/^{14}C$
0
1000
3000
5000
7000
20
40
60
0.2
0.6
1.0

CHAPTER III

BIOSYNTHESIS OF GLYCOPROTEIN, GANGLIOSIDES AND MUCOPOLYSACCHARIDE IN BRAINS OF CHICKS FED D-GALACTOSE

Introduction

Glycoproteins, known to be abundant in neuronal and synaptic membranes, are thought to play a role in intercellular recognition by neurons (Gesner and Ginsberg, 1964; Crandall and Brock, 1968; Barondes, 1970) and hence, in the establishment of neural pathways. Abnormal glycoprotein metabolism, particularly at the synapse, might be expected to have adverse effects on normal brain development. Previous studies have shown an enhanced incorporation rate of [^{3}H] glucosamine into the glycoprotein fraction of brains of chicks suffering from galactose toxicity while free and protein bound levels of glucosamine were essentially unaltered (Chapter II), suggesting the possibility of a faster turnover rate of the carbohydrate moieties. There are, however, disadvantages to using glucosamine as a precursor for glycoprotein synthesis because of the cell's ability to metabolize glucosamine

by more than one pathway and the existence of several intermediates between glucosamine and glycoprotein. In the latter case, a change in the pool size of any one of the intermediates could affect the incorporation rate of the labelled precursor into glycoprotein without an actual change in true rate occurring. Glucosamine is also a precursor for ganglioside and mucopolysaccharide synthesis; hence the increased incorporation of radioactive tracer into glycoprotein may be secondary to an increased biosynthesis of either one of these classes of molecules.

To better characterize in vivo glycoprotein synthesis rate in chicks fed galactose, use has been made of other radioactively labelled precursors of glycomacromolecules. [^{3}H] Mannosamine, a precursor to N-acetylneuraminic acid, demonstrated an enhanced incorporation rate into both glycoproteins and gangliosides while $Na^{35}SO_4$ utilization for mucopolysaccharide biosynthesis appeared to be normal. In addition, [^{3}H] glucosamine incorporation into gangliosides and mucopolysaccharide was measured.

Materials and Methods

Animals and Materials

Day-old cockrels were purchased from Klagers (Manchester, Michigan) and housed in a brooder. Animals were placed on synthetic diets described by Rutter, Krichevsky, Scott, and Hansen (1953) with 40 per cent

galactose (w/w) in place of a corresponding quantity of cerelose in the experimental group. Fresh water was provided, ad libitum. D-[6-^{3}H] glucosamine (3.6 Ci/mmole); Na_2 $^{35}SO_4$ (496 mCi/mmole) and D-[6-^{3}H] mannosamine (3 Ci/mmole) were all purchased from New England Nuclear (Boston, Massachusetts). All other chemicals were of reagent grade.

In Vivo Tracer Studies

Animals were fed their respective diets for 44 to 48 hours. At the end of this period, tracers were injected intracrainally in 20 µl of 0.154 N NaCl. In the amino sugar experiments, 1 µCi was given to each chick while 5 µCi of Na_2 $^{35}SO_4$ was injected per animal to the mucopolysaccharide study. Twelve chicks were used per time period per experimental group for the glucosamine experiment while groups of 10 animals per time period were used in the rest of the studies. At the end of the appropriate time period (see legends to figures for details) the chicks were sacrificed and brains removed, pooled and placed in ice cold water. Tissue was drained of excess liquid and then homogenized in 6 volumes of water in a Potter-Elvehjem homogenizer. When subcellular fractions were to be prepared, brains were placed in 0.32 M Sucrose, with 1 mM Tris, pH 7.4. These samples were homogenized in a Potter-Elvehjem homogenizer in 10 volumes of the 0.32 M sucrose tris buffer solution.

Isolation of Subcellular Fractions

Portions of the crude homogenate, equivalent to 1 gram of the original tissue, were centrifuged at 850 g for 10 minutes to remove cell debris. The supernate was further centrifuged at 20,000 g to obtain a crude mitochondrial pellet. The pellet was resuspended in 7 mls of homogenization buffer and 5 mls were layered on a discontinuous sucrose gradient consisting of 1.2 M sucrose and 0.8 M sucrose (Whittaker, 1969). The gradients were centrifuged at 20,000 rpm for 2 hours in a SW22 Rotor with a Beckman Model L2 centrifuge. Synaptosomes (or nerve ending particles) band between 0.8 and 1.2 M Sucrose solutions while the pellet contains partially purified mitochondria. The synaptosomal fraction was carefully removed by pipette and centrifuged at 100,000 g for 90 minutes to pellet the particles. Microsomes were also isolated by centrifuging the 20,000 g supernate fraction at 100,000 g for 90 minutes.

Isolation and Determination of the Specific Radioactivity of Glycoproteins

Glycoprotein was isolated according to the method of Robinson, Molnar and Winzler (1964). The ether dried glycoprotein material was hydrolyzed in 0.25 N NaOH at 100°C for 20 minutes. Aliquots were counted in a 4 per cent Cabosil scintillation fluid described previously

(Chapter II, Materials and Methods) and protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Acid hydrolysis of the glycoprotein pellet from chicks injected with [^{3}H] mannosamine in 0.1N H_2SO_4 at 80°C for 30 minutes (Neuberger and Marshall, 1966) released essentially all the radioactivity, indicating that only N-acetyl neuraminic acid was labelled.

Isolation and Determination of Radioactivity in Mucopolysaccharides

The glycoprotein fraction, isolated as described above, was treated by modification of the method described by Brunngraber, et al. (1969) for isolation of a mucopolysaccharide fraction. Following digestion of the protein pellet with papain and brief centrifugation of the solution to remove undigested debris, cetylpyridinium chloride was added (2 mg/mg original protein). Samples were allowed to stand for 1 hour at 23°C and then were centrifuged. The supernatant fraction was discarded and the pellet was washed with 5 ml of H_2O. The precipitate was then resuspended in 2.5 M sodium acetate (10 ml/gm original wet tissue) and 3 volumes of ethanol were added (Katzman, 1971). The precipitate was allowed to collect overnight at -20°C and then, following centrifugation, was dissolved in H_2O (6 mls/gm original tissue). Aliquots

were taken for radioactive determination (described above) and quantitation of hexosamine (see below).

When $Na_2{}^{35}SO_4$ was used as a precursor, only the crude glycoprotein-containing fraction was measured for ^{35}S. Glycoprotein contains no sulfur; ^{35}S being incorporated into mucopolysaccharides of this fraction.

Isolation of Gangliosides

Samples of the crude homogenate equivalent to one gram of original tissue were treated with 5 mls of 10 per cent TCA. The precipitate was resuspended in 6 mls of methanol and 12 mls of chloroform were then added. Extraction of the lipids was carried out at 45°C for 30 minutes in a Buchler Rota-Vap. The tissue was re-extracted chloroform-methanol (1:2, v/v) by the same procedure and the two organic extracts were combined and dried. Lipids were redissolved in 10 ml of chloroform-methanol (2:1, v/v) and gangliosides were extracted according to the procedure described by Kanfer (1969). The ganglioside fraction was dialyzed overnight against 8 liters of H_2O and lypholyzed. These samples were redissolved in 4 ml of H_2O and kept for determinations of hexose, hexosamine, and sialic acid.

Quantitation and Specific Radioactivity Determination of Sialic Acid in Gangliosides

Samples were heated for one hour at 80°C in 0.1N H_2SO_4 and the sialic acid liberated was measured by

the method of Warren (1959). To determine the distribution of tritium in the ganglioside fraction when [^{3}H] mannosamine was used as a precursor, the H_2SO_4 digest from the two-hour time period (Figure 5) was neutralized with 0.3N $Ba(OH)_2$ and an aliquot was chromatographed on a Dowex 2 column (5 cm x 0.4 cm diameter, formate form) according to a modification of the method described by Yamashina (1956). The column was washed with 25 mls of H_2O to remove desialylated gangliosides and sialic acid was then eluted with 20 mls of 2.4N formic acid. The sample was taken to dryness and the radioactivity was counted. Essentially all the counts applied to the column were eluted in this fraction, indicating that only sialic acid is isotopically labelled. A similar procedure was used when [6-^{3}H] glucosamine was utilized as a precursor (see Results).

Quantitation of Bound Hexosamine and Hexose

Ganglioside, glycoprotein, or mucopolysaccharide samples were hydrolyzed for 3 hours in 4N HCl. Hexosamine was determined by a modification of the Elson and Morgan method for amino sugars (Boas, 1953). For quantification of glucose or galactose in gangliosides, samples were hydrolyzed in 2N HCl for 3 hours. The HCl was removed by repeated evaporation on a rotary flash evaporator and

derivitized for gas chromatography (Sweeley, Wells and Bentley, 1966). α-Methyl mannoside was used as an internal standard.

The extent of *in vivo* labelling of neutral sugars in gangliosides from intracerebrally injected [^{3}H] glucosamine was determined by chromatographing acid hydrolyzates described above on Whatman 3MM paper with a butanol: pyridine: H_2O solvent (6:4:3, v/v). The region on the chromatogram with an R_f equivalent to standard glucose and galactose was eluted and counted in Bray's scintillation fluid (10g of 2,5-diphenyloxazole, 100g-of naphthalene and 1 liter of dioxane). No radioactivity above background was detected.

Isolation of Free [^{3}H] Mannosamine

Aliquots of the crude homogenate equivalent to one gram of brain tissue were deproteinized with an equal volume of 10 per cent TCA and extracted with 4 successive volumes of ether to remove the TCA. The neutralized extract was applied to a Dowex 50X-8 colum (100-200 mesh; 7 cm by 0.9 cm, diameter) in the ammonium form which is known to bind only basic amino acids and amino sugars (Thompson, Morris and Gering, 1959). The column was washed with 30 ml of H_2O and [H^3] mannosamine was eluted with 25 ml of 2N NH_4OH. The eluent was taken to dryness with a flash evaporator, redissolved in H_2O and an aliquot counted in dioxane based scintillation fluid.

Results

Incorporation of [^{3}H] Glucosamine Into Gangliosides

Gangliosides from brain tissue homogenates of chicks injected intracranially with [6-^{3}H] glucosamine were extracted and their specific radioactivities determined (Figure 6). Initially, tritium was incorporated faster into the gangliosides of the galactose-fed animals but by 60 minutes the total amount utilized was slightly less than that observed in control chicks. The percentage of the total radioactivity present in the form of sialic acid was approximately 30 per cent for both experimental and control animals at 20 and 60 minutes. Since no radioactivity could be detected in glucose or galactose from gangliosides, it was concluded that approximately 70 per cent of the tritium was present as galactosamine. The levels of glucose, galactose, galactosamine, and sialic acid were measured and found to be essentially unaltered in the galactose-fed chicks (Table 3).

Biosynthesis of Mucopolysaccharides

The utilization of [^{3}H] glucosamine for mucopolysaccharide synthesis differed slightly from that observed for gangliosides (Figure 7). Tritium incorporation was approximately 40 per cent faster in the galactose intoxicated chicks than in controls for the first 30 minutes.

However, there was a leveling off of tritium incorporation in the control animals by 60 minutes while the rate remained unchanged in the experimental animals. Levels of hexosamine in the mucopolysaccharide fraction were not found to be statistically different between the two dietary groups (Table 3).

In contrast, the incorporation rate of $Na^{35}SO_4$ into chondroitin sulfate, a subclass of mucopolysaccharides, in galactose-fed chicks was essentially identical to that of controls (Figure 8). It is generally accepted that sulfation of hexosamine residues of acid polysaccharides occurs as the polysaccharide chain grows (White, Handler and Smith, 1968). This suggests that augmented flux rate of [^{3}H] glucosamine into chondroitin sulfate is not due to an actual increase in the rate of biosynthesis although sulfation could possibly lag behind an increased glycosylation rate. A third study was attempted using [^{14}C] glucuronic acid as a precursor but no radioactivity was detected in a trichloroacetic acid precipitate two hours after intracerebral injection.

Incorporation of [6-^{3}H] Mannosamine Into Glycoprotein and Gangliosides

As seen in Figure 9, [^{3}H] mannosamine incorporation into glycoprotein was biphasic and markedly faster in the galactose-fed chicks. Over the first 60 minutes the rate

was three times that of the control animals. Likewise, the ganglioside fraction was labelled faster in the experimental chicks, although the difference in the initial rate was not as great as in the glycoprotein fraction (Figure 10). Figure 6 demonstrates the labelling of glycoprotein in the subcellular fractions. The microsomal fraction (Figure 11, B) had the highest specific radioactivity in both control and experimental groups. In both the mitochondrial and microsomal fractions from galactose-fed chicks, [^{3}H] mannosamine incorporation was twice that found in controls over the first 60 minutes while in the synaptosomal fraction the rate of labelling was somewhat less.

In Table 4 are tabulated the levels of radioactivity in the TCA soluble and free mannosamine fractions. As can be seen, mannosamine appears to be metabolized faster in the galactose-fed chicks. However, the total radioactivity decreases faster in the control brain (Table 4, TCA soluble fraction at 120 minutes) suggesting that some of the radioactivity in the brain of control animals may be equilibrating faster with the blood at later times.

Discussion

Uridine diphospho N-acetylglucosamine (UDPAG) is the common intermediate for N-acetylglucosamine transfer into glycoprotein and mucopolysaccharides.

UDPAG 4-epimerase (E.C.5.1.3. 2d) converts UDPAG to UDP N-acetylgalactosamine, the immediate donor in ganglioside and glycoprotein biosynthesis. A faster synthesis rate of any one or two of these glycomacromolecules in the galactose-fed chick would in turn affect a faster turnover rate of the common metabolic pools in the pathway from glucosamine to UDPNG or UDP N-acetylgalactosamine. Introduction of radioactively labelled glucosamine under such conditions would result in a more rapid equilibrium of the tracer with the uridine amino sugar intermediates and give the appearance of a greater initial rate of incorporation of radioactivity into all fractions when compared with control animals under normal conditions.

This phenomena may explain the [^{3}H] glucosamine incorporation into the mucopolysaccharide fraction (Figure 7) which, like that shown for gangliosides (Figure 6) and glycoprotein (Chapter II, Figure 4), is observed to occur at a faster rate in the galactose-fed chicks. Normal $^{35}SO_4$ utilization in the animals (Figure 8) suggests that chondroitin sulfate synthesis is not altered and the enhanced flux of [^{3}H] glucosamine into the whole acidic polysaccharide fraction could well be secondary to increased demands on UDPAG for glycolipid and/or glycoprotein biosynthesis.

The levels of sialic acid measured in glycoprotein (Table 3) agree fairly closely with those found in rat brains (1.2 μmoles/gm tissue) by Quarles and Brady (1970). Garrigan and Chargaff (1963) found sialic acid levels in gangliosides from 2-day-old chicks to be approximately 2.0 μmoles/gm tissue which is somewhat higher than those detected in these studies (Table 3). When expressed on a molar basis, the ratios of glucose:galactose:galactosamine:sialic acid in gangliosides are 1:1.9:1.3:2.3 respectively and are in close agreement with the theoretical ratios of 1:2:1 for glucose:galactose:galactosamine.

The pathway for conversion of mannosamine to N-acetylneuraminic acid has not been elucidated in the brain. It is presumed that mannosamine is phosphorylated and acetylated to N-acetylmannosamine 6-phosphate (a sequence parallel with glucosamine metabolism) where it enters the established pathway for conversion of UDPAG to CMP-Nacetylneuraminic acid, the latter of which is the sialyl donor to gangliosides and glycoprotein. Raisys and Winzler (1970) demonstrated phosphorylation of the 6 position of mannosamine in Sarcoma 180 cells. However, no further metabolic products were detectable. In the same study, neither rat liver nor kidney exhibited any metabolic activity toward [^{14}C] mannosamine. Most of the radioactivity was excreted in the urine as [^{14}C] mannosamine within two hours

following intraperitoneal injection. Hence, mannosamine conversion to sialic acid in the brain appears to be unique to the tissues studied to date. A specific kinase and transacetylase could be involved in its metabolism, although the normally low levels or absence of free mannosamine in the brain would suggest that a nonspecific kinase and acetyltransferase could play the needed catalytic roles.

The enhanced incorporation rate of [^{3}H] mannosamine into glycoprotein and glycolipid of the neurointoxicated chicks in consistent with the earlier observations of increased [^{3}H] glucosamine utilization for ganglioside and glycoprotein synthesis. Since protein and lipid bound amino sugars do not appear to be altered in whole brain, a faster turnover rate of one or both of these fractions remains a possibility.

The more rapid metabolism of free [^{3}H] mannosamine could be interpreted as a response to the greater requirements for sialic acid incorporation to glycomacromolecules in the galactose treated animals. However, if mannosamine is phosphorylated by a hexokinase not under regulatory control by mannosamine 6-phosphate or other intermediates in the sialic acid biosynthetic pathway, a more trivial explanation could be advanced for the observation. Brain glucose levels in the galactose-fed chick are markedly lower than in controls (Kozak and Wells, 1969).

[^{3}H] Mannosamine competition with different pool sizes of glucose for the same enzyme could be reflected in a faster phosphorylation rate for the amino sugar in the smaller glucose pool, free mannosamine metabolism being then a function of intracellular glucose concentrations. Several lines of evidence argue against this possibility. Raisys and Winzler (1970) found that mannosamine 6-phosphate formation in Sarcoma cells was faster in the presence of glucose than in its absence. In their subsequent studies with rat liver and kidney, hexokinase was unable to phosphorylate mannosamine. The turnover rate of [^{14}C] glucose has been measured in chick brain and appears to be much slower in the galactose-fed animals than controls suggesting that hexokinase turnover number *in vivo* is actually slower in the brains of the galactose neurotoxic chicks (Granett, Kozak, McIntyre and Wells, 1972).

Other radioactive precursors for glycoprotein and gauglioside biosynthesis such as glucose and galactose suffer from the disadvantages of alternate pathways by which they can be metabolized and a large disproportionation in the intracellular levels between experimental and control chicks. Although the mannosamine tracer studies support the concept of a faster turnover of glycoprotein and gangliosides, a lack of knowledge of its route of metabolism and the levels and specific

activities of the intermediates in its metabolism precludes this as conclusive evidence. The possible role of lysosomal enzymes in contributing to abnormal glycoprotein and ganglioside metabolism will be discussed in the following chapter.

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TABLE 3.--Levels of Neutral and Amino Sugars in Gangliosides, Glycoprotein and Mucopolysaccharides.

	Levels nmoles/brain ± s.d.	
	Control	Galactose
Gangliosides		
Sialic Acid	1380 ± 82	1440 ± 76
Galactosamine	840 ± 53	907 ± 50
Galactose	1130 ± 609	1240 ± 100
Glucose	640 ± 35	600 ± 40
Glycoprotein		
Sialic Acid	1500 ± 100	1400 ± 160
Hexosamine	4500 ± 400	4700 ± 600
Mucopolysaccharide		
Hexosamine	154 ± 20	139 ± 12

TABLE 4.--Change in Radioactivity in Free Mannosamine and the TCA Soluble Fraction with Time Following Intracerebral Injection of 1 μCi of [6-^{3}H]-D-Mannosamine.

Time	Mannosamine (in dpm)		TCA Soluble (in dpm)	
	Control	Galactose	Control	Galactose
30	825,000	550,000	1,071,000	1,208,000
60	603,600	393,000	814,000	1,286,000
120	264,400	182,000	480,000	996,000

Above values are subject to approximately 10 per cent range of variability.

Figure 6. Incorporation of [^{3}H] galactosamine into gangliosides.

Gangliosides, purified from homogenates of brain tissue as described in methods, were re-dissolved in H_2(and duplicate aliquots counted in Bray's scintillation fluid. Separate aliquots were hydrolyzed and galactosamine content determined. Range of error < 10 per cent.

Control, ●——●; Galactose, O----O.

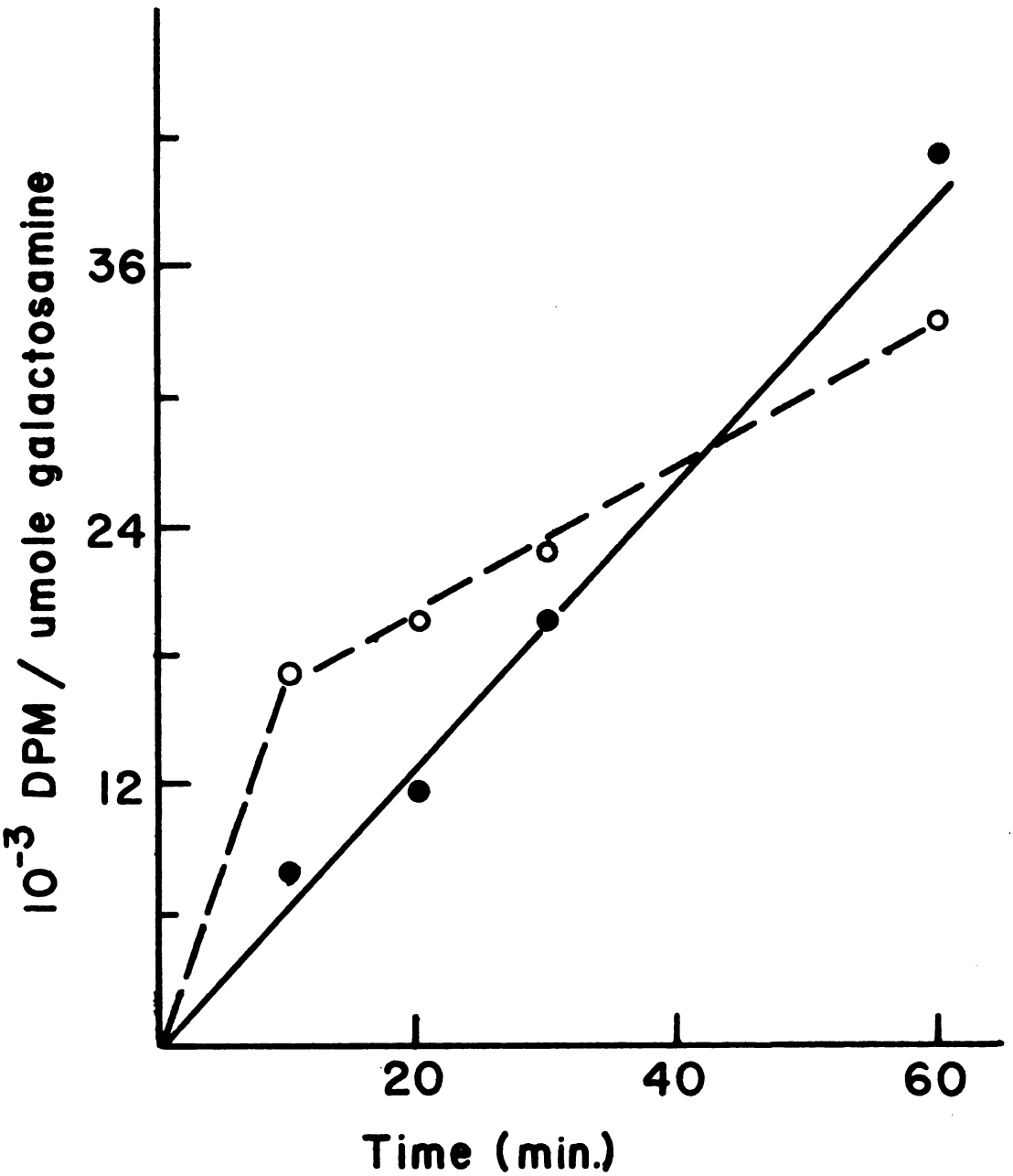

10^{-3} DPM / umole galactosamine
12
24
36
20
40
60
Time (min.)

Figure 7. Incorporation of [^{3}H] hexosamine into mucopolysaccharides.

Vertical bars indicate the range of error in determining the specific radioactivity from two separate extractions of the acid polysaccharides.

Control ●——●; Galactose O----O.

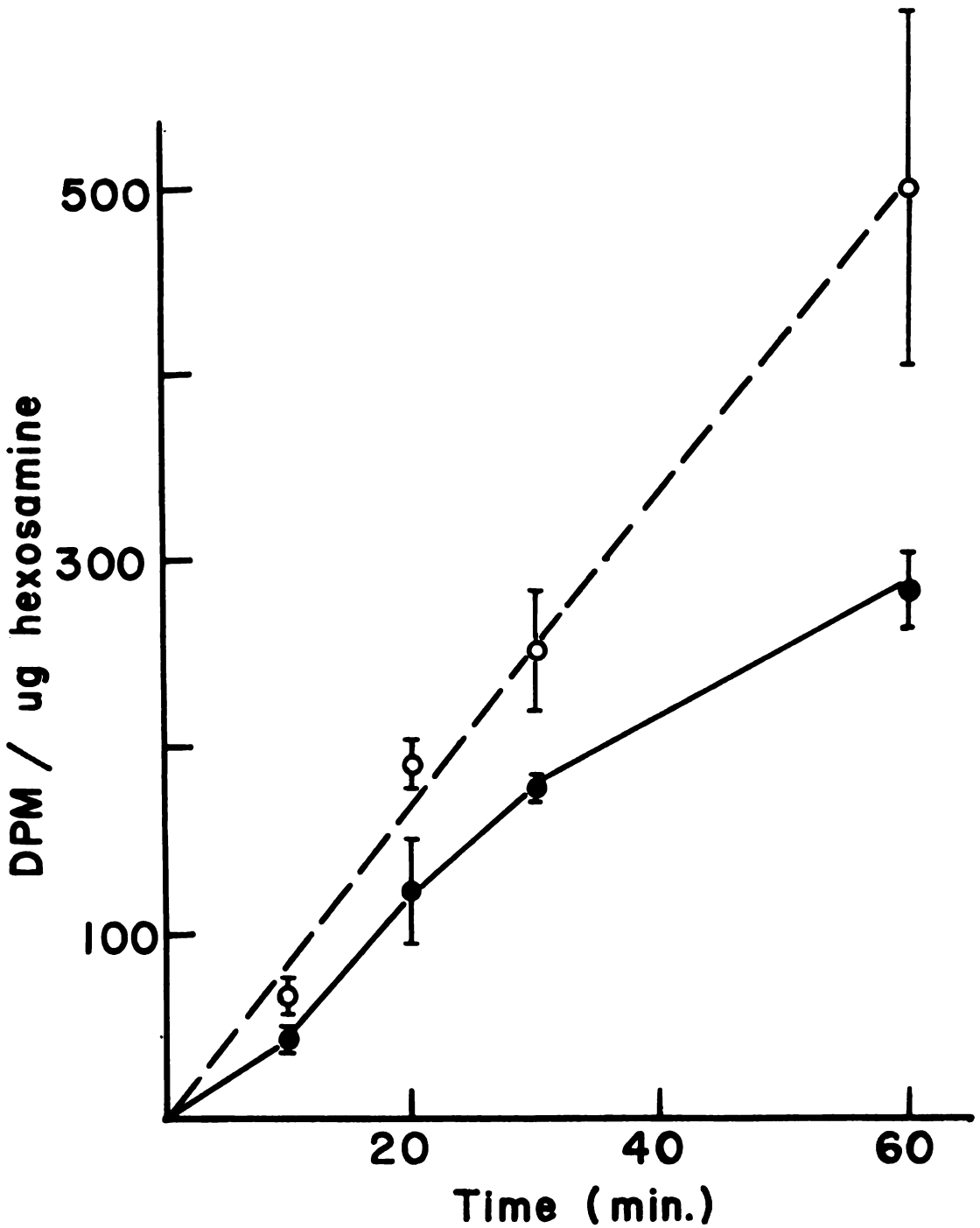

500
300
100
DPM / ug hexosamine
20
40
60
Time (min.)

Figure 8. $^{35}SO_4$ incorporation into chondroitin sulfate. Radioactivity was determined in triplicate aliquots from the crude homogenator as described in Methods. Standard deviation $\leq$ 10 per cent.

Control ●——●; Galactose O----O.

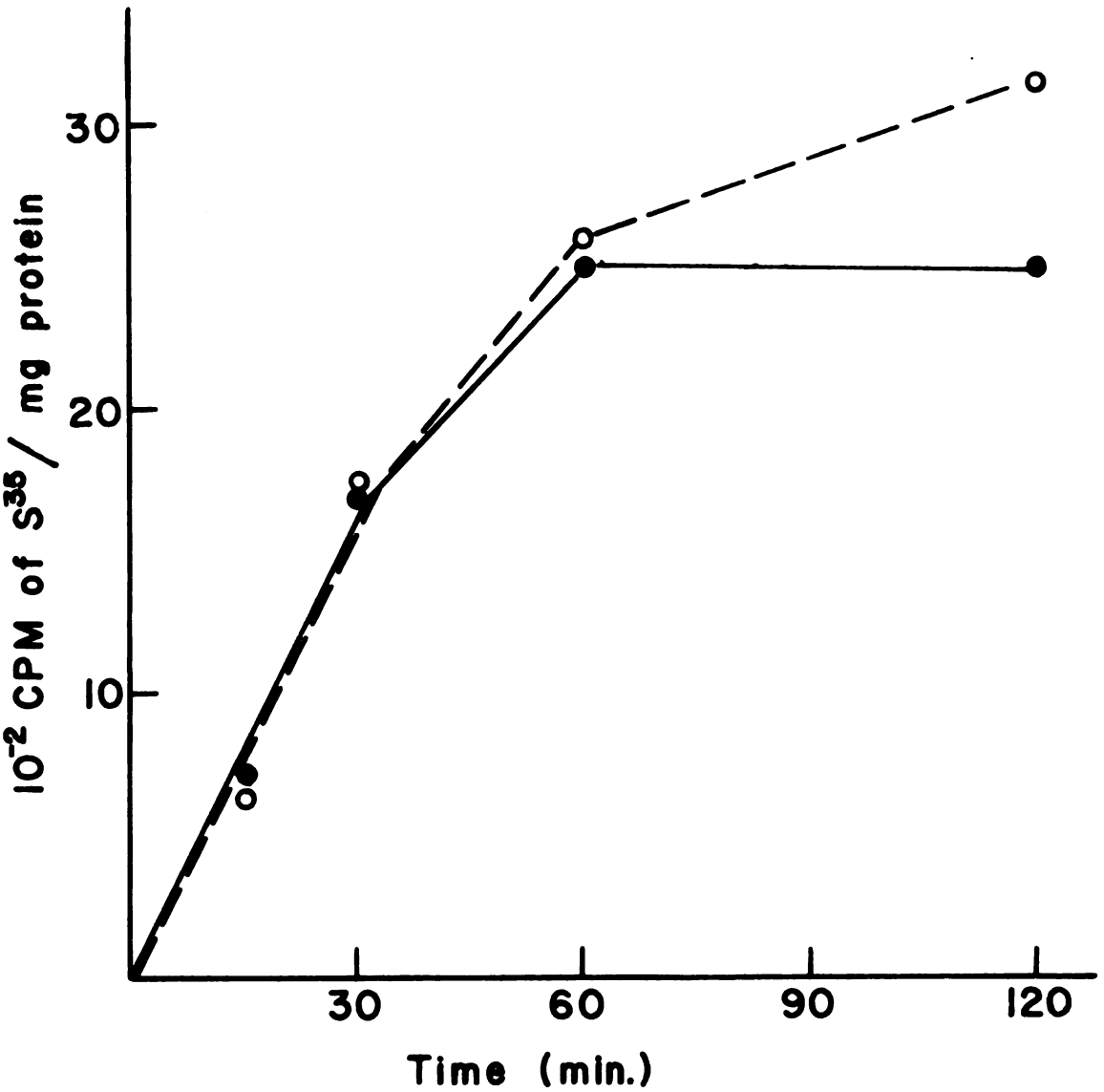

30
20
10
10^{-2} CPM of S^{35} / mg protein
30
60
90
120
Time (min.)

Figure 9. [^{3}H] Mannosamine incorporation into glycoprotein as sialic acid.

Triplicate aliquots from the crude homogenates of each time period indicated were assayed as described in Methods. Standard deviation approximately 10 per cent. A second [^{3}H] mannosamine incorporation study gave essentially identical results.

Control ●——●; Galactose O----O.

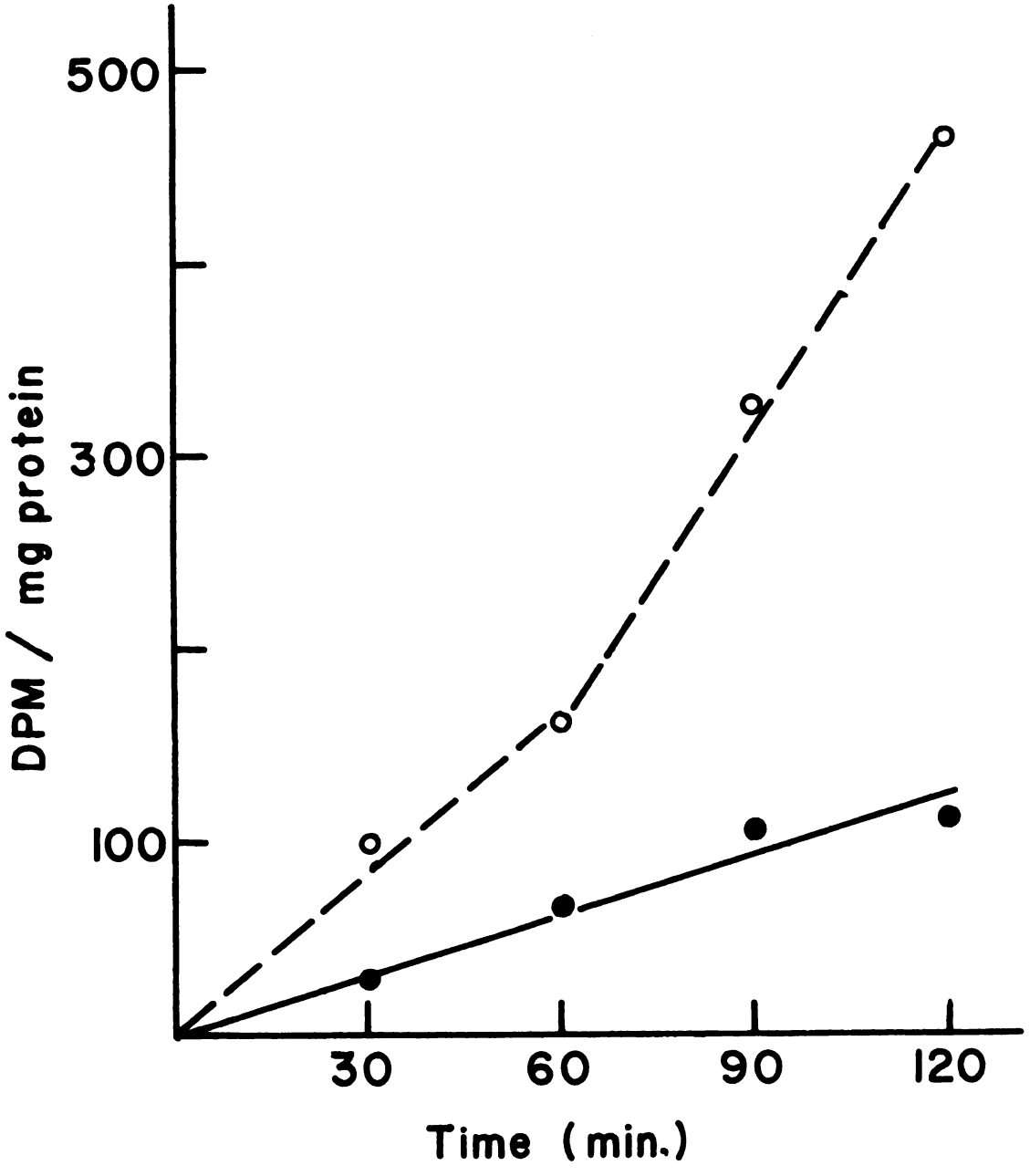

500
300
100
DPM / mg protein
30
60
90
120
Time (min.)

Figure 10. [^{3}H] Mannosamine incorporation into gangliosides as sialic acid.

Hydrolyzates of the ganglioside fraction were neutralized and duplicate aliquots taken for determination of radioactivity and sialic acid. Range of error approximately 15 per cent. Control ●——●; Galactose O----O.

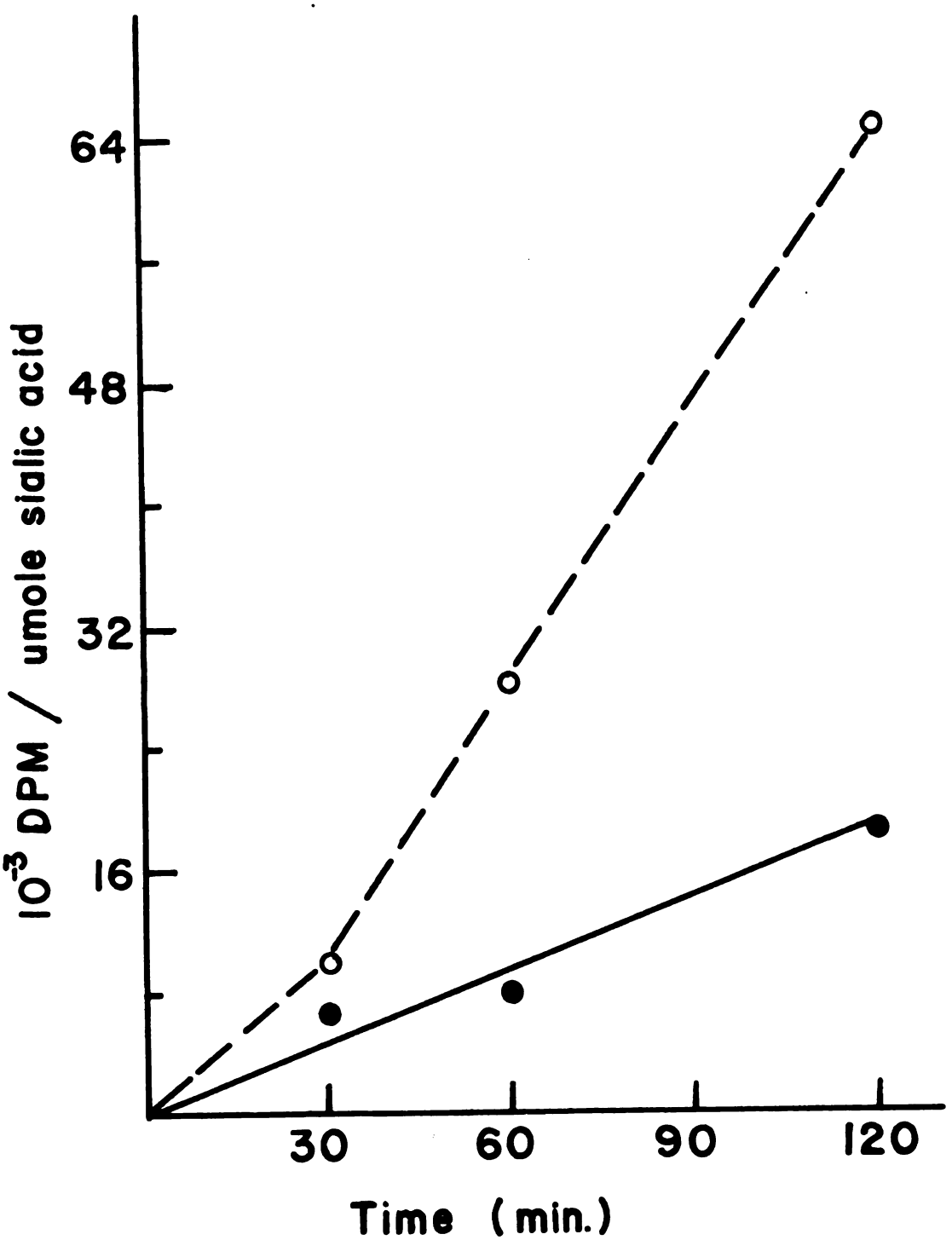
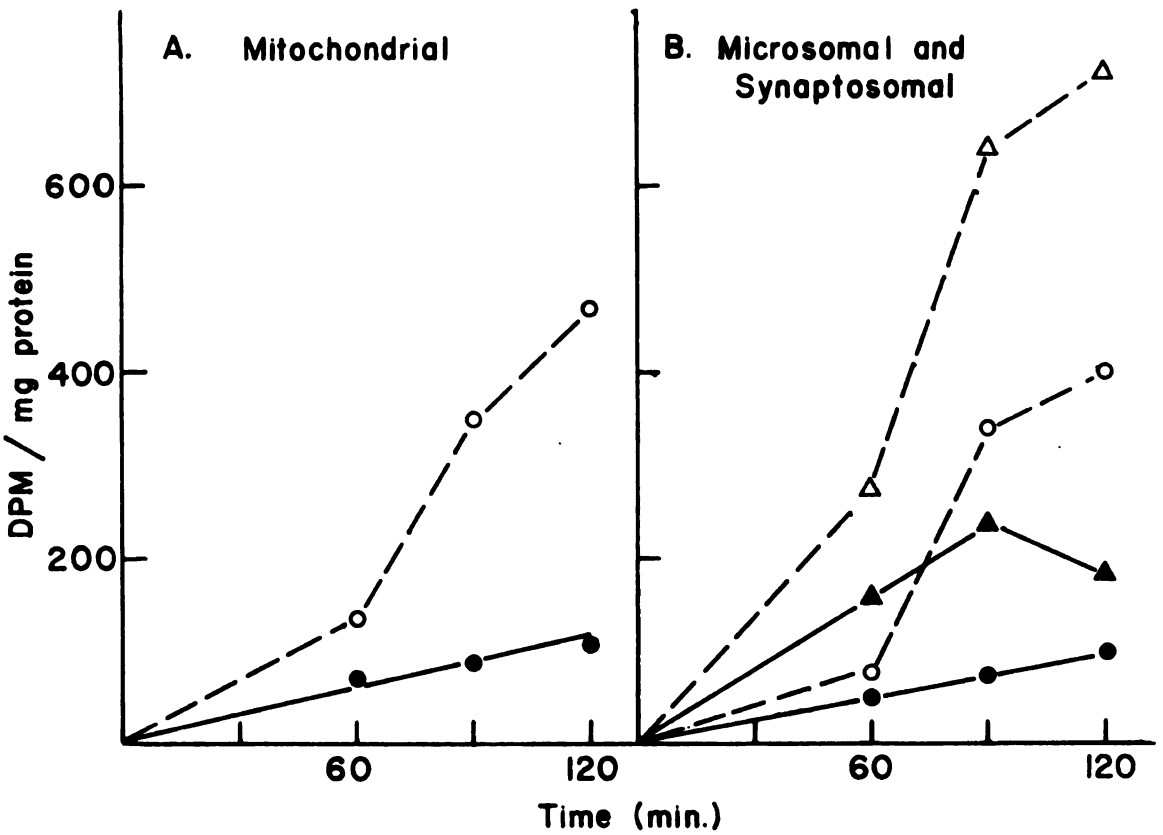

64
48
32
16
10^{-3} DPM / umole sialic acid
30
60
90
120
Time (min.)

Figure 11. Incorporation of [^{3}H] Mannosamine into glycoprotein of subcellular fractions.

All samples quantitated in duplicate. Range of error $\leq$ 15 per cent.
Colored circles, control; Open circles, galactose fed.
B: Microsomes (triangles), Synaptosomes (circles).


A. Mitochondrial
B. Microsomal and Synaptosomal
600
400
200
DPM / mg protein
60
120
60
120
Time (min.)

CHAPTER IV

ENHANCED FRAGILITY OF NEURAL LYSOSOMES FROM CHICKS SUFFERING FROM GALACTOSE TOXICITY

Abstract

The stability of neural lysosomes to osmotic and temperature shock and the free (non-sedimentable) activities of selected lysosomal hydrolases from chicks suffering from galactose neurotoxicity were investigated. The neural lysosomes from chicks fed galactose demonstrated enhanced fragility to both elevated temperature and hypo-osmotic media in comparison to the behavior of neural lysosomes isolated from control animals. The increased lability to osmotic shock could be duplicated by pre-incubation of normal lysosomes in solutions of galactose or galactitol. Further, the increased fragility induced *in vivo* by galactose feeding could be reversed by removing the chicks from the diet for 8 hours, and such removal was accompanied in the brain by large reductions in levels of galactose and galactitol. The free activities of both β-galactosidase (EC.3.2.1.23) and β-N-acetyl hexosaminidase (ED.3.2.1.30) were elevated above those of controls, and the percent increases were proportional

to the combined brain levels of galactose and galactitol. Our data suggest that increased fragility of lysosomes is a function of the accumulation of galactose and galactitol in the brains of chicks fed toxic amounts of galactose. Alteration of lysosomal integrity represents an attractive role for galactitol, as well as galactose, in the causation of galactose neurotoxicity in chicks.

Introduction

Recent studies in our laboratory (Knull, Blosser, and Wells, 1971; Blosser and Wells, 1972) have demonstrated a faster rate of incorporation of [^{3}H] glucosamine into glycoprotein in galactose-fed chicks, while protein bound glucosamine levels remained unchanged, an observation suggestive of a faster rate of turnover of protein-bound glucosamine. Such a phenomenon could well involve dysfunction of lysosomal degradative processes. It is characteristic of galactose toxicity in chicks fed a diet containing 40 per cent D-galactose that there is accumulation of significant levels of galactose and galactitol (Kozak and Wells, 1971). Aldohexoses and hexitols have been shown to permeate lysosomes from rat liver (Lloyd, 1969) and from Tetrahymena pyriformis (Lee, 1970). This effect is associated with a decrease in the latent (detergent-activated) activity and a corresponding increase in the free activity of various acid

hydrolases from lysosomal preparations. When cell cultures are incubated in the presence of aldohexoses, hexitols or disaccharides, the acid-phosphatase-containing particles become extensively vacuolated (Dingle, Fell, and Glauert, 1969; Nyberg and Dingle, 1970). Our present study was undertaken to assess the effects of galactose and galactitol on the stability of neural lysosomes and on the free activities of selected lysosomal acid hydrolases.

Materials and Methods

Animals and Materials

Day-old Leghorn cockerels were purchased from either Cobbs, Inc. (Goshen, Ind.) or Klagers (Manchester, Mich.) and kept in a brooder at 32°C. Animals were placed on synthetic diets described by Rutter, Krichevsky, Scott, and Hansen (1953) containing 40 per cent (w/w) D-galactose in the place of a corresponding quantity of cerelose. The substrates used in the assays of the enzymes examined were: ρ-nitrophenyl β-D-galactopyranoside for β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23), ρ-nitrophenyl-2-acetylamino-2-deoxy-β-D-glucopyranoside for N-acetyl hexosaminidase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxy glucohydrolase, EC 3.2.1.30), and ρ-nitrophenyl phosphate for acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), all

purchased from Sigma Chemical Co. (St. Louis, Mo.). Other chemicals were of analytical grade.

Preparation of Lysosomal Fraction

Brains were removed from decapitated chicks and placed in 10 ml of ice-cold homogenizing solution (250 mM sucrose with 1 mM EDTA). All of the following preparative operations were carried out at 0-4°C. The solution was discarded and the brains were minced with a razor blade. The tissue was then placed in a Potter-Elvehjem homogenizer in 8 vol of homogenizing solution and disrupted by three up-and-down strokes with a loose-fitting, motor-driven pestle at slow speed. The resulting homogenate was centrifuged at 800 g for 10 minutes to remove cell debris. To obtain a crude lysosomal pellet, the 800 g supernatant fluid was then centrifuged at 20,000 g for 15 minutes. Enzyme assays were carried out on the post lysosomal supernatant fraction or the crude lysosomal fraction, which was gently resuspended by hand in 4 vol of the appropriate carbohydrate solutions (see Legends to Figures) with a Potter-Elvehjem homogenizer as already described.

Enzymatic Assays

All enzymatic activities were assayed according to a modification of the method described by Frohwein and Gatt (1969) for β-N-acetyl hexosaminidase. The

reaction mixture contained in final concentrations: 0.8 mM for the appropriate substrate, 50 mM sodium phosphate-citrate buffer (pH 5.0, or for the β-N-acetyl hexosaminidase assay, pH 3.8); homogenate; and water to 0.5 ml. Homogenate was added at several concentrations to the assays to substantiate linearity of the reaction rate with respect to enzyme concentration. For estimation of total sedimentable enzyme activity, 20 µl of 5 per cent (w/v) Triton X-100 were added to a reaction mixture containing portions of the resuspended crude lysosomal pellets. All assay mixtures for enzymes were incubated in a 37°C water bath for 1 hour except for acid phosphatase, in which case the incubation was for 20 minutes. The reactions were stopped by addition of 1 ml of 2.7 per cent (w/v) TCA, and the mixtures were centrifuged for clarification and neutralized with 0.2 ml of 1 N NaOH. Sodium borate (1.3 ml of a 0.125 M solution) was added, and ρ-nitrophenol was measured at 410 nm (ε_{410} = 15.5 X 10^3 M^{-1} cm^{-1}) with a Gilford 300 spectrophotometer. Protein was estimated according to the method of Lowry, Rosebrough, Farr, and Randall (1951).

Free or released enzyme activity refers to that which did not sediment at 20,000 g for 15 minutes. Total activity refers to that detected in the 800 g supernate in the presence of 0.2 per cent (w/v) Triton X-100, whereas total sedimentable activity is defined as activity

measured in the 20,000 g resuspended pellet in the presence of 0.2 per cent (w/v) Triton X 100. Soluble activity refers to that remaining in the 100,000 g supernatant.

Quantification of Galactose and Galactitol

Portions of the crude homogenates were deproteinized by the Somogyi technique and assayed for galactose and galactitol by gas-liquid chromatography (Sweeley, Wells and Bentley, 1966) or for galactose by Galactostat (Worthington Biochemical Corp., Freehold, N.J.). Essentially identical amounts of galactose were detected by either method.

Results

Stability of Lysosomes to Osmotic and Temperature Shock

To investigate possible alterations in the structural integrity of lysosomes during galactose neurotoxicity, crude lysosomal fractions were prepared from brains of chicks fed experimental or control diets for 48 or 64 hours and the fractions were subjected to hypotonic sucrose solutions. The enhanced release of β-N-acetyl hexosaminidase, acid phosphatase, and β-galactosidase from the lysosomes of galactose-intoxicated chicks (Figure 12) reflected an increased susceptibility to osmotic shock. That the increased release of activity

was not attributable to more total enzyme could be shown by demonstration of equal activities of all three enzymes in both dietary groups in lysosomal fractions resuspended in isotonic sucrose and assayed in the presence of 0.2 per cent (w/v) Triton X-100.

Further evidence for increased instability of neural lysosomes from galactose-fed chicks was obtained by the demonstration of enhanced release of β-N-acetyl hexosaminidase from lysosomes preincubated at various temperatures (Figure 13A). Increased temperatures affected acid phosphatase to a lesser extent (Figure 13B); however, more enzyme was released from the lysosomes of the galactose-fed animals. β-Galactosidase did not appear to be released to any appreciable extent, even with temperatures up to 40°C.

Stability of Lysosomes Preincubated in Galactose or Galactitol to Osmotic Shock

To ascertain whether galactose or galactitol alone could cause the increased lability, lysosomes from control chicks were preincubated in galactose, galactitol, or sucrose solutions, supplemented with sucrose for a total concentration of 250 mM, and then subjected to osmotic shock. As illustrated in Figure 14, preincubation with galactitol and, to a slightly lesser extent, with galactose (50 mM concentrations) had an appreciable effect on the release of both β-N-acetyl hexosaminidase

and β-galactosidase. As control experiments, galactose and galactitol at similar concentrations were added to enzymes released by detergent or H_2O and were shown to have no stimulatory or inhibitory effect on the activities of either of these enzymes. Similarly, when compared with isotonic KCl, 250 mM sucrose did not affect any of the enzyme activities. Although β-N-acetyl hexosaminidase was the most active of the enzymes studied, water extracted only 15 to 25 per cent of the total activity (Figure 14A). When pellets of water-shocked lysosomes were resuspended and assayed for β-N-acetyl hexosaminidase in the presence of Triton X-100, 75 and 85 per cent of the original total activity was detected as still bound to the particles from the galactose and control groups, respectively. On the other hand, over 85 per cent of β-galactosidase and 50 to 60 per cent of the acid phosphatase activity was released by osmotic shock in H_2O. In contrast to the increased fragility to osmotic shock, control lysosomes preincubated with 50 to 100 mM galactose or galactitol for 45 minutes at temperatures from 0 to 38°C did not exhibit increased lability (as monitored by release of acid phosphatase or β-N-acetyl hexosaminidase) in comparison to those samples incubated similarly in sucrose.

To determine whether the increased lability of lysosomes was reversible, chicks were fed the diet containing galactose for 44 hours and then placed on the

control diet for 8 hours. Stability of lysosomes from "recovered" animals closely approximates those of controls (Figure 15), except at extreme hypotonicity where the extent of fragility was intermediate between that of lysosomes from control and those from galactose-fed chicks. Levels of galactose and galactitol in the galactose-fed animals were 8.8 and 6.4 μmoles/g wet weight of tissue, respectively, whereas in the "recovered" animals, galactose could not be detected and levels of galactitol had dropped to 3.5 μmoles/g of tissue.

Activities of Free Acid Hydrolase as a Function of In Vivo Levels of Galactose and Galactitol

Chicks fed galactose or control diets were sacrificed 18 to 64 hours later and the post-lysosomal supernatant fractions were prepared from the brains and assayed for β-galactosidase and β-N-acetyl hexosaminidase. The results of 7 experiments are summarized in Table 5. The activities of both enzymes from the brains of galactose-fed chicks were elevated above those measured in preparations from controls. The variation in the levels measured did not correlate directly with increasing time periods of dietary feeding or increasing levels of galactose or galactitol alone. However, a good proportional relationship appeared to exist between the enzyme activities and the sum of the levels of galactose and galactitol. The calculated correlation coefficients (Steel and Torrie,

1960) were 0.82 for β-galactosidase and 0.85 for β-N-acetyl hexosaminidase which are significant at the 5 and 1 per cent levels, respectively. The amount of free activity for β-galactosidase and β-N-acetyl hexosaminidase was low and typically 5 to 9 per cent of the total activity. In similar studies, in which galactitol levels were not measured, there was little or no increase in the free activities of acid phosphatase, whereas β-galactosidase and β-N-acetyl hexosaminidase exhibited increases in free activity similar to those presented in Table 5.

To determine the degree of solubility of the released enzyme activity measured in the post-lysosomal supernatant fractions, portions of these preparations from the 43 and 64 hour periods (Table 5) were centrifuged at 100,000 g for 60 minutes. Approximately 65 per cent of the β-galactosidase remained soluble (Table 6). However, the difference in the enzyme levels between galactose-fed chicks and their corresponding controls compared closely with those differences seen in the respective 20,000 g supernatant fractions. This would indicate that the increases in the levels of β-galactosidase observed in the 20,000 g preparations from the galactose-intoxicated animals are due to enzyme released from the lysosomes and not the result of increased de novo synthesis of the enzyme in the microsomes. Previous studies have indicated no alteration in general protein synthetic rates

in the galactose-intoxicated chicks (Blosser and Wells, 1972). Only 5 to 8 per cent of the β-N-acetyl hexosaminidase found in the 20,000 g supernatnat fractions was recovered in the 100,000 g supernate although the levels from the experimental animals were markedly higher than those measured from controls (see Discussion and Table 6).

Plasma levels of N-acetyl hexosaminidase, β-galactosidase, and acid phosphatase in galactive and control-fed chicks were measured to ascertain whether acid hydrolases might be released by tissue as a result of cell damage. As seen in Table 7, concentrations of free acid hydrolases are essentially identical from the control and experimental chicks.

Discussion

As indicated by the enhanced release of lysosomal enzymes by osmotic and temperature shock, lysosomes from the brains of galactose-fed chicks are more fragile, presumably as a result of the uptake and concentration of galactose and galactitol by the lysosomes. This latter conclusion is based on (1) the demonstration of enhanced lability of normal lysosomes to osmotic shock after preincubation in galactose or galactitol, (2) a correlation between the sum of *in vivo* levels of galactose and galactitol and the increase in free activities of β-galactosidase and β-N-acetyl hexosaminidase, and (3) the loss of increased fragility accompanying

reduction of levels of galactose and galactitol in the brain by 8 hours after the animals were removed from the experimental diet.

The enzymes studied exhibit differential responses to osmotic or temperature shock (Figures 12 and 13). Such phenomena have been observed previously with these or other acid hydrolases in lysosomes from rat liver (Baccino, Rita and Zuretti, 1971) or rat brain (Sellinger and Nordman, 1969; Sellinger and Hiatt, 1968; Bowen and Radin, 1969). These latter investigations have suggested differential structural affinities of the acid hydrolases for the lysosomal matrix and, hence, a variation in the ease with which disruptive agents may effect solubilization of different hydrolases. Following disruption of brain tissue in hypotonic sucrose and differential centrifugation, Sellinger and Nordman (1969) concluded that much of the β-N-acetyl hexosaminidase was still bound to small membrane fragments of disrupted lysosomes, and in adsorbability experiments, Bacinno et al. (1971) demonstrated that previously solubilized β-N-acetyl hexosaminidase was almost completely readsorbed to particulate material from rat liver homogenates. Such a high degree of membrane adherence by the soluble enzyme could explain the loss of most of the activity of the β-N-acetyl hexosaminidase in the 20,000 g supernatant fraction in our studies when this fraction was further centrifuged at 100,000 g for 1 hour.

One inconsistency between the behavior of lysosomes from galactose-fed chicks and those from control animals preincubated with galactose in galactitol is the apparent absence of an augmented heat lability in the latter case. This difference may reflect an insufficient concentration of galactose or galactitol in the supplemented control lysosomes for enhanced response to a rather gentle disruption procedure such as elevated temperature. There also may be a difference in the mode by which lysosomes become fragile in vivo and in vitro. In vivo, increased lability could result, in part, from the vacuolation produced by monosaccharides in the lysosomes (Dingle et al., 1969), whereas in vitro, the much shorter incubation periods with monosaccharides may cause simple swelling of the lysosomes by imbibition of water in response to the inward diffusion of monosaccharides (see Lloyd, 1969). Hence, distortion of the lysosomal matrix in different manners could affect the responsiveness to different types of stress. Perhaps the relative lengths of exposure of lysosomes to galactose and galactitol compounded with radically different environments in vitro and in vivo may also explain the need for levels of monosaccharides in vitro several times those attained in vivo to elicit a similar response to osmotic shock.

From present experiments, it is difficult to ascertain whether the increases in free enzyme activities in brains of galactose-fed chicks (Table 5) represents leakage of the enzymes from the lysosome *in vivo* or release that occurs during homogenization of the tissue as a result of increased fragility. Even though most of the β-N-acetyl hexosaminidase was observed to be membrane bound, it could be argued either that it comes from the ruptured lysosomes, supporting the release-during-homogenization interpretation, or that it represents enzyme released *in vivo* which had adhered to, for example, microsomal membrane. The lack of increase of free levels of acid phosphatase over control free values might suggest a localization in or affinity to the lysosomal matrix such that acid phosphatase cannot be released readily *in vivo* or, on the other hand, the lack of increase might suggest that the careful procedures used for disruption of the tissue were not sufficient stress to enhance the release of the enzyme from more fragile lysosomes.

An abnormal release of acid hydrolases from lysosomes has been implicated in the pathology of a variety of disorders, including rheumatoid arthritis (reviewed by Lack, 1969), muscular dystrophy (reviewed by Weinstock and Iodice, 1969), vitamin E deficiency (reviewed by Roels, 1969), and poisoning by such agents

as vitamin A, bacterial toxins, or UV irradiation (reviewed by Slater, 1969). Whether increased lysosomal fragility or possible leakage of hydrolases into the cytoplasm would disrupt the cellular degradative processes in galactose neurotoxicity remains to be elucidated. However, independent findings lend support to such a possibility. Neurohistological studies on chicks during galactose toxicity demonstrated degeneration of neurons in the basal ganglia, medulla, and occipital lobes (Rigdon, Couch, Creger and Ferguson, 1963). Levels of hexosamine in mucopolysaccharides and, in gray matter, in glycoprotein were found to be significantly lower from an individual suffering from human hereditary galactosemia than those found in normal adults (Haberland, Perou, Brunngraber, and Hof, 1971). Another neurologic disorder, human neurolathyrism, is caused by ingestion of β-N-oxalyl-L-α,β diamino propionic acid from the seeds of Lathyrus sativus. Injection of this amino acid into animals is thought to increase catabolism of protein and nucleic acids and has also been shown to increase the free levels of acid phosphatase, ribonuclease and cathepsin, all lysosomal enzymes (Lakshmanan, Cheema and Padmanaban, 1971).

Disruption of lysosomal integrity and increases in the free levels of acid hydrolases is supportive of the hypothesis of a faster turnover of carbohydrate

constituents of glycolipid and glycoprotein. That free levels of acid phosphatase did not increase over control values while the levels of the other two enzymes studied did, suggests that not all lysosomal enzymes would exhibit increased free activities in the brains of galactose-fed chicks. Several additional acid hydrolases have been measured, using ρ-nitrophenol substrate derivitives, and were found to be absent or present in low levels in lysosomal fractions from chick brain. Activities of α-L fucosidase, β-glucuronidase (E.C.3.2.1.31) and β-D mannosidase (E.C.3.2.1.24) were quite low in comparison to β-galactosidase while arylsulfutase (E.C.3.1.6.1) and β-glucosidase (E.C.3.2.1.21) could not be detected in the lysosomal fraction in the presence of Triton X-100. With the exception of β-glucuronidase, none of these enzymatic activities were found in the 20,000 g supernatant fraction. This would suggest that increased catabolism of only specific types of glycosidic bands or cellular macromolecules might be expected to occur during galactose neurotoxicity.

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TABLE 5.--*In Vivo* Levels of Galactose and Galactitol and Free Activities of β-Galactosidase and β-N-Acetyl hexosaminidase in Brains of Chicks Fed a High Galactose Diet.

Time on Diet	Galactose	Galactitol	Galactose + Galactitol	β-N-Acetyl Hexosaminidase	β-Galactosidase
h		μmol/g tissue		ρ-nitrophenol/h/100 mg tissue	
18	4.5	1.3	5.8	372	25.8
18	6.0	1.4	7.4	384	23.5
42	4.0	3.4	7.4	356	23.3
46	4.0	4.0	8.0	378	23.3
22	6.2	2.9	9.0	360	28.4
43	8.7	3.5	12.2	452	27.0
64	7.5	5.5	13.0	557	41.3

Four chicks from each dietary group were sacrificed at the times indicated; their brains were pooled, and 20,000 g supernatant fractions prepared (see Methods), and assayed. Mean control values (± S.D.) for β-N-acetyl hexosaminidase and β-galactosidase were 300 ± 37 and 16.7 ± 1.8, respectively. Differences in specific activities were observed whether expressed per gram of fresh tissue or per mg of brain. Assays on each separate fraction were determined in triplicate with a range of variability ≤ 5 per cent.

TABLE 6.--Soluble Activities of β-Galactosidase and β-N-Acetyl hexosaminidase in Brains of Chicks Fed Control or Galactose-Containing Diets.

Time on Diet	Galactose + Galactitol	β-N-Acetyl hexosaminidase		β-Galactosidase	
h	μmol/g tissue	nmoles ρ-nitrophenol released/h/100 mg tissue			
		Control	Galactose	Control	Galactose
43	12.2	12	27	9	18
64	13.0	12	37	11	32

Triplicate assays were made on each separate fraction with a range of variability ≤ 5 per cent.

TABLE 7.--Levels of Selected Acid Hydrolases in Plasma.

	Control	Galactose
	(nmoles ρ-nitophenol released/ ml plasma)	
β-N-acetylhexosaminidase	876 ± 200 (2)	866 ± 107 (5)
β-Galactosidase	135 ± 5 (2)	131 ± 18 (5)
Acid phosphatase	105 ± 8 (3)	98 ± 10 (3)

Chicks were anesthetized with dithylether and exsanguinated with a heperinized seringe. Blood was pooled from groups of 4 chicks and centrifuged to remove red blood cells. The plasma was assayed for the enzymes indicated as outlined in methods. Values are expressed ± s.d. The numbers in parentheses represent the number of pools assayed.

Figure 12. Effect of osmotic shock on stability of lysosomes from chick brains. Lysosomes from 5 chicks from each of the two dietary groups were prepared as described in Methods, divided into equal portions corresponding to approximately 0.57 g of fresh tissue, resuspended in 4 ml of the appropriate sucrose solution at 0°C, and recentrifuged at 20,000 g for 10 minutes. Enzyme assays were made on the supernatant fractions. A: ●——● control, O----O galactose-fed for β-galactosidase; B: ●——● control, O----O galactose-fed for β-N-acetylhexosaminidase; C: ●——● control, O----O galactose-fed for acid phosphatase. Each point represents the average of duplicate assays. Range of variability was approximately 5 per cent.

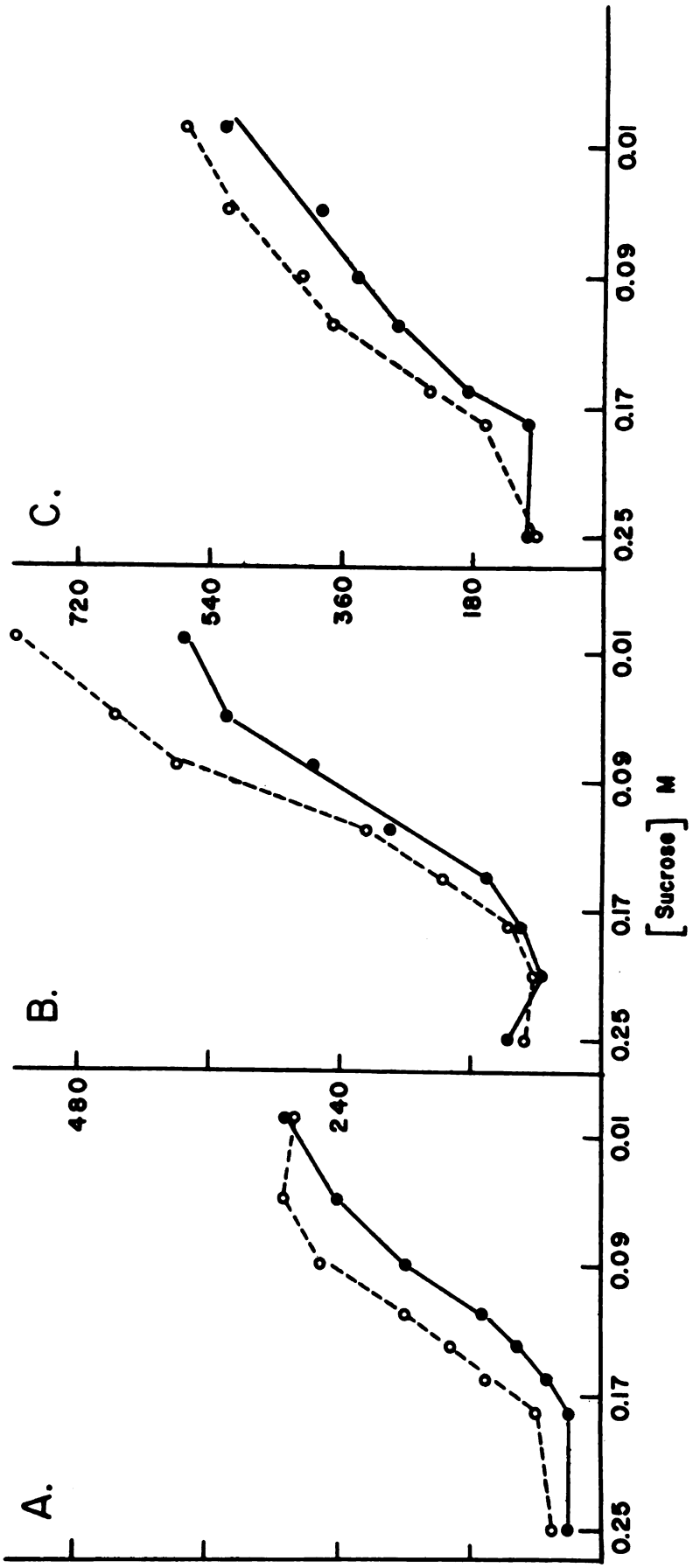

A.
B.
C.
nmoles p-nitrophenol/hour/100mg tissue
240
180
120
60
480
240
720
540
360
180
0.25
0.17
0.09
0.01
[Sucrose] M

Figure 13. Effect of increasing temperature on stability of neural lysosomes. The lysosomal pellets from 5 pooled brains of chicks on the respective diets for 44 hours were resuspended in 250 mM sucrose (1:4, w/v suspension, referred to original tissue wt.) and portions were incubated at the temperatures indicated for 45 minutes. Samples were then centrifuged at 20,000 g for 10 minutes, and the supernanant fractions were assayed for enzyme activity. Total enzyme activity, measured by resuspending sedimented lysosomes and assaying for the respective enzymes in the presence of 0.2 per cent (w/v) Triton X-100, was essentially identical in each sample. Each point represents the average of duplicate assays with a 5 per cent range of variability.
A: β-N-acetylhexosaminidase; ●——● control, O----O galactose-fed.
B: acid phosphatase; ●——● control, O----O galactose-fed.

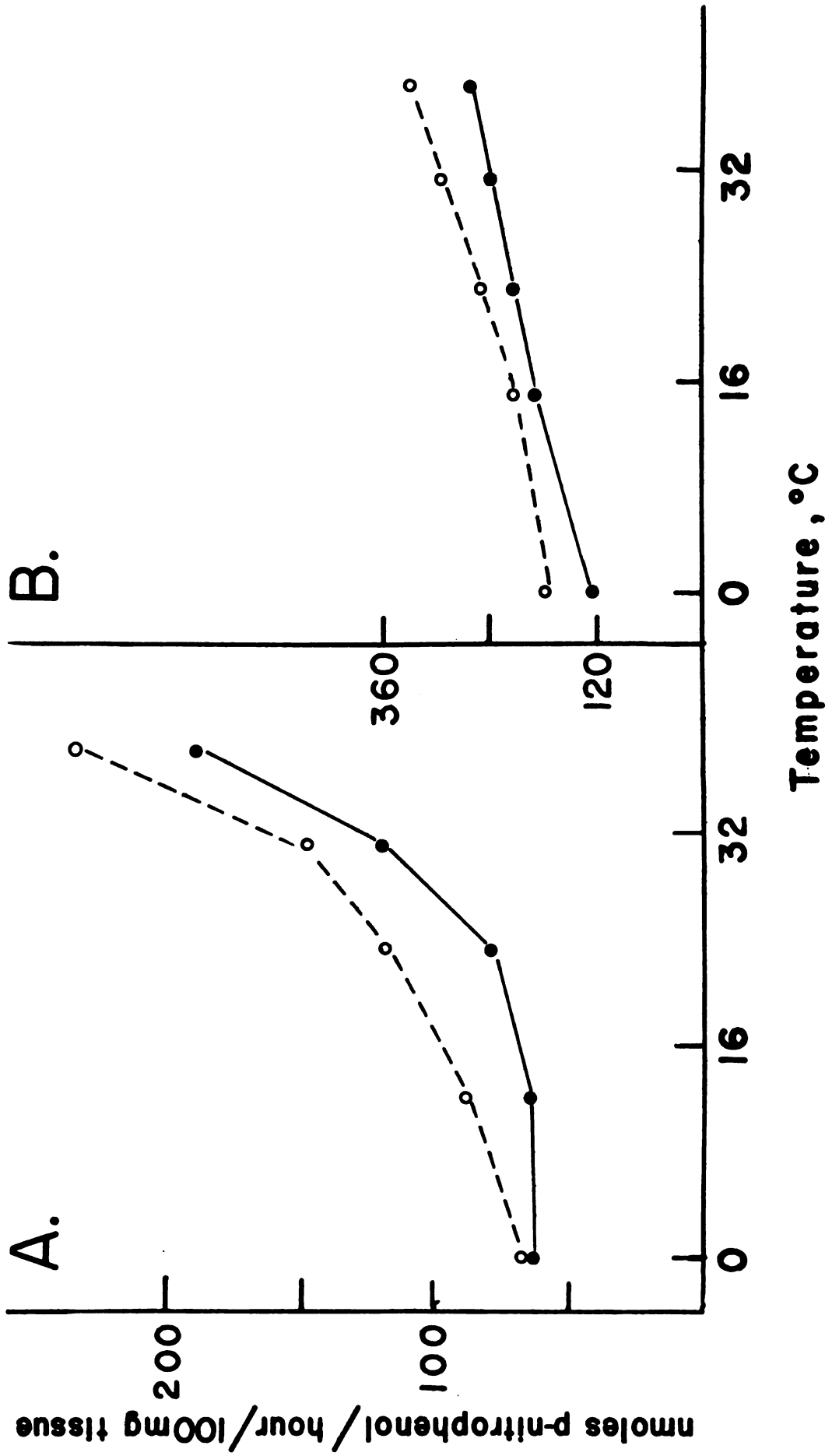

A.
B.
200
100
360
120
0
16
32
0
16
32
nmoles p-nitrophenol / hour / 100mg tissue
Temperature, °C

Figure 14. Osmotic stability of neural lysosomes preincubated in galactose, galactitol, or sucrose. Lysosomes, isolated from a pool of 5 brains from chicks fed the control diet, were divided into equal portions and resuspended in 6 vol (6 vol/g original tissue wt) of either 250 mM sucrose, 200 mM sucrose + 50 mM galactose, or 200 mM sucrose + 50 mM galactitol and incubated at 15°C for 1 hour. Lysosomes were then sedimented at 20,000 g for 15 minutes, subjected to osmotic shock, and assayed for free enzyme activity, as described in Figure 1. Each point represents an average of duplicate assays with a 5 per cent range of variability. A: β-N-acetylhexosaminidase; ●——● control, Δ----Δ galactose, O----O galactitol. B: β-galactosidase; ●——● control, Δ----Δ galactose, O----O galactitol.

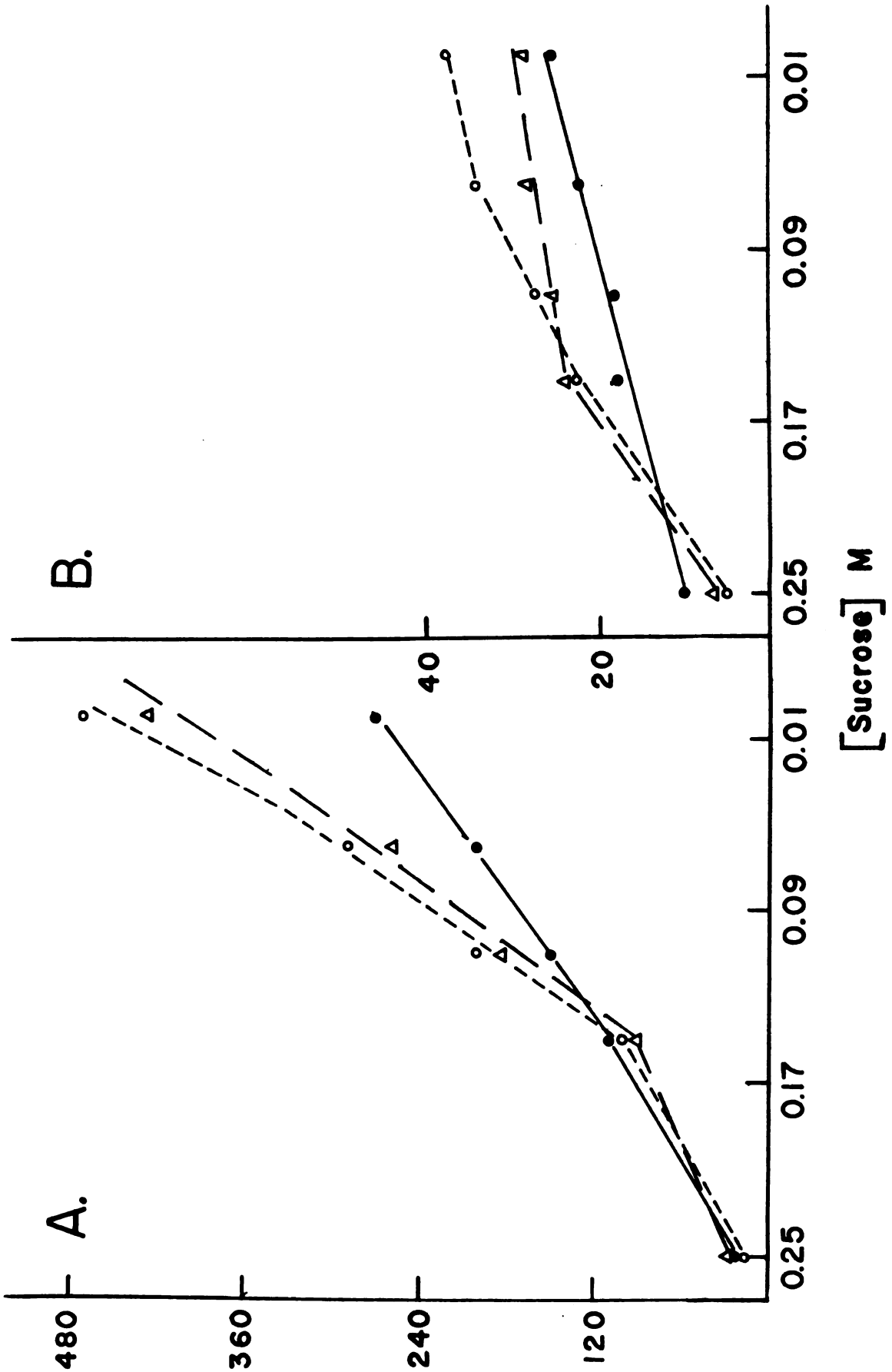
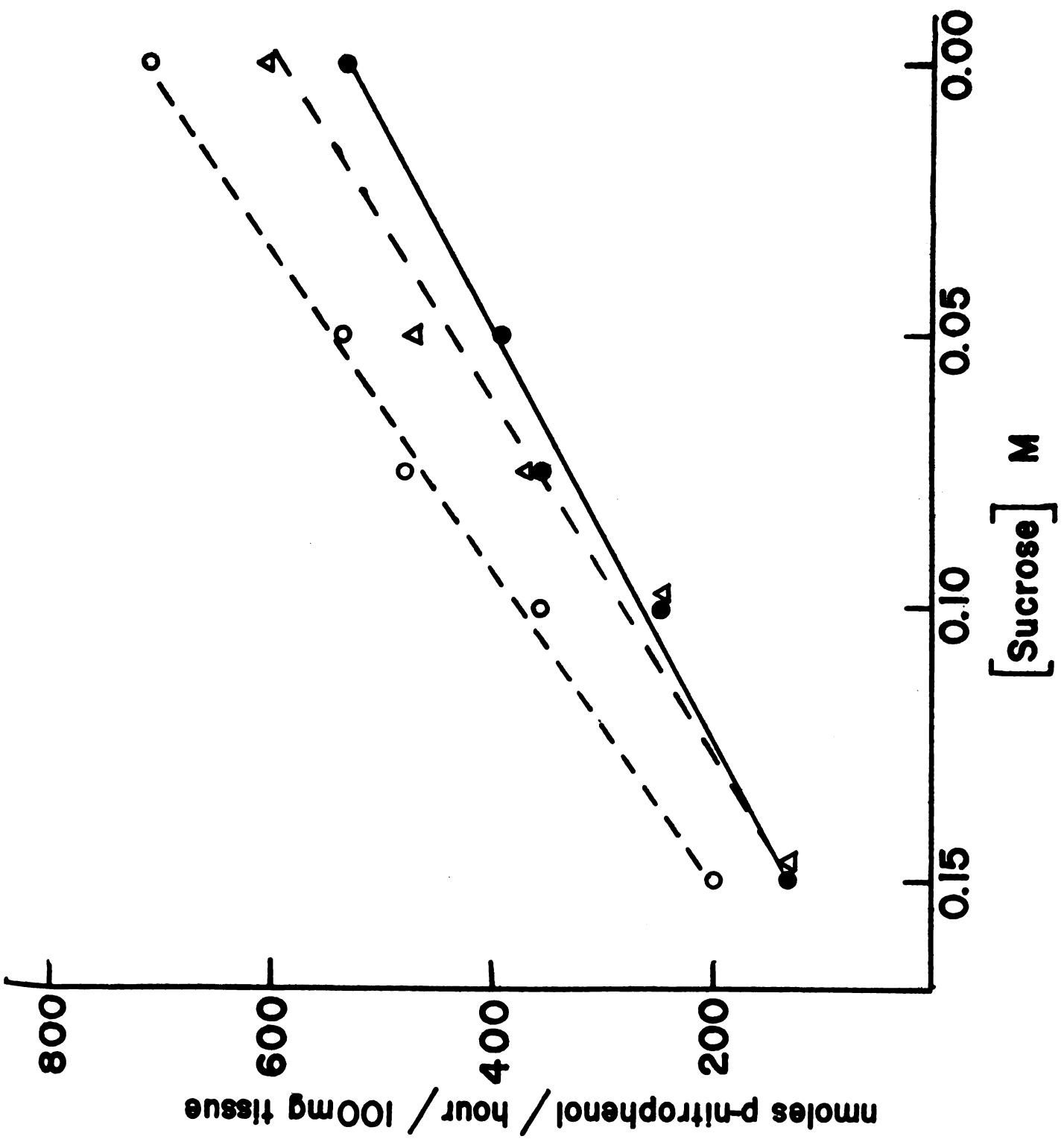

A.
B.
480
360
240
120
40
20
0.25
0.17
0.09
0.01
[Sucrose] M
nmoles p-nitrophenol / hour / 100 mg tissue

Figure 15. Reversal of neural lysosomal fragility induced by the galactose diet. Lysosomes were isolated as described in Methods from pools of 6 brains from chicks fed control or galactose diets for 52 hours, or fed galactose diets for 44 hours and then switched to the control diet for 8 hours. Exact aliquots of the lysosomal fractions (as determined by measuring total β-N-acetylhexosaminidase activity) were resuspended in hypotonic sucrose solutions and the suspensions were centrifuged at 20,000 g for 15 minutes. The supernatant fraction was assayed for activity of β-N-acetylhexosaminidase. Each point is an average of duplicate assays with a 5 per cent range of variability. Control, ●——●; galactose, O----O; reversed, Δ----Δ.


nmoles p-nitrophenol / hour / 100mg tissue
800
600
400
200
0.15
0.10
0.05
0.00
[Sucrose] M

SUMMARY

Rates of synthesis of protein and carbohydrate containing macromolecules do not appear to be decreased in response to the moderate reductions in ATP and phosphocreatine levels and the energy charge in the brains of galactose-fed chicks. This lack of effect is in contrast to suggestions by other workers on the influence of the levels of ATP and energy charge on protein synthesis rate (see Chapter II, Introduction). It appears that under conditions of these studies, biosynthesis of these macromolecules have high priority with respect to utilization of available energy reserves. These findings do not exclude the possibility of an individual protein having an altered synthesis rate. However, such a change in the normal synthesis or degradation rate of a protein would likely involve perturbation of specific control mechanisms and not the availability of ATP for polypeptide formation. Likewise, moderate decreases in the concentrations of several amino acids do not affect protein synthetic rates. Of those amino acids affected, those whose metabolism are closely associated with the tricarboxylic acid cycle (alanine, glutamate, glutamine,

aspartate) are most appreciably affected. Changes in concentration of these amino acids may be in response to the depressed energy reserves status and glycolytic rate of the galactose-fed chick.

The half-lives of brain protein in the galactose intoxicated chick, measured by the loss of [^{14}C-guanidino] arginine, are not affected over the time period studied. An average half-life of 36 hours was calculated for total protein which is shorter by nearly an order of magnitude than those determined in an adult brain of other species (Chapter II, Discussion). Elevated ammonia levels, a commonly used indicator for increased protein or amino acid breakdown, was not observed in the galactose-treated animals.

The stability of neural lysosomes in the galactose-fed chicks to hypoosmotic solutions and incubation at high temperatures is decreased. The increased lability to hypoosmotic shock can be duplicated by preincubation of normal lysosomes in solutions of galactose and galactitol. In addition, the observed increases in the extra-lysosomal activities of β-galactosidase and β-N-acetylhexosaminidase correlate with the summation of the in vivo concentrations of galactose and galactitol. These data strongly suggest that uptake of galactose and galactitol by the lysosomes leads to increased fragility. Contrary to the carbohydrase enzymes, acid phosphatase levels did not increase

in the cytoplasm consistent with the data of other workers (Chapter IV, Discussion) that there is a differential binding of the acid hydrolases to the lysosomal matrix. It cannot be concluded unequivically that increased lysosomal enzyme activities in the 20,000 or 100,000 g supernatant preparations are a result of release of enzymes in vivo. Homogenation of the tissue may cause some artificial lysis of the more fragile lysosomes.

Radioactively labelled glucosamine and mannosamine were both incorporated into brain glycoprotein and gangliosides at significantly greater rates in galactose-fed chicks than in controls. Although [^{3}H] glucosamine uptake for mucopolysaccharide biosynthesis was also enhanced, normal incorporation rates of $^{35}SO_4$ into chondroitin sulfate implies that this subclass of the acid polysaccharides may not be affected.

Levels of bound sialic acid and hexosamine did not appear to be significantly altered in any of these fractions. These observations have led to formulation of a hypothesis of increased turnover rate of carbohydrate units on glycoprotein and gangliosides in galactose intoxicated chicks. The possibility of lysosomal dysfunction as a result of increased lability or release of acid hydrolases into the cytoplasm is consistent with this hypothesis. In view of the presently held opinions on the central roles played by carbohydrate moieties of

glycoprotein and gangliosides in intercellular communication, aberrant metabolism of the carbohydrate residues in plasma or nerve ending membranes could disrupt normal development of neural pathways and hence, neural function.