PERTURBATIONS OF ENERGY METABOLISM IN CHICK BRAIN INDUCED BY HYPERPHENYLALANEMIA AND GALACTOSEMIA

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SANDRA SPIEKER GRANETT
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This is to certify that the

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Perturbations of Energy Metabolism in Chick Brain

Induced by Hyperphenylalanemia and Galactosemia

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Sandra Elizabeth Granett

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William W. Wells

Major professor

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ABSTRACT

PERTURBATIONS OF ENERGY METABOLISM IN CHICK BRAIN INDUCED BY HYPERPHENYLALANEMIA AND GALACTOSEMIA

By

Sandra Spieker Granett

Cerebral energy metabolism was investigated in the chick treated with L-phenylalanine or D-galactose.

Hyperphenylalanemia was produced in the day-old chick by 3 hourly intraperitoneal injections with neutralized 0.18 M L-phenylalanine and maintained for 12 - 18 h; controls were injected similarly with 0.154 M saline. Shortly after the last injection the L-phenylalanine-treated animals were observed to be sedated; normal righting reflexes were impaired and the severely debilitated chicks were prostrated. Levels of phenylalanine in the plasma reached 10 - 15 μmol/ml (normal: 0.16); tyrosine increased from 0.10 μmol/ml to 0.6 μmol/ml. In the brain phenylalanine and tyrosine were elevated to 2.5 - 3.5 μmol/g (normal: 0.05) and 0.15 - 0.30 μmol/g (normal: 0.05), respectively.

Examination of several glycolytic intermediates and high energy phosphate compounds in the brain at a time

when levels of phenylalanine were greatest revealed a 15 - 25% increase in phosphocreatine accompanied by a reciprocal decrease in inorganic phosphate; levels of fructose-1,6-diphosphate decreased by 15 - 25% and levels of lactate and L-α-glycerol phosphate by 25%. Glucose and glucose-6-phosphate were either unchanged or increased, whereas glycogen concentrations did not differ significantly from those seen in the controls. The levels of the adenine nucleotides were unaffected. The rate of expenditure of cerebral high energy phosphates was depressed by 50 - 79% when determined as a function of metabolic rate during ischemia by the "closed-system" technique. A 15% hypoglycemia was consistently observed. Phenylacetic acid accumulated in the brain to levels of 0.01 to 0.06 μmol/g.

The state of narcosis was reversible within 24 h, although the animals had not regained their appetite. Phenylalanine and tyrosine concentrations in the brain decreased to normal levels by 18 h, and most of the altered metabolites approached concentrations in control tissue 8 - 12 h after the final injection. The changes observed in the metabolites coincided with cerebral phenylalanine levels of 2 μmol/g or more.

No *in vitro* inhibition of anaerobic glycolysis by 18 mM L-phenylalanine could be demonstrated with high speed supernatants prepared from chick brain. Commercially

available creatine kinase activity purified from muscle was unaffected by 9 mM L-phenylalanine.

The profile of cerebral amino acids was markedly altered in the phenylalanine-treated chicks. Lysine, histidine, phosphorylethanolamine, taurine, aspartic acid, threonine, serine, glutamic acid, alanine, valine, methionine, isoleucine and leucine were decreased. Glutamine, glycine, phenylalanine and tyrosine were elevated. Neither arginine nor ammonia were affected. The summation of free amino acids and ammonia did not differ between the phenylalanine and saline-treated chicks.

That the phenylalanine-injected chicks were metabolically depressed was supported by the delay in tonic extension observed upon injection of the animals with pentylenetetrazol or picrotoxin. In some cases recovery from the convulsions occurred or convulsive activity was eliminated.

Injection of either phenylalanine or saline did not change plasma osmolality or levels of sodium or calcium in comparison to uninjected control animals; however, potassium was increased by 60% regardless of the solute.

A survey of other amino acids administered in a similar manner disclosed D-phenylalanine, L-histidine, L-methionine and L-tryptophan to reduce activity in the chick, often resulting in prostration and death.

The changes produced in cerebral glycolytic metabolism in the chick fed a diet 40% in D-galactose for 46 h were compatible with those previously observed: levels of phosphocreatine, ATP, glucose, glycogen, fructose-1,6-diphosphate and lactate were depressed; AMP levels were elevated. Analysis of the utilization of actual and potential high energy phosphate compounds for various intervals of postmorten ischemia (zero time to 10 min) according to the "closed-system" technique indicated a slower rate of glycolysis in the galactose-fed animals in comparison to controls. Galactose and galactitol were not utilized and did not constitute energy sources. ATP expenditure began after 12 sec of ischemia in the galactose-toxic chick in contrast to 24 sec in the chicks fed control diet. Cerebral glucose levels were initially 0.3 μmol/g in the galactose-fed chicks compared with 1.0 - 1.5 μmol/g in control animals and decreased to 0.15 μmol/g 6 sec after initiation of the ischemia, remaining at this level for the 10 min. Glycogen reserves were depleted sooner and lactate formation was insignificant until after 1 min of ischemia in contrast to the control situation in which accumulation of lactate was immediate. No regulation of glycolysis at the phosphofructokinase point as evidenced by an increase in the level of fructose-1,6-diphosphate during the first 30 sec of ischemia could be demonstrated in chicks fed

galactose. Citrate levels did not differ between the two groups of animals.

Determinations of cerebral glycogen levels by acid hydrolysis were found to be erroneously high due to the presence of a glucose-containing, non-glycogen material. A more specific method for hydrolysis of glycogen, employing amyloglucosidase, was proposed. The degradation was accomplished in 50 mM citrate buffer, pH 5, and the glucose released was quantified at pH 8 spectrophotometrically with hexokinase and glucose-6-phosphate dehydrogenase. The procedure was demonstrated as accurate, precise and convenient, and sensitivity could be enhanced by fluorometric analysis.

PERTURBATIONS OF ENERGY METABOLISM IN CHICK BRAIN INDUCED BY HYPERPHENYLALANEMIA AND GALACTOSEMIA

By

Sandra Elizabeth Spieker Granett

A THESIS

Submitted to
Michigan State University
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This work

is dedicated

to

my husband, Jeff,

to my mother,

Mildred Spieker

and to the

spirit of my father,

Rudolph Spieker

GENERAL ACKNOWLEDGMENTS

I should like to acknowledge my committee members, Dr. Richard Anderson, Dr. Loran Bieber, Dr. Richard Rech, Dr. John Wilson and my major professor Dr. William Wells, for their conscientious criticism of this work.

In particular I wish to recognize Dr. Wells for his encouragement and his confidence in me as a developing scientist. His consistently optimistic, imaginative, and vigorous approach to research was refreshing.

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The work was conducted in the second and third years of my marriage. For this I acknowledge my husband whose love for me extended to my professional fulfillment, though it necessitated a year's separation. I am indebted to him for my inner satisfaction and contentment and I cherish him more deeply for this.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CPM	Counts per minute
EDTA	Ethylenediaminetetraacetic acid
GABA	Gamma-Aminobutyric acid
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PHE	Phenylalanine
Tris	Tris(hydroxymethyl)aminomethane

I. STATEMENT OF ORGANIZATION

Galactosemia and phenylketonuria, two genetic diseases of man, are easily distinguished in that the former is a disorder of carbohydrate metabolism and the latter of amino acid metabolism. However, associated with both is mental retardation. For the studies described herein day-old chicks were treated with D-galactose or L-phenylalanine and investigated as model systems for the respective human conditions; galactose proved toxic to the chick, producing convulsions, whereas treatment with phenylalanine was accompanied by sedation. It was of special interest to examine some biochemical parameters of brain function in these animals for two reasons: involvement was indicated by the chicks' behavioral signs and impairment of normal mental capacities is an outstanding clinical symptom shared by both galactosemics and phenylketonurics. Perturbations in high energy phosphate compounds and glycolytic intermediates had been previously characterized for the galactose-intoxicated chick by Kozak and Wells (1969 and 1971). In Section I evidence is presented for a depressive effect of phenylalanine on

energy metabolism in the chick brain. In Section II, further studies on the effects of galactose on glycolytic flux are described. Also a new method for quantification of glycogen employed in these investigations is presented.

This thesis is divided into two parts, two chapters each, as a matter of convenience; Chapter I of Section I has been published in the form presented here; Chapter I of Section II in conjunction with other pertinent work.

II. SECTION I

INTRODUCTION

Several animals notably the mouse, rat and monkey have received considerable attention as model systems for the study of the human genetic disorder, hyperphenylalanemia or phenylketonuria. The chick, though, had been conspicuously neglected until the late sixties when Tamimie and Pscheidt (1966 a, b and c; 1968) published a series of papers on the effects of feeding for four weeks a diet 5 or 8% in phenylalanine to the young chick. They found aberrant morphological changes associated with poor growth and development, but no mortality was reported and post-mortem examination indicated no gross pathology in any internal organs. Symptoms were reversible. Analysis of any biochemical alterations, particularly in the brain, was not extensive. Subsequently, further studies were conducted (Granett, 1970) to elucidate this latter point. The brain was considered, primarily, since irreversible neurologic disturbances are the most outstanding clinical features of phenylketonuria.

L-Phenylalanine was administered through the diet (7-1/2 and 10%) to newly hatched chicks for periods of two or three weeks. Abnormally low weight gain was recorded; the animals appeared alert on gross observation. Investigation into cerebral high energy phosphate metabolism and glycolysis revealed the phenylalanine chicks to have consistently lower lactate levels than controls. Phosphocreatine levels were either unchanged or elevated. Changes in concentration of glucose and fructose-1,6-diphosphate were variable; glucose-6-phosphate decreased or did not change. Upon treatment of the chicks with larger amounts of L-phenylalanine by serial intraperitoneal injections, though, they entered a state of narcosis, reversible with time, resembling in some way that produced with barbiturates. In this section, then, the cerebral energy metabolism in the narcotized animals during the highest phenylalanine levels and during the recovery period is discussed; the pattern of free amino acids is characterized; and evidence for anti-convulsive activity of phenylalanine is presented. Also, the in vivo observations are discussed in terms of a direct effect of L-phenylalanine on glycolysis. The work presented in Chapter I has been published under the title "Energy Metabolism in the Brains of L-Phenylalanine-Treated Chicks," by Sandra E. Granett and W. W. Wells, Journal of Neurochemistry 19, 1089 (1972).

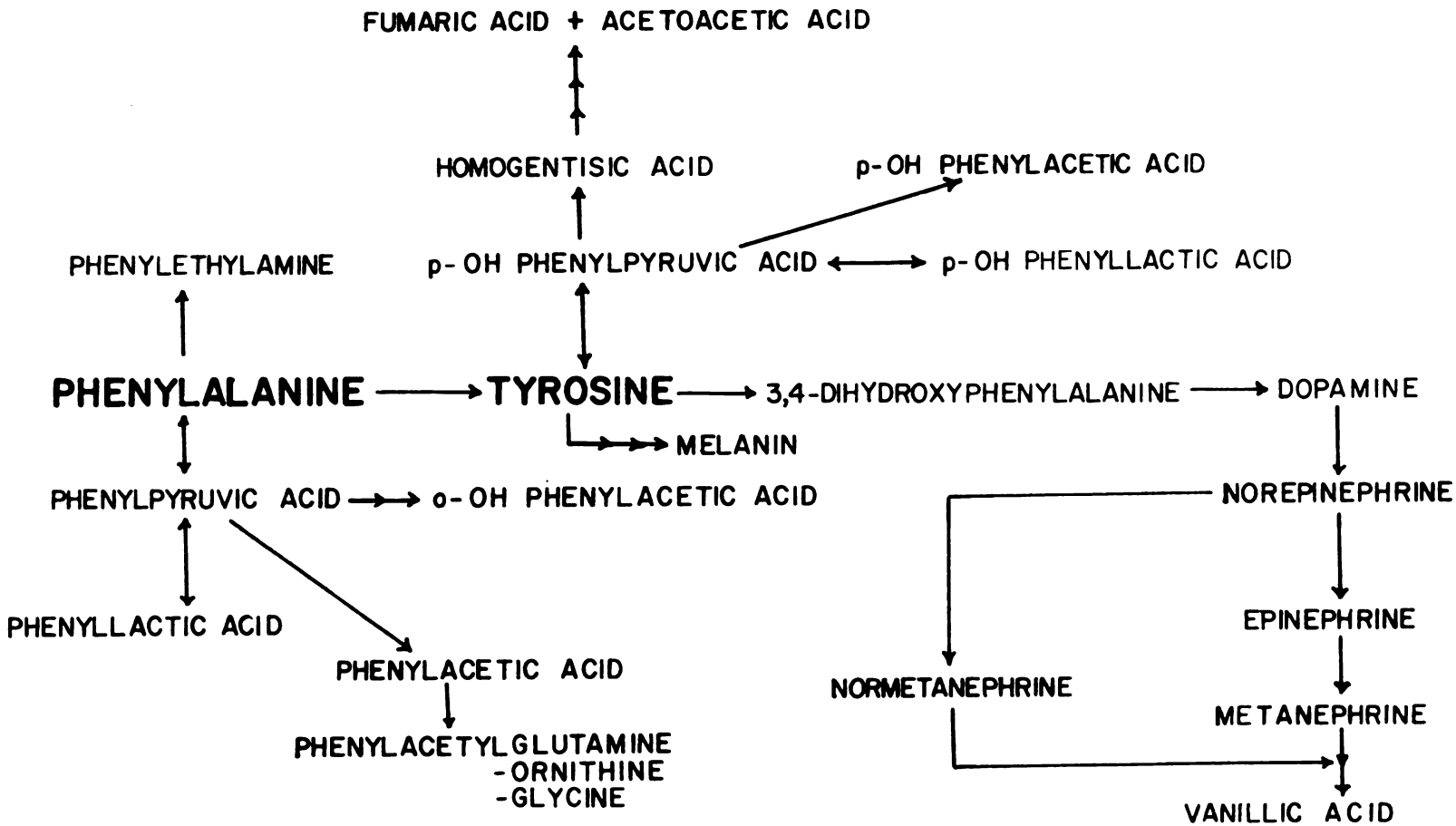
LITERATURE REVIEW

Phenylketonuria

Description of the disease in man

Phenylketonuria is a genetic disorder characterized as autosomal recessive in which the enzyme phenylalanine hydroxylase, responsible in part for the conversion of phenylalanine to tyrosine, is absent (Jervis, 1939 and 1953). The second enzyme required for the hydroxylation, dihydropteridine reductase, is present in normal amounts. Fölling first recognized the condition in 1934. Clinical features of the homozygote include microcephaly, hyperkinesis, electroencephalographic abnormalities, seizures, mental retardation, eczema and deficient pigmentation. L-Phenylalanine accumulates in the plasma of patients to levels as high as 2-6 μmol/ml, as compared to a normal range of 0.054-0.12 (Knox, 1972). Alternative metabolites resulting from the diversion of the amino acid through other pathways (Fig. 1) are detected. Plasma concentrations of phenylpyruvic acid and o-hydroxyphenylacetic acid measuring 6-64 μmol/liter and 7-40 μmol/liter, respectively, have been reported (Jervis and Drejza, 1966).

Figure 1. Enzymatic metabolism of L-phenylalanine and L-tyrosine


FUMARIC ACID + ACETOACETIC ACID
HOMOGENTISIC ACID
p-OH PHENYLACETIC ACID
PHENYLETHYLAMINE
p-OH PHENYLPYRUVIC ACID
p-OH PHENYLLACTIC ACID
PHENYLALANINE
TYROSINE
3,4-DIHYDROXYPHENYLALANINE
DOPAMINE
MELANIN
PHENYLPYRUVIC ACID
o-OH PHENYLACETIC ACID
NOREPINEPHRINE
PHENYLLACTIC ACID
EPINEPHRINE
PHENYLACETIC ACID
NORMETANEPHRINE
METANEPHRINE
PHENYLACETYLGLUTAMINE
-ORNITHINE
-GLYCINE
VANILLIC ACID

The phenyl or phenolic acid derivatives, though, appear to be largely excreted in the urine and have been quantified by gas chromatography (Hoffman and Gooding, 1969). Attempts to correlate the various degrees of mental retardation observed with plasma levels of phenylalanine or urinary levels of the phenyl or phenolic acids have not been fruitful.

The most significant pathologic findings occur in the nervous system. Frequently the brains of phenylketonurics weigh about 40% less than normal. The three major morphological alterations seen in the tissue are impairment of normal maturation, defective myelination and cystic degeneration of the white matter (Wiltse and Menkes, 1972). Associated with these are decreased content of cholesterol and cerebrosides in white matter (Prensky, Curr, and Moser, 1968) and a reduction in the amount of some proteolipid fractions of cerebral cortex (Menkes, 1968). Although the postulate is made that synthesis of protein and lipid is disturbed under conditions of hyperphenylalanemia, a specific molecular defect in myelin has yet to be demonstrated.

Analysis of free amino acids in the plasma (Perry, Hansen, Tischler, Bunting and Diamond, 1970) cerebrospinal fluid (McKean and Peterson, 1970; Van Sande, Mardens, Adriaenssens and Lowenthal, 1970), and brain (McKean and Peterson, 1970) of phenylketonurics demonstrates marked differences in comparison with control samples.

The pharmacodynamic amines serotonin (Pare, Sandler and Stacey, 1958) and epinephrine (Weil-Malherbe, 1955) are reduced in the plasma.

The disorder is diagnosed qualitatively through the detection of phenylpyruvate or phenylacetate in urine, both salts imparting a unique aromaticity. The presence of phenylpyruvate can also be confirmed by the ferric chloride test. The quantification, though, of phenylalanine in the serum is the superior method. Heterozygotes with intermediate levels of phenylalanine hydroxylase are detected with phenylalanine tolerance tests. The symptoms associated with phenylketonuria are reversible in part by restriction of protein diet to low phenylalanine containing food preparations. The accumulation of the amino acid and its alternative metabolites subsides. The eczema and imperfect pigmentation are corrected and serotonin levels rise in the blood. The treatment ameliorates the hyperactivity, restlessness and irritability associated with phenylketonurics and electroencephalographic abnormalities and seizures often disappear. However, any retardation incurred during the course of the disease is not reversible and thus early dietary treatment is of extreme importance (Knox, 1972).

At this point in the study of the disease, the molecular event responsible for the neuropathology has not been defined. The deficiency in phenylalanine

hydroxylase cannot be directly invoked since the enzyme normally is found in the liver and not in the nervous system. There is no indication that any of the metabolites of phenylalanine, excepting possibly phenylethylamine (Oates, Nirenberg, Jepson, Sjoerdsma and Udenfriend, 1963) are present in high enough concentrations to be toxic to the brain. This type of circumstantial evidence would indict the high level of phenylalanine.

The melanin insufficiency commonly observed in patients has been resolved and attributed to phenylalanine inhibition of the tyrosinase system (Miyamoto and Fitzpatrick, 1957; Boylen and Quastel, 1962).

Hyperphenylalanemia in animal models

Since investigations into the biochemical abnormalities in the phenylketonuric patient are at best superficial, the animal models of hyperphenylanemia have provided the best clues as to actual mechanisms for the pathology. These model systems are accomplished either by dietary supplementation with phenylalanine or by treatment with an inhibitor of the hydroxylation such as p-chlorophenylalanine (Lipton, Gordon, Guroff and Udenfriend, 1967; Guroff, 1969), esculin (DeGraw, Cory, Skinner, Theisen and Mitoma, 1968) or amethopterin (McKean, Boggs and Peterson, 1968).

From studies with animals, evidence has accumulated for disturbed synthesis of proteins and lipids, which could account for the irreversible neurologic damage seen in phenylketonurics. L-phenylalanine significantly reduces the incorporation *in vivo* of radioactively labeled amino acids into myelin proteins in the rat (Agrawal, Bone and Davison, 1970) and into ribosomal protein in the brains of the rabbit (Swaiman, Hosfield and Lemieux, 1968). Peterson and McKean (1969) demonstrated inhibition of protein synthesis by phenylalanine with rat brain homogenates. Thus far three possible sites in protein synthesis have been recognized as susceptible to inhibition by the amino acid: transport at the cell membrane, synthesis of the amino acyl t-RNA, and polysome formation and integrity. *In vivo* and *in vitro* evidence is available that demonstrates an altered free amino acid pattern in several tissues including brain (Carver, 1965; McKean, *et al.*, 1968; Lowden and LaRamée, 1969) and L-phenylalanine has been shown to competitively inhibit the transport of amino acids across the cell membrane (Blasberg and Lajtha, 1965 and 1966). Appel (1966) observed *in vitro* a 70% inhibition of the synthesis of tyrosine acyl t-RNA under a phenylalanine/tyrosine ratio similar to that in phenylketonuric patients. Dissociation of brain polysomes occurred in young rats injected with L-phenylalanine and was

correlated with the phenylalanine-induced depletion of tryptophan (Siegel, Aoki and Colwell, 1971).

With regard to lipid metabolism, Shah, Peterson and McKean (1968) reported that phenylalanine impairs the synthesis of cholesterol from mevalonate in rat brain *in vivo*; however, to achieve the inhibition *in vitro* the concentration of L-phenylalanine had to be increased to 1.5 µmol/ml or more, five times that observed in hyperphenylalanemic rat brain; at this higher level the synthesis was also inhibited in liver. The phenyl acids proved to be more potent inhibitors *in vitro* (Shah *et al.*, 1969) at 3 mM concentrations. Using a minced preparation of rat brain, Barbato and Barbato (1969) observed decreased acetate and glucose incorporation into lipids with 60 mM L-phenylalanine. Decreased glucose incorporation into lipids was confirmed *in vivo* by Shah *et al.* (1970) and *in vitro* the phenyl acids proved better inhibitors than phenylalanine. Again the discrepancy between inhibitory concentrations of phenylalanine under different experimental conditions was apparent; the phenylalanine concentration required for *in vitro* inhibition was 360 times that required for an *in vivo* effect. Glazer and Weber (1971) found phenylpyruvate to reduce the incorporation of glucose into lipids in rat cerebral slices. Clarke and Lowden (1969) demonstrated a delay in the accumulation of galactolipid in suckling rats; and O'Brien and Ibbot

(1966) observed a reduction in levels of total lipids and cerebrosides in the brain of the infant monkey; in contrast, Geison and Waisman (1970) found in chronic feeding studies with 21 day old rats that phenylalanine did not significantly alter the synthesis or deposition of major brain lipids. Such studies suggest that the undeveloped brain is more sensitive to high levels of the amino acid. With purified myelin preparations Shah et al. (1972) reported that total myelin, cholesterol and galactolipid were reduced in hyperphenylalanemic rat brain and that the levels of the latter two myelin components were present in normal ratios. This would indicate that phenylalanine affects the net amount of myelin synthesized, not the integrity.

Attempts made to produce symptoms of the mental retardation characteristic of phenylketonurics in model systems have been successful in part. Animals loaded with phenylalanine have displayed reduced learning abilities (Averbach, Waisman and Wycoff, 1958; Waisman, Wang, Palmer and Harlow, 1960; Yuwiler and Louttit, 1961; McKean, Schanberg and Giarman, 1967).

Cerebral levels of serotonin are decreased in phenylalanine-fed rats; inhibition by the amino acid has been suggested at three sites critical to the synthesis of the amine: 5-hydroxytryptophan transport into the brain (Yuwiler and Geller, 1969), hydroxylation of

tryptophan to 5-hydroxytryptophan and decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine (Davidson and Sandler, 1958).

It is also possible that synthesis of other neurotransmitter sybstances as GABA or norepinephrine is adversely affected by conditions in which phenyl or phenolic acids are accumulated. Inhibition by aromatic acids of glutamate decarboxylase was suggested by Hanson (1958 and 1959). Fellman (1956) demonstrated inhibition of the formation of epinephrine from dihydroxyphenylalanine by phenylpyruvate, phenyllactate and phenylacetate. In interpreting these data, though, some caution is necessary. Little information is available on the levels of the acid metabolites of phenylalanine in tissues, as their presence has been mainly recognized in urine, and the question remains whether millimolar concentrations demanded for inhibition *in vitro* are met *in vivo* or are even required.

An experimental animal that may afford certain advantages over the rat or monkey as a model system is the dilute-lethal mouse which naturally has a defective phenylalanine hydroxylating system and thus resembles more closely in its genetics the human phenylketonuric (Coleman, 1960; Kelton and Rauch, 1962; Rauch and Post, 1963; Zannoni, Weber, VanValen, Rubin, Bernstein and LaDu, 1966; Zannoni and Cuperman, 1972).

CHAPTER I

ENERGY METABOLISM IN THE BRAINS OF L-PHENYLALANINE-TREATED CHICKS

ABSTRACT

When day-old chicks were injected intraperitoneally with 1.62 mmol of L-phenylalanine, they developed a condition resembling narcosis. Simultaneously, whole brain levels of phenylalanine were 2 - 4 μmol/g, whereas those in control brain were 0.06 μmol/g. Examination of some glycolytic intermediates in the brain revealed significant decreases in fructose-1,6-diphosphate, L-α-glycerol phosphate and lactate in comparison to the levels of these compounds in the saline-injected, control animals. Levels of glucose and glucose-6-phosphate either increased or did not change, whereas levels of glycogen did not differ significantly. Phosphocreatine increased reciprocally with the decrease in inorganic phosphate. The levels of adenine nucleotides (energy charge) were not affected. Utilization of cerebral high-energy phosphates was depressed by 50 - 79% when determined as a function of metabolic rate in the brain at 15- and 30-sec periods of

ischemia according to the "closed-system" technique. Explanations for these data have been examined, such as toxicity of phenylacetate and inhibition of glycolytic enzymes by phenylpyruvate and L-phenylalanine, and their relevance to this study is discussed.

INTRODUCTION

Previous findings in our laboratory have suggested that the state of energy metabolism in the brain is an important consideration in the study of impaired cerebral function induced by galactose (Kozak and Wells, 1969; 1971). In these investigations, a reduction was observed in the adenine nucleotide energy charge and the glycolytic intermediates in the brains of chicks fed high levels of D-galactose. To evaluate whether perturbation of energy control might be identified with other metabolic disturbances associated with common genetic disorders in man, we initiated studies in which young male chicks were given high levels of L-phenylalanine by diet (Granett, 1970) or intraperitoneal injection (Granett and Wells; 1971).

Inhibition of energy metabolism by phenylalanine has been suggested by other investigators. Weber (1969) reported the inhibition of pyruvate kinase (EC 2.7.1.40) and hexokinase (EC 2.7.1.1) in human and rat brains by L-phenylalanine and phenylpyruvate, respectively. Furthermore, lactic acid production was reduced in rat and human cerebral cortical slices by L-phenylalanine and

phenylpyruvate (Weber, Glazer and Ross, 1969). Shah, Peterson and McKean (1970) observed decreased rates of incorporation of [U-^{14}C] glucose into cerebral lipids of rats injected with L-phenylalanine. Barbato and Barbato (1969) found oxygen consumption and CO_2 production to be inhibited in minces of rat brain incubated with concentrations of L-phenylalanine of 15 mM or greater. Also, incorporation of [U-^{14}C] glucose into aspartate, glutamate, GABA, alanine and lipids was reduced.

In this study, we have analyzed the narcosis produced in the chick by the intraperitoneal injections of L-phenylalanine, particularly the effects of the amino acid on high-energy phosphate reserves and glycolytic intermediates. Also, evidence for a reduced cerebral metabolic rate is presented from "closed-system" studies.

MATERIALS AND METHODS

Animals and materials

Day-old White Rock cockerels were obtained from Cobbs, Inc. (Goshen, Ind.) and housed in a brooder at 32°C. The animals were placed on a commercial chick starter mash received through the Michigan State University Poultry Department. L-Phenylalanine was obtained in the free form from Sigma Chemical Co. (St. Louis, Mo.). L-[U-^{14}C]-Phenylalanine, 376 μCi/μmole, was purchased from New England Nuclear Corp. (Boston, Mass.). The amino acid was pure as judged by chromatography on paper developed in a butanol:acetic acid:water (120:30:50, by vol) system. Hexokinase (ATP:D-hexose-6-phosphotransferase; EC 2.7.1.1), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase; EC 1.1.1.49), pyruvate kinase (ATP: pyruvate phosphotransferase; EC 2.7.1.40), adenylate kinase (ATP:AMP phosphotransferase; EC 2.7.4.3), creatine kinase (ATP:creatine phosphotransferase; EC 2.7.3.2), aldolase (fructose-1,6-diphosphate:D-glyceraldehyde-3-phosphate-lyase; EC 4.1.2.b), glycerol-1-phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase; EC 1.1.1.8) and triose phosphate isomerase (D-glyceraldehyde-3-phosphate

ketol-isomerase; EC 5.3.1.1) were obtained from Boehringer Mannheim (New York, N.Y.), and lactic dehydrogenase (L-lactate:NAD oxidoreductase; EC 1.1.1.27) was obtained from Sigma Chemical Co.

Production of hyperphenylalanemia

Experimental chicks were injected intraperitoneally thrice, at hourly intervals, with 3 ml of 0.18 M L-phenylalanine for a total of 1.62 mmoles of the amino acid. A 2-h interval after the third injection ensued before the animals were sacrificed. Groups of control chicks were treated similarly with either a 0.180 M or a 0.154 M NaCl solution; the difference in NaCl concentration did not affect the biochemical parameters under consideration.

Preparation of extract

The chicks were decapitated directly into liquid N_2, and powdered brain tissue was obtained at -20°C. The tissue was treated as described by Kozak and Wells (1969). The labile phosphates were quantified immediately, and the remaining extract was stored at 4°C for a maximum of 2 days. Immediately after decapitation, blood was obtained from carcasses partially frozen at the neck region from the decapitation process. The blood flowed into heparin-coated plastic caps without contamination from gastric contents. Plasma was separated by centrifugation at 3,000 g for 10 min and stored at -20°C.

Analytical methods for glycolytic metabolites

ATP and phosphocreatine were assayed spectrophotometrically according to the following alteration in the procedure of Lamprecht and Trautschold (1963a and b). The assay medium comprises: 0.1 M Tris-HCl (pH 7.5); 5 mM $MgCl_2$; 2.5 mM $NADP^+$; 5 mM glucose; 20 µg of yeast hexokinase/ml; 10 µg of glucose-6-phosphate dehydrogenase/ml. After completion of the ATP reaction, 0.5 µmole of ADP and 40 µg of creatine phosphokinase were added per ml of assay solution for reaction of phosphocreatine. ADP, AMP and glucose-6-phosphate were quantified fluorometrically, and lactate was measured spectrophotometrically (Lowry, Passonneau, Hasselberger and Schulz, 1964). Free glucose was measured spectrophotometrically according to Slein (1963). The method of Bücher and Hohorst (1963) was modified for fluorometry for the quantification of fructose-1, 6-diphosphate. Final reagent concentrations in the reaction cuvette were: 20 mM recrystallized triethanolamine (pH 7.4); 1 mM EDTA; 3 µM NADH; 5 µg of aldolase per ml; 1 µg triose phosphate isomerase per ml; 5 µg glycerol-1-phosphate dehydrogenase per ml. L-α-Glycerol phosphate was quantified according to the method of Lowry et al. (1964) except for the use of 0.2 M glycine-0.4 M hydrazine. HCl (pH 9.5), and 0.4 mM NAD^+. Inorganic phosphate was measured colorimetrically (Schulz, Passonneau and Lowry,

1967). Glycogen was purified and hydrolyzed according to the method of Walaas and Walaas (1950) and the resulting glucose was quantified fluorometrically (Lowry et al., 1964).

Quantification of phenylalanine and tyrosine

Before deproteinization, a known amount of L-[U-^{14}C]-phenylalanine was added to the biological material to permit correction for losses incurred before sample preparation. A perchlorate extract of tissue or plasma (5 ml of acid per g or ml) was prepared and neutralized with 1.2 N KOH. The neutral solution was desalted on a Dowex 50 (H^+) x 8 column (100-200 mesh; 1 x 8 cm) according to Block and Weiss (1956). The 4 N NH_4OH eluant containing the amino acids was reduced in volume; spotted on Whatman 3MM paper, and the chromatogram was developed in butanol: acetic acid:water (120:30:50, by vol) for 17 h. The area corresponding to phenylalanine was eluted with a total of 2 ml of 50% (v/v) aqueous pyridine. A portion was counted in a dioxane base fluid (10 g of 2,5-diphenyloxazole, 100 g of naphthalene and 1 liter of dioxane) in the Beckman CPM 100 liquid scintillation spectrophotometer. The remainder of the eluant was prepared for analysis on a Hewlett-Packard Model 402 gas chromatograph according to Gehrke and Stalling (1967); phenylalanine was quantified on a 2% (w/w) OV-17 column (Applied Science Laboratories, Inc., State College,

Pa.) or a 3% (w/w) OV-225 (Supelco, Inc., Bellefonte, Pa.) as the N-trifluoroacetyl n-butyl ester, with hydroxyproline as the internal standard. Recovery of radioactivity ranged from 40-70%. Tyrosine was measured according to the fluorometric procedure of Ambrose, Sullivan, Ingerson and Brown (1969).

Separation and assay of pyruvate kinase

The enzyme was obtained from the brains of rats 30 days of age. The tissue homogenate and supernatant fraction were prepared essentially according to Schwark, Singhal and Ling (1971), with the use of 0.12 M KCl (pH 7.4) in 1 mM dithiothreitol. Their enzymatic assay was also used with the exception that the KCl concentration was increased 4-fold to 100 mM. Changes in optical density at 340 nm were recorded on a Gilford 2000 recording spectrophotometer at 37°C; enzyme activity was calculated as μmoles of ADP phosphorylated/h per g of tissue.

"Closed-system" studies

Heads from animals sacrificed by decapitation were maintained at 41°C for 15- or 30-sec periods of anoxia before freezing in liquid nitrogen. Metabolic rate expressed in terms of the rate of high-energy phosphate utilization was calculated according to the formula (Lowry et al., 1964); 2 ΔATP + ΔADP + Δphosphocreatine + Δlactate, where Δ refers to the changes in the metabolites during

the 15 or 30 sec of anoxia. The Δlactate was used to represent loss in potential high-energy phosphate rather than 2 Δglucose + 1.45 (Δlactate-2 Δglucose), since in some cases we observed a decrease in glucose and glycogen greater than the increase in lactate. Gatfield, Lowry, Schulz and Passonneau (1966) have attributed this situation to the accumulation of intermediates that have not produced ATP.

Quantification of phenylacetic acid

In these studies, the chicks were decapitated, and the brains were removed within 15 sec, frozen in liquid N_2 and powdered. Tissue samples (300 to 400 mg) were deproteinized with 0.6 M $HClO_4$ (0.5 ml/100 mg) and centrifuged at 12,000 g for 10 min. The supernatant fractions were saved, and the pellets were washed with 1 ml of the perchlorate solution. Subsequently, fractions were combined and neutralized with 2 M $KHCO_3$. After centrifugation at 2,000 g for 5 min, the neutralized extracts were saturated with NaCl and acidified to approximately pH 1 with HCl. The aromatic acids were then extracted thrice with 5 ml of ethyl acetate. The organic phases were combined and taken to dryness at room temperature with a rotary evaporator. Phenylbutyric acid, dissolved in dry pyridine, was added as an internal standard. The acids were converted to trimethylsilyl esters with bis-(trimethylsilyl)-acetamide (Pierce Chemical Co., Rockford, Ill.) and

chromatographed on a 6-ft column of 100/120 mesh Chromosorb W coated with 3% (w/w) OV-1 (Applied Science Laboratories, Inc., State College, Pa.) at 115°C and an optimal carrier gas flow rate. Recovery experiments have demonstrated that the above procedure permits an 85% recovery of phenylacetic acid from chick brain (Granett, 1970).

RESULTS

Unlike the control animals, the chicks injected with L-phenylalanine exhibited a narcosis, evident by the third injection. Preliminary evidence suggested that the depression was reversible within 18 h. The levels of phenylalanine were observed in the plasma and brain for three experiments (Table 1). The highest concentrations were attained in the first experiment (plasma, 14.11 ± 1.15 mM). The plasma-to-brain ratios of phenylalanine for each experiment were rather similar (I, 3.81; II, 3.84; III, 5.65). Plasma levels of tyrosine increased, but not in proportion to those of phenylalanine. The cerebral level of phenylalanine for control animals was similar to that obtained on an amino acid analyzer (0.061 μmoles) (Blosser and Wells, 1971).

Brain high-energy phosphates and glycolytic intermediates

The effects of the increased concentrations of phenylalanine on brain high-energy phosphates were examined (Table 2). ATP and ADP did not change significantly. However, AMP was decreased by 32% in Experiment 1. Not

Table 1.--Plasma and Brain Levels of Phenylalanine and Tyrosine in Chicks Injected Intraperitoneally with L-Phenylalanine or Saline.

Exp.	Plasma				Brain	
	Phenylalanine		Tyrosine		Phenylalanine	
	Control	Experimental	Control	Experimental	Control	Experimental
	µmol/ml		µmol/ml		µmol/g	
I	--	14.77 ± 1.15	0.09 ± 0.01	0.57 ± 0.02	--	3.87 ± 0.61
II	--	10.28, 8.13*	0.11 ± 0.01	0.60 ± 0.01	--	2.40 ± 0.36
III	0.16 ± 0.04	11.91 ± 1.10	---	---	0.08 ± 0.03	2.11 ± 0.27

Note: Except where indicated, the phenylalanine levels represent averages of single determinations on 3 samples; the tyrosine levels are averages of determinations on 4 samples. In experiments I, II and III, the analyses were done on tissue pooled from 15, 10 and 5 chicks, respectively. The one-day-old chicks were injected intraperitoneally thrice at hourly intervals with 3 ml of 0.18 M L-phenylalanine (total dose 1.62 mmoles) or with saline vehicle and decapitated into liquid N_2 2 h after the last injection. See text for details of sample preparations and analyses.

*Individual values for 2 determinations.

Table 2.--Effect of Intraperitoneally-Injected L-Phenylalanine on Energy-Related Phosphates in Chick Brain.

Substrate	Exp.	Control	L-Phenylalanine Injected	% of Control
		μmol/g		
ATP	I	1.76 ± 0.15	1.81 ± 0.06	103
	II	1.73 ± 0.05	1.83 ± 0.12	106
ADP	I	0.79 ± 0.10	0.66 ± 0.03	84
	II	0.86 ± 0.09	0.75 ± 0.06	87
AMP	I	0.16 ± 0.04	0.11 ± 0.03	68†
	II	0.18 ± 0.02	0.16 ± 0.01	90
Total adenine nucleotides	I	2.71 ± 0.15	2.58 ± 0.06	95
	II	2.77 ± 0.11	2.74 ± 0.13	98
Energy charge[δ]	I	0.80	0.83	104
	II	0.78	0.81	103
Phosphocreatine	I	1.79 ± 0.06	2.20 ± 0.06	123*
	II	1.96 ± 0.15	2.25 ± 0.18	115**
Inorganic phosphate	I	3.37	2.74	81‡
	II	3.61 ± 0.27	3.25 ± 0.10	90††
Sum of phosphates	I	12.18	11.81	97
	II	12.65	12.64	100

Note: Each value, except where indicated, represents the average ± SD of 1 or 2 determinations on 4 or 5 samples from pools described for Table 1. See text section on METHODS for details of sampling and analyses.

*P<0.001. **P<0.01. ††P<0.05. †P<0.1.

‡Average of 2 analyses.

δEnergy charge is defined as $\frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$, as taken from Atkinson (1968).

shown here was a third experiment in which a 26% decrease in AMP was observed for the L-phenylalanine-injected group. However, the fluctuations in AMP were insufficient to significantly affect the total adenine nucleotide level. The energy charge (Atkinson, 1968) likewise did not differ between the control and experimental group. However, an increase in phosphocreatine and a reciprocal decrease in inorganic phosphate was consistently associated with the L-phenylalanine-injected animal. These differences reflected increases in phosphate esters. The total phosphate represented by these components was virtually identical between experimental groups (e.g., in Experiment I, 12.18 and 11.81 μmoles/g of tissue for control and phenylalanine-treated groups, respectively). In both experiments, fructose-1,6-diphosphate, L-α-glycerol phosphate and lactate were decreased significantly (Table 3). Glucose and glucose-6-phosphate increased in the L-phenylalanine-injected chicks only in Experiment I. Changes in the levels of glycogen were not considered significant.

"Closed-system" anoxia

To characterize further the narcotic state induced in the chicks by phenylalanine, we analyzed the status of the cerebral metabolic activity with the "closed-system" technique described by Lowry et al. (1964) in the study of barbiturate-induced anesthesia. ATP, ADP, AMP,

Table 3.--Effect of L-Phenylalanine on Intermediates of Carbohydrate Metabolism in Chick Brain.

Substrate	Experiment I		Experiment II	
	NaCl Injected	L-Phenylalanine Injected	NaCl Injected	L-Phenylalanine Injected
	µmol/g		µmol/g	
Glucose	1.170 ± 0.140	2.070 ± 0.210*	1.970 ± 0.160	1.930 ± 0.230
Glucose-6-phosphate	0.036 ± 0.000	0.044 ± 0.003**	0.029 ± 0.003	0.030 ± 0.006
Fructose-1,6-diphosphate	0.153 ± 0.023	0.129 ± 0.015†	0.143 ± 0.008	0.106 ± 0.008*
L-α-Glycerol phosphate	0.127 ± 0.012	0.095 ± 0.004**	0.148 ± 0.010	0.087 ± 0.010*
Lactate	2.790 ± 0.110	2.110 ± 0.250**	2.190 ± 0.290	1.780 ± 0.100†
Glycogen	1.420 ± 0.260	1.710 ± 0.150	1.670 ± 0.320	1.790 ± 0.070

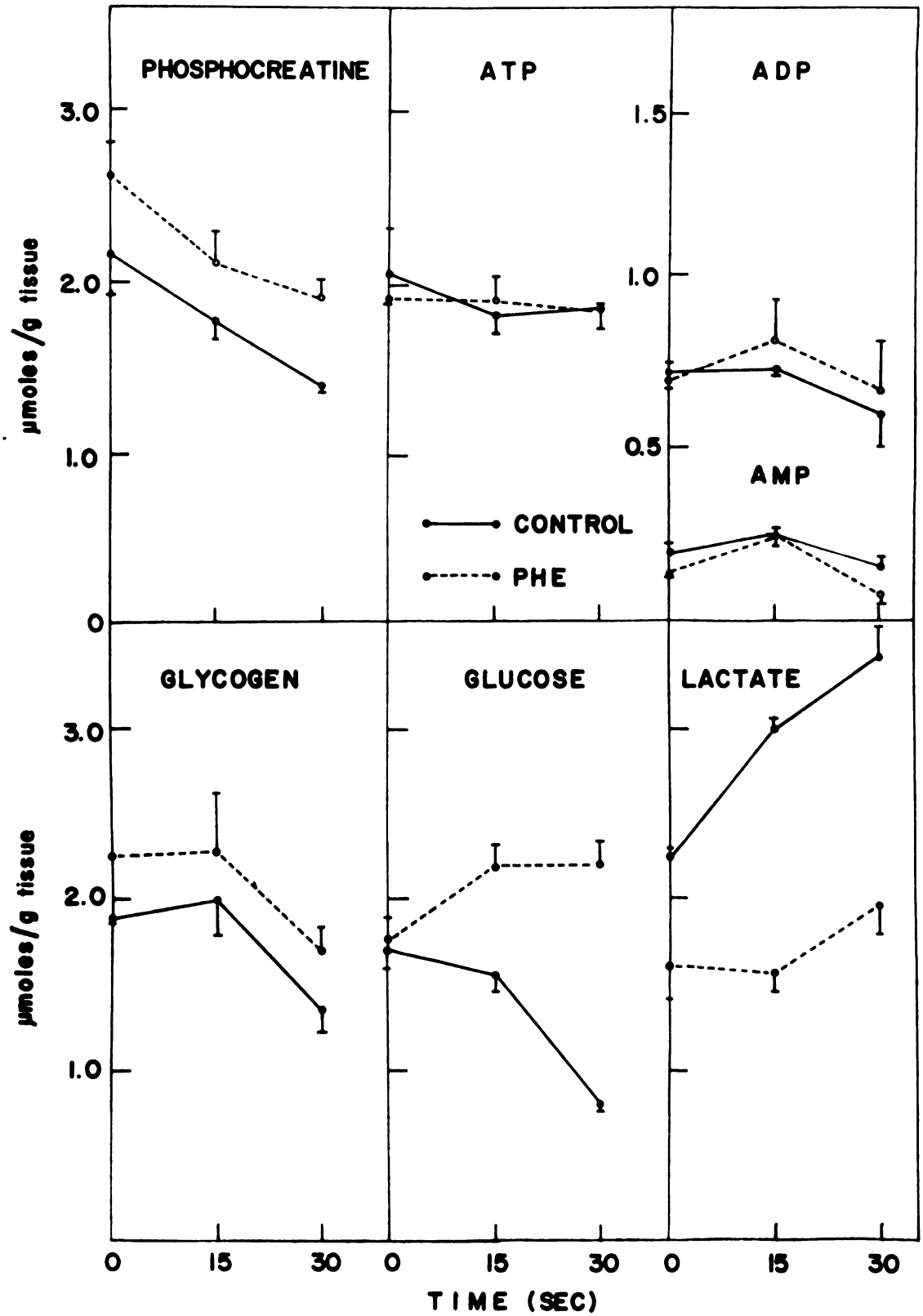
Note: Each value represents the average of 1 or 2 determinations on 4 or more tissue samples from pools, as described for Table 1. See text section on METHODS for details of sampling and analyses.

*$P<0.001$. **$P<0.01$. †$P<0.05$.

phosphocreatine, lactate, glucose and glycogen were determined in the brain after 15 and 30 sec of anoxia (Figure 2). In both the control and experimental situations, phosphocreatine appeared to be quickly hydrolyzed, while virtually no net utilization of ATP occurred and neither ADP nor AMP accumulated. A significant dissimilarity occurred in the cases of lactate and glucose. In the control group, lactate increased considerably within the first 15 sec; however, the increase was delayed in the L-phenylalanine-injected group. Similarly, the control levels of glucose decreased significantly after 15 sec of anoxia, whereas essentially no utilization of glucose was demonstrated in the L-phenylalanine-treated group, even at 30 sec. Examination of the stoichiometry for the control group during the first 15 sec of anoxia revealed that the increase in lactate could not be accounted for by changes in glucose and glycogen. Jongkind and Bruntink (1970) noted a similar imbalance under closed-system conditions in rat brain. They suggested that substrates other than glucose were utilized for lactate production; however, they did not measure depletion of glycogen. In our system, it is unlikely that the lactate carbons originate from alanine since Yoshino and Elliott (1970) have shown that the levels of alanine in rat brain are not altered by a 1-min period of anoxia. Conversely, the 30-sec anoxia period in our control group produced a decrease in glucose and glycogen

Figure 2.--Fluxes of metabolites during ischemia in the brains of chicks previously injected with L-phenylalanine or saline.

The animals were injected as described in METHODS. PHE represents the phenylalanine group. Each value is the average ± SD for 1 or 2 determinations each on 3 samples of brain tissue from a pool of 5 chicks, excepting the zero-time PHE glycogen value which is the average of 2 analyses. After decapitation, the heads were maintained at 41°C for 15- or 30-sec periods of anoxia before being frozen in liquid N_2, essentially according to the "closed-system" technique of Lowry *et al.* (1964).


PHOSPHOCREATINE
ATP
ADP
AMP
CONTROL
PHE
GLYCOGEN
GLUCOSE
LACTATE
μmoles/g tissue
μmoles/g tissue
3.0
2.0
1.0
0
1.5
1.0
0.5
0
15
30
TIME (SEC)

that was greater than the increase in lactate. Glycogen depletion was evident in both groups after 30 sec of anoxia. In the experimental group, this might have been the source of the lactic acid accumulation since glucose was still unchanged. The rate of utilization of high-energy phosphates (Table 4) was consistently lower in the phenylalanine-treated chick in comparison to that in the control animals at both periods.

Studies of the inhibition of pyruvate kinase by phenylalanine

Weber (1969) suggested a decline in the production of ATP could occur when the levels of phenylalanine *in vivo* approach that of the K_i (6 - 10 mM) for the inhibition of pyruvate kinase by phenylalanine. In our present work with the chick, 2 to 4 mM phenylalanine accumulated in the brain, yet there was no shortage of high-energy metabolites. Indeed, on the contrary, decreased utilization of energy characterized the status of cerebral metabolism. This situation suggested that the pyruvate kinase from chick brain was less sensitive to phenylalanine or that the inhibition was insufficient to affect levels of ATP. Our investigation of pyruvate kinase from chick whole brain has revealed that the enzyme exhibited no inhibition by phenylalanine, even when the concentration of phenylalanine was 400-fold higher than that of phosphoenolpyruvate. As a control, pyruvate kinase from a young rat brain was assayed under identical

Table 4.--High-Energy Phosphate Utilization in Brains of Chicks Given L-Phenylalanine.

Time Interval After Decapitation	Control	L-Phenylalanine Injected
sec	μmol/g/min	
0 - 15	6.52	1.36
0 - 30	4.96	2.49

Note: The utilization was calculated from the average values represented in Figure 2, according to the formula (Lowry et al., 1964):

2 ΔATP + ΔADP + Δphosphocreatine + Δlactate,

as described in the text section on METHODS. The one-day-old chicks were injected intraperitoneally thrice, at hourly intervals, with 3 ml of 0.18 M L-phenylalanine (total dose 1.62 mmoles) or with saline vehicle and decapitated 2 h after the last injection. The heads were maintained at 41°C for 15- or 30-sec periods of anoxia before being frozen in liquid N_2. See text for further details.

conditions, and the kinetic parameters were determined. The V_{max} was calculated to be 6.7×10^{3} µmoles of ADP phosphorylated/h per g of tissue; the K_m was calculated as 3.7×10^{-5} M, and the K_i for phenylalanine was 1×10^{-3} M. These values agree with those reported by Schwark et al. (1971) and Weber et al. (1969).

Levels of phenylacetic acid

The extent to which the metabolite, phenylacetic acid, accumulated in the brains of the chicks treated with L-phenylalanine was determined (Table 5). Considerable variation in the levels of the acid was observed (0.006-0.065 µmol/g), whereas levels of phenylalanine were relatively constant (3.04 ± 0.39 µmol/g).

Table 5.--Levels of Phenylacetic Acid in the Brains of L-Phenylalanine-Treated Chicks.

	Phenylalanine	Phenylacetic Acid
		µmol/g
Control	0.09 ± 0.02*	None detected
Phenylalanine-treated	3.04 ± 0.39**	0.010, 0.065, 0.0006†

Note: The one-day-old chicks were injected intraperitoneally thrice, at hourly intervals, with 3 ml of 0.18 M L-phenylalanine or with saline vehicle and decapitated 2 h after the last injection. The brains were immediately removed and frozen in liquid N_2. See text for details of the analyses.

*An average of single determinations from 3 pools of 3 brains each ± SD.

**An average of duplicate determinations from 3 pools of 3 brains each.

†Individual averages for duplicate determinations from each of the 3 pools of brains.

DISCUSSION

The induction of narcosis in the chick by phenylalanine has not, to our knowledge, been previously reported. Tamimie and Psheidt (1966) fed diets 5 and 8% (w/w) in L-phenylalanine to day-old chicks for a 4-week period. They observed reduced weight gain, poor feather development and abnormal development of leg joints with consequent poor motor coordination in the animals. No mortality occurred, and the effects were reversible. In similar experiments with a diet 10% (w/w) in L-phenylalanine for 2 weeks, we noted the failure to gain weight (Granett, 1970), and observed at termination of these experiments, a rise in brain phenylalanine levels to 2 μmol/g, with plasma levels rising to 4 mM, or a plasma-to-brain ratio lower than in the acute injection experiments described here. The levels of phenylalanine attained in the brain (e.g., 2 μmol/g of brain; Granett, 1970) were comparable to those attained in 2 of our 3 injection experiments; yet narcosis was not observed, a finding that suggested that a gradual adjustment to the increased levels of the amino acid had occurred. In the injection studies, tyrosine accumulated

in the blood to a considerably lesser extent than that observed in the dietary experiments (plasma tyrosine level of 2 mM; Granett, 1970). Thus, the association of the narcosis with the acute studies rather than the chronic does not seem to be related to the absolute level of phenylalanine itself, but perhaps to the ratio of phenylalanine to tyrosine or the rapidity of the rise in the levels of phenylalanine or a metabolite thereof.

The toxicity of phenylacetic acid, a metabolite of phenylalanine, has already been demonstrated in the hen, monkey, dog, mouse and human (Sherwin and Kennard, 1919; Berthelot and Dieryck, 1939). In the dog, large doses of phenylacetate are accompanied by drowsiness, inability to stand and coma. We have observed similar reactions with cockerels. When fed phenylacetate, the chicks die after the fourth day, and chicks injected intraperitoneally become sedated within 30 min. Cerebral levels of phenylacetate are at least 2 μmol/g in these animals. However, it is doubtful that this compound could be the toxic agent in our phenylalanine-injection experiments since phenylacetate did not accumulate to levels greater than 0.07 μmol/g.

In studies on phenobarbital-induced anesthesia in mice, Lowry et al. (1964) observed an increase in phosphocreatine and decreases in inorganic phosphate, fructose-1, 6-diphosphate, L-α-glycerol phosphate and lactate in the

brain. Similar changes occurred in the brains of our chicks after injection of phenylalanine. The phenobarbital-induced anesthesia also resulted in increases in glucose, glucose-6-phosphate and glycogen. Elevated levels of brain glucose have been confirmed by Mayman, Gatfield and Breckenridge (1964) and Gatfield et al. (1966). We observed significant increases in glucose and glucose-6-phosphate and slightly elevated glycogen only in experiment I (Table 3) in which the brain phenylalanine level was highest. Lowry et al. (1964) found that the barbiturates affected neither the level of the adenine nucleotides nor the energy charge (0.866 for control versus 0.860 for the phenobarbital-treated group), observations with which our findings agree. Recently, Nilsson and Siesjö (1970) reported no effect of various volatile anesthetics and barbiturates on levels of phosphocreatine in rat brain. However, they did substantiate the decrease in lactate associated with barbiturate anesthesia.

It is generally accepted that anesthesia lowers the metabolic rate in the brain (Himwich, Homburger, Maresca and Himwich, 1947; Wechsler, Dripps and Kety, 1951). Lowry et al. (1964) and Gatfield et al. (1966) demonstrated the utility of the "closed-system" technique for approximating the reduction in cerebral aerobic energy flux induced by phenobarbital. Likewise, we found that the acute phenylalanine intoxication is associated with a

reduction; utilization of actual and potential high-energy phosphates was lowered 50 - 79% at the two time periods examined. The reduced metabolic activity in the brain of the phenylalanine-injected chick is compatible with the increase in phosphocreatine and the decreases in fructose-1,6-diphosphate and inorganic phosphate, which exert glycolytic control of the enzyme phosphofructokinase. Uyeda and Racker (1965a) demonstrated inhibition of rabbit muscle phosphofructokinase at 1 - 5 mM concentrations of phosphocreatine, and Krzanowski and Matschinsky (1969) showed that the enzyme from sheep brain is more sensitive to the inhibition than the muscle enzyme. The stimulation of phosphofructokinase by fructose diphosphate and inorganic phosphate is well known (Uyeda and Racker, 1965a and b; Passonneau and Lowry, 1962).

In contrast to the pyruvate kinase isolated from rat brain (Weber, 1969 and Schwark et al., 1971), prostate, seminal vesicles, uterus and skeletal muscle (Vihayvargiya, Schwark and Singhal, 1969), our finding that chick brain pyruvate kinase is not inhibited by L-phenylalanine appears to be a species difference. The inhibition of hexokinase by phenylpyruvate (K_i = 2 - 13 mM in postpartum rat brain) also reported by Weber (1969) was not investigated in the chick. The concentration of phenylpyruvate in the brains of the phenylalanine-injected chicks was not determined; however, it did not accumulate

to levels greater than 0.07 - 0.15 mM in plasma of chicks in dietary studies (Granett, 1970).

The mechanism of induction of narcosis by phenylalanine in the chick is not understood. As a possible explanation, the recent observation that L-phenylalanine can influence the activity of $(Na^+ + K^+)$-ATPase from chick microsomes *in vitro* (Ting-Beall and Wells, 1971) is being further investigated.

ACKNOWLEDGMENTS

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CHAPTER II

FURTHER STUDIES ON CEREBRAL METABOLISM IN L-PHENYLALANINE AFFECTED CHICKS

INTRODUCTION

This chapter represents a continuation of the previous work, more fully characterizing alterations in cerebral metabolism by L-phenylalanine. A 24-h recovery period following the serial injections of the amino acid is examined for the purpose of relating the duration of the changes in cerebral glycolytic metabolites or high energy phosphates with that of the increased levels of phenylalanine or tyrosine. Changes in cerebral levels of amino acids are described. Seizure threshold is shown to be elevated. Direct inhibition of glycolysis by phenylalanine is investigated as a mechanism for the reduced metabolic rate. Also, the effect of the amino acid on creatine kinase is examined. Several other amino acids are tested for narcotic effects similar to phenylalanine's. In light of the extremely large volume of water and

quantity of sodium chloride or phenylalanine administered, changes in plasma osmolality and cationic composition are determined.

MATERIALS AND METHODS

Animals and materials

Day-old Leghorn cockerels from Rainbow Trail Hatcheries (St. Louis, Mich.) were housed in a brooder at 32°C and fed a commercial chick starter mash received through the Michigan State University Poultry Department. L-Phenylalanine (free form) and picrotoxin were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-α-Glycerol phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase; EC 1.1.1.8) and creatine kinase (ATP:creatine phosphotransferase; EC 2.7.3.2) were obtained from Boehringer Mannheim (New York, N.Y.); glucose oxidase, from Worthington (Freehold, N.J.). Pentylenetetrazol, a product of the Knoll Pharmaceutical Company (Orange, N.J.) was a gift from Dr. Steve Baskins, Pharmacology Department, Michigan State University. D-(6-^{14}C)-Glucose (specific radioactivity, 2.9 mCi/mmol) was purchased from Nuclear Chicago (Chicago, Ill.) and D(U-^{14}C)-glucose (specific radioactivity, 60 mCi/mmol) from Calatomic (Los Angeles, Cal.). All adenine and pyridine nucleotides used in the assays were obtained from either Sigma or Boehringer-Mannheim.

Recovery Studies

Chicks, 1-day old, were injected intraperitoneally three times at hourly intervals with 3 ml of neutralized 0.18 M L-phenylalanine or 0.154 M saline. After various periods of time had elapsed (2, 5, 8, 12, 18 or 24 h) following the last injection, the animals were sacrificed. They were deprived of food and water during these times to avoid possible drowning of the sedated chicks, except for the last 60 min before the termination of the 24-h period, at which time the animals' hunger responses were checked. Those animals used for quantification of cerebral high-energy phosphates or glycolytic intermediates were decapitated directly into liquid N_2, and the brain tissue was later chipped out and powdered over dry ice in the cold room. Three or four individual brains were analyzed at each point. Brains for analysis of phenylalanine and tyrosine levels were removed from the skull and then frozen in liquid nitrogen and pooled, three brains per point. Blood was obtained from these animals by drainage at the neck and was likewise pooled. Serum was separated from the red cells by centrifugation at 3000 *g* for 10 min. All tissue samples were stored at -90°C. They were extracted with perchloric acid; ATP, phosphocreatine, L-α-glycerol phosphate and cerebral glucose were quantified as described in METHODS, Chapter I. Free glucose in the serum was measured on zinc sulfate-barium hydroxide

filtrates using glucose oxidase as described for Worthington's semi-micro method; 0.5 ml of reagent was reacted with 0.5 ml of extract.

Amino acid analyses

The amino acids were quantified using a Beckman 121 automatic analyzer. Separation of the acidic and neutral amino acids was accomplished at 55°C on resin AA-15 with two 0.2 N sodium citrate buffers (normality with respect to sodium) pH 3.25 and 4.25, respectively; separation of the basic amino acids was accomplished on resin AA-35 with 0.35 N sodium citrate, pH 5.26. Chicks injected with saline or L-phenylalanine according to the usual protocol were decapitated 2-h after the last injection, brains removed, frozen in liquid nitrogen and powdered over dry ice. Weighed tissue was homogenized in 1% picric acid (15 ml/g) using a motor-driven Potter-Elvehjem homogenizer (Stein and Moore, 1954). After centrifugation at 12,000 g for 10 min, the picrate was removed from the supernatant by batch-wise mixing with Dowex 2-X8 resin (chloride form), 4 g of resin per g of original tissue. The resin was filtered and washed with four 2-ml volumes of 0.02 N HCl per g of brain. To remove glutathione which chromatographs in the region between aspartic acid and proline, the filtrate was neutralized to pH 7.2-7.5 with 1 N NaOH and freshly prepared 0.5 M sodium sulfite was added (80 µl/g

of tissue). The mix was allowed to stand open to the air at room temperature for 4 h, at which time the pH was readjusted to 2.0-2.2 with 1 N HCl. Subsequently, the sample was taken to dryness, redissolved in a known volume of water and aliquots were removed. Typically 1-1.5 g of tissue were extracted; the final residue was then dissolved in 10 ml of water; two 4-ml aliquots (equivalent to 0.4-0.6 g of brain) were removed, taken to dryness and redissolved in 0.6 ml of 0.2 N sodium citrate, pH 2, 0.125 mM with respect to the internal standard, norleucine or α-amino-β-guanidinopropionic acid for the acid-neutral or basic column, respectively. This sized aliquot would allow for quantification of amino acids less than 1 μmol/g of brain. For those greater than this, a 0.2-ml aliquot (20-30 mg of tissue) was removed from the original 10-ml volume, taken to dryness on a rotoevaporator and dissolved in 1 ml of citrate-norleucine buffer. Asparagine and N-acetyl aspartic acid and glutamine were quantitated by difference in the aspartate and glutamate levels before and after acid hydrolysis. Also, acid hydrolysis unmasked the serine-threonine peaks beneath glutamine. As an example, a 0.5-ml aliquot (50-75 mg of tissue) from the 10-ml volume was diluted to 2 N HCl with 4 N acid, heated at 100°C for 2 h, taken to dryness, redissolved in water, and again dried to remove HCl. The residue was finally dissolved in 0.8-1.0 ml of citrate-norleucine buffer. For

all analyses, a 0.5-ml aliquot of the final solution was injected on top of the column. Standard amino acid solutions were chromatographed intermittently throughout the series of samples. Calculations of unknown quantities were based on the area ratios of the compound in question to the internal standard; area was determined by multiplication of peak height by width at half height. When only tyrosine and phenylalanine were quantified, the oxidation of glutathione was omitted as its presence did not interfere with either of these amino acids.

Quantification of ammonia

Levels of ammonia were determined chemically by a diffusion method described by Weil-Malherbe (1969). A small glass rod coated with 0.1 N sulfuric acid was extended through a rubber seal into a 25-ml flask containing 2 ml of saturated potassium carbonate and 0.5 ml of saturated potassium bicarbonate. A 0.4 ml aliquot of a 0.6 M perchlorate extract of brain (0.5 ml/100 mg) was injected into the basic solution, and the flasks were shaken for 1 h at room temperature. The rods were then immersed into 3 ml of phenol (1%)-sodium nitroferricyanide (5 mg%) reagent and to this were added 3 ml of an alkaline hypochlorite solution (0.34 ml of 4.6% sodium hypochlorite in 100 ml of 0.5% NaOH). Color was allowed to develop at room temperature for 40 min; absorbancy was read at 635 nm.

Calculations were based on a standard ammonium chloride solution assayed in the same manner as the samples.

Seizure threshold studies

The day-old chicks, weighing 40-50 g, were injected 2 or 3 times at hourly intervals with 3 ml of 0.18 M neutralized L-phenylalanine or physiological saline. Approximately 2 h after the last injection when narcotic effects were visible in the phenylalanine-treated group, the animals were injected intraperitoneally with 1 ml or less of picrotoxin or pentylenetetrazol solutions. The picrotoxin was dissolved either in water (250 mg/100 ml) or 95% ethanol (250 mg/10 ml); the pentylenetetrazol was used full strength (100 mg/ml) or diluted 1 to 10 with water. The interval between injection of the convulsant agent and the first tonic extension was measured.

Study of the effect of L-phenylalanine on anaerobic glycolysis

Day-old chicks were decapitated, brains removed and homogenized in 1.15% neutralized KCl (9 ml/g tissue). After an initial 10-min centrifugation at 12,000 g, the fraction containing the soluble glycolytic enzymes was prepared by centrifugation at 100,000 g for 30 min in a No. 40 rotor using a Beckman L2 ultracentrifuge. The glycolytic mixture (Weber, Glazer and Ross, 1970) contained the following components given as final concentration: ATP, 1 mM; $MgSO_4$, 2 mM; KH_2PO_4, 5 mM; NAD^+, 1 mM; nicotinamide, 30 mM;

glycylglycine, pH 7.4, 50 mM; glucose, 1 mM; a minimum of 20,000 cpm of D-(^{14}C)-glucose per ml; 0.2 ml of high-speed supernatant per ml. Neutralized L-phenylalanine was added at the expense of water to a final concentration of 9 or 18 mM. The samples were incubated for periods of 0 to 60 min at 37°C in a Dubnoff shaker bath, after which the reaction was stopped by the addition of 10% trichloroacetic acid (1 ml per ml of incubate). Radioactive lactate was recovered as the acetaldehyde-dimedone derivative according to the method of Long, Mashimo and Gump (1971). Into a wide-mouthed jar were placed 15 ml of a 0.4% aqueous dimedone solution, pH 6. An aliquot of the deproteinized sample (0.5 or 1.0 ml) was combined with 1 ml of a lactate solution (10 mg/ml) in a 15-ml beaker; to this was added 1 ml of 20% cerric sulfate in 2 N H_2SO_4. The beaker attached to a rubber stopper by tape was immediately suspended in the jar; the jar was stoppered and shaken for 3 h at room temperature, during which time the lactate was chemically converted to acetaldehyde which, upon diffusion, reacted quantitatively with the dimedone. The product was precipitated upon acidification to pH 4 with 2 N acetic acid and recovered by filtration on preweighed Whatman No. 540 filter papers (2.4 cm). The air-dried precipitate was then weighed and counted on a Beckman CPM-100 scintillation spectrometer in 10 ml of scintillation fluid (10 g

of 2,5-diphenyloxazole, 100 g of naphthalene and 1 leter of dioxane). No quenching was observed.

Assay of creatine kinase in the presence of L-phenylalanine

Commercial creatine kinase from muscle was assayed at 25°C by a Gilford recording spectrophotometer with and without L-phenylalanine according to the procedure of Dawson (1970). The assay medium consisted of the following expressed as final concentrations: Tris-HCl, pH 7, 65 mM; glucose, 3 mM; $NADP^+$, 0.125 mM; ADP, 2 mM; $MgCl_2$, 6.25 mM; 3.33 µg each of hexokinase and glucose-6-phosphate dehydrogenase per ml; 10 µg of creatine kinase per ml. L-Phenylalanine was added to yield either 3.6 or 9 mM. A reaction attributed to ATP contamination of the ADP was encountered and allowed to go to completion before addition of creatine kinase and phosphocreatine. The levels of phosphocreatine assayed ranged from 20 to 80 nmol/ml.

Plasma osmolality determinations

Hyperphenylalanemia was produced in the chicks by intraperitoneal injection of L-phenylalanine as previously described. Saline-injected animals served as controls. Osmolality was measured on plasma obtained by heart puncture, employing a Fiske G-66 osmometer.

Plasma electrolyte determinations

The levels of potassium, sodium and calcium were measured on a Coleman flame photometer for the same samples used in the osmolality studies. As an additional control, the plasma from uninjected chicks fed a mash diet was included. A 1/100 dilution of the plasma in 0.02% Sterox was made for the potassium determination; 1/200 for sodium; 1/25 for calcium.

RESULTS

Observations on the narcotic state induced in the chick by L-phenylalanine

Photographs were taken of the animals 2 h after the third injection of the amino acid. The drowsy state is evident in Figure 3; the more alert, wide-eyed chick is representative of the saline-control group. Some of the phenylalanine-treated animals were more severely incapacitated (Figure 4). A loss of motor activity could easily be demonstrated by challenging the animal's normal righting reflexes. If a control chick were placed on its back, it would immediately maneuver to its feet again; however, the chick injected with L-phenylalanine had great difficulty in accomplishing this and would remain on its back for 5 sec or more (Figure 5). The legs of the chicks in this position could easily be flexed.

Recovery studies

When chicks were injected intraperitoneally with a total of 1.62 mmole of L-phenylalanine, levels of the amino acid greater than 2 μmol/g of tissue were maintained in the brain for a period of 12 h after treatment; the increase in tyrosine followed that of phenylalanine,

Figure 3.--Photograph illustrating the drowsy state induced in chicks by the intraperitoneal injection of L-phenylalanine.

The chicks were injected intraperitoneally 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline and observed 2 h after the final injection. The more alert animal is representative of controls.

Figure 4.--Photograph of a chick prostrated by serial injections of L-phenylalanine.

The animals were treated as described for Figure 2. The animal standing is representative of the control group.

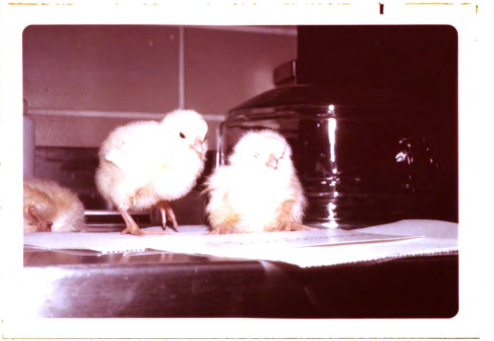


Figure 3.

Figure 4.

Figure 5.--Photograph demonstrating loss of motor activity in chicks injected with L-phenylalanine.

The injection procedure was identical to that described for the animals of Figure 2; 2 h following the last injection the chicks were placed on their backs and their righting reflexes were observed. The animals were capable of standing.

Figure 5.

peaking at 12 h (Figure 6). Both amino acids appeared to be rather rapidly metabolized and levels returned to normal by 18 h. Maximally, phenylalanine increased about 60 times the control value, whereas tyrosine was elevated 5 fold.

Levels of ATP in the brain were not observed to change in the phenylalanine-injected group throughout the 24 h period; however, phosphocreatine increased by 15 - 20% after 2 h and the rise was maintained for about 12 h (5 h, $p<0.02$; 8 h, $p<0.1$) (Figure 7). L-α-Glycerol phosphate (Figure 8) was affected earliest; levels remained depressed by 30 - 45% for 12 h compared to the saline-control values (2 h, $p<0.02$; 5 h, $p<0.02$; 8 h, $p<0.10$). The levels of fructose-1,6-diphosphate fell 30 - 40% after 2 h had elapsed (5 h, $p<0.05$; 8 h, $p<0.10$) and appeared to return to near normal levels after 8 h (Figure 8). Cerebral levels of glucose in the control chicks decreased with time, rising slightly between 18 and 24 h (Figure 9); the rise correlated well with an increase in glucose in the plasma and is probably related to the intake of mash 60 min before the sacrifice. This was not observed in the phenylalanine group, but these animals did not eat when given the food. A very slight hypoglycemia (15%) was detected in the phenylalanine group at the early times studied (Figure 9). It appears that the phenylalanine-treated chicks maintained higher cerebral levels of glucose than the controls.

Figure 6.--Cerebral levels of phenylalanine and tyrosine in chicks during a 24 h period following intraperitoneal injections of L-phenylalanine.

Day-old chicks were injected intraperitoneally 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline. The tissue was obtained as described in METHODS. Each value represents one determination on tissue pooled from 3 animals. Analyses were done on an amino acid analyzer, utilizing 400 mg of brain.

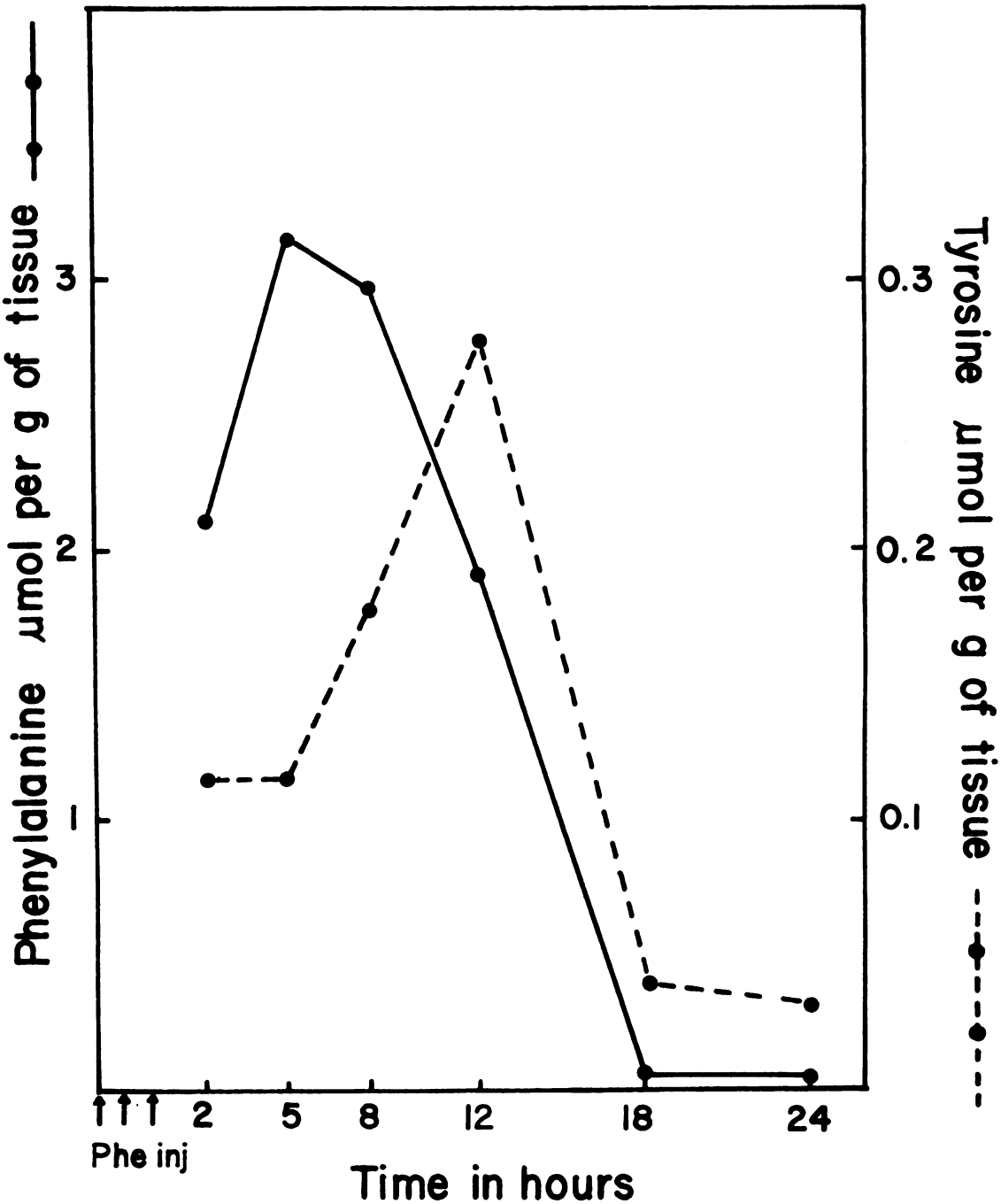

Phenylalanine µmol per g of tissue
Tyrosine µmol per g of tissue
3
2
1
0.3
0.2
0.1
2
5
8
12
18
24
Phe inj
Time in hours

Figure 7.--Cerebral levels of ATP and phosphocreatine in the chick during a 24 h period following injections with L-phenylalanine.

Day-old chicks were injected intraperitoneally 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline. Values represent the average of single determinations ± standard deviation on 4 animals with the exception of the control values at 12 and 24 h for which n is equal to 3. Tissue was obtained and assays performed as described in METHODS.

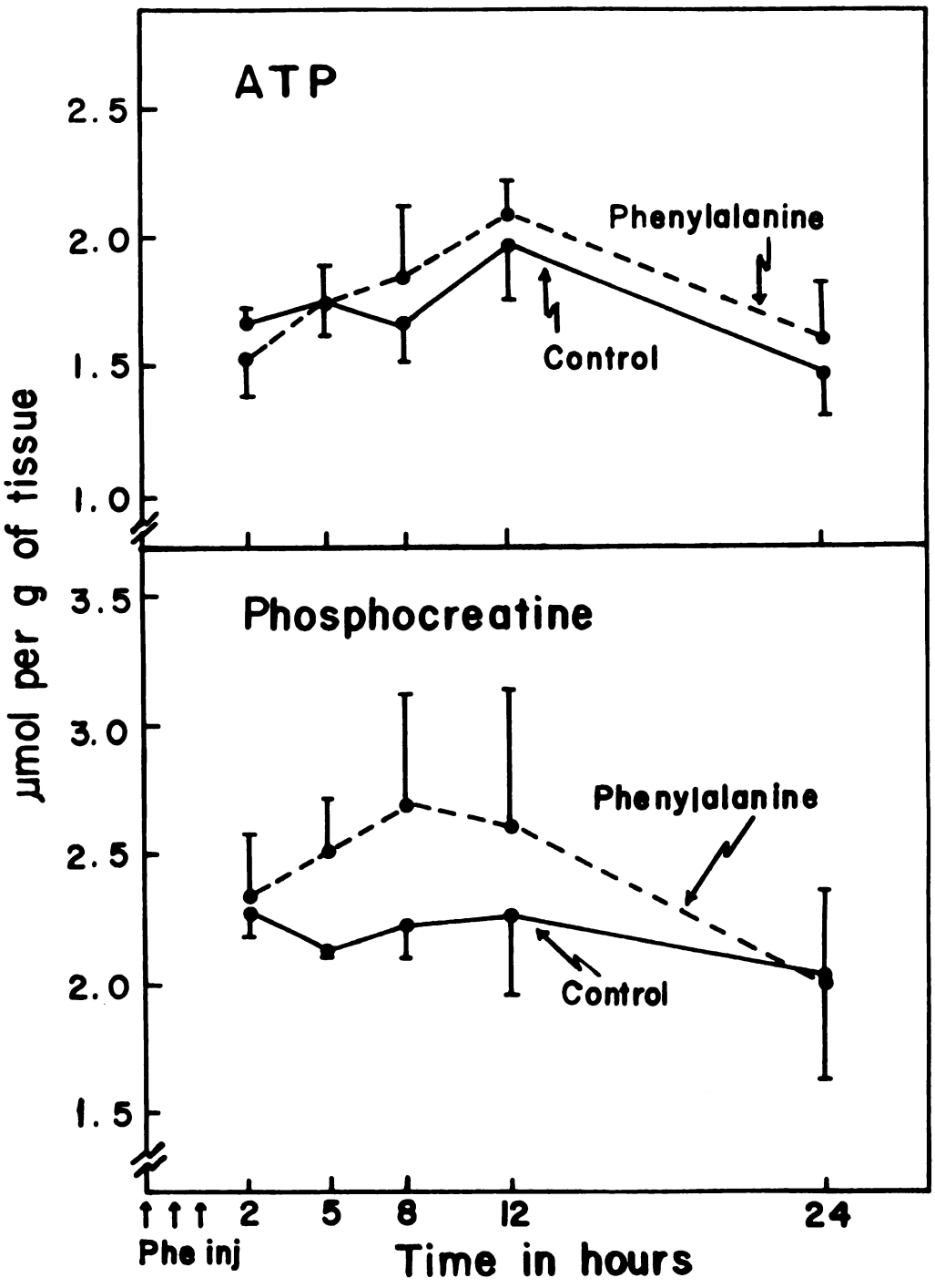

ATP
2.5
2.0
1.5
1.0
Phenylalanine
Control
Phosphocreatine
3.5
3.0
2.5
2.0
1.5
Phenylalanine
Control
µmol per g of tissue
Phe inj
2
5
8
12
24
Time in hours

Figure 8.--Cerebral levels of L-α-glycerol phosphate and fructose-1,6-diphosphate during a 24 h period following L-phenylalanine injections in the chick.

Refer to the description of Figure 4 for experimental details.

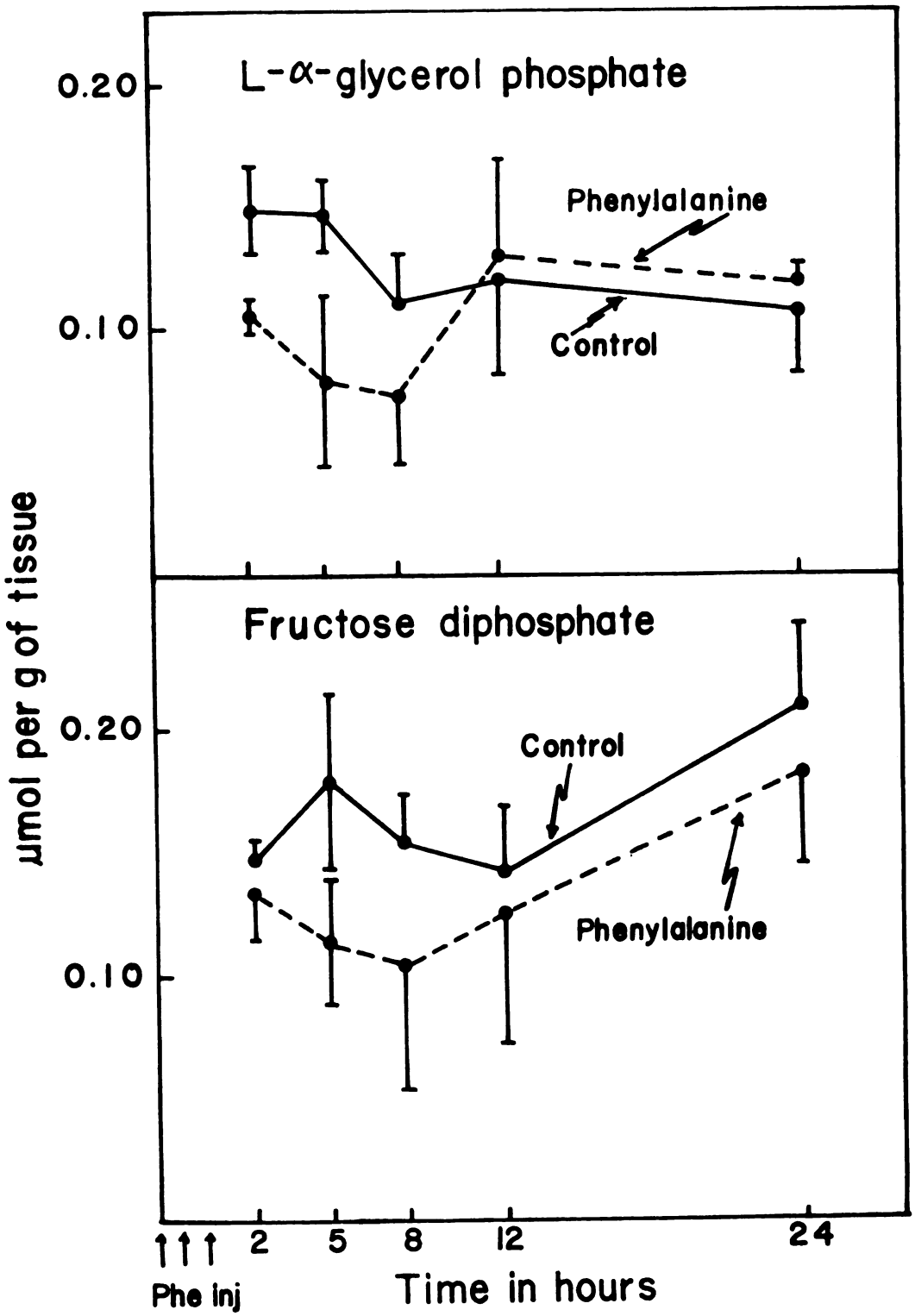
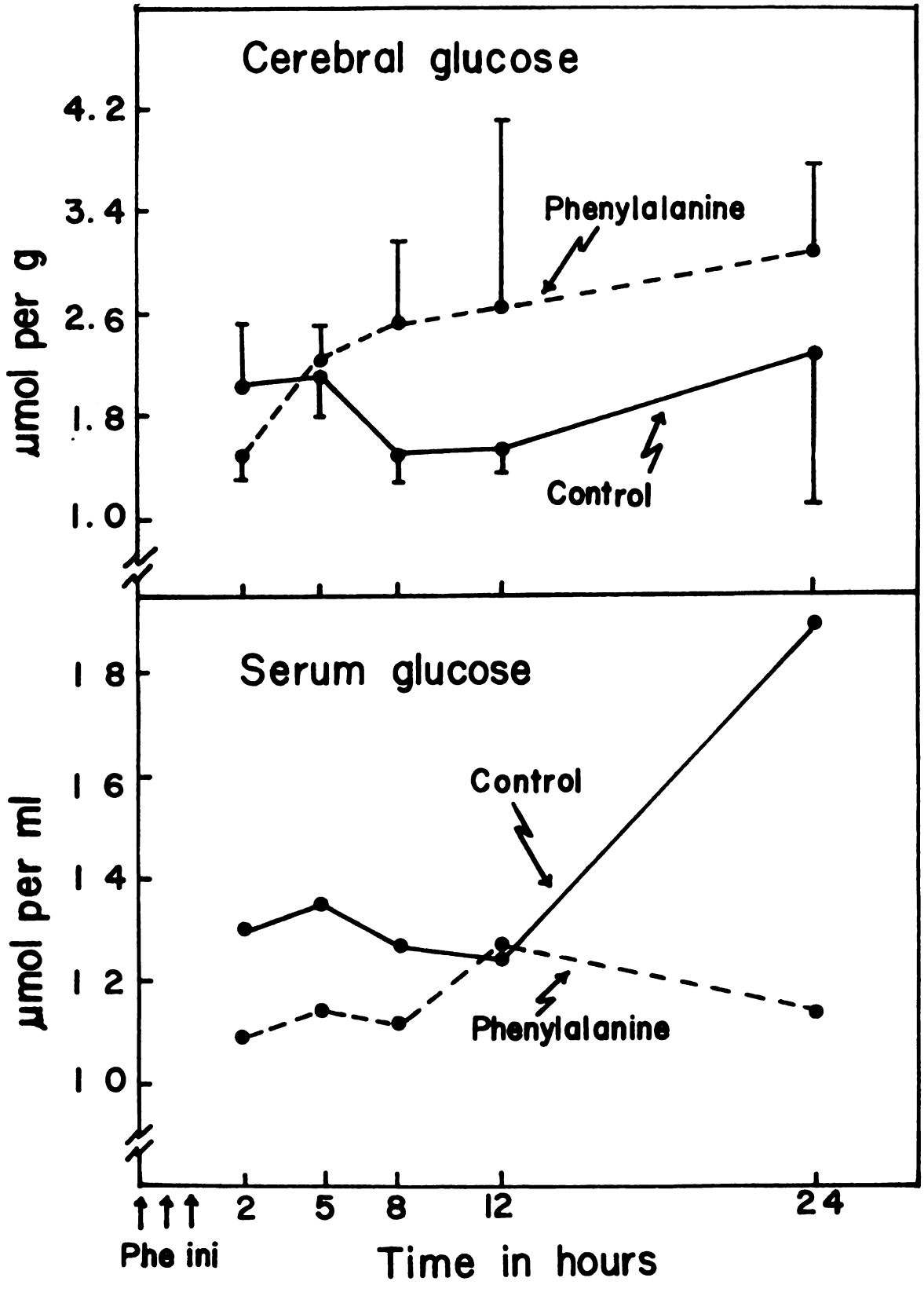

L-α-glycerol phosphate
Phenylalanine
Control
0.20
0.10
Fructose diphosphate
Control
Phenylalanine
0.20
0.10
µmol per g of tissue
2
5
8
12
24
Phe inj
Time in hours

Figure 9.--Levels of glucose in the brain and serum of chicks during a 24 h period following injections with L-phenylalanine.

Experimental details are identical to those for Figure 4. The serum glucose levels represent single determinations on blood pooled from 3 animals.


Cerebral glucose
4.2
3.4
2.6
1.8
1.0
µmol per g
Phenylalanine
Control
Serum glucose
1 8
1 6
1 4
1 2
1 0
µmol per ml
Control
Phenylalanine
2
5
8
12
24
Phe ini
Time in hours

Amino acid analyses

The large dose of L-phenylalanine administered to the chicks produced very drastic changes in the profile of cerebral amino acids at two hours after the final injection (Table 6). Lysine, histidine, phosphorylethanolamine, taurine, aspartic acid, threonine, serine, glutamic acid, alanine, valine, methionine, isoleucine and leucine were decreased. Neither arginine nor ammonia were affected. Only glutamine, glycine, phenylalanine and tyrosine were elevated. The level of histidine as calculated represents a minimum value in the saline-injected animals. A second component that chromatographed beneath the histidine peak may possibly have contained some histidine but was not included in the calculations. In the phenylalanine group the histidine level was below limits for accurate quantification (less than 0.015 μmol/g of tissue). The summation of free amino acids and ammonia in the control and phenylalanine-treated groups was 36.944 and 36.787 μmol/g of brain, respectively (Table 6).

Seizure threshold studies

Convulsions produced in the chicks with either picrotoxin or pentylenetetrazol appeared similar; the animals underwent tonic-clonic movements followed by a tonic extension of limbs. In the experiments in which the chicks were injected with picrotoxin, the animals pretreated with either 1.08 or 1.62 mmol of L-phenylalanine

Table 6.--Levels of Free Amino Acids and Ammonia in the Brains of Chicks Injected with Saline or L-Phenylalanine.

Compound	Control	Phenylalanine-Injected	% of Control	
	μmol/g of wet wt.			
Lysine	0.593 ± 0.022*	0.414 ± 0.051	70	
Histidine	0.040 ± 0.004*	Not measurable		
Arginine	0.067 ± 0.001*	0.073 ± 0.002	109	
Phosphorylethanolamine	1.910 ± 0.100*	1.610 ± 0.056	84	
Taurine	8.750 ± 0.310*	7.510 ± 0.182	86	
Aspartic Acid	2.450 ± 0.136	1.930 ± 0.136	79	($p<0.005$)
Asparagine and N-Acetyl Aspartic Acid	4.130 ± 0.897	4.220 ± 0.375	102	(NS)
Threonine	0.383 ± 0.041	0.240 ± 0.034	63	($p<0.010$)
Serine	0.576 ± 0.043	0.441 ± 0.006	77	($p<0.025$)
Glutamic Acid	8.820 ± 0.342	5.480 ± 0.371	62	($p<0.001$)
Glutamine	6.880 ± 1.340	9.480 ± 0.729	138	($p<0.050$)
Glycine	1.160 ± 0.045	1.290 ± 0.042	111	($p<0.025$)
Alanine	0.451 ± 0.028	0.318 ± 0.015	71	($p<0.005$)
Valine	0.097 ± 0.004	0.044 ± 0.004	45	($p<0.001$)
Methionine	0.029 ± 0.008	0.014 ± 0.003	48	($p<0.050$)
Isoleucine	0.060 ± 0.009	0.041 ± 0.005	68	($p<0.050$)
Leucine	0.083 ± 0.011	0.042 ± 0.005	51	($p<0.005$)
Tyrosine	0.049 ± 0.006	0.160 ± 0.022	327	($p<0.001$)
Phenylalanine	0.048 ± 0.010	3.140 ± 0.414	6542	($p<0.001$)
Ammonia	0.368 ± 0.127†	0.340 ± 0.103	92	(NS)
Total	36.944	36.787	100	

Note: The 1-day-old chicks were injected intraperitoneally 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or with 0.154 M saline, and decapitated 2 h after the last injection. The brains were immediately removed and frozen in liquid N_2.

*These values represent the average of single or duplicate analyses ± range from 2 pools of 5 brains each. All other values are averages of single or duplicate determinations from 3 pools of tissue, 5 brains each. Statistical significance was determined according to the student's t-test.

†Quantified spectrophotometrically (Weil-Malherbe, 1969).

(Figure 10) underwent tonic extension at a later time than controls. This delay was more apparent with the higher amino acid treatment. The dose response curve for the convulsant agent was straight over a 50 fold concentration range in the controls. Some variation in control values was observed between experiments at the lower doses of picrotoxin. The survival rates of the saline and phenylalanine (1.08 mmol) injected groups differed markedly at 5 mg/kg picrotoxin. Only 1/5 controls recovered from the convulsions; the average time of death of the remaining four was 44 ± 8 min; whereas 4/8 of the phenylalanine-treated animals did not undergo convulsions; 2/8 underwent convulsions but recovered completely and the other two chicks died after 45 and 60 min. No such protective effect could be demonstrated at any other doses of picrotoxin. Levels of phenylalanine and tyrosine determined from a pool of brains from animals treated with 1.08 mmol of amino acid were 0.68 and 0.46 μmol/g of tissue, respectively; phenylalanine measured in 3 pools of brains from animals treated with 1.62 mmol was 2.96 ± 0.30 and tyrosine was 0.20 ± 0.05 μmol/g. This anti-convulsive effect of L-phenylalanine was also shown with pentylenetetrazol (Table 7). At a pentylenetetrazol dose of 200 mg/kg, the tonic phase was delayed by 20 min over the control ($p<0.001$). The brains from these animals were selectively pooled, 3 - 5 per pool and phenylalanine and tyrosine

Figure 10.--Seizure threshold to picrotoxin in the chick after intraperitoneal injections of L-phenylalanine.

Day-old chicks were injected intraperitoneally 2 or 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline; 2&1/2-3 h after the final injection, the animals were injected with picrotoxin as described in METHODS and the interval before the onset of tonic extension was noted. The values ± standard deviation represent averages from 5 animals excepting one phenylalanine group mentioned in RESULTS.

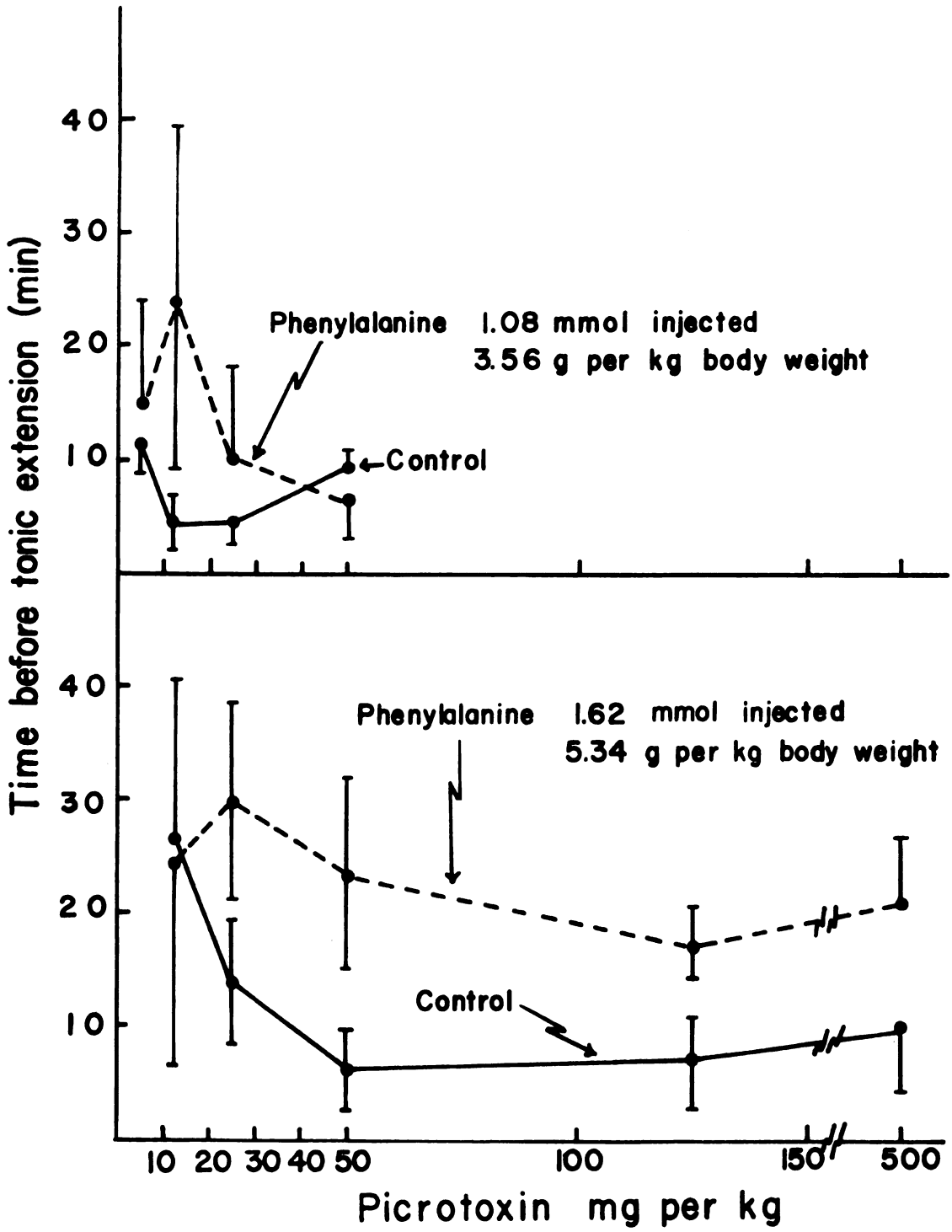

Time before tonic extension (min)
40
30
20
10
Phenylalanine 1.08 mmol injected
3.56 g per kg body weight
Control
40
30
20
10
Phenylalanine 1.62 mmol injected
5.34 g per kg body weight
Control
10 20 30 40 50 100 150 500
Picrotoxin mg per kg

Table 7.--Seizure Threshold to Pentylenetetrazol after Intraperitoneal Injections of L-Phenylalanine.

Pentylenetetrazol	Interval Before Tonic Extension	
	Saline	Phenylalanine
200 mg/Kg	8.5 ± 4.2 min (n = 15)	28.8 ± 21.2* min (n = 16)
400 mg/Kg	3.0 ± 2.7 min (n = 6)	3.1 ± 1.6† min (n = 6)

Note: Chicks were injected intraperitoneally 2 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline; 2&1/2 - 3 h after the last injection, the pentylenetetrazol was administered. The amino acids were determined on representative tissue samples using an automated analyzer.

*$p<0.001$; cerebral level of phenylalanine is 1.52 ± 0.69 µmol/g; level of tyrosine, 0.21 ± 0.07 µmol/g.

†Cerebral level of phenylalanine is 1.01 and tyrosine, 0.26 µmol/g.

were determined for 4 such pools. Phenylalanine was present at levels of 1.52 ± 0.69 μmol/g and tyrosine at 0.21 ± 0.07 μmol/g.

Effect of L-phenylalanine on anaerobic glycolysis and creatine kinase activity

It was of interest to determine if any of the *in vivo* observations could be demonstrated in simple *in vitro* systems. Production of lactate was studied for the 100,000 g soluble fraction of chick brain with and without L-phenylalanine (Table 8). Concentrations of the amino acid as high as 18 mM exerted no inhibition. Also 9 mM L-phenylalanine had no effect on creatine kinase catalyzed phosphorylation of ADP (Figure 11).

Studies of the effects of other amino acids on the chick

Several amino acids were injected into the chick according to the same procedure used with L-phenylalanine, and the animals were observed for a 24 h period after the third injection (Table 9). The animals treated with L-histidine, L-methionine, D- or L-phenylalanine and L-tryptophan were most severely debilitated; they were most inactive and in a majority of instances prostrated. L-Leucine, L-lysine, L-threonine, L-valine or hydroxyproline had slight or no effect. The mortality rates correlated with the severity of the conditions.

Table 8.--*In vitro* Study of Anaerobic Glycolysis in Chick Brain in the Presence of L-Phenylalanine.

	Conversion of [^{14}C] Glucose to [^{14}C] Lactate				
	Control	9 mM L-Phenylalanine	% of Control	18 mM L-Phenylalanine	% of Control
	cpm/mg*			cpm/mg*	
Exp. I†					
15 min	35	36	103	36	103
30 min	134	132	99	148	110
45 min	250	291	116	297	119
60 min	391	420	107	496	126
Exp. II‡					
15 min	1.4	1.8	129	1.4	100
30 min	7.8	6.8	87	6.8	87
45 min	17.0	20.0	118	19.4	114

*[^{14}C]lactate was isolated as the acetaldehyde-dimedone derivative. See METHODS. cpm/mg indicates the total radioactivity in this final product.

†290,000 cpm of [6-^{14}C]glucose/1 ml incubation.

‡18,750 cpm of [U-^{14}C]glucose/1 ml incubation.

Figure 11.--Activity of creatine kinase in the presence of L-phenylalanine.

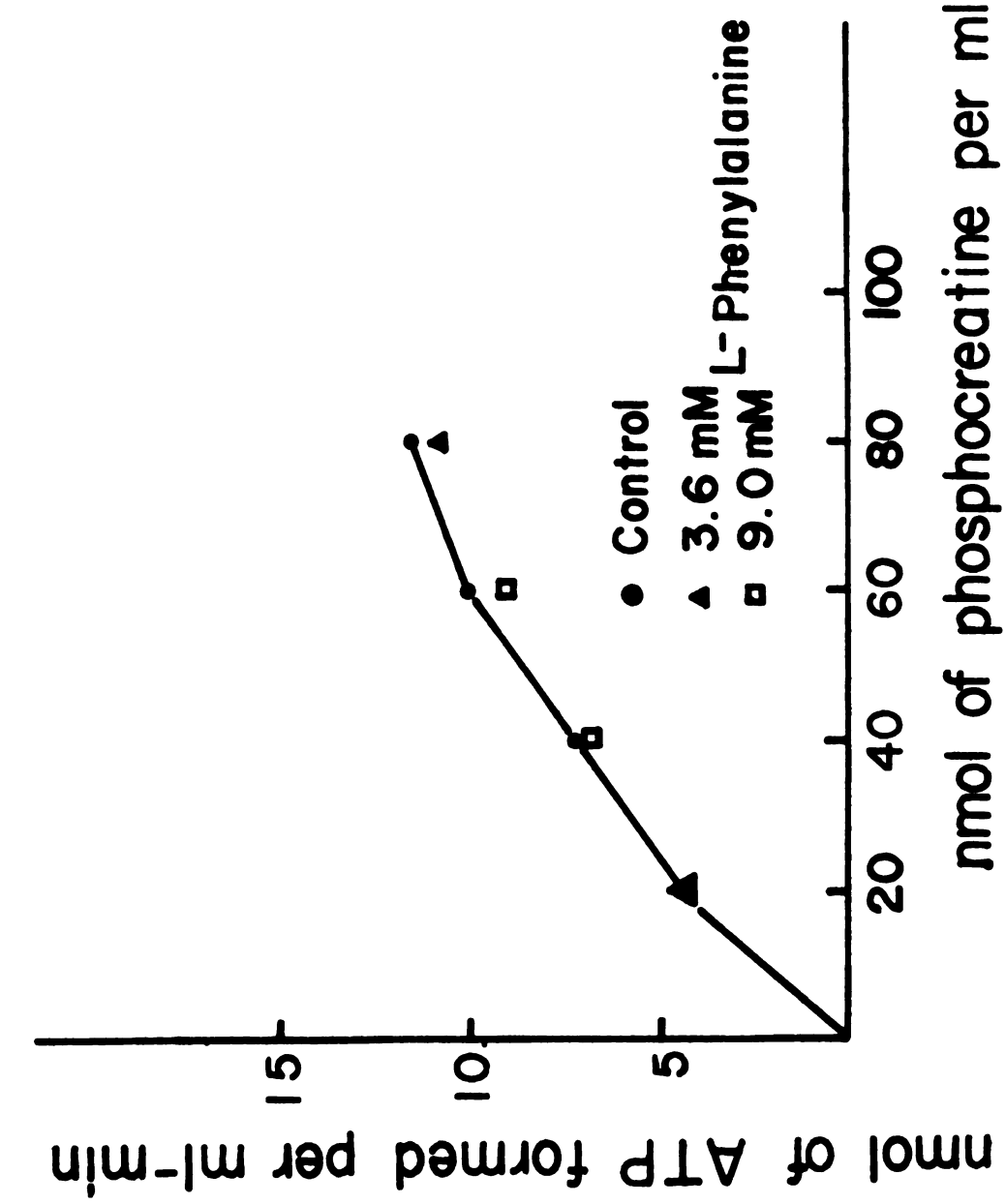

nmol of ATP formed per ml-min
15
10
5
20
40
60
80
100
nmol of phosphocreatine per ml
Control
3.6 mM L-Phenylalanine
9.0 mM

Table 9.--Survey of the Effects of Several Amino and Imino Acids on the Chick.

Compound	Effect on Physical Activity	Deaths in 24 h Period
L-Leucine	Slight Reduction	0/5
L-Lysine	Slight Reduction	0/5
L-Histidine	Severe Reduction	1/4
L-Methionine	Severe Reduction	3/5
L-Phenylalanine	Severe Reduction	2/5
D-Phenylalanine	Severe Reduction	---
L-Tryptophan	Severe Reduction	4/5
L-Threonine	No Change	0/5
L-Valine	No Change	0/5
Hydroxyproline	No Change	1/5

Note: The chicks were injected intraperitoneally 3 times with 3 ml of a 0.18 M neutralized amino acid solution, except for tryptophan which was 0.09 M. The animals were observed for a 24-h period.

Effect of the injection procedure on plasma osmolality and electrolyte concentrations

A considerable volume of water (9 ml) and quantity of sodium chloride or L-phenylalanine was injected into the chicks. Previously the level of phenylalanine in the plasma of the chicks was observed to increase from 0.16 to 10 - 15 μmol/ml (Granett and Wells, 1972). The extent to which the treatment alters blood electrolytes was investigated by determining plasma osmolality and the levels of several cations. The absorption of the water, NaCl or amino acid by the chick did not modify the osmolality (Table 10) compared to a value of 309 ± 7 milliosmol/kg obtained by Knull, Wells and Kozak (1972) for uninjected chicks. The levels of potassium, sodium and calcium did not differ in the two injected groups; but when compared to the uninjected animals, potassium was 160% higher regardless of the solute injected (Table 11).

Table 10.--Comparison of Plasma Osmolalities in Phenylalanine and Saline-Injected Chicks.

	Osmolality
	milliosmol/Kg
Saline Group	306 ± 7
Phenylalanine Group	307 ± 4

Note: Chicks were injected intraperitoneally 3 times at hourly intervals with 3 ml of 0.18 M L-phenylalanine or 0.154 M saline. Blood was obtained by heart puncture 4 h after the last injection. Each value represents the average of duplicate determinations on 3 separate pools of plasma, 5 animals per pool.

Table 11.--Plasma Concentrations of Sodium, Potassium and Calcium in Uninjected Chicks and Chicks Injected with Saline or L-Phenylalanine.

Injection	K^+	Na^+	Ca^{++}
	milliequivalents/liter		
None*	5.03 ± 0.36	136 ± 1	5.80 ± 0.06
Saline†	7.98 ± 0.80	139 ± 2	6.56 ± 0.08
L-Phenylalanine†	8.65 ± 0.35	135 ± 2	6.16 ± 0.08

Note: The values represent averages of duplicate determinations on 3 different samples, except for the value for potassium in the uninjected group for which n = 6.

*The chicks were mash-fed, uninjected.

†These same samples were used in the osmolality studies.

DISCUSSION

The recovery studies confirm many of the earlier observations (Granett and Wells, 1972); that is, the level of phosphocreatine is elevated and the concentrations of fructose diphosphate and L-α-glycerol phosphate are depressed. Standard deviations are larger than previously recorded since analyses were performed on individual brains rather than on pooled tissue and thus variability amongst animals is quite prominent. A very slight hypoglycemia was evident throughout the time course, which is compatible with the report of Fajans, Floyd, Knopf and Conn (1967) that many essential amino acids including phenylalanine are capable of invoking an insulin release and thus lowering plasma levels of glucose in humans and rats. The abrupt rise in glucose concentrations in the control plasma, also reflected in the brain, corresponds to the intake of food 60 min before the 24 h sacrifice. Up to this time the animals had been deprived of food and water. The phenylalanine-treated animals refused the food and were abnormal in this respect, although they did appear more alert at the end of the

experiment. No decline was observed in the glucose levels in the brains of the phenylalanine-treated chicks as in controls. Evidence has been presented (Lowry, Passonneau, Hasselberger and Schulz, 1964; Mayman, Gatfield and Breckenridge, 1964; Brunner, Passonneau and Molstad, 1971) that barbiturate or volatile anesthetics increase serum or cerebral levels of glucose and the brain/serum glucose ratio. The levels of glucose-6-phosphate also increase in the brain. It has been difficult with our system to consistently demonstrate an increase in glucose or glucose-6-phosphate (Granett and Wells, 1972), but in these recovery studies it would appear that the brain/serum glucose ratio is increased in the phenylalanine-treated group and that the glucose is not being depleted from the brain as in control animals, perhaps because of a postulated reduced utilization.

Of the metabolites examined L-α-glycerol phosphate was affected earliest. The most significant changes in this compound, fructose diphosphate and phosphocreatine were associated with the highest concentrations of phenylalanine in the brain and their levels appeared to return to those observed in control animals as the amino acid concentration fell. The data implies that phenylalanine may be the active compound, directly or indirectly, causing the altered state of energy metabolism; however,

the participation of a metabolite of phenylalanine following a similar concentration profile cannot be ruled out.

Evidence has been presented from the rat and human brain systems that phenylalanine directly inhibits glycolysis at pyruvate kinase (Weber, 1969). This inhibition was not demonstrable in the chick (Granett and Wells, 1972). Furthermore, L-phenylalanine did not inhibit lactate formation in a fortified 100,000 g supernatant fraction prepared from chick brain. Inhibition was reported by Weber, Glazer and Ross (1970) for such a system purified from human brain and in human and rat brain slices (Ki of 2 and 17 mM for 2 and 7 day-old rats, respectively). In some preliminary work with brain slices from the chick, we found L-phenylalanine (3.6 mM) to inhibit lactate formation by 10 - 30%; in one experiment with 10 mM L-phenylalanine both stimulation and inhibition were observed. More work is needed before any significance can be attached to these findings in slices, but it would appear that L-phenylalanine in vitro does not affect glycolysis in chick brain as it does in rat brain. Any direct interaction of phenylalanine with a glycolytic component in chick brain remains unproven.

Recently levels have been reported for cerebral amino acids in the young chick (Levi and Morisi, 1971; Blosser and Wells, 1972). In general our analyses are in good agreement with their work. Discrepancies may be due

to differences in strain (White Leghorn versus White Livorno). Also the validity of equating our saline-treated control animals with uninjected controls is questionable. It is known that brain slices under certain conditions will swell in saline with sodium being taken up and potassium being extruded from the tissue (Franck and Schoffeniels, 1972). If such were occurring in our animals in which elevated plasma levels of potassium were observed, it is reasonable that the concentrations of other cellular constituents would be altered. Dobkin (1972) demonstrated that cerebral levels of glutamine were altered by intraperitoneal injection of 0.9% NaCl in the rat.

The differences between the amino acid levels in the control and phenylalanine-treated animals suggest inhibition at transport sites. In vitro L-phenylalanine (2 - 10 mM) has been shown to reduce the initial rate of influx or the steady-state accumulation of glycine, alanine, leucine, arginine, lysine, methionine (Blasberg and Lajtha, 1965 and 1966), histidine (Neame, 1964) and serine (Abadom and Scholefield, 1962) in cerebral slices. On the basis of their studies Cohen and Lajtha (1972) distinguished separate transport sites for at least eight classes of amino acids; namely, small neutral, large neutral, small basic, large basic, acidic, GABA, amido and imino acids. L-Phenylalanine was described as having a significant affinity for the transport systems of other neutral amino

acids and basic amino acids, but as not affecting the influx of the dicarboxylic group. This corresponds well to our finding except for arginine and glycine which were not decreased in concentration and aspartic and glutamic acids which were decreased. Since the decrease in glutamate balances the increase in glutamine, the formation of glutamine may represent an attempt of the cell to alleviate an accumulation of ammonia resulting from metabolism of phenylalanine in the liver. However, plasma levels of ammonia were not quantified and therefore cannot be assumed to be elevated. Should the detoxification mechanism be active, it would appear to be adequate as ammonia levels are essentially equal in the brains of both saline and phenylalanine-treated groups. Since aspartate and alanine are synthesized by transamination of oxaloacetate and pyruvate, respectively, by glutamate, a reduction in their levels could be due to the decrease in amine donor. An alternative explanation for the decreased cerebral levels of aspartic and glutamic acids and alanine and serine as well is related to their metabolic proximity to glycolysis and the tricarboxylic acid cycle; the levels may reflect slower carbon flow. The rate of glycolysis has been shown to be lower in the phenylalanine-injected group (Granett and Wells, 1972) (see Chapter I, this section), although the rate of the tricarboxylic acid cycle was not examined. The synthesis of these amino

acids appears to be rapid enough to be affected within the few hours of the acute study as Yoshino and Elliott (1970) demonstrated considerable radioactive labeling in the amino acids in rat brain 15 min after injection of [^{14}C] glucose in the tail vein.

In addition to influx and metabolic alterations, another dimension to the steady-state levels of cerebral amino acids possibly disturbed under our conditions is the efflux of amino acids from the central nervous system. Active transport of amino acids between the blood and cerebrospinal fluid has been demonstrated in vitro with choroid-plexus tissue (Lorenzo and Cutler, 1969) and in vivo with the ventricular perfusion technique (Cutler and Lorenzo, 1968).

The effects of excess phenylalanine on cerebral amino acids have been described most frequently for the rat. Significant reductions in cerebral concentrations of threonine, valine, methionine, isoleucine, histidine and leucine were observed by McKean, Boggs and Peterson (1968) in acute injection studies with both immature and mature rats. No change was observed in the level of arginine. Most of these observations were confirmed by Castells, Zischka and Addo (1971). Lowden and LaRamèe (1969) examined non-essential amino acids and found alanine, serine, glutamic acid and aspartic acid decreased in the brains of 10 day-old rats. Analyses of the brain

tissue from untreated phenylketonuric subjects (McKean and Peterson, 1970) demonstrated a reduction in threonine and an elevation in glutamine, but the paucity of data available disallows any extensive comparison with the chick.

The depressed physical response of the chick to extremely high levels of L-phenylalanine is unlike that of the rat. Rats subjected to a rigorous injection series with the amino acid reacted in a manner characteristically sympathomimetic (Goldstein, 1961). Heart and respiratory rate were increased; surface blood vessels were constricted; gastrointestinal tone was reduced; and analgesia was displayed. Most severely affected animals demonstrated tremors. Injection of the rats with the amino acid and a monoamine oxidase inhibitor, iproniazid (also an antidepressant), produced similar and more potentiated effects. In contrast the phenylalanine-treated chicks at no time were hyperactive. Noteworthy is the fact that high phenylalanine concentrations in the brain greater than 3 μmol/g of tissue were necessary to produce either of the two behavioral states; these different responses would indicate separate mechanisms for toleration of this amino acid in the rat and chick.

More compatible with our observations is a depletion in brain catecholamines. It has been reported that rats treated with non-toxic doses of α-methyl tyrosine, an

inhibitor of tyrosine hydroxylase, become depressed as quantified in behavioral studies, and the cerebral catecholamines are reduced (Rech, Borys and Moore, 1966; Rech, Carr and Moore, 1968). This particular aspect has not as yet been examined in the phenylalanine-treated chick, but it is possible that the extremely high concentrations of the amino acid could alter synthesis or storage of norepinephrine. Phenylalanine has been reported to competitively inhibit the formation of 3,4-dihydroxyphenylalanine from tyrosine with tyrosine hydroxylase from adrenal gland ($Ki = 1 - 3 \times 10^{-4}$ M), heart and caudate nucleus (Ikeda, Levitt and Udenfriend, 1967). Phenylalanine could also inhibit synthesis by chelating copper (Albert, 1950) at the dopamine B-hydroxylase step. Cu (++) chelators have been found to inhibit this enzyme (Goldstein and Nakajima, 1967).

The studies with picrotoxin and pentylenetetrazol confirm the hypoactive state produced in the chick by phenylalanine. Decreased cerebral excitability was evidenced by the delayed tonic phase of the convulsion and occasional total elimination of clinical signs; a rather large dose of L-phenylalanine was required. In this respect the chick also differs from the rat, for using the convulsant, hexafluorodiethyl ether, Gallagher (1970) demonstrated that phenylalanine reduced seizure threshold in immature rats. The effect appeared to be independent

of derived tyrosine or phenylpyruvate (Gallagher, 1971). However, his experiments differed from ours in that DL-phenylalanine was utilized and the levels of phenylalanine did not exceed 1 μmol/g of brain. In agreement more with phenylalanine's effect in the chick than in the rat is Nigam, Watson and Marcus' (1968) study of electroencephalographic effects of L-phenylalanine in the cat. Generalized slowing was observed when plasma phenylalanine levels were elevated beyond 1.1 μmol/ml.

Utilization of varied methods for production of convulsions should aid in elucidating the mechanism of phenylalanine's effect. However, the studies with picrotoxin and pentylenetetrazol thought to induce cerebral excitability by two distinct pharmacological actions (namely, picrotoxin blockage of presynaptic inhibition and pentylenetetrazol shortening neuronal recovery; Esplin and Lablicka, 1967) would indicate a general rather than specific effect since phenylalanine suppressed convulsive activity induced by either agent. Attempts to measure phenylalanine's protection against a third seizure inducer, electroshock, were unsuccessful due to technical difficultires involving the eyelid closure reflex of the chick displacing the corneal electrodes.

Several substances including GABA and alanine injected into mice as hyperosmolar solutions (1 M) have offered protection against convulsions. Mild dehydration

of the brain with increased plasma osmolarity followed the treatment (DeFeudis and Elliott, 1967; DeFeudis, 1971), and it was suggested that the anti-convulsive activity was a consequence of the loss of water from the brain and unrelated to the particular compound injected. This theory is not new. In 1929 Fay used fluid restriction as therapy against convulsions and McQuarrie, Anderson and Ziegler (1942) introduced as a diagnostic tool a hydration test for inducing seizures. Toman and Goodman (1948) emphasized the concommitant changes in electrolyte balance. Our experimental conditions differ from those of DeFeudis in that our solutions of L-phenylalanine were slightly hypo-osmolar and regardless of the increased plasma levels of phenylalanine to 10 - 15 μmol/ml (Granett and Wells, 1972) the plasma was iso-osmolar. Also the elevated levels of potassium in the plasma were compatible with water uptake by various tissues, not dehydration (Franck and Schoffeniels, 1972). Since the saline-injected controls, which were alert, exhibited the same elevated potassium levels, it is unlikely that water exchange is instrumental in producing the sedated state in the animals treated with L-phenylalanine.

Another factor worth consideration is the role of ketone bodies in our system. Phenylalanine is catabolized eventually to acetoacetate and fumarate. Ketosis induced by fasting has been a classic treatment for seizures since

ancient times in lieu of sophisticated therapy. Currently high fat and low carbohydrate diets are being used with children with seizure disorders who haven't responded positively to normal anticonvulsive drugs. Uhlemann and Neims (1972) demonstrated significant protection against maximal electroshock using ketotic mice, but they were unable to quantitatively relate blood ketones and the positive or negative response. Levels of β-hydroxybutyrate presently being determined in the plasma of the phenylalanine-treated chicks have been found to be about 100% higher than in controls. Acetoacetate hasn't been quantified as yet. Mechanisms by which ketosis exerts anti-convulsive activity are not well defined. Various laboratories have emphasized the ketone bodies, per se, utilization of ketone bodies by the brain for energy instead of glucose, dehydration, electrolyte imbalance, metabolic acidosis, or hyperlipemia in one way or another (refer to Uhlemann and Neims, 1972, for specific references). Fischer (1951) concluded from studies of ketosis in rabbits and humans that ketone bodies probably are not the direct cause of the depressed state in diabetic coma. It was reported that fatty acids and not β-hydroxybutyrate reduced muscular tone and activity, producing unconsciousness in the rat (Samson, Dahl and Dahl, 1956) and altered electroencephalographic patterns in the rabbit, suggestive of sleep (White and Samson, 1956); of carbon chains ranging

in length from acetate to decanoate, the longer chain fatty acids were demonstrated more effective. Acetoacetate was not examined. Thus at this point it is very difficult to definitively relate the presence of the ketone bodies in the chick to the sedation associated with phenylalanine, but it is a factor deserving additional experimentation.

At present nothing is known about the steady-state levels of lipids in the phenylalanine-treated chick; if insulin were being secreted, a lowering in fatty acids could be expected; but it is an interesting area in view of the narcotic effect reported with fatty acids. Already a difference is apparent between that system and ours. Dahl (1968) correlated the effective narcotic concentration of the various fatty acids *in vivo* with *in vitro* inhibition of the $(Na^+ + K^+)$-ATPase, postulating that the two phenomena rather than being causally related were linked through a common action of the fatty acid on the membrane lipids. This is in contrast to the observation of Ting-Beall and Wells (1971) with L-phenylalanine, which stimulates $(Na^+ + K^+)$-ATPase, *in vitro*, probably through a mechanism involving chelation of divalent cations that would otherwise inhibit the enzyme. Reversible blockage of the action potential in vagus nerve preparations was demonstrated with sodium octanoate (Dahl, Shirer and Balfour, 1966). Now in progress in this laboratory are experiments by Dr. Ting-Beall to determine the effect of

L-phenylalanine on the action potential in peripheral nerve. It has been demonstrated that L-phenylalanine complexes with calcium which suggests that the amino acid could displace the calcium from the axonal membrane surface (Ting-Beall and Wells, 1971). Tasaki, Watanabe and Lerman (1967) have shown that calcium is necessary for axonal membrane excitability.

ACKNOWLEDGMENTS

Dr. Hyram Kitchen is acknowledged for the use of his amino acid analyzer and Miss Irene Brett for her very fine technical assistance. I am indebted to Mrs. Betty Schoepke for the electrolyte analyses. Also I would like to thank Mr. Willie Taylor for his energies on the anti-convulsion studies.

SUMMARY

The chick was investigated as a model system for the study of phenylketonuria, the genetic disease in which the hydroxylation of phenylalanine to tyrosine is blocked. Hyperphenylalanemia was produced by hourly intraperitoneal injections of 3 ml of 0.18 M L-phenylalanine over a 3 h period, resulting in the administration of 1.62 mmoles of the amino acid. Shortly following the final injection, the animals were observed to enter a state of narcosis which was reversible within 24 h. The animals lost normal righting reflexes. Levels of phenylalanine reached 10 - 15 μmol/ml of plasma (control value, 0.16) and 2.5 - 3.5 μmol/g of brain tissue (control value, 0.05). Examination of several cerebral glycolytic intermediates and high energy phosphate compounds at a time when the concentration of phenylalanine in the brain was highest disclosed elevated levels of phosphocreatine and lowered levels of fructose-1,6-diphosphate, lactate and L-α-glycerol phosphate. Glucose and glucose-6-phosphate were either unchanged or elevated; glycogen remained unchanged and adenine nucleotide energy charge was not affected. Some

of these data are compatible with those observed for mouse brain under conditions of barbiturate anesthesia. The "closed-system" technique revealed that the rate of utilization of high energy phosphates was depressed as is also characteristic of the anesthetized state. Attempts to demonstrate in vitro an effect of phenylalanine on anaerobic glycolysis with high-speed supernatant fractions prepared from chick brain or on creatine kinase were negative. In recovery studies the changes in the metabolites indicated previously coincided quite well with cerebral phenylalanine levels of 2 μmol/g or more. A very slight hypoglycemia was associated with the phenylalanine treatment.

The large increase in phenylalanine in brain was shown to significantly reduce the levels of all the essential amino acids, with the exception of tryptophan which was not quantified. A probable explanation is that phenylalanine inhibited entry or exit of the amino acids at translocation sites on the membrane. Some of the non-essential amino acids were also decreased which may be attributed to the reduction in carbon flow through glycolysis. Only phenylalanine, tyrosine, glutamine and glycine were elevated. Ammonia levels were unaltered.

In the animals treated with L-phenylalanine the tonic phase of pentylenetetrazol and picrotoxin induced

convulsions was delayed and in some cases eliminated which is compatible with barbiturate actions.

When the injected animals were compared with uninjected ones, the injection procedure was found not to alter plasma osmolality; however, potassium was elevated in the plasma of both the saline control and phenylalanine-injected groups, while sodium and calcium were unchanged. It was concluded that the technique alone was not a factor in the response observed with the chicks injected with phenylalanine.

This investigation has revealed two major differences between the chick and the rat as model systems for phenylketonuria. Firstly, the two species react to the amino acid loads in different ways. The rat does not become sedated as does the chick. Phenylketonurics do not display hypoactivity, but more often hyperkinesis, agitated behavior, tremors and seizures. Secondly, phenylalanine appears to inhibit brain pyruvate kinase in both rats and humans (Weber, 1969) but is ineffective in the chick. Thus it would appear that studies on the effects of phenylalanine in the chick may be less relevant to hyperphenylalanemia in humans and more significant for the demonstration of sedation, a previously unknown action of the amino acid. Indications are that studies with histidine, tryptophan and methionine would be useful from a comparative point of view as these amino acids also

showed on gross observation similar toxicity in the chick.

In conclusion, from indirect evidence the state of narcosis induced in the chick may be mediated by an activity of L-phenylalanine on transmission of nerve impulses either by interference with the synthesis of the transmitter species, norepinephrine, or by impairment of the propagation of the impulse along the nerve. The alterations in the glycolytic intermediates that were observed may reflect a depressed electrical activity in the brain and a decreased demand for energy.

III. SECTION II

CHAPTER I

STUDIES ON CEREBRAL GLYCOLYTIC FLUX IN GALACTOSE-TOXIC CHICKS

INTRODUCTION

A study was made of certain aspects of the cerebral energy metabolism in chicks intoxicated with galactose. Previously, the levels of various glycolytic intermediates had been found to be lower in the brains of cockerels fed a diet containing galactose (40% w/w), and it was of interest to know to what extent the rate of glycolysis was affected. The "closed-system" technique described in the previous section was used to estimate the flux. In combination with other relevant work, the data presented here is currently in press under the title, "Studies on Cerebral Energy Metabolism during the Course of Galactose Neurotoxicity in Chicks," by S. E. Granett, L. P. Kozak, J. P. McIntyre, and W. W. Wells, in Journal of Neurochemistry. Striving to provide the reader with a complete dimension of the problem of galactose-toxicity in the

chick, in the following literature review I have summarized various efforts in the area, including those perhaps only indirectly related to cerebral energy metabolism.

LITERATURE REVIEW

Rutter, Krichevsky, Scott and Hanson (1953) reported that chicks fed a diet greater than 10% in galactose displayed ataxia, tremors and convulsions, followed by death. Degeneration of neurons was associated with the toxicity (Rigdon, Couch, Creger and Ferguson, 1963). Galactose and its metabolites, galactitol, UDP galactose and galactose-1-phosphate, accumulated in the brain as well as in other tissue (Wells and Segal, 1969; Kozak and Wells, 1971).

It has been established that the female is more sensitive to the high dietary levels of galactose than the male (Nordin, Wilken, Bretthauer, Hansen and Scott, 1960). Mayes, Miller and Myers (1970) demonstrated that the levels of galactose-1-phosphate were higher and the activity of galactose-1-phosphate uridyl transferase lower in females than males and suggested these differences might account for the higher mortality rate in female chicks.

The capacity of chick brain to oxidize galactose was found to be low in comparison to that for glucose, and the activity was 5 to 10% of that observed for the kidney (Wells, Gordon and Segal, 1970).

Kozak and Wells (1969) reported a perturbation in high energy phosphate metabolism (fall in adenylate energy charge) and glycolysis in galactose-toxic chicks. The cerebral levels of phosphocreatine, ATP and many glycolytic intermediates were depressed, while ADP, AMP and Pi were elevated. The incorporation of ^{32}P into galactose-1-phosphate was rapid and very similar to that for ^{32}P into [γ-^{32}P]-ATP while little galactose was being incorporated into glycogen, lipid, or glutamate. To explain this enigma and account for hydrolysis of ATP, the following mechanism of a futile ATPase was proposed:

$$\text{Galactose} + \text{ATP} \overset{\text{Galactokinase}}{\rightleftarrows} \text{Galactose-1-Phosphate} + \text{ADP}$$

$$\text{Galactose-1-Phosphate} \overset{\text{Phosphatase}}{\rightleftarrows} \text{Galactose} + \text{Pi}$$

A time course study of the dietary period revealed that ATP and glucose-6-phosphate decreased early in the feeding period (9 h), whereas reductions in other glycolytic metabolites were not observed until 18 or more hours had elapsed. The cerebral level of glucose fell from 1 - 2 µmol/g of tissue to as low as 0.2 - 0.3 (Granett, Kozak, McIntyre and Wells, in press). No hypoglycemia has been observed (Rutter et al., 1953).

Chicks administered galactose via drinking water as opposed to diet display hyperosmolality (160% of control), and dehydration of nervous tissue has been suggested as a

mechanism for the convulsions (Malone, Wells and Segal, 1972). However, Knull, Wells and Kozak (1972) have produced seizures under more mild hyperosmolar conditions (110% of control) using the synthetic diet 40% by weight in galactose and shown that symptoms of excited motor activity and alterations in the cerebral energy metabolites can be reversed by treatment with glucose without simultaneously decreasing the slight hyperosmolarity.

Rates of protein synthesis and degradation were examined in the brains of the galactose-toxic chick and found to be similar to those in the control animal. Analysis of the free amino acids revealed alanine and leucine most significantly reduced and aspartate elevated. Polyribosomal profiles were unaffected by galactose diet (Blosser and Wells, 1972), Glycoprotein metabolism, however, appeared to be altered as judged by a faster rate of incorporation of [^{3}H]-glucosamine which could not be attributed to a difference in specific activity of precursor pools (Knull, Blosser and Wells, 1971; Blosser and Wells, 1972). Lysosomes from galactose-toxic chicks were found to be more labile to hypo-osmotic shock and temperature in contrast to those isolated from control chicks, and the fragility was associated with the accumulation of galactose and galactitol (Blosser and Wells, in press).

MATERIALS AND METHODS

Animals and materials

Male White Leghorn chicks (1 to 2 days old) were purchased from Cobbs, Inc., Goshen, Indiana, and housed in a brooder at 32° for the experimental period. As previously described (Kozak and Wells, 1969), the diets were either free of galactose or contained 40% (w/w) of D-galactose (General Biochemicals, Chagrin Falls, Ohio) in place of an equal amount of cerelose (D-glucose monohydrate). Enzymes and coenzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Boehringer Mannheim (New York, N.Y.).

Substrate analysis

Perchlorate extracts of tissue were prepared as described by Kozak and Wells (1969). The acid-soluble phosphates and glycolytic intermediates were quantified enzymatically from neutralized extracts according to methods previously described (Granett, 1970; Kozak and Wells, 1969). Citrate concentrations were determined by the fluorometric procedure of Williamson and Corkey (1969) utilizing citrate lyase (EC 4.1.3.6) and malate dehydrogenase (EC 1.1.1.37).

The gas liquid chromatographic procedure of Sweeley, Bentley, Makita and Wells (1963) was employed to measure galactose and galactitol levels on perchlorate extracts desalted with MB-3 resin (Rohm and Haas Co., Philadelphia, Pa.).

"Closed-system" studies

The chicks, 3 to 4 days of age, were fed their respective diets for 46 hours. Anoxic conditions were produced by decapitation, and the heads were frozen in liquid N_2 after periods of up to 10 minutes. For intervals beyond 18 sec, the heads were incubated at 40°C; otherwise, they were maintained at room temperature. For each period of anoxia, there were four control and four experimental chicks and individual brains were chipped out and pooled. Metabolic rate, defined in these studies as the rate of utilization of actual and potential high energy phosphates, was calculated according to the formula (Lowry, Passonneau, Hasselberger and Schulz, 1964):

$$2\,\Delta ATP + \Delta ADP + \Delta phosphocreatine + \Delta lactate$$

where Δexpresses the change in the level of the metabolite during the period of anoxia. The term, Δlactate, was used instead of 2 Δglucose + 1.45 (Δlactate - 2 Δglucose) since glucose exhibited considerable variation early in the time course (see RESULTS).

Glycogen analysis

Brain glycogen was purified and hydrolyzed according to the method of Walaas and Walaas (1950), which involves base digestion of the perchlorate pellet, ethanol precipitation of the glycogen, followed by acid hydrolysis. The glucose released was then quantified according to the fluorometric procedure of Lowry et al. (1964). After 10 min of anoxia, glucose-yielding material was still isolated in amounts (0.5 μmol glucose/g of brain) similar to those observed at 2 and 5 min of anoxia. This value was observed for both dietary groups. The possibility that the basal level of 0.5 μmol released glucose/g of brain may be due to sources other than glycogen was investigated since glycogen should have been depleted under our conditions of anoxia. An attempt was made to demonstrate that the material isolated at the 10 min period was not glycogen using highly purified α-1,4-glucan glucohydrolase (EC 3.2.1.3) (Pazur, Simpson and Knull, 1969), an enzyme previously shown to hydrolyze α-1,4- and α-1,6-glucosidic linkages (Pazur and Kleppe, 1962). The 10 min sample proved to be completely resistant to treatment with the hydrolase, whereas material representing the zero time was not. Thus, all the glycogen data presented here has been corrected for contamination from non-glycogen, glucose-containing polymers. Refer to the following chapter for a more complete report on the analysis of glycogen with the glucohydrolase.

RESULTS

Employing the "closed-system" technique of Lowry et al. (1964), the levels of the adenine nucleotides, phosphocreatine, glycogen, glucose, fructose-1,6-diphosphate and lactate were determined in the brains of chicks fed, respectively, control or galactose-containing diets for 46 hours at various intervals of anoxia (Figures 12-16). As expected, utilization of phosphocreatine occurred promptly in both the control and galactose-fed groups, the phosphocreatine being lower with the onset of anoxia in the brains of chicks fed galactose (Figure 12). The ATP expenditure was initially delayed in both groups, the ATP presumably being replenished by the action of creatine phosphokinase (EC 2.7.3.2), but utilization was apparent as phosphocreatine became limiting. This occurred at an earlier period (12 sec) in the brains of chicks fed galactose than in those fed the control diet (24 sec) (Figure 13). One minute after decapitation, the level of ATP was only 10% of the initial in chicks fed galactose, whereas 57% remained in brains of animals fed the control diet. AMP levels were elevated in animals fed galactose at the onset of ischemia as previously noted (Kozak and Wells, 1969)

Figure 12.--Change as a function of the duration of post-mortem ischemia in phosphocreatine in brains of chicks fed control or galactose-containing diets.

The chicks were fed their respective diets for 46 h. The determinations were carried out on individual tissue samples from a pool of 4 brains. The vertical bars represent ± SD for 3 or more determinations. See METHODS for experimental details.

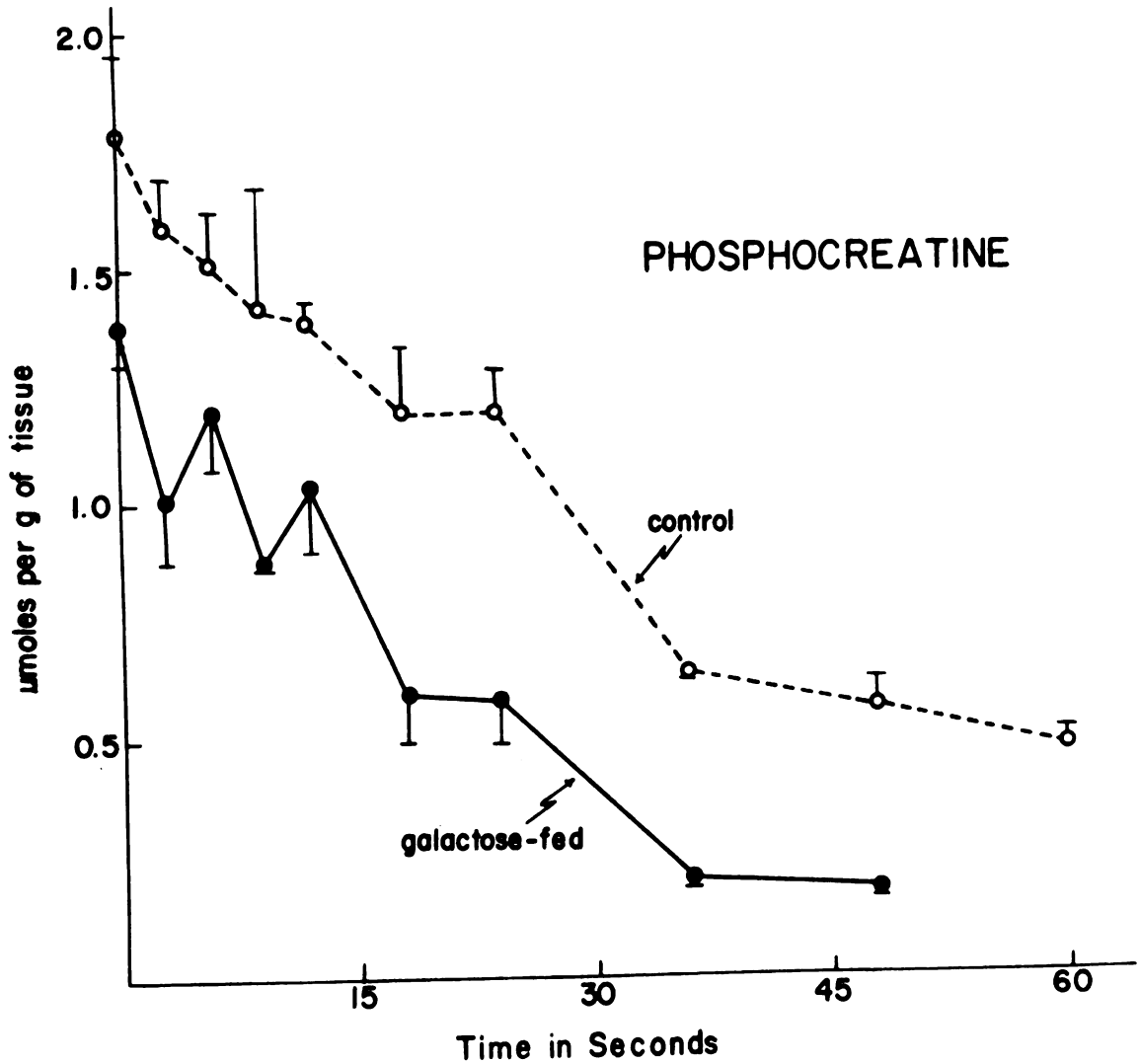

PHOSPHOCREATINE
2.0
1.5
1.0
0.5
μmoles per g of tissue
control
galactose-fed
15
30
45
60
Time in Seconds

Figure 13.--Changes as a function of the duration of postmortem ischemia in ATP and AMP in the brains of control or galactose-fed chicks.

The means ± SD for 3 or more determinations are plotted. Experimental conditions were the same as for Figure 12.

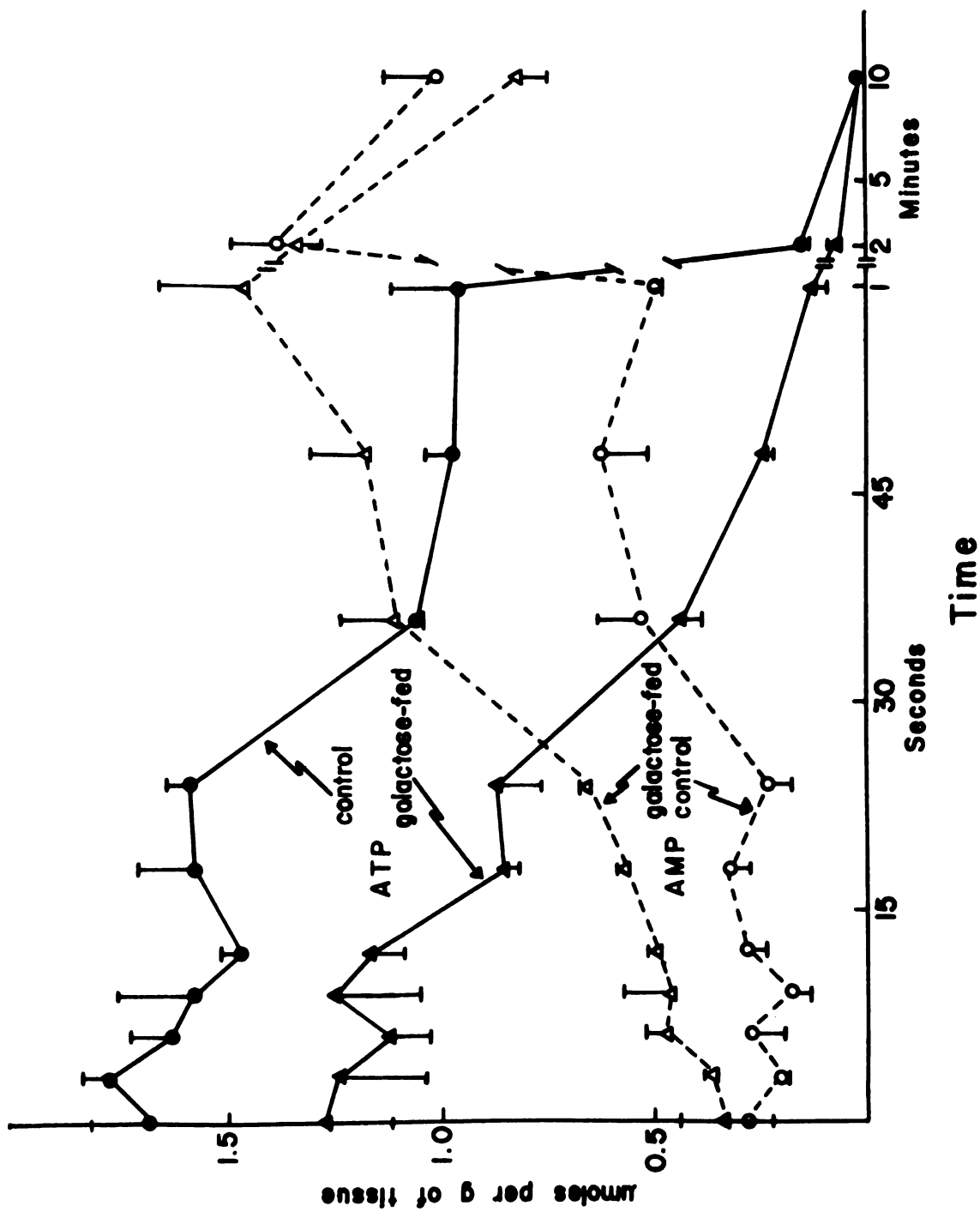

μmoles per g of tissue
1.5
1.0
0.5
ATP
control
galactose-fed
AMP
galactose-fed
control
15
30
45
Seconds
1
2
5
10
Minutes
Time

Figure 14.--Change in cerebral ADP levels under ischemic conditions.

See Figure 12 for experimental details.

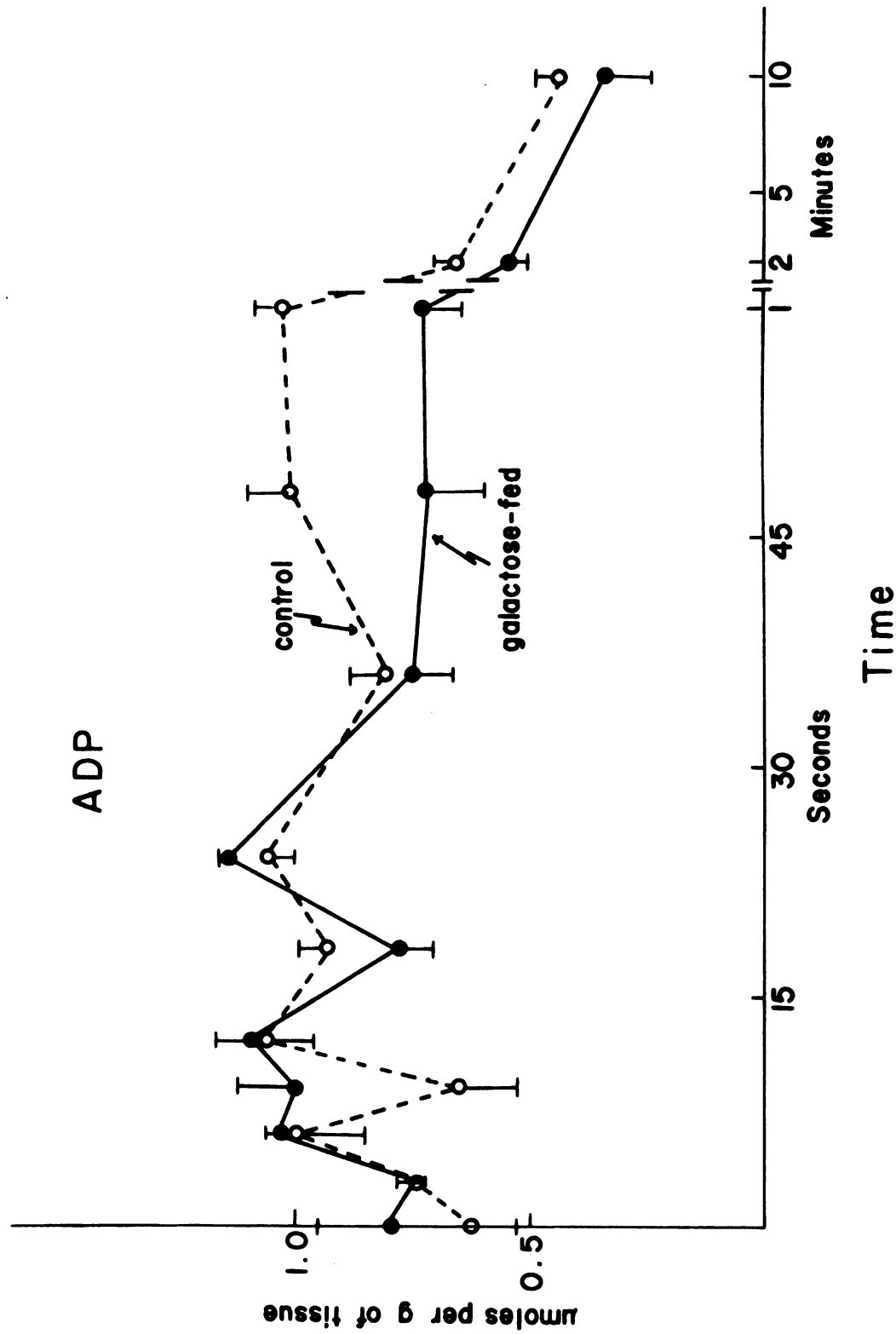

ADP
control
galactose-fed
1.0
0.5
μmoles per g of tissue
15
30
45
Seconds
1
2
5
10
Minutes
Time

Figure 15.--Effect of postmortem ischemia on total level of adenine nucleotides and energy charge in the brains of control and galactose-fed chicks.

Calculations were based on the averages presented in Figures 13 and 14. Energy charge (Atkinson, 1968) is defined by the following formula:

$$\frac{[ATP] + 0.5\ [ADP]}{[ATP] + [ADP] + [AMP]}$$

Adenine nucleotides are indicated with O,● and energy charge with △, ▲.

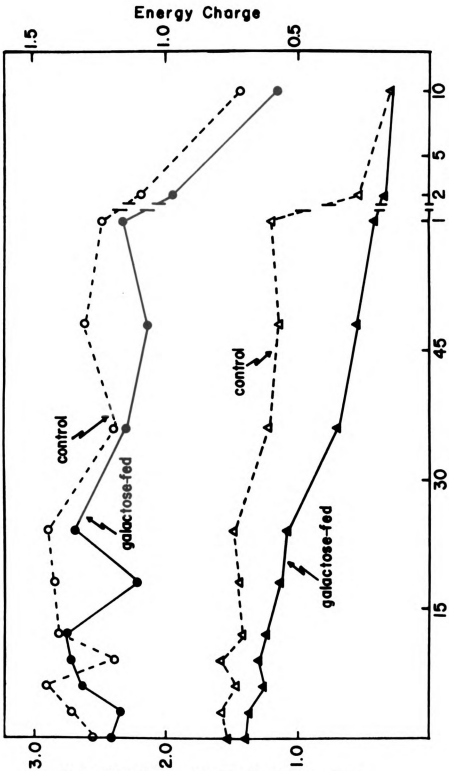
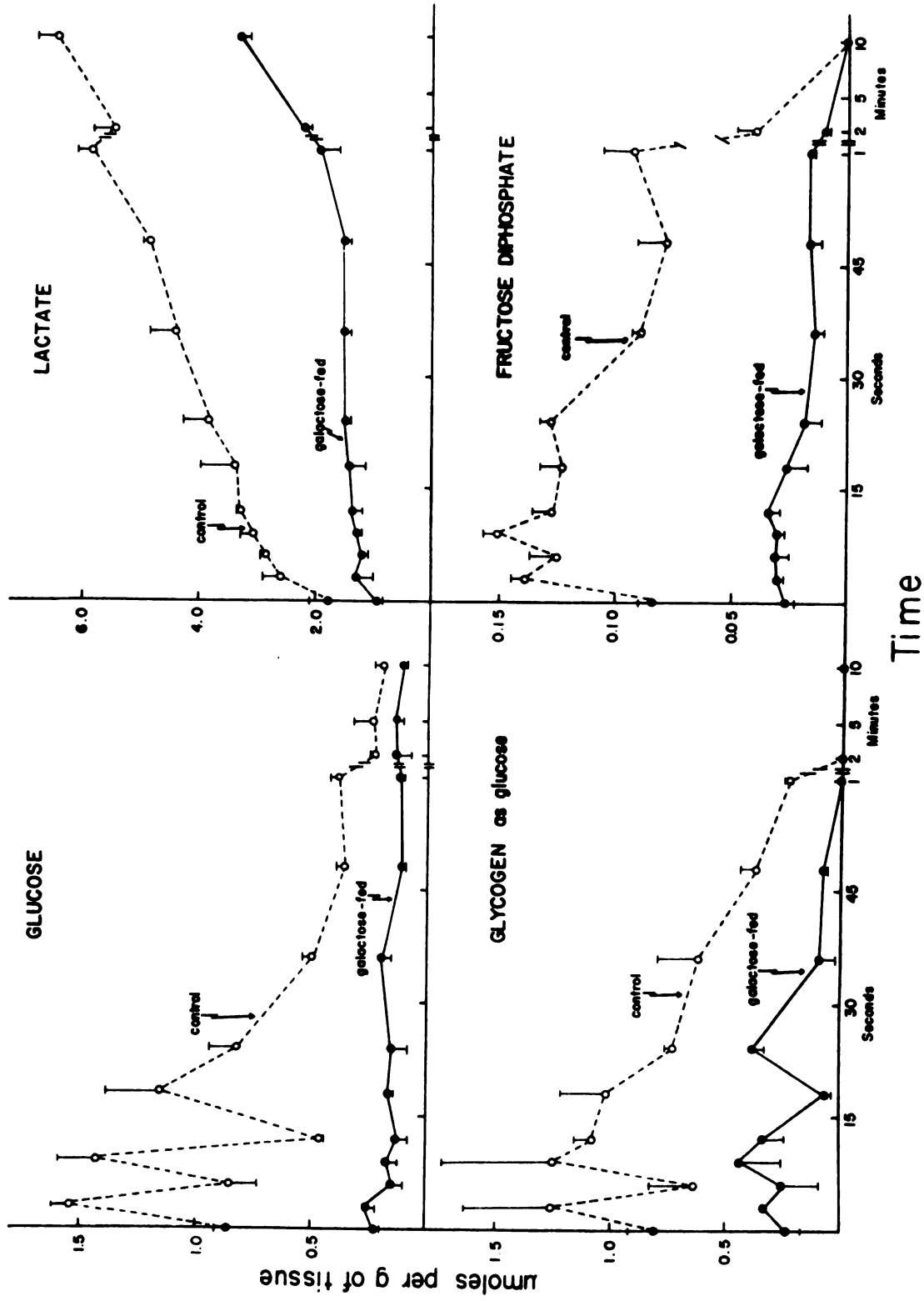

Energy Charge
1.5
1.0
0.5
Total Adenylate in μmoles per g of tissue
3.0
2.0
1.0
control
galactose-fed
control
galactose-fed
15
30
45
1
1½
2
5
10
Seconds
Minutes
Time

Figure 16.--Changes in various cerebral glycolytic metabolites and cerebral glycogen as functions of prolonged postmortem ischemia in control and galactose-fed chicks.

Plotted points represent means ± SD for 3 or more tissue samples. Experimental conditions were the same as for Figure 12.


LACTATE
GLUCOSE
FRUCTOSE DIPHOSPHATE
GLYCOGEN as glucose
control
galactose-fed
μmoles per g of tissue
Time
Seconds
Minutes

and increased in proportion to the decrease in ATP levels observed in either group of chicks. In contrast, although fluctuations were apparent, the levels of ADP (Figure 14) were generally unchanged up to one minute after decapitation; then they gradually decreased until at 10 minutes, low but detectable amounts remained. The ratio [ATP][AMP]/[ADP]^2 was calculated from data for adenine nucleotides at the various periods after decapitation, and gave an average of 0.60 ± 0.25 and 0.58 ± 0.19 for chicks fed galactose and control diets, respectively. These are in good agreement with values calculated for mouse brain by Lowry et al. (1964). As seen in Figure 15, the total adenine nucleotide content remained stable. The differences in the onset of expenditure of high energy phosphate between the dietary groups can be seen by the more rapid decline of whole brain energy charge in chicks fed galactose as compared with those fed control diets.

Analysis of brain glucose and glycogen revealed the low supply of these vital carbohydrate reserves in brains of chicks fed galactose (Figure 16). Immediately after onset of ischemia, the levels of glucose and glycogen in the brains of control chicks decreased synchronously in sawtooth-like patterns which were strikingly similar. A study was made of the variability of the glucose levels in brains of chicks fed the control diet during ischemia. Analyses of 4-5 animals per time period up to and including

18 sec revealed considerable standard deviation; e.g., 2.14 ± 0.72 μmol glucose/g of fresh tissue at zero time, 3.66 ± 1.31 at 3 sec after decapitation, and 1.13 ± 0.31 at 18 sec (Table 12). The wide standard deviation would indicate that the patterns for the levels of glucose and glycogen (Figure 16) are due to animal variability and do not suggest synthesis of either of the two molecules during ischemia. Large variation in cerebral levels of glucose (2.5 ± 1.2 μmol/g) among individual chicks has been previously noted, while the variations in ATP (1.9 ± 0.1 μmol/g) and lactate (3.0 ± 0.4 μmol/g) were less (Kozak and Wells, 1969). The large percentage of carbohydrate in the diet (60%) may also cause the individual variability to be more striking. Glycogen in the brains from chicks fed galactose was completely exhausted after one min of anoxia, while minor amounts of glucose remained (0.15 μmol/g of tissue). This level of glucose had been maintained in the animals fed galactose since 6 sec of anoxia had elapsed and persisted for the remaining experimental times. The fact that this concentration approximates 20 times the K_m of hexokinase for glucose (Crane and Sols, 1955) suggests that this residual glucose may be compartmentalized, e.g., due to trapped blood in the cerebrum, and unavailable to the enzyme. In fact, Dr. H. R. Knull (unpublished results) has demonstrated using [^{14}C]-inulin that there is contamination of powdered brain from blood.

Table 12.--Variation of Cerebral Glucose Levels in the Chick During Anoxia.

Period of Anoxia	Glucose
sec	µmol/g tissue
Zero time	2.14 ± 0.72
3	3.66 ± 1.31
6	2.21 ± 0.41
9	1.92 ± 0.84
12	1.60 ± 0.73
18	1.13 ± 0.31

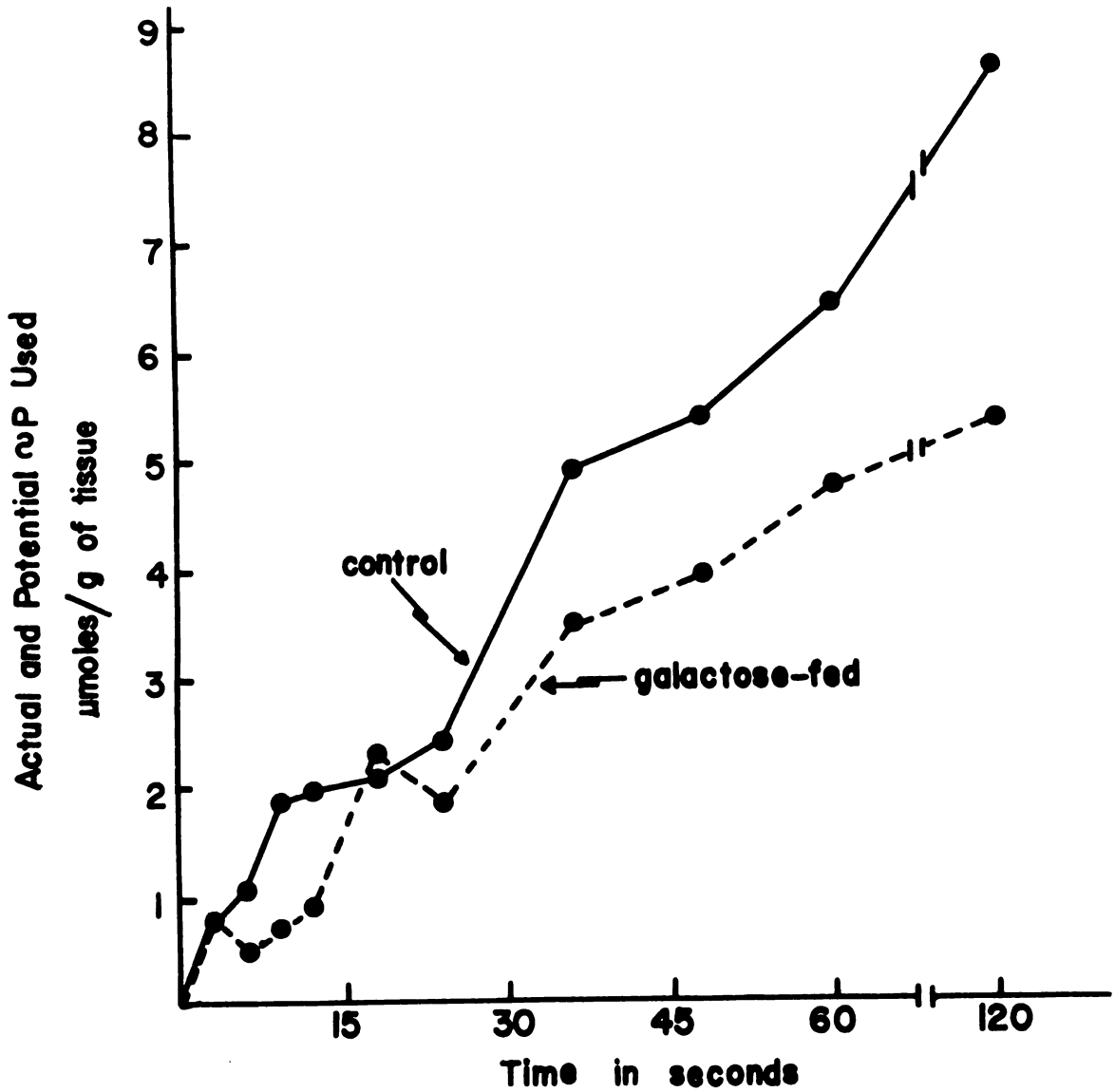
Note: The chicks were fed the control diet for 46 h, at which time they were sacrificed according to the "closed-system" techniques. The values represent the average ± standard deviation of determinations on 4 or 5 individual brains.

If the cerebral glucose is then corrected for blood glucose content, the zero time concentration in brain would be 0.1 μmol/g and the levels after 6 sec closer to the K_m for hexokinase than previously estimated.

That less energy could be derived from glucose in the animals fed galactose than in the controls after decapitation was supported by analyses of lactate. The level of lactate initially was depressed in brains from galactose-fed chicks and remained relatively constant throughout the first minute of anoxia, increasing significantly only between 2 and 10 minutes. The increase cannot be accounted for by corresponding decreases in glucose, glycogen, or glycolytic intermediates. In control tissue, a rapid increase in lactate, inversely proportional to the rate of utilization of glucose and glycogen, was observed following decapitation, in agreement with the results of Lowry et al. (1964) for normal mouse brain. Similarly, the concentration of fructose-1,6-diphosphate increased initially, then declined as carbon sources were depleted. No similar increase in the levels of fructose-1,6-diphosphate immediately after decapitation was observed in the brains of chicks fed galactose, presumably because of the paucity of carbohydrate reserves prior to decapitation. Inspection of the actual plus potential high energy phosphate utilization (Figure 17) reveals a generally depressed rate of energy expenditure in brains of chicks fed galactose.

Figure 17.--Utilization of actual and potential high-energy phosphates by chick brain during postmorten ischemia.

Calculations were based on the data presented in Figures 12, 13, 14 and 16, according to the formula specified in the text section on METHODS.


Actual and Potential ∿P Used
μmoles/g of tissue
9
8
7
6
5
4
3
2
1
control
galactose-fed
15
30
45
60
120
Time in seconds

During the entire "closed-system" study period, cerebral levels of galactose and galactitol (Table 13) were virtually unchanged at 9 periods of anoxia between "zero" time and 10 min, verifying the inability of either carbohydrate to act as a significant energy source (Kozak and Wells, 1971). However, variability among the various pools was again evident as previously noted for glucose.

Citrate, known to act as a modulator of phosphofructokinase (EC 2.7.1.11) activity, was quantified in brains of chicks of both dietary groups. At "zero" time, brain citrate levels were 0.183 ± 0.012 and 0.170 ± 0.012 μmol/g fresh tissue for control and galactose-fed groups, respectively.

Table 13.--Cerebral Levels of Galactose and Galactitol in the Chick after Various Periods of Anoxia.

Period of Anoxia	Galactose	Galactitol	Summation of Galactose and Galactitol
		µmol/g tissue	
Zero time	4.90	5.14	10.04
3 sec	2.87	2.92	5.79
6 sec	1.14	2.77	3.91
9 sec	2.26	2.61	4.87
18 sec	4.93 ± 0.29	6.71 ± 0.32	11.64
1 min	3.37	2.28	5.65
2 min	7.11 ± 0.18	4.19 ± 0.26	11.30
5 min	1.65 ± 0.23	4.61 ± 0.35	6.26
10 min	4.15	4.94	9.09

Note: The tissue was the same as used for the analyses in Figures 12-17. Where standard deviations are given, the values represent averages of 3 determinations on the pooled tissue; otherwise, 2 values were averaged.

DISCUSSION

The "closed-system" represents an attempt to evaluate the ability of the brain in the galactose-fed animal to carry out glycolysis sufficiently well to meet the requirements for energy production. Consideration of the expenditure of potential ~P points out that animals treated with galactose are unable to replenish significantly the energy expended because of the limiting amount of substrate available. This depletion of substrate effectively slows glycolysis down. In the control animals, 0.5 μmol glucose/g tissue (Figure 16) appears to be the critical level at which the brain can no longer maintain near-normal concentrations of ATP (Figure 13). During ischemia, in the animal treated with galactose, lactate does not accumulate until after 48 sec of anoxia have elapsed. Furthermore, fructose-1,6-diphosphate did not increase throughout the period of ischemia. Possibly the intrinsic ability of the glycolytic pathway to oxidize glucose in the chicks fed galactose is not impaired, and if more substrate were provided, metabolic control of phosphofructokinase and increased formation of lactate could be demonstrated.

The very severe lowering of the cerebral level of glucose simultaneous with normal plasma levels suggests that the translocation of glucose across the blood-brain barrier is limited. Additional evidence is provided by experiments in which the specific activity of brain glucose was monitored after intraperitoneal injection of [^{14}C]-glucose. A nearly 10-fold higher amount of [^{14}C]-glucose from plasma pools virtually equivalent in glucose concentrations and specific radioactivity was found in the brains of control-fed chicks compared with those of galactose-fed animals 5 min after administration of the tracer (Granett et al., in press). Bidder (1968) previously presented evidence suggesting a common transport mechanism for glucose and galactose based on their inhibition of 3-0-methylglucose transport across the blood-brain interface. In our studies, plasma galactose may reach values of greater than 22 mM in the galactose-toxic animals, whereas in the control animal, the free sugar is absent from the blood. The observation that treatment of the galactose-fed chicks with glucose, which doubles the plasma level of glucose, increases cerebral fructose diphosphate, lactate, glycogen and glucose to near normal levels also supports the concept that the intracellular concentration of glucose is limited by the competition of galactose with glucose at transport sites (Knull, Wells and Kozak, 1972).

Although the etiology for the galactose-induced neurotoxicity in the chick is undoubtedly complex, it is reasonable that the exhausted metabolic state of the brain described here is intimately involved in the abnormal motor activity and neuronal degeneration associated with the toxicity. The brain is unique in that it depends almost completely on carbohydrate furnished it by the blood for its energy sources. Its utilization represents 25% of the total bodily consumption of glucose, and the metabolic rate may be 20 times the average for the body as a whole (Bachelard, 1970). Thus impairment of glucose uptake by the brain which appears to occur in the galactose-toxic chick would be of major significance.

Comparison of the changes induced in energy metabolism in chick brain by galactose versus phenylalanine reveals an interesting similarity. Both agents produced a depressed cerebral metabolic state in the animals, galactose probably limiting the substrate available for glycolysis; however, no carbohydrate paucity in the brain was associated with the phenylalanine treatment and no evidence was obtained for a direct interaction of the amino acid with glycolysis. Thus the two compounds, galactose and phenylalanine, appear to exert primary and secondary effects on carbohydrate metabolism.

CHAPTER II

DETERMINATION OF GLYCOGEN FROM BRAIN WITH AMYLOGLUCOSIDASE

INTRODUCTION

After various periods of ischemia the concentration of glycogen in the brains of normal chicks decreased from 1.5 to 0.5 μmol of glucosyl units/g after 1 min and remained at this level after 10 min had elapsed (Granett, Kozak, McIntyre and Wells, in press). Since simultaneously glucose and high energy phosphate compounds were exhausted, it was logical that glycogen should have been utilized more completely. For these studies the glycogen had been isolated from perchlorate insoluble material, digested in base, precipitated in ethanol and quantified by enzymatic determination of the glucose released upon acid hydrolysis. Employing similar analytical methods Lowry, Passonneau, Hasselberger and Schulz (1964) also observed a glucose-containing material stable to ischemia. An attempt was made to demonstrate that the material isolated at 10 min was not glycogen using amyloglucosidase (α-1,4-glucan glucohydrolase), an enzyme shown to hydrolyze

α-1,4- and α-1,6-glucosidic linkages (Pazur and Kleppe, 1962). Incubation of partially purified glycogen with the enzyme revealed that glucose was released from the zero time sample and not from the 10 min sample, indicating that a non-glycogen substance containing glucose was present.

Of the methods available for glycogen quantification, the procedure employing phosphorylase degradation coupled with conversion of the glucose-1-phosphate to 6-phosphogluconate with phosphoglucomutase and glucose-6-phosphate dehydrogenase is most specific (Passonneau, Gatfield, Schulz and Lowry, 1967). The non-specific methods are based on a Pflüger type procedure (Pflüger, 1909; Good, Kramer and Somogyi, 1933) for isolation of the glycogen by a KOH digestion of the tissue and ethanol precipitation of the polysaccharide. This is followed by measurement directly with anthrone reagent (VanHandel, 1965) or indirectly by acid hydrolysis and enzymatic determination of the glucose with hexokinase and glucose-6-phosphate dehydrogenase (Slein, 1963) or glucose oxidase (Hugget and Nixon, 1957).

In the following chapter a method for quantification of glycogen utilizing amyloglucosidase is described. The assay is specific for glycogen in the brain and is equally as sensitive as the phosphorylase assay. Also it is very convenient; only one enzyme is required for the

degradation of the glycogen and no activation is necessary as with the phosphorylase method. While this work was in progress Jongkind, Corner and Bruntink (1972) published an abbreviated method for glycogen determination employing amyloglucosidase.

MATERIALS AND METHODS

Materials

Rabbit liver glycogen (Type III) and amyloglucosidase from Rhiszopus and Aspergillus were obtained from Sigma Chemical Co. (St. Louis, Mo.). The purified amyloglucosidase (α-1,4-glucan glucohydrolase; EC 3.2.1.3) (Pazure, Simpson and Knull, 1969) was a gift from Dr. Harvey Knull. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP oxidoreductase; EC 1.1.1.49) hexokinase (ATP : D-hexose-6-phosphotransferase; EC 2.7.1.1), phosphoglucomutase (α-D-glucose-1,6-diphosphate : α-D-glucose-1-phosphate phosphotransferase; EC 2.7.5.1), phosphorylase a (α-1,4-glucan : orthophosphate glucosyltransferase; EC 2.4.1.1) and nucleotides were obtained from Boehringer Mannheim (New York, N.Y.) or Sigma.

Isolation of glycogen from brain

Chicks were decapitated directly into liquid N_2; brain tissue was chipped out and powdered over dry ice in a cold room and later stored at -90°C. The tissue was homogenized in 0.6 M perchlorate-1 mM EDTA (0.5 ml/100 mg)

and the homogenate centrifuged for 10 min at 12,000 g. The pellet was digested in 5 N KOH (0.4 ml/100 mg original tissue) for 30 min at 100°C, whereupon 3% Na_2SO_3 (0.1 ml/100 mg) and absolute ethanol (1 ml/100 mg) were added. Glycogen precipitated upon cooling of the samples to -20°C for 1 h or overnight and was recovered by centrifugation and washed in 70% ethanol (0.5 ml/100 mg) (Walaas and Walaas, 1950). The pellet was then dried with warming under a N_2 stream.

Quantification of glycogen by acid hydrolysis

A standard solution of glycogen (8mg/50 ml) was diluted 1:1 with 2 N H_2SO_4 and hydrolysed in a sealed ampule for 3 h at 100°C. Partially purified glycogen from brain was dissolved in 1 N H_2SO_4 (0.6 ml/100 mg) in an ampule and hydrolysed similarly. The hydrolysates were neutralized with NaOH and the glucose content was determined enzymatically.

Quantification of glucose

Free glucose was determined enzymatically according to a modification of the method of Slein (1963). The reaction cuvette contained the following components expressed as final concentration in a 0.25 ml volume: 320 mM Tris-HCl, pH 8; 6 mM $MgCl_2$; 1 mM ATP; 0.32 mM $NADP^+$; 10 µg/ml glucose-6-phosphate dehydrogenase; 20 µg/ml

hexokinase. The production of NADPH was monitored on a Gilford 2400 recording spectrophotometer at 25°C at 340 nm.

Quantification of glycogen with amyloglucosidase

A standard glycogen solution (8 mg/50 ml) was diluted 1:1 with 0.1 M citrate buffer, pH 5, or glycogen isolated from tissue was dissolved in 50 mM citrate, pH 5 (100 mg original tissue/ml). Amyloglucosidase dissolved in buffer (20 mg/ml) was added, 20 µg/ml. If crude enzyme were used, the undissolved material was first removed by centrifugation. Samples were incubated with shaking at 37°C for 2 h or as specified, placed in ice and assayed for glucose released by the enzymatic method previously described. Tissue samples were centrifuged to clarity before being assayed.

Recovery experiments

Standard glycogen (8 mg/50 ml) was added to the perchlorate pellet obtained upon deproteinization of 100 mg of tissue, and the glycogen was isolated and quantified as previously described. Addition of glycogen before the perchlorate step resulted in incomplete precipitation of the standard presumably because of inefficient trapping of the glycogen by tissue proteins. If tissue samples were deproteinized both in the presence and absence of standard glycogen and the perchlorate extracts incubated

with amyloglucosidase, glucose release could be demonstrated only for those samples to which glycogen had been initially added.

Enzymatic assay for debranching activity

The method of Passonneau et al. (1967) was employed except that BSA was omitted. The assay consisted of the following components expressed as final concentration: 0.05 M recrystallized imidazole, pH 7; 0.5 mM MgAcetate; 1 mM $NADP^+$; 0.1 mM AMP; 5 mM inorganic phosphate; 0.25 mM glycogen (as glucosyl units); 6 µg/ml phosphoglucomutase; 4 µg/ml glucose-6-phosphate dehydrogenase. Phosphorylase a and accompanying debranching activity (Sigma) were activated with BSA, dithiothreitol, AMP and heating at 38°C for 60 min as described (Passonneau et al., 1967). The phosphorylase was added to the reaction to give a final concentration of 10 µg/ml. The reaction was initiated by addition of the enzymes and increase in absorbancy at 340 nm at 25°C was monitored. The initial rate of the reaction due to phosphorylase activity is very rapid (0.04 OD units/min) and gradually slows as the outer tiers of the glycogen are cleaved and becomes limited by slow debranching activity.

Purification of debranching activity

Amylo-1,6-glucosidase was purified from frozen rabbit muscle according to the method of Cori (1955) through the first ammonium sulfate precipitation and dialysis. The procedure of Nelson, Kolb and Larner (1969) was also employed, isolating the activity from fresh rabbit muscle. The enzyme was used after the ammonium sulfate and dialysis steps. Upon assay of the activity, a significant reaction in the absence of added glycogen was observed. This was attributed to entrapment in the ammonium sulfate crystals of glycogen that had been added during purification. This component was reduced considerably by reprecipitation of the dialysate with ammonium sulfate (45% saturation). A portion of this material was then dissolved in 0.005 M Tris - 0.0005 M EDTA - 0.01 M 2-mercaptoethanol without further dialysis and was used in the assays.

Purification of pullulanase activity

Pullulanase was purified as an extracellular enzyme from the culture filtrate of batchwise-cultivated Aerobacter aerogenes according to the procedure of Bender and Wallenfels (1966). The enzyme was precipitated with acetone, dissolved in buffer (50 mg/ml) and dialysed against 0.02 M phosphate buffer, pH 6.8 for 24 h. This enzyme catalyzes the hydrolysis of α-1,6 linkages in the branched

α-glucan, pullulan. It also attacks glycogen and was of interest for this reason.

Assay of pullulanase activity on glycogen

To 12.7 mg of glycogen dissolved in 0.1 ml of distilled water was added 0.1 ml of the pullulanase solution. The pH was corrected from 6.8 to 5 with 1 N HCl. The mix was incubated with shaking at 37°C for up to 3 h. Aliquots measuring 10 µl were spotted at selected intervals on Whatman No. 1 paper and developed twice with ascending chromatography in butanol : pyridine : water (6 : 4 : 3, v/v/v). Oligosaccharides were detected with silver nitrate dip reagent (Mayer and Larner, 1959) as follows: the dry chromatogram was dipped into a solution containing 1 ml of a saturated silver nitrate solution, 6 ml of water and 200 ml of acetone, air dried and then placed into a tray containing 40 ml of 10% NaOH and 200 ml of methanol. After black spots indicative of reduced Ag^{+} appeared, the chromatogram was placed in 0.05 M $Na_2S_2O_3$ to wash out remaining silver ions. It was then air dried. Dilution of commercially available Karo syrup provided maltooligosaccharide references.

RESULTS

Quantification of standard glycogen by acid hydrolysis and amyloglucosidase methods

Comparison of standard glycogen determined as glucosyl units/μg either by acid hydrolysis followed by enzymatic quantification of glucose or with purified amyloglucosidase revealed the two methods to be identical (Figure 18). Assay of glycogen with crude amyloglucosidase from either Rhizopus or Aspergillus resulted in similar release of glucose.

Time course study of the release of glucose from glycogen by amyloglucosidase

Glycogen isolated from tissue was hydrolysed by amyloglucosidase with and without added glycogen (Figure 19). The reaction was completed within 1 h but samples were routinely incubated for 2 h.

Recovery of glycogen added to tissue

Table 14 illustrates that standard glycogen added to brain tissue after perchlorate deproteinization could be recovered and quantified by the amyloglucosidase method.

Figure 18.--Comparison of standard glycogen degradation by acid hydrolysis and amyloglucosidase.

Hydrolysis in sulfuric acid or enzymatic cleavage with amyloglucosidase was conducted as described in METHODS.

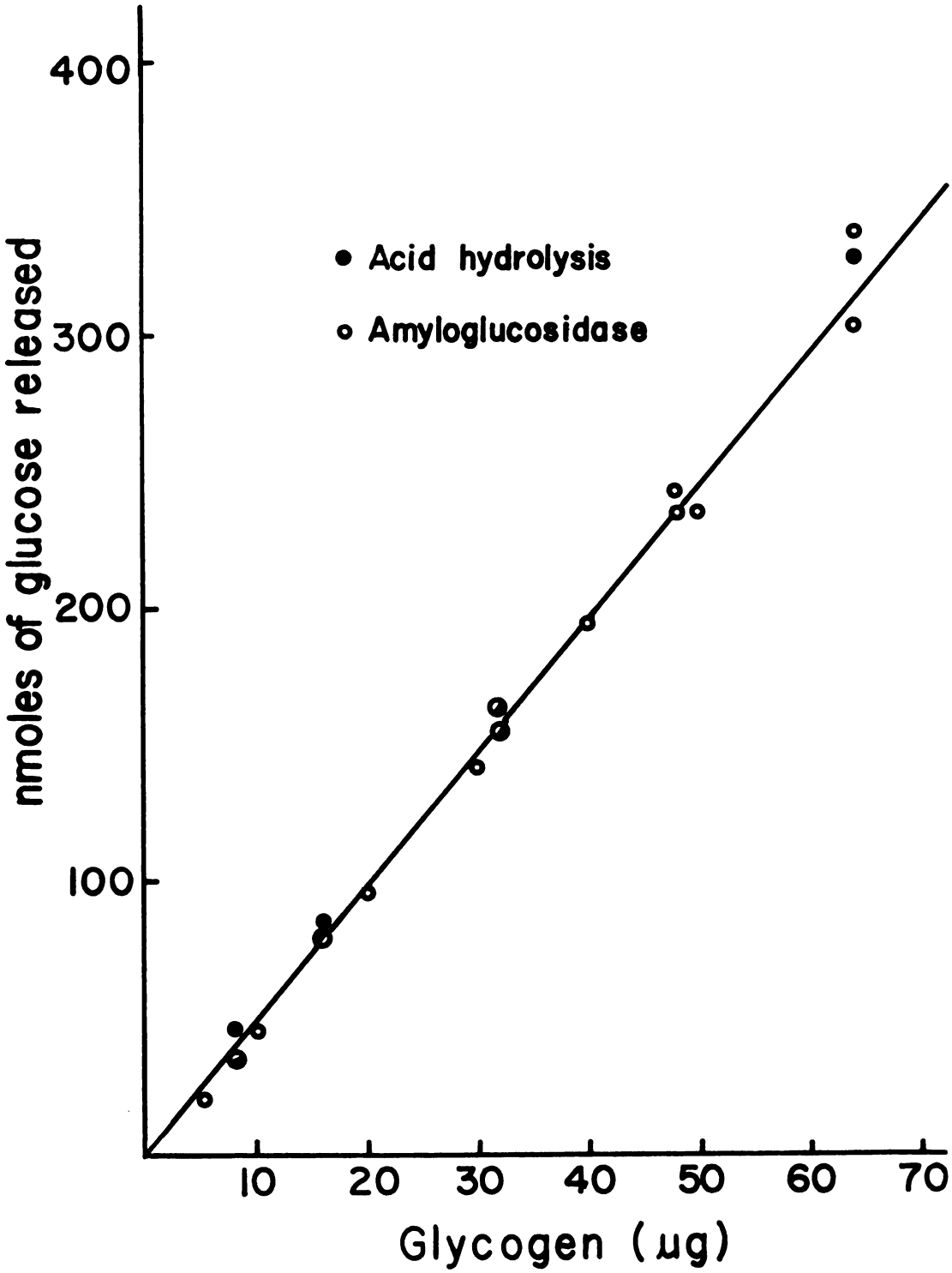

400
300
200
100
nmoles of glucose released
● Acid hydrolysis
○ Amyloglucosidase
10
20
30
40
50
60
70
Glycogen (µg)

Figure 19.--Analysis with time of the hydrolysis of glycogen by amyloglucosidase.

Glycogen isolated from brain tissue was incubated with amyloglucosidase in the presence or absence of 49.5 µg of standard glycogen over a 4 h period. At hourly intervals an aliquot was removed and assayed for glucose.

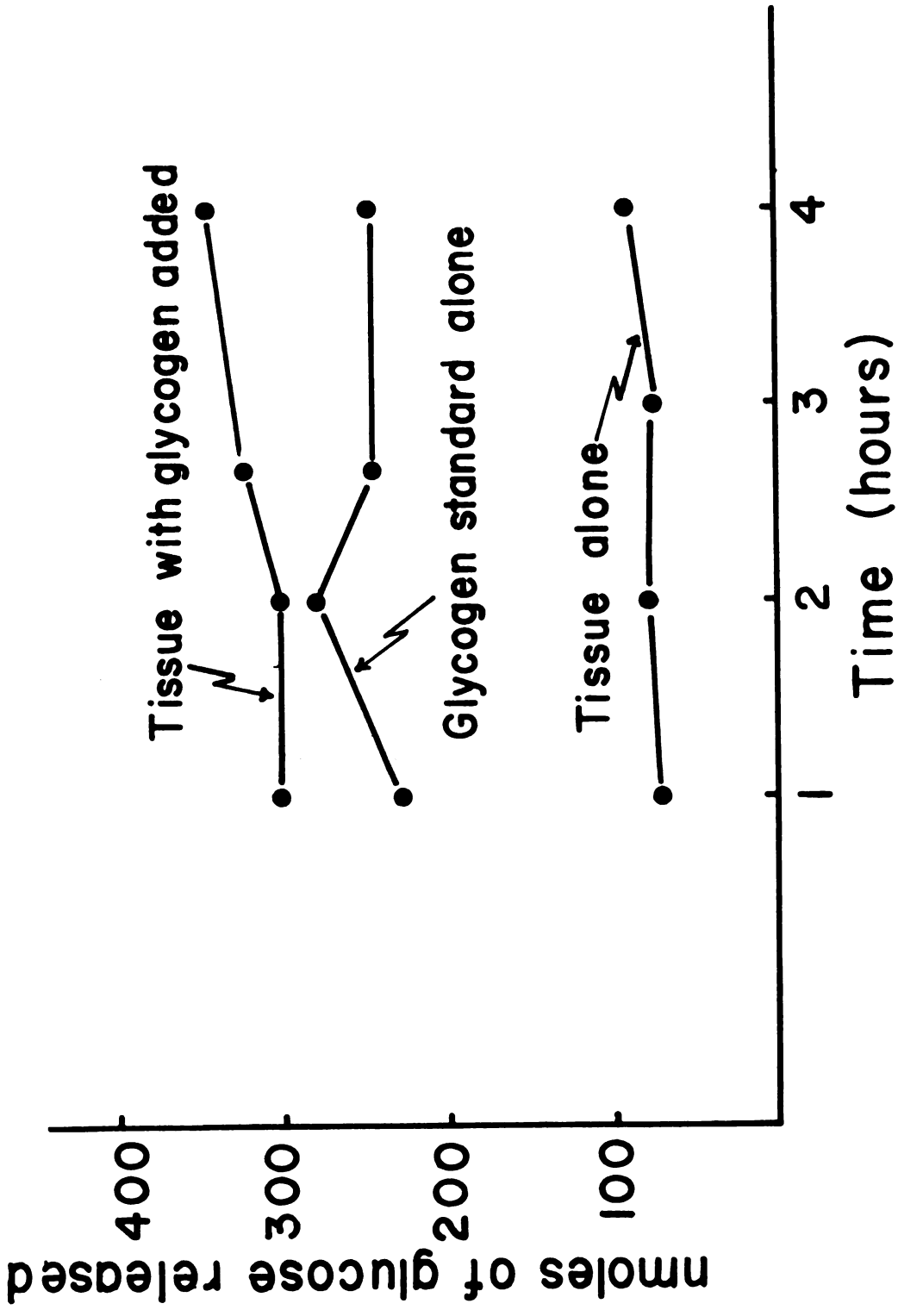

Tissue with glycogen added
Glycogen standard alone
Tissue alone
nmoles of glucose released
400
300
200
100
1
2
3
4
Time (hours)

Table 14.--Recovery of Standard Glycogen from Tissue Samples.

Sample No.	Glycogen added (glucosyl units)	Tissue glycogen (glucosyl units) With	Tissue glycogen (glucosyl units) Without	%Recovery
1	79 nmol	204 nmol	109 nmol	124
2	79	200	108	116
3	79	167	73	119
4	79	142	68	94
5	119	221	111	93
6	119	232	106	105
7	119	228	101	107
				av $\overline{108} \pm 12$

Note: Glycogen was isolated from brain tissue and standard was added as described in METHODS. The glycogen was hydrolysed with amyloglucosidase.

Quantification of glycogen levels in brain by acid hydrolysis and amyloglucosidase methods

Tissue samples from the "closed-system" studies described in the previous chapter were used. A discrepancy was noted between the levels of glycogen determined by the two methods; the values resulting from the amyloglucosidase method were consistently lower than those obtained by acid hydrolysis (Table 15). The differences measured approximately 0.5 μmol glucosyl units/g with the exception of the samples at 24 and 48 sec which differed by 0.2 μmol/g.

Assay of standard glycogen with phosphorylase

Phosphorolysis of standard glycogen with phosphorylase a (Sigma) resulted in incomplete degradation as calculated from the weight of glycogen assayed (assuming a molecular weight of 162 for the glucosyl unit). This was attributed to inactive debranching enzyme. Attempts made to purify the debranching complex from rabbit muscle yielded active phosphorylase but the α-1,6-glucosidase-transglucosylase was inactive in the assay system we were using. Degradation of glycogen standards was consistently 42 ± 8% (n=7), which corresponds to the figure of 40% reported for the percentage of glucosyl units composing the outer tier of the glycogen molecule. It would appear then that the phosphorylase was not proceeding beyond the first branch point.

Table 15.--Comparison of Glycogen Levels in Tissue Determined by Acid Hydrolysis and Amyloglucosidase Methods.

Tissue Sample	Acid Hydrolysis	Amyloglucosidase
	µmol of glucose released/g of brain	
3 sec	1.77 ± 0.38	1.09
12 sec	1.59 ± 0.08	1.08
24 sec	1.24 ± 0.04	0.97
36 sec	1.12 ± 0.19	0.71
48 sec	0.87 ± 0.08	0.68
5 min	0.55 ± 0.01	0.00
10 min	0.52 ± 0.04	0.05

Note: Tissue samples were taken from pools of control brains subjected to the periods of ischemia indicated. The values for acid hydrolysis represent averages of 3 determinations ± SD; those for the amyloglucosidase method are averages of 2 differing from each other by 10% or less.

The assay was also supplemented with pullulanase, an enzyme that hydrolyses the α-1,6-linkages in pullulan, a highly branched α-glucan. The enzyme itself was judged active by the appearance of materials chromatographing with maltose, maltotriose, maltotetrose and maltopentose upon incubation with glycogen at 37°C for 1 to 3 h. However, inclusion of it in the glycogen assay resulted in no further release of glucose-1-phosphate above that obtained with phosphorylase alone.

Assay of tissue glycogen with phosphorylase and amyloglucosidase

The partially purified glycogen from 100 mg of brain was dissolved in 1 ml of water and centrifuged to clarity. Aliquots of the supernatant were subsequently assayed for glycogen using the phosphorylase and amyloglucosidase methods. As indicated in Table 16 glucose release was observed for both methods for the 3 sec ischemic tissue but not for the 5 min samples. More glucose was released with amyloglucosidase as would be expected if degradation was more complete than with the phsophorylase.

Table 16.--Degradation of Glycogen Isolated from Tissue by Phosphorylase and Amyloglucosidase.

Tissue Sample		Phosphorylase Assay	Amyloglucosidase Assay
		nmol of glucose released	
3 sec	1	45	194
	2	50	161
5 min	1	7	none detectable
	2	2	none detectable

Note: The tissue for these analyses was taken from pools of control brains subjected to the periods of post-mortem ischemia indicated. The assays performed with phosphorylase and amyloglucosidase are described in METHODS.

DISCUSSION

Glycogen isolated from 100 mg of brain by a modified Pflüger technique contains impurities that are detectable from the milligram quantity of material recovered; the glycogen component itself would consist of 15 - 30 micrograms. Consequently a fairly specific method must be utilized for accurate quantification of the polysaccharide. The enzyme amyloglucosidase was investigated for this purpose.

It was demonstrated to hydrolyze standard glycogen as quantitatively as does 1 N H_2SO_4. The reaction was conducted at pH 5, the pH optimum of the enzyme. If the reaction were run at pH 7, enabling coupling with hexokinase and glucose-6-phosphate dehydrogenase, the amyloglucosidase activity became limiting, having been reduced by 80%. Lowering of the pH to 6, better favoring the glucosidase (decreased in activity by 25%) resulted in an incomplete conversion of the glucose. Thus the assay consisted of two steps. Determination of the glucose released from tissue samples was accomplished with hexokinase and glucose-6-phosphate dehydrogenase rather than with

glucose oxidase, as a slight turbidity remained in the incubate after centrifugation. Also an important advantage is that due to the fluorescence of NADPH the method can be adapted for fluorometry for greater sensitivity (Lowry et al., 1964).

Unfortunately because of the incomplete degradation of glycogen obtained with phosphorylase, any direct, quantitative comparison cannot be made between that procedure and the one utilizing amyloglucosidase described here.

The method is reproducible as illustrated by the good replication obtained both with standard glycogen (Figure 18) and tissue samples (Table 15).

Comparison of the procedure with acid hydrolysis degradation of tissue glycogen (Table 15) reveals the greater specificity obtained with amyloglucosidase. The hydrolysis of the tissue glycogen is presumed to be complete since glycogen added to tissue could be quantitatively recovered. It is postulated that the additional glucosyl units released by H_2SO_4 at 100°C may originate from glycoproteins that survived the alkali-digestion. Brunngraber (1970) estimated that 2.7 µmol of hexose are derived from the glycoproteins in 1 g of rat brain. Qualitatively, a non-glycogen component is indicated by the absence of any substantial release of glucose from the 5 min tissue samples reacted with phosphorylase (Table 16).

The ratio $\frac{\text{glycogen level at 5 min}}{\text{" " " 3 sec}}$ based on acid hydrolysis degradation approximates 1/3. If the 5 min material is indeed glycogen, this same relationship should be maintained regardless of assay method employed, and from the data in Table 16 this did not occur.

In conclusion, the data presented here suggests that values reported for the levels of glycogen in various tissues determined by acidic degradation of the polymer may be erroneously high, depending on the amount of non-glycogen, glucose-containing material present.

ACKNOWLEDGMENTS

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SUMMARY

Feeding a diet 40% in D-galactose (w/w) to newly hatched chicks results in a syndrome characterized by shivering, seizures, ataxia and eventual death. The rate of glycolysis was examined in the brains of these animals, employing the "closed-system" technique. Cerebral levels of phosphocreatine, ATP, ADP, AMP, fructose-1,6-diphosphate, glucose, glycogen and lactate were quantified for various periods of post-mortem ischemia ranging from "zero" time to 10 min. The changes in the metabolites at "zero" time confirmed the previous observations: phosphocreatine, ATP, fructose-diphosphate, glucose, lactate and glycogen were reduced in the galactose group. Concentrations of phosphocreatine declined immediately in both galactose-fed and control animals upon production of ischemia. ATP expenditure occurred in the brains of animals fed galactose at 12 sec of ischemia whereas this occurred significantly later (24 sec) in control animals. In both situations AMP levels increased in proportion to the decrease in ATP and reflected the utilization of ATP more accurately than did ADP. The adenine nucleotide energy

charge declined more rapidly in the chicks fed galactose than in those fed control diets. Glycogen reserves were depleted sooner in the galactose-fed group. Cerebral glucose levels were initially low in the chicks fed galactose, 0.3 μmol/g of brain; they decreased to 0.15 μmol/g after 6 sec of ischemia and were maintained at this level for the remaining time. Metabolic control of cerebral glycolysis at the phosphofructokinase point could be demonstrated for the control animals but not for the galactose-fed chicks. Lactate accumulation characteristic of the anoxic state was rapid and immediate in controls but delayed for 1 min in the galactose-intoxicated chicks. Inspection of the utilization of actual and potential high energy phosphates disclosed a depressed rate of energy expenditure in the brains from galactose-fed chicks. Galactose and galactitol were quantified and observed not to decrease during the 10 min ischemia, indicating they were not energy sources. The level of citrate in the brain, of interest as a modulator of phosphofructokinase, did not differ between the two groups.

The striking paucity of utilizable carbohydrates in the brains of the galactose-fed animals appears to be major factor in the slow-down of glycolysis and in the inability of the chicks to replenish the high energy phosphates expended. Interference of glucose entry into

the brain by galactose is postulated as responsible for the severe lowering of the cerebral levels of glucose.

Analysis of partially purified glycogen by acid hydrolysis at the 10 min period of ischemia revealed significant release of glucose (0.5 μmol/g of tissue) that was not detected upon degradation of the sample with amyloglucosidase, an enzyme that hydrolyzes α-1,4- and α-1,6- glucosidic linkages. This material also was unreactive with phosphorylase. Degradation by amyloglucosidase was proposed as a method more specific than acid hydrolysis for quantification of glycogen from brain. Conditions for hydrolysis of the glycogen consisted of incubation of the glycogen in citrate buffer, pH 5, with the enzyme, followed by enzymic quantification of the glucose released by reaction with hexokinase and glucose-6-phosphate dehydrogenase at pH 8. The method was demonstrated to be accurate and precise and is capable of the sensitivity obtained with the phosphorylase assay.

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