

ENERGY METABOLISM IN
PHENYLKETONURIC MODELS,
RELEVANCE AS A MECHANISM FOR
PHENYLALANINE TOXICITY

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Sandra Elizabeth Granett
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ABSTRACT

ENERGY METABOLISM IN PHENYLKETONURIC MODELS: RELEVANCE AS A MECHANISM FOR PHENYLALANINE TOXICITY

By

Sandra Elizabeth Granett

Cerebral energy metabolism was investigated in the following phenylketonuric models: (1) chicks fed diets 7-1/2 and 10% in L-phenylalanine; (2) chicks fed diets 5 and 10% in phenylacetic acid; (3) a dilute lethal strain of mice possibly deficient in phenylalanine hydroxylase.

It was found that brain ATP and creatine phosphate levels did not decrease in any of the three situations, as would be expected under an energy stress condition; and fluctuations in glycolytic intermediates could be ascribed to factors other than an inhibition of glycolysis. In the mice, the decrease in L-alpha-glycerol phosphate was more than expected from the decrease in glucose. In the phenylacetic acid feeding experiments, preliminary observations indicate that phenylacetic acid may interfere with glucose transport across the blood brain barrier.

All three series of animals suffered from caloric insufficiency. Chick weight gain was depressed and liver glycolytic intermediates were very much reduced as characteristic of gluconeogenesis. Hepatic ATP levels in the phenylacetic acid fed chicks were decreased, possibly due to the liver utilizing ATP in the conjugation of the acid with

ornithine. Monitoring uric acid concentrations as an indication of purine catabolism was unsatisfactory.

A perchlorate-ethylacetate extraction procedure for blood and tissue was devised for simultaneous quantitation of aromatic acids from blood or tissue by gas liquid chromatography. Phenylacetic acid, p-OH phenylacetic acid, and phenyllactic acid are satisfactorily recovered and these acids can be validly quantified. Recoveries of o-OH phenylacetic acid, phenylpyruvic acid, and p-OH phenylpyruvic acid are acceptable for detection purposes only. A survey of the aromatic acids present in the plasma and brain of chicks fed L-phenylalanine was made, and the significance of aromatic acids to the mechanism of mental retardation is discussed.

A convenient method for amino acid determination from tissue employing paper chromatographic and gas liquid chromatographic methods is presented.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1970

G-64421
10-23-70

This is dedicated to my parents
and
to Jeffrey

ACKNOWLEDGEMENTS

I want to express my appreciation to Dr. William W. Wells for his assistance in carrying out the experiments and for the helpful discussions.

My committee members: Dr. W. W. Wells, Dr. R. L. Anderson, Dr. L. L. Bieber, Dr. J. E. Wilson, and Dr. P. J. Gehring are acknowledged for their patience with me and for the insight into research this experience has given me.

I thank Jeff (my husband) for his encouragement, willingness to help wherever possible, and cheery love. This research was conducted in the first year of our marriage.

My parents, Rudolph and Mildred Spieker, are thanked for their belief in education.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BSA	N,O bis - (trimethylsilyl) - acetamide
BSTFA	bistrifluorotrimethylsilylacetamide
CNS	central nervous system
Cr-P	creatine phosphate
DOPA	dihydroxyphenylalanine
F-6-P	fructose-6-phosphate
FdP	fructose diphosphate or fructose-1,6-diphosphate
GABA	gamma-aminobutyric acid
GLC	gas liquid chromatography
Glu	glucose
G-6-P	glucose-6-phosphate
α -GP	alpha-glycerol phosphate
Glut	glutamate
Gly	glycogen
HSCoA	coenzyme A
Lac	lactate
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)
PA	phenylalanine
PAA	phenylacetic acid
PLA	phenyllactic acid
PPA	phenylpyruvic acid
PKU	phenylketonuria
PPI	pyrophosphate
3-PGA	3-phosphoglyceric acid
PPO	2-5 diphenyloxazole
POPOP	1,4 bis 2- (50phenyloxazolyl)-benzene
TMS	trimethylsilyl
Tris	tris (hydroxymethyl) aminomethane

INTRODUCTION

The genetic lesion in phenylketonuria has been identified as a mutation in the phenylalanine hydroxylating system that converts L-phenylalanine to L-tyrosine. Accumulation of phenylalanine and aromatic acids in the urine and blood of the phenylketonurics has been demonstrated, though the concentration of these acids in the tissues of these patients is unknown or of doubtful accuracy because of post mortem changes. Also patients usually display some grade of mental retardation, the mechanism of which has not been elucidated. The synthesis of proteins, lipids, and neurologic transmitter substances under high concentrations of phenylalanine and aromatic acids have received considerable attention; however, energy metabolism has only recently been emphasized.

This research was initiated to study cerebral energy metabolism in three model systems pertinent to phenylketonuria:

1. Chicks fed high levels of L-phenylalanine;
2. Chicks fed high levels of phenylacetic acid, an aromatic acid metabolite of phenylalanine known to produce toxicity;
3. Dilute lethal strain of mice, possibly deficient in the phenylalanine hydroxylase system.

LITERATURE REVIEW

Phenylketonuria

A. Characteristics of phenylketonuria

Between 1939 and 1953 Jervis demonstrated that phenylketonuria is inherited through a single autosomal recessive gene (1) and that phenylalanine accumulates in the patients (2). He subsequently identified the genetic defect as the enzyme system oxidizing phenylalanine to tyrosine (3). In a series of phenylketonuric patients the plasma phenylalanine level ranged from 1.03 mM to 6 mM with an average of 2.26 mM as compared to a normal range of 0.054 to 0.12 mM (4). Incidence of occurrence in the general population is estimated at 2 to 6 per 100,000 (5). The most dominant, though not unique, symptom is mental retardation. Two independent studies on intelligence conclude that most I.Q.'s are less than 20 (idiot class) and that the remainder are less than 50 (imbecile class) (4). A few high grade patients are found however. Table 1 summarizes the clinical features.

The melanin insufficiency indicated by the blond hair and blue eyes has been attributed to the phenylalanine inhibition of the tyrosinase system (6-7).

Defective myelination is generally observed (4). This was substantiated by the decrease in proteolipid protein, cerebroside, and myelin lipids observed by Prensky (8)

Table 1: Major Clinical Findings in Phenylketonuria (4)

Finding	Incidence, per cent	Occurrence in low-grade patients
Agitated behavior	90-32	+
EEG abnormalities	80	- /
Muscular hypertonicity	75	-
Microcephaly	68	+
Hyperactive reflexes	66	+
Blond hair, blue eyes	62	-
Inability to talk	63	+
Hyperkinesis	50	+
Inability to walk (and usually incontinence)	35	+
Tremors	30	+
Eczema	19-34	-
Seizures	26	+

Those findings occurring more frequently or severely in low-grade patients are marked +.

/ The incidence of EEG abnormalities is not different in high- and low-grade patients, but the latter may have more obviously abnormal tracings.

upon autopsy examination of frontal white matter in brains of phenylketonurics. Menkes (9) detected peculiarities in the proteolipid fraction from a brain of a phenylketonuria patient; however, total lipids, phospholipids, cholesterol, lipid hexose, cholesterol esters, and gangliosides were normal. Oteruelo et al. (10) reported electron microscopy studies on the brains of two PKU patients, observing changes in myelin sheaths, glial cells, synaptic processes, and extracellular space.

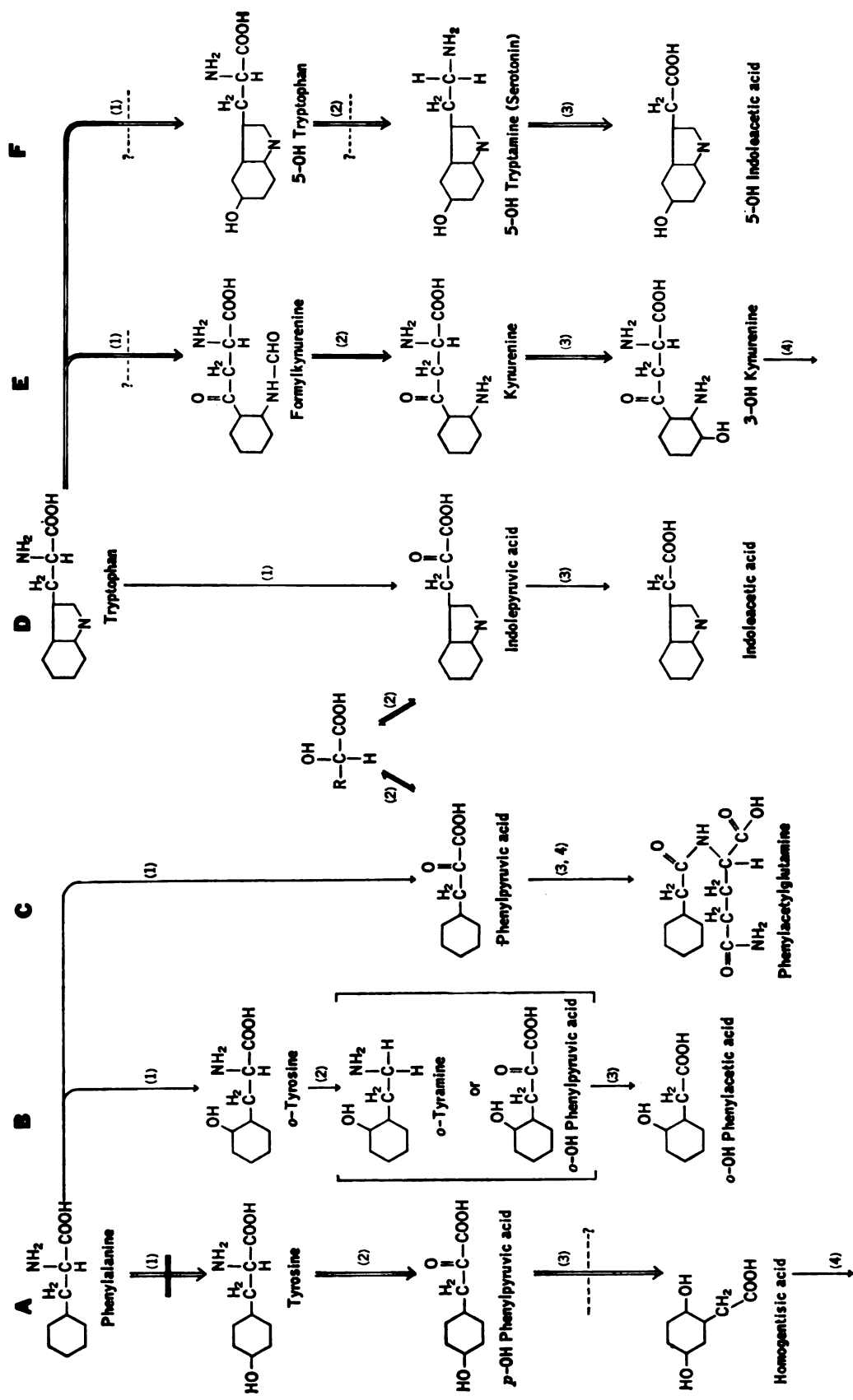
Serotonin and epinephrine (12) are depressed in the plasma of phenylketonurics; however the epinephrine decrease was found in other mental defectives as well.

Since the normal route for phenylalanine metabolism is blocked by the genetic defect, the patient depends on other pathways to relieve the phenylalanine accumulation. These are outlined in Figure 1. o-Hydroxyphenylacetic acid, phenyllactic acid, phenylpyruvic acid, and p-hydroxyphenylpyruvic acid have been quantitated in phenylketonuria-urine by Hoffman and Gooding (13). Woolf (14) detected phenylacetic acid and phenylacetyl-glutamine in urine. Jervis and Drejza (15) reported values for phenylpyruvic acid and o-OH phenylacetic acid in blood plasma of phenylketonuria patients: 0.16 to 1.05 mg/100 ml phenylpyruvic acid, and 0.11 to 0.6 mg/111 ml o-OH phenylacetic acid.

In addition to phenyl- and phenolic acids, analogous indole acids are excreted by the phenylketonuria patient. Indolepyruvic acid (16), 5-OH indoleacetic acid (17), indoleacetic acid (17), and indolelactic acid (17) have been

Figure 1: Map of the metabolic routes of phenylalanine, tyrosine, and tryptophan

The double arrows indicate the main routes; single arrows, the alternate routes producing the metabolites abnormally observed in phenylketonuria. The real block and apparent blocks are indicated. The drawing was taken from Knox's review article (4). The letters and numbers marking the reactions refer to the original text.

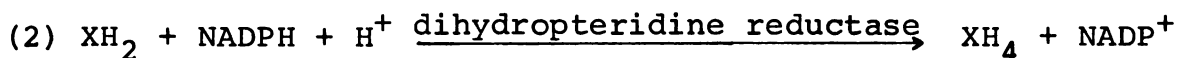
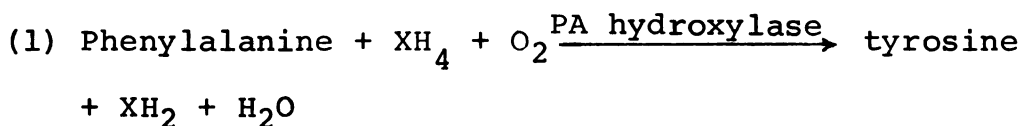


discovered in abnormal amounts in patients' urines. Further indication of peculiar tryptophan metabolism is increased excretion of indican (18).

There is very little information available on the concentrations of these acids in body tissue, especially the brain, due to lack of specific and sensitive assay methods. Unfortunately, no correlation has been observed between blood or urine concentrations of any of the acid metabolites and severity of the mental retardation.

B. Metabolic Pathways in Phenylalanine Metabolism

The reaction catalyzed by phenylalanine hydroxylase and blocked in phenylketonuria occurs in the liver. The tetrahydrobiopterin cofactor, TPNH, and molecular oxygen are required, and the reaction has the following stoichiometry (19):

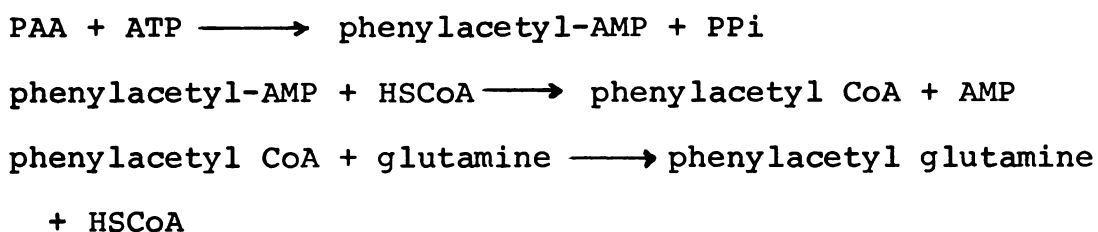


Enzyme activity is found in fetal and neonatal liver (20-21). The enzyme is inhibited by p-chlorophenylalanine (22), esculin (23) or amethopterin (50). The genetic defect in phenylketonuria is limited to the hydroxylase enzyme; the reductase system is not involved. It has been well substantiated that there is no phenylalanine hydroxylase in brain (24-25). However Bagachi and Zarycki (26) reported in vivo formation of tyrosine from phenylalanine in rat brain; but it appears that tyrosine hydroxylase is partially responsible for this conversion.

The primary alternative phenylacid, phenylpyruvic acid, is produced in a transamination reaction with alpha-ketoglutarate. Jacoby and LaDu (27) reported that tyrosine alpha-ketoglutarate transaminase from liver transaminates tryptophan and phenylalanine in addition to tyrosine. Thus if this enzyme activity were increased in a phenylketonuria situation, the indole acid formation would be reasonable. Fonnum, Haavaldsen, and Tangen (28) reported transaminase activity in rat brain also. If phenylpyruvic acid were to accumulate in cerebral tissue of phenylketonurics, it could originate via the circulation or from in situ synthesis.

Weber and Zannoni (29) isolated and described an aromatic alpha-keto acid reductase, occurring in heart, skeletal muscle, kidney, adrenal gland, liver, blood, and thyroid. This enzyme is responsible for the major production of phenyllactic acid from phenylpyruvate; lactic acid dehydrogenase is 1/4 to 1/5 as active.

The reactions involved in phenylacetic acid formation are probably analogous to those for pyruvate to acetate or alpha-ketoglutarate to succinate. Exact mechanisms are unknown. The conjugation of phenylacetic acid with glutamine for the human liver system, though, was elucidated by Moldave and Meister (30) according to the following scheme:



It has been reported that the conjugation activity is adapted

in phenylketonuria to accomodate increased substrate levels (31).

Three pathways of o-OH phenylacetic acid formation have been postulated:

1. Phenylalanine $\rightarrow$ o-tyrosine $\rightarrow$ o-tyramine $\rightarrow$ o-hydroxy-phenylacetic acid
2. Phenylalanine $\rightarrow$ o-tyrosine $\rightarrow$ o-OH phenylpyruvic acid $\rightarrow$ o-OH phenylacetic acid
3. Phenylalanine $\rightarrow$ phenylpyruvic acid $\rightarrow$ o-OH phenylacetic acid

Mitoma demonstrated path 1 (32); Flatow, path 2 (33); Taniguchi and Armstrong, path 3 (34). The mechanism for the third reaction is similar to that of p-OH phenylpyruvic acid to homogentisic acid.

C. The Application of Model Systems

To study phenylketonuria it is necessary to create a model system that best resembles the human patient. In the absence of an animal counterpart, a technique often used is subjection of a laboratory animal to high concentrations of phenylalanine or a metabolite through regular dietary feeding or injection. Another alternative is treatment of the animal with a phenylalanine hydroxylase inhibitor; however, inhibitors are not specific. p-Chlorophenylalanine, for example, also inhibits tryptophan decarboxylase. Dolan (35) has summarized discrepancies encountered between models and the authentic phenylketonuria system: (1) Plasma tyrosine levels are increased in models fed phenylalanine. There is a definite flux of phenylalanine to tyrosine. In the human

tyrosine is not increased. Longenecker et al. (36) have succeeded in stomach-tube feeding neonatal rats and maintaining phenylalanine/tyrosine plasma ratios similar to those in phenylketonuria patients. (2) Excretion of acidic metabolites is variable. A decrease in urinary indole acids was observed in rats fed phenylalanine, contrary to the phenylketonuric system (37). (3) The distortion pattern of plasma amino acids in the hyperphenylalaninemic model differs from that in the phenylketonuric.

A correlation has been made between high phenylalanine diet and decreased learning ability for the rat (38) and for the monkey (39); Yuwiler and Louttit (40) and McKean (41) observed a decrease in brain serotonin and a retardation in the animal's learning ability, but the two phenomena were not causally related.

The chick model used in this research will be discussed in more detail. The phenylalanine hydroxylase activity is lower in chick than in the rat (20), so the system may provide higher phenylalanine and phenylacid accumulations. Tammie and Pscheidt (42-45) fed diets 5 and 8% in phenylalanine to 1-day old chicks for a 4 week period. The "phenylketonuric" chicks displayed reduced weight gain, abnormal hock and feather development, discoloration in the shank, face, and eyelids, and poor motor coordination. No mortality was observed. Post mortem examination indicated no gross organ pathology, although organs were smaller in the phenylalanine fed than in the controls. The symptoms were reversible if the phenylalanine fed animals were switched to control

diet. Plasma phenylalanine concentration in the experimental group was 20 times that of the control value. Brain serotonin concentration decreased 33%, whereas norepinephrin was unaffected. Female chicks developed more severe deformities at an earlier time than the males. Feeding a diet 5% in phenylalanine to pullets decreased fertility, hatchability and hatching weight, but no deformed and viable chicks hatched.

D. Mechanisms for Mental Retardation

1. Protein synthesis

Swaiman et al. (46) using phenylalanine-fed rabbits injected with labelled lysine observed a decrease in the specific activity of ribosomal protein. Petersen (47) found that excess phenylalanine inhibited labelled tyrosine incorporation into brain homogenate protein. Aoki and Siegel (48) demonstrated disaggregation of brain polyribosomes and inhibition of protein synthesis in a cell free system from 7 day old rats injected with phenylalanine. At 4 weeks of age the effects did not occur. Agrawal, Bone, and Davison (49) reported inhibition of (^{35}S)-methionine incorporation into rat brain protein by phenylalanine administered intraperitoneally or subcutaneously, 2 mg/g body weight; myelin proteins were most adversely affected. Furthermore, the inhibition of transport of labelled methionine by phenylalanine equalled the inhibition of incorporation. An amino acid whose transport system was distinct from that of phenylalanine was incorporated normally into protein. Amino acid analyses on the brains of the phenylketonuria models

also suggest transport problems. McKean et al. (50) demonstrated depletions of threonine, valine, methionine, isoleucine, leucine, histidine, tryptophan, and tyrosine in infant or adult rat brain post intraperitoneal injection of phenylalanine, 0.5 or 1 mg/g body weight. In the pups, plasma amino acid levels increased or remained the same, whereas in the adult, the levels decreased or were unchanged. Lowden and LaRamee (51) confirm some of these observations in rat brain, but not all. In addition they studied the non-essential amino acids, finding the most significant change to be a decrease in alanine. Since alanine can be metabolized via pyruvate and the citric acid cycle, energy stress may be an important causative factor. Van Sande et al. (52) reported free amino acid patterns in the cerebrospinal fluid of treated and untreated phenylketonuria patients. Serine, tyrosine, phenylalanine, histidine, homocarnosine, and citrulline increased; threonine and GABA decreased.

2. Lipid Metabolism

Lipids are a significant component in neural membranes, especially myelin, the lamellar material associated with the nerve axons. Sterols, sphingolipids and phospholipids accumulate in the brain during the myelination period. Abnormal myelin deposition interferes with neural activity.

Shah et al. (53) reported a cholesterol synthesizing preparation from the brains of rats injected with phenylalanine that incorporated 40 to 77% less mevalonate than the control. The liver system for cholesterol synthesis was unaffected. When phenylalanine was added to a control brain

system in concentrations observed in the hyperphenylalaninemic rat brain no inhibition occurred; for inhibition, 5 times the concentration of phenylalanine was needed. This concentration also caused inhibition in the liver system. Phenylpyruvate was reported to be a more potent inhibitor in the in vitro cholesterol synthesizing system.

Using a minced preparation of rat brain, Barbato (54) observed decreased acetate and glucose incorporation into lipids and reduced glucose incorporation into aspartate, glutamate, GABA, and alanine at 60 mM phenylalanine or tryptophan. O_2 consumption and CO_2 evolution were inhibited by phenylalanine, tryptophan and leucine. The effects of tryptophan and leucine suggest nonspecificity and transport disturbances. Shah et al. (55) demonstrated in vivo and in vitro inhibition of glucose incorporation into cerebral lipids of hyperphenylalanine-treated rats; however, the phenylalanine concentration required for in vitro inhibition was 360 times greater than the in vivo phenylalanine concentration. Phenyllactic acid, phenylpyruvic acid, and phenylacetic acid were better in vitro inhibitors at lower concentrations than phenylalanine.

Feeding 5 and 7% phenylalanine to 21-day old rats for 3 weeks, Geison and Waisman (56) observed no significant effects on synthesis and deposition of major brain lipids. However, in suckling rats injected with phenylalanine, galactolipid and cholesterol accumulation was delayed (57). O'Brien and Ibbot (58) reported a reduced concentration of total lipids and cerebrosides in the brain of an infant monkey

fed excess phenylalanine. This suggests the importance of age and extent of cerebral development.

3. Phenylacid Toxicity

A major difficulty in interpreting information from the models is lack of data on physiological levels of the various acids in phenylketonuric patients especially in tissues such as the brain. Also, inhibitions by the phenylacids are seldom demonstrated on human systems. Weber (59), though, has demonstrated phenylalanine inhibition of human brain pyruvate kinase and phenylpyruvate inhibition of human brain hexokinase; K_i 's are 8.5 mM and 2 mM respectively for the adult enzymes and are similar for the fetal enzymes. However, the absolute activities of both enzymes in the fetus are only 10% of the adult levels. This would cause the fetal brain to be more vulnerable to the effects of phenylalanine and phenylpyruvate accumulations. But if the activity of phenylalanine hydroxylase in the fetus is low (20), the significance of its deficiency to the fetus is questionable. Knox (4) states: "The effects of treatment are in complete accord with the view that the phenylketonuric infant is normal at birth and that in the first months of life retardation begins and becomes progressively more severe."

The alternative phenylalanine metabolites have been implicated in many other enzyme systems pertinent to normal brain function, but these will be summarized in a later section. Of particular interest is in vitro inhibition of tryptophan decarboxylase and DOPA decarboxylase, jeopardizing serotonin and epinephrine synthesis.

E. Treatment

Treatment of phenylketonuria is simply exclusion of unnecessary phenylalanine from the diet. Blood phenylalanine is subsequently decreased as well as phenyl, phenolic and indole acid excretion in the urine. Some of the symptoms will reverse themselves; pigmentation improves; many of the neurologic signs disappear. Intellectual impairment is irreversible, though; it can be prevented if dietary treatment is begun before the retardation has developed.

Phenylacetic Acid Toxicity

A. Toxicity and Clinical Features

Sherwin and Kennard (60) in 1919 recorded phenylacetic acid toxicity in the hen, monkey, dog, and human; the mouse also displayed adverse effects (61). The toxicity in the dog has been characterized most completely by the early investigators (60). Given an initial dosage of 1 gm and an increase of 1 gm per day, the dog underwent great thirst, loss of appetite, drowsiness, loss of weight, inability to stand, and on the sixth day of the diet a semicomatose condition. The animal died on the seventh day after undergoing a series of convulsions. Analysis of the etiology of the toxic state was limited to post mortem examination. Microscopic examination indicated destruction of epithelium in the proximal convoluted tubule and the loop of Henle in the kidney. Changes in the liver were secondary; spleen and intestinal tract were normal. The brain was not studied. The symptoms observed in humans were similar to those in the

dog, except the coma and convulsions.

B. Detoxification

The detoxification of phenylacetic acid is effected by a conjugation mechanism, differing with species. In the human 95% of ingested acid is excreted as phenylacetyl glutamine; the remaining 5%, as the glucuronide derivative (62). In most other mammals the acid is conjugated with glycine to form phenylaceturic acid. In the bird, the detoxification product is phenacetornithuric acid (63). The conjugation mechanism requires ATP and coenzyme A (30).

C. Inhibition of Cell Functions and Enzyme Activities by the Aromatic Acids

Howell and Lee (64) have shown that phenylacetic acid and phenylpyruvic acid at 10 mM concentrations depress O_2 consumption in rat brain slices by 80 to 84%; however, 1 mM phenylacetic acid does not affect respiration in rat kidney cortex slices (65). Also demonstrated in the in vitro kidney cortex system by Krebs and deGasquet (65) was a 50% inhibition in gluconeogenesis from lactic acid by 1 mM phenylacetic acid, phenyllactic acid, phenylpyruvic acid, p-hydroxyphenylpyruvic acid. This would be insignificant in the brain, though, since gluconeogenesis is not a cerebral function.

Since serotonin, epinephrine, and gamma-aminobutyric acid are postulated as transmitter substances in the brain, interference with their metabolism would certainly jeopardize normal brain activity.

Davison and Sandler (66) have shown in vitro a 50%

inhibition of 5-OH tryptophan decarboxylase in guinea pig kidney by 16.5 mM phenylpyruvic acid, 33 mM phenyllactic acid, and 68% with 16.5 mM phenylacetic acid. Justice and Hsai (67) using guinea pig brain homogenates demonstrated a competitive inhibition of the 5-OH tryptophan decarboxylase by the following acids with K_i 's given: phenylpyruvic acid, 0.63 mM; phenyllactic acid, 4.5 mM; phenylacetic acid, 2.5 mM; p-hydroxyphenylpyruvic acid, 3.0 mM; p-hydroxyphenyllactic acid, 5.2 mM; p-hydroxyphenylacetic acid, 10.4 mM. This inhibition was not observed in vivo in rats fed 3 and 5% phenylacetic acid for 1, 2, or 3 weeks. Brain serotonin levels were higher in the experimental group than in the control. The serotonin level in the liver was reduced in the experimental group, but this was attributed to decreased caloric intake. At the levels of feeding mentioned no toxicity was noted in the rat (68).

Fellman (69) has pointed out that the aromatic acids may inhibit epinephrine synthesis at the dihydroxyphenylalanine decarboxylase point. With an in vitro beef adrenal medulla system, 3.3 mM phenylpyruvic acid inhibited the enzyme by 77%, 3.3 mM phenyllactic acid by 50%, and 33.3 mM phenylacetic acid by 50%. No inhibition by phenylacetic acid was observed at a 16 mM concentration.

Rat brain glutamic acid decarboxylase was inhibited by a series of phenylalanine metabolites. K_i 's for phenylpyruvic acid, p-OH phenylpyruvic acid, phenylacetic acid, p-OH phenylacetic acid and o-OH phenylacetic acid are, respectively: 30, 120, 55, 25, 100 μ Moles/l (70). Some

confusion arises, though, as to the actual potential of the inhibitors. Another report specified a 28 mM concentration of phenylpyruvic acid as necessary for a 61% inhibition of the enzyme; 28 mM phenylacetic acid inhibited by 96%; p-OH phenylacetic acid at 28 mM, by 92% (71).

Phenylacetic acid was observed to inhibit phenylalanine hydroxylase in vitro at 1 mM concentration; however, the effect could be minimized by addition of the pteridine cofactor (72). This particular activity of the acid has no import on phenylketonuria itself, though, since hydroxylase is essentially absent.

Jacoby and LaDu (73) reported a 30% inhibition of tyrosine transaminase from rat liver by 12 mM phenylacetic acid.

The effect of phenylacetic acid on total nitrogen balance was studied in the rabbit by Hijikata (74). Small doses of the compound resulted in aminoaciduria; free amino acids, though, were not distinguished from the glycine in the conjugated form. Total urinary N and NH_3 were not changed. Large doses produced an increase in total N excreted; NH_3 and amino acids were increased both in relative and absolute amounts; urea also rose but not proportionately to the total N. Buck and Berg observed a decrease in blood urea in the rabbit post phenylacetic acid ingestion (75). Accumulation of NH_3 in the urine by way of increased deaminase activity is questionable. It has been reported that phenylacetic acid inhibits the deamination of L and D amino acids in the kidney (76).

Uric acid levels were reported to decrease in the urine of humans after administration of either phenylacetic acid or benzoic acid (77).

Mice Deficient in Phenylalanine Hydroxylase: Model System for Phenylketonuria

The laboratory animal that best approaches the phenylketonuric patient and which would be the most ideal model in studying the disease is one with a mutation affecting its own phenylalanine hydroxylating system. In 1960 Coleman (78) reported three genotypes (Dd, dd, dldl) of mice strains (DBA/1J and DBA/2J) to have a 30 to 86% reduction in phenylalanine hydroxylase activity and an increased capacity to form the alternative metabolite, phenylacetic acid. The reduction in hydroxylase activity is caused by an inhibitor confined to the 15,000 x g precipitate. The mice are characterized by dilute coat color; in addition, the dilute lethals (dldl) demonstrate severe CNS disorder in the form of spontaneous convulsions and die at 3 weeks of age. The lowering in hydroxylase activity is greatest in the dilute lethals. The peculiarly light pigmentation is not due to a decrease in the absolute amount of pigment as could be expected under conditions of improper aromatic amino acid metabolism, but is rather caused by an unnatural configuration of the melanin granules around the melanocyte nucleus (79). Kelton and Rauch (80) reported in 1962 on the myelination process in the dilute lethal mice. The formation of the myelin fibers is normal; however, degeneration occurs

rapidly. The abnormality in enzyme activity was later confirmed by the work of Rauch and Yost (81) who also report accumulation of phenylalanine in the blood to levels 10 times the normal.

In 1966 Zannoni et al. (82), in a series of studies on dilute lethal homozygotes of 3 different stocks (Bar Harbor, Oak Ridge, and Harwell), challenged the previous findings on the following points: (1) Neither blood phenylalanine levels nor urinary phenylpyruvate levels were elevated; (2) Phenylalanine hydroxylase activity was normal; (3) The hydroxylase was inhibited only if the animals were first primed with a feeding of either phenylalanine or phenylpyruvic acid. Concomitantly phenylalanine and phenylpyruvate concentrations rose. The extent of inhibition of the hydroxylase system in the dilute lethals by the 15,000 x g sediment factor was no greater than in other dilute genotypes or in other species such as rat, dog, or guinea pig. These mice exhibited the same type of neurological dysfunction, but it is now possible that the CNS problems may be independent of the phenylalanine hydroxylase system, concentrations of phenylalanine and other metabolites.

MATERIALS AND METHODS

Chemicals

The following materials were obtained commercially: cerelese (D-Glucose monohydrate), vitamin free casein, gelatin, cotton seed oil, Wesson salt mixture, vitamin mixture, DL methionine, choline chloride, and phenylacetic acid from Nutritional Biochemicals Corporation, Cleveland, Ohio; stock diet "Purina Laboratory Chow" for mice and rats from Ralston Purina Company, St. Louis, Missouri; scintillation grade 2,5-diphenyloxazole (PPO), $^{14}\text{C}(\text{U})$ tyrosine and $^{14}\text{C}(\text{U})$ phenylalanine with specific activity of 0.05 mc/0.02 mg in 0.5 ml 1N HCl from New England Nuclear, Boston, Massachusetts; scintillation grade 1,4 bis 2-(5-phenyloxazolyl)-benzene (POPOP) from Packard Instrument Company, Downers Grove, Illinois; o-hydroxyphenyl acetic acid, 4-phenylbutyric acid, phenylacetic acid, and 1-nitroso-2-naphthol from Aldrich Chemical Company, Milwaukee, Wisconsin; p-hydroxyphenylacetic acid and p-hydroxyphenylpyruvic acid from K and K Laboratory, Incorporated, Plainview, New York; phenylpyruvic acid and L-leucyl-L-alanine from Mann Research Laboratory, Incorporated, New York, New York; hexamethyldisilazane and N,O bis-(trimethylsilyl)-acetamide (BSA) from Pierce Chemical Company, Rockford, Illinois; trimethylchlorosilane from General Electric Silicone Products Department, Waterford, New York; OV-17

80/100 mesh Chromosorb W, and 100/120 mesh 3% OV-1 on Chromosorb W from Applied Science Laboratory, Incorporated, State College, Pennsylvania; 80/100 mesh GC Grade Se-30 on Suplecoport from Supelco, Incorporated, Bellefonte, Pennsylvania; trifluoroacetic anhydride in methylene chloride from Regis Chemical Company, Chicago, Illinois. The methanol-HCl and butanol-HCl were prepared by passing HCl gas into the alcohol, and determining the amount added by weight change. The gaseous HCl was liberated from the acid by concentrated sulfuric acid. Final normality was determined by titration. In preparing trimethylsilylating reagents the reagent grade pyridine was first redistilled over barium oxide and stored over potassium hydroxide pellets. All enzymes were purchased from either Sigma Chemical Company, St. Louis, Missouri or Boehringer Mannheim Corporation, New York, New York. Acetyl NAD⁺, NAD⁺, NADH, NADP⁺, ATP and ADP were also purchased from Boehringer Mannheim Corporation. Commercial tri-ethanolamine was recrystallized from benzene before use. Worthington Biochemical Corporation, Freehold, New Jersey furnished the glucose oxidase assay materials. The phenyl-acetic acid sodium salt was prepared by neutralizing an aqueous solution of the acid with sodium hydroxide and lyophilizing.

Experimental Animals

Genetic deficient mice were obtained from Dr. V. G. Zannoni and Dr. B. N. LaDu, New York University Medical Center. The dilute lethal mutants were 18-23 days of age; they had

been accompanied by one lactating mother; the dd controls ranged in age from 23 to 47 days and were fed a commercial pellet diet. The mice were sacrificed one day after arrival.

White Rock cockerels (1 to 2 days old) were obtained from Cobbs, Incorporated, Goshen, Indiana and housed in a brooder at 32°. The diet was prepared according to Rutter et al. (83).

Cerelose (glucose monohydrate)	62.36%
Casein	18.00
Gelatin	10.00
Cotton seed oil	5.34
DL Methionine	0.30
Wesson salt mixture	4.01
Vitamin mixture	1.00
Choline chloride	0.10

In the experimental diets the L-phenylalanine, phenylacetic acid, or phenylacetic acid - sodium salt was added in place of some cerelose. The controls were fed ad libitum or a restricted quantity of diet (paired feeding) to compensate for the reduced dietary intake in the experimental group.

Methods

A. Procedure for Securing Tissue and Plasma Samples

The brain tissue for metabolite analysis was obtained from the animal as follows: (1) The animal was decapitated directly into liquid nitrogen and skulls were stored at -90°; (2) Tissue was chiselled out in a cold room at -20°, powdered with mortar and pestle, and stored at -90°. Liver was obtained from ether-anesthetized animals by in situ freezing the tissue with tongs precooled in liquid nitrogen, subsequently powdered, and stored at -90°. Blood was

secured by heart puncture with a heparinized syringe or by drainage from the neck into a heparin-coated cap. Care was exerted with the latter method to avoid contamination with gut contents. Plasma was immediately separated from red cells by centrifugation and stored at -20° .

B. Assay Procedures

1. Determination of Phenylalanine in Plasma

The McCaman and Robins (84) fluorometric procedure was used. For a typical analysis, 50 μ l of plasma was deproteinized with 50 μ l of 0.6 N trichloroacetic acid, followed by centrifugation. Up to 20 μ l of the supernatant fraction was incubated at pH 5.8, 60° , with 60 μ moles succinate, 2.4 μ moles ninhydrin, and 0.2 μ moles L-leucyl-L-alanine in a total volume of 0.36 ml. To control non-specific plasma fluorescence, filtrate blanks including all reagents except the dipeptide were run. Phenylalanine standard solutions (0.05 and 1.0 mM) were prepared in 0.3 N trichloroacetic acid and 0.5 to 10 μ moles were assayed. Reported linearity range is 0.1 μ M to 30 μ M final concentration. For the incubation, 10 x 100 mm culture tubes with teflon lined caps were used. After a 2 hour period, the tubes were cooled and contents were diluted with 2 ml copper reagent: 1.6 g Na_2CO_3 , 0.065 g potassium sodium tartrate, 0.060 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter of water. After 15 minutes the fluorescence was read in 2 ml cuvettes in a fluorometer with excitation wave length set at 365 m μ and emission wave length at 495 m μ . A 5 minute period was necessary for the standards and samples high in phenylalanine to reach a

constant fluorescence.

2. Determination of Tyrosine in Plasma

The fluorometric method of Ambrose et al. (85) was employed. For example, 50 ul of plasma was deproteinized with 50 ul of 0.6 N trichloroacetic acid followed by centrifugation. For assay 30 ul of the supernatant fraction was diluted 1/5 with water and the volume was adjusted to 0.5 ml with 0.06 N trichloroacetic acid in culture tubes with teflon lined screw tops. Linearity was observed with 1 to 5 ug of standard tyrosine. To 0.5 ml of standard or sample was added 1 ml of nitric acid reagent (1 part 2.5 N HNO_3 to 2 parts nitrosonaphthol - sodium nitrite reagent to 1.5 parts phosphoric acid reagent), and the contents were mixed and incubated for 6 minutes at 85°; finally the tubes were cooled at 33° for 10 minutes and 5 ml of 95% ethanol were added. After 30 minutes at 33°, the fluorescence was read with the excitation wave length set at 436 mu and the emission wave length at 540 mu. Compounds that also will yield considerable fluorescence under these conditions are tyramine, p-OH phenyllactic acid, p-OH phenylacetic acid, 5-OH indoleacetic acid, p-OH phenylpyruvic acid, and DL-m-tyrosine.

3. Determination of Phenylalanine and Tyrosine in Brain Tissue

At the time of sacrifice the brain was removed from the skull and quickly frozen in liquid nitrogen. Routinely 1 g of powdered tissue was homogenized in 5 ml of 0.6 M perchloric acid in a Potter-Elvehjem type homogenizer.

¹⁴C(U) tyrosine (New England Nuclear: 0.05 mc/0.0203 mg in

0.5 ml 1 N HCl) (1 ul) and $^{14}\text{C}(\text{U})$ phenylalanine (New England Nuclear: 0.05 mc/0.022 mg in 0.5 ml 1 N HCl) (1 ul) were added to the homogenate. After being well mixed, the homogenate was centrifuged at 12,000 g for 10 minutes. The supernatant fraction was neutralized with 2.5 ml of 1.2 N KOH. For part A (6 days) of the second phenylalanine feeding experiment the neutralized sample was taken to dryness on a rotoevaporator, redissolved in 50% pyridine/water, and spotted on Whatman 3M chromatography paper prewashed in the solvent system. The spots were developed with descending chromatography in butanol : acetic acid : water (120:30:50, v/v/v) for 15 hours. The phenylalanine and tyrosine positions were detected with a Model 7201 Packard radiochromatogram scanner. The two amino acids were eluted with 2 to 3 ml of 50% pyridine/water into a graduated conical tube. An aliquot of the eluant was counted on a Beckman CPM scintillation spectrometer in a scintillation fluid containing 5 g PPO and 0.3 g POPOP per liter of toluene.

Phenylalanine and tyrosine were quantitated by gas liquid chromatography as the butyl ester, trifluoroacetamide derivatives according to Gerke's (86) method. Experimental conditions were as follows: (1) 3 to 5 ug of internal standard (stearic acid, OH proline, or glutamate) were added and the sample was dried on a rotoevaporator with 2 or 3 washes of 1 ml methylene chloride to insure dryness. (2) If necessary, the methyl ester was formed by a 1-hour incubation at room temperature with 1 ml of methanol-1.25 N HCl. This was important for the subsequent transesterification of

stearic acid. The excess methanol-HCl was removed by evaporation. (3) The butyl ester was formed by a 2.5 hour incubation at 90° (oil bath) with 1.5 ml of butanol-1.25 N HCl, and the excess reagent was removed by evaporation. (4) The butyl ester was dissolved in 0.2 ml of methylene chloride and transferred to a screw top culture tube with a teflon lined cap. Trifluoroacetic anhydride in methylene chloride (0.08 ml) was added and the sample was incubated at 150° (paraffin bath) for 5 minutes. (5) The volume was reduced to 20 to 40 μ l with a N_2 stream for amino acids in the 0.1 mM range, and 3 or 4 μ l were injected into a F and M 402 high efficiency gas liquid chromatograph equipped with a H_2 flame detector and nitrogen carrier gas. The derivatives were separated on a 6 foot 2% OV-17 (50% phenyl methyl-silicone) column with 80/100 mesh Chromosorb W support. Internal column diameter was 3 mm. Column temperature favorable for phenylalanine quantification with OH proline as internal standard was 140°; for tyrosine quantification, 185° was used with glutamate and stearate as internal standards. Standards with known amounts of amino acids and internal standards were prepared. The amino acids were quantified using the following formula:

$$\frac{\text{weight of amino acid standard}}{\text{weight of internal standard}} = \frac{\text{unknown amino acid amount}}{\text{amount of internal standard added}}$$

$$\frac{\text{peak height of amino acid standard}}{\text{peak height of internal standard}} = \frac{\text{peak height of sample amino acid}}{\text{peak height of sample internal standard}}$$

The amino acid quantitation from gas liquid chromatography was

corrected for loss by radioisotope dilution analysis.

The spotting of the sample immediately after neutralization without desalting interfered with chromatography. So in part B (14 days) the sample was first put through a Dowex 50 (H^+) x 8, 100-200 mesh column 1 x 8 cm in dimension (37). Before application of the extract, pH was checked and corrected if greater than pH 7.8. The column was washed with 10 ml of water and the amino acids were eluted with 10 ml of 4 N NH_4OH . The volume was reduced to 200 μl on the rotoevaporator; paper chromatography and gas liquid chromatography were conducted as previously described. Radioactivity recoveries ranged from 40 to 70% for phenylalanine and from 60 to 70% for tyrosine.

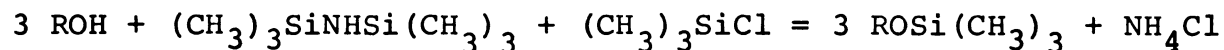
4. Determination of Glucose in Plasma

Several methods were used to assay for glucose and the results agreed well.

Method I: Gas liquid chromatography

The GLC method of Wells (88) was followed. Preliminary to chromatography plasma proteins were precipitated according to Somogyi (89). ZnSO_4 (2%) and $\text{Ba}(\text{OH})_2$ (1.8%) were balanced at the phenolphthalein end point. In a typical preparation, 0.5 ml of ZnSO_4 and a balancing quantity of $\text{Ba}(\text{OH})_2$ were added to 0.1 ml of plasma. The volume was adjusted to 2.0 ml with water and the mixture was centrifuged. An aliquot (50 μl) of the supernatant containing 50 μg of methylmannoside as internal standard was dried on a flash evaporator. Trimethylsilyl reagent (100 μl) was added to form trimethylsilyl ethers. The reagent contained 15 parts

pyridine to 4 parts hexamethyldisilazane to 1 part trimethylchlorosilane. The following reaction occurs upon derivatization:



A 4 foot 3% OV-1 (dimethylsilicone) column, 80/100 mesh

Chromosorb W support at 180° was used for separation and quantification of glucose. Standard glucose and methyl mannoside samples were run, and typical retention times in minutes are: methylmannoside, 2.4; alpha-D-glucose, 3.6; beta-D-glucose, 5.4. The amount of glucose present was calculated on the basis of the peak height ratio.

Method II: Fluorometry

The method of Lowry et al. (90) was employed. The reaction cuvette contained the following components expressed as final concentration in a 2 ml volume: 100 mM Tris-HCl, pH 7.5; 0.3 mM ATP; 0.03 mM NADP⁺; 5 mM MgCl₂; 2.5 ug/ml yeast hexokinase; 1.25 ug/ml glucose-6-phosphate dehydrogenase; 1 to 5 ul plasma. The reaction was initiated by the addition of both enzymes; correction was made for fluorescence of the dehydrogenase. Fluorescence was quantitated according to a standard NADH or glucose-6-phosphate solution. Exciting wave length was set at 355 mu, and the emitting wave length was at 448 mu. Temperature of the reaction was 25°.

Method III: Spectrophotometry

The glucose oxidase micromethod described by Worthington (91) was followed. A 1:20 plasma filtrate was prepared as described under the gas liquid chromatography section and was diluted 1:1 with water. Aliquots (100 to 200 ul) of

this were added to 200 ul of the glucose oxidase-chromagen reagent; the final volume was adjusted to 400 ul with water. After a 10 minute incubation at room temperature, the mixture was acidified with 1 drop 3 N HCl. The optical density was read at 400 mu on a Gilford 2000 spectrophotometer after 5 minutes. Standard glucose was linear over the range, 3 to 18 ug.

5. Determination of Lactic Acid in Plasma

The Lowry (90) method was employed. A 1:20 Somogyi plasma filtrate was prepared (89). The test solution contained the following components expressed as final concentration in a 0.4 ml volume: 200 mM carbonate, pH 9.7: 1 mM acetyl-NAD⁺; 12 ug/ml beef heart lactic acid dehydrogenase; 50 or 100 ul of filtrate. After the addition of the dehydrogenase, the optical density change was determined on a Gilford 2000 at 363 mu at 25°. The molar extinction coefficient of the reduced nucleotide is 9.3×10^6 .

6. Extraction of Glycolytic Intermediates and Adenine Nucleotides from Brain

The extraction of the metabolites was similar to that of Lowry (90) except for a few modifications. Generally 100 mg of frozen tissue powder were weighed into a tube containing 0.2 ml of 0.6 M HClO₄-1 mM EDTA, previously frozen. HClO₄-EDTA (0.2 ml) was added above and the tissue was homogenized directly in the tube with a teflon homogenizer. Material adhering to the homogenizer was rinsed into the tube with 0.1 ml of the HClO₄-EDTA, and the homogenate was centrifuged at 12,000 g for 10 minutes. The supernatant fraction

was decanted and neutralized with 0.25 ml of 2 M KHCO_3 . After centrifugation at 2000 g for 10 minutes the supernatant fraction was assayed according to the procedures to be described. The original perchlorate pellet was saved for glycogen determination.

7. ATP and Creatine Phosphate Spectrophotometric Determination

The methods of Lamprecht and Trautschold (92-93) were used. The reaction cuvette contained the following components expressed as final concentration in a 0.25 ml volume: 0.1 M Tris-HCl, pH 7.5; 5 mM MgCl_2 ; 2.5 mM NADP^+ ; 5 mM glucose; 20 ug/ml yeast hexokinase; 10 ug/ml yeast glucose-6-phosphate dehydrogenase; 20 or 40 ul perchlorate extract. The reaction for the ATP determination was initiated by the addition of the hexokinase and dehydrogenase; at the termination of the reaction, 0.125 umoles ADP and 10 ug creatine phosphokinase were added in a small volume for the creatine phosphate determination. The optical density change at 339 mu was recorded on a Gilford 2000 equipped with a multiple cuvette changer. The molar extinction coefficient of the reduced nucleotide is 6.22×10^6 at 339 mu.

8. ADP and AMP Fluorometric Determination

The Lowry (90) procedure was employed. The assay contained the following components expressed as final concentration in a 2.0 ml volume: 50 mM potassium phosphate, pH 7; 3 uM NADH; 25 uM phospho-enolpyruvate; 12 uM ATP; 2 mM MgCl_2 ; 5 ug/ml beef heart lactic acid dehydrogenase; 1 ug/ml pyruvate kinase; 1 ug/ml myokinase; 10 ul perchlorate extract.

Pyruvate kinase addition initiated the ADP reaction; after completion, myokinase was added to initiate the AMP reaction. Fluorometric response was standardized with NADH. Excitation wave length was set at 355 mu; emission wave length, at 448 mu. Temperature was maintained at 25°.

9. Glucose Spectrophotometric Determination

The procedure of Slein (94) was modified. The reaction cuvette contained the following components expressed as final concentration in a 0.25 ml volume: 32 mM Tris-HCl, pH 8; 6 mM $MgCl_2$; 1 mM ATP; 0.32 mM $NADP^+$; 20 ug/ml hexokinase; 10 ug/ml glucose-6-phosphate dehydrogenase; 30 to 60 ul perchlorate extract. The reaction was monitored on a Gilford 2000 at 25° at 339 mu, UV source.

10. Glucose-6-phosphate and Fructose-6-phosphate Fluorometric Determination

Essentially Lowry's (90) method was used, omitting the cycling procedure in the fructose-6-phosphate determination. The test solution consisted of the following components expressed as final concentration in a 2.0 ml volume: 0.1 M Tris-HCl, pH 8; 0.01 mM $NADP^+$; 1.25 ug/ml glucose-6-phosphate dehydrogenase; 100 ul perchlorate extract. After the glucose-6-phosphate conversion, phosphohexose isomerase was added, 2.5 ug/ml. Enzyme fluorescence was determined. Fluorometric response was standardized with glucose-6-phosphate or NADH solutions. Excitation wave length was set at 355 mu; emission wave length, at 448 mu.

11. Fructose-1,6-diphosphate and Dihydroxyacetone Phosphate Fluorometric Determination

The spectrophotometric procedure by Bucher and Hohorst (95) was modified for fluorometry. The reaction cuvette contained the following components expressed as final concentration in a 2.0 ml volume: 20 mM recrystallized triethanolamine, pH 7.4; 1 mM EDTA; 3 μ M NADH; 5 ug/ml L-alpha-glycerol phosphate dehydrogenase; 1 ug/ml triose phosphate isomerase; 5 ug/ml rabbit muscle aldolase; 30 μ l perchlorate extract. The reaction was initiated by the addition of glycerol phosphate dehydrogenase and the isomerase; after the dihydroxyacetone phosphate had completely reacted, the aldolase was added for conversion of the fructose diphosphate. Correction was made for the fluorescence of the dehydrogenase. Fluorometric response was standardized with a NADH or dihydroxyacetone phosphate solution. Excitation wave length was 355 mu; emission wave length, 448 mu.

12. L-alpha-glycerol Phosphate Fluorometric Determination

A modification of Lowry's (90) procedure was employed. For the test solution the following components expressed as final concentration in a 2.0 ml volume were added: 0.2 M glycine-0.4 M hydrazine, pH 9.5; 1 mM EDTA; 0.4 mM NAD^+ ; 2.5 ug/ml L-alpha-glycerol phosphate dehydrogenase; 50 μ l perchlorate extract. The reaction was begun by the addition of the glycerol phosphate dehydrogenase. A correction had to be made for its fluorescence. The fluorometric response was standardized with NADH. Excitation wave length, 355 mu; emission wave length, 448 mu.

13. 3-phosphoglyceric Acid Fluorometric Determination

Lowry's (90) procedure was essentially used. The reaction cuvette contained the following components expressed as final concentration in a 2.0 ml volume: 20 mM recrystallized triethanolamine, pH 7.4; 3 μ M NADH; 0.3 mM ATP; 5 mM MgCl_2 ; 5 mM mercaptoethanol; 25 μ g/ml glyceraldehyde-3-phosphate dehydrogenase; 4 μ g/ml phosphoglycerate kinase; 100 μ l perchlorate extract. The assay was started by the addition of the two enzymes whose fluorescence was determined. The fluorometric response was standardized with NADH. Excitation wave length, 355 μ ; emission wave length, 448 μ .

14. Lactic Acid Spectrophotometric Determination

The assay procedure described for the determination of lactate in plasma was followed, employing 0.02 ml perchlorate extract.

15. Glutamate Spectrophotometric Determination

Bernt's (96) procedure was used. The reaction cuvette contained the following components expressed as final concentration in a 0.4 ml volume: 0.375 M glycine-0.3 M hydrazine, pH 9; 37.5 μ M NAD^+ ; 30 μ g glutamate dehydrogenase; 0.01 or 0.02 ml perchlorate extract. The reaction commenced after the addition of the dehydrogenase. The optical density change was determined on a Gilford 2000 at 339 μ .

16. Glycogen Determination

The glycogen was purified and hydrolyzed according to the method of Walaas and Walaas (97). The perchlorate pellet was digested in 0.4 ml of 5 N KOH at 100° for 30 minutes. For standard conditions 0.1 ml of 3% Na_2SO_4

followed by 1 ml absolute ethanol were added; the mix was allowed to stand overnight at -20° . The precipitate was centrifuged at 12,000 g and washed with 0.5 ml of 70% ethanol to remove residual glucose. The pellet was dried under a N_2 stream, transferred to a 5 ml hydrolysis tube, and dissolved in 0.6 ml 1 N H_2SO_4 . The ampule was sealed, and the contents were hydrolyzed at 100° for 3 hours. The solution was removed and neutralized with 1 N NaOH. The neutralized solution was then assayed for glucose by the fluorometric assay previously described or by the glucose oxidase method. Generally 3 or 5 μ l was required for the former. Using the latter method required filtration of the solution to remove charred particles resulting from the hydrolysis. For this purpose a millipore apparatus served well (plain, white filters, 25 mm in diameter with a 0.45 μ pore size). In this instance a 50 μ l aliquot was assayed. To insure that the pH for the glucose oxidase reaction was neutral, the final volume was adjusted to 400 μ l with 0.1 M phosphate buffer, pH 7.

17. Determination of Glycolytic Intermediates and Adenine Nucleotides in the Liver

The extraction and assay procedures were followed as for the brain with these exceptions:

- (1) A glutathione reductase activity in the extract was reflected by the disappearance of the NADPH or NADH fluorescence. This was reduced considerably by the addition of mercaptoethanol, 1.67 mM final concentration in the test solution.

(2) The glycogen pellet was washed twice with chloroform to remove lipid material before hydrolysis.

18. Determination of Uric Acid in Plasma

The method of Praetorius (98) was followed. For this analysis 50 μ l of plasma was diluted to 2 ml with 0.07 M glycine buffer, pH 7.3. Aliquots (0.4 ml) of this were assayed directly or diluted 1:1 with buffer. The reaction was initiated by the addition of 1 μ g of uricase, and optical density change was determined at 293 m μ at 25° on a Gilford 2000 spectrophotometer. The oxidation of 1 μ g of uric acid/ml corresponds to an optical density decrease of 0.075 at 293 m μ ; or 1 optical density unit equals 0.0794 μ moles of uric acid oxidized/ml.

19. Determinations of Aromatic Acids in Plasma and Tissue by Gas Liquid Chromatography

The acids in which we were interested were phenylacetic (PAA), o-OH phenylacetic (o-OH PAA), phenyllactic (PLA), p-OH phenylacetic (p-OH PAA), phenylpyruvic (PPA), and p-OH phenylpyruvic (p-OH PPA). Isolation from urine and quantification of some of these acids by gas liquid chromatography has been discussed by Sweeley and Williams (99) and Horning and Horning (100).

Since some of the acids are volatile, the amount lost during drying on a flash evaporator was important. This was determined with and without cyclohexylamine. The cyclohexylamine, by forming a salt with the acids, should diminish their volatility. Mannitol was used as the internal standard. Three series of tubes were prepared. For the first,

a solution containing 50 ug of mannitol was taken to dryness in a conical tube. To the residue were added 50 ug each of PAA, PLA, p-OH PAA, and p-OH PPA in pyridine. The contents were trimethylsilated directly with TMS reagent (10 ml pyridine to 4 ml hexamethyldisilazane to 1 ml trimethylchlorosilane). For the second series, mannitol and the acids in a volume of 0.5 ml were taken to dryness with mild heating conditions and derivatized. The third series was treated like the second series with the addition of 0.3 ml cyclohexylamine. Four samples were prepared in each series. The derivatives were separated and quantified on a 6 foot 3% SE-30 (methylsilicone) column with an internal column diameter of 3 mm; column support was Supelcoport, 80/100 mesh. A temperature gradient at 5° per minute was run from 125 to 210°. Quantification was according to the peak height ratio method.

The following procedure proved satisfactory for determining the aromatic acids in plasma and brain tissue. An aliquot of plasma (0.1 to 0.5 ml) was deproteinized with 0.6 M perchloric acid (0.5 ml acid per 0.1 ml plasma) and the sample was centrifuged at 12,000 g for 10 minutes. The supernatant fraction was saved, and the precipitate was washed with 1 ml of the perchlorate and recentrifuged. The two supernatant fractions were combined and neutralized with 2 M KHCO_3 (0.25 ml KHCO_3 per 0.5 ml perchlorate) and centrifuged at 12,000 g for 10 minutes. The supernatant fraction was decanted and the precipitate was washed with 1 ml water. After recentrifugation, the original supernatant and wash

fractions were combined, acidified with HCl to pH 1 or slightly below pH 1, and saturated with NaCl in a 50 ml glass stoppered centrifuge tube. The aromatic acids were then extracted 3 times with 3 to 5 ml redistilled ethylacetate and once with 5 to 10 ml diethyl ether. The organic phases were combined and taken to dryness on a rotoevaporator at room temperature. Phenylbutyrate, the internal standard dissolved in dry pyridine, was added at this point. It proved to be a more versatile internal standard than mannitol because it chromatographed at a lower temperature and could accommodate isothermal runs for PAA, PLA, or PPA. Also phenylbutyrate was derivatized well by the BSA (bis-trimethylsilylacetamide) reagent, whereas mannitol reacted slowly with this reagent. The acids were derivatized as the trimethylsilyl ethers and esters using the TMS reagent described in the previous paragraph or the BSA reagent diluted 1 to 4 with dry pyridine. The BSA was preferred as it gave no reagent peaks. A 6 foot 3% SE-30 column or a 4 foot 3% OV-1 column with a Chromosorb W support, 100/120 mesh were utilized. Internal column diameter was 3 mm. Relative separation characteristics on the two columns were the same, though the temperature for the 3% OV-1 column was usually set 5 to 10 degrees lower than the temperature necessary with the SE-30 column. Initially a temperature gradient was usually run to survey the acids present. When only PAA was quantified, isothermal chromatography at 125° (SE-30) was conducted, and when only the PLA and PPA area was inspected, isothermal runs were at 150° (SE-30).

Brain tissue samples were prepared similarly. The frozen powder was homogenized with 0.6 M perchloric acid (0.5 ml per 0.1 g) with a Potter-Elvehjem type homogenizer. Usually 200 to 400 mg tissue were extracted. The neutralization and organic solvent extractions and final derivatizations were then followed as described for the plasma.

Standards with known amounts of acid and phenylbutyrate as internal standard were chromatographed with the samples, and calculations were based on the peak height ratio method. All standard solutions of the free acid were prepared in dry pyridine. TMS reagent or BSA was added directly without prior flash evaporation.

Experiments were conducted to determine recovery of aromatic acids from plasma and brain tissue. Additions of 50 and 100 ug of PAA, p- and o-OH PAA, PLA, PPA, and p-OH PPA were made to 0.5 ml rabbit serum, and the mixture was deproteinized with perchlorate. For brain tissue 50 and 100 ug of each acid were added to the perchlorate homogenate of 200 mg of tissue powder.

RESULTS

Procedural: Aromatic Acid Recoveries

The method developed for aromatic acid determinations required perchlorate extraction, KHCO_3 neutralization, HCl acidification and saturation with NaCl, followed by ethyl acetate and ether extraction of the acids. The organic phases were taken to dryness on the flash evaporator at room temperature. Loss of the acids through flash evaporation was small and is summarized in Table 2 with and without cyclohexylamine. Cyclohexylamine was not incorporated into the general procedure even though it improved some recoveries because phenylpyruvate and p-OH phenylpyruvate were difficult to derivatize in the salt form. TMS 10:4:1 reagent was used with the cyclohexylamine salt; BSA and BSTFA (bistrifluorotrimethylsilylacetamide) diluted 1:4 with pyridine were tried with the sodium salt of phenylpyruvate and proved to be unsuccessful.

Recoveries of 50 or 100 ug of the acids from rabbit plasma are given in Table 3. Washing the protein and potassium perchlorate precipitates improved the recovery. Values of PAA, PLA, and p-OH PAA recoveries are acceptable; the keto acids, though, present difficulties. Recoveries from chick brain were slightly better, especially for o-OH PAA (Table 4). This indicates a co-precipitation problem since

Table 2: Effect of Flash Evaporation on Aromatic Acid Recoveries

	% recovered	
	flash evaporation	flash evaporation with cyclohexylamine
Phenylacetic acid	88	96
Phenyllactic acid	93	99
p-OH phenylacetic acid	97	109
Phenylpyruvic acid	88	none recovered
p-OH phenylpyruvic acid	81	none recovered

Table 3: Recovery of Aromatic Acids from Rabbit Plasma

	% recovered	
	without washing precipitates*	with washing
Phenylacetic acid	74 $\pm$ 3	81 $\pm$ 15
o-OH phenylacetic acid	37 $\pm$ 23	40 $\pm$ 17
Phenyllactic acid	70 $\pm$ 3	76 $\pm$ 8
p-OH phenylacetic acid	79 $\pm$ 1	88 $\pm$ 3
Phenylpyruvic acid	40 $\pm$ 13	47 $\pm$ 23
p-OH phenylpyruvic acid	50 $\pm$ 11	30, 100

*The potassium perchlorate precipitate and protein precipitate were washed as described in the methods section. 50 and 100 ug of each acid had been added to 0.5 ml plasma. The percentages except for PPA washed represent an average of 4 values $\pm$ standard deviation.

Table 4: Recovery of Aromatic Acids from Chick Brain*

	% recovered
Phenylacetic acid	85 $\pm$ 2
o-OH phenylacetic acid	93 $\pm$ 3
Phenyllactic acid	95 $\pm$ 8
p-OH phenylacetic acid	105 $\pm$ 2
Phenylpyruvic acid	greater than 100
p-OH phenylpyruvic acid	greater than 100

*50 or 100 ug of each acid were added to about 200 mg of tissue. The percentages represent an average of 4 values $\pm$ standard deviation.

the acids were added to the plasma before deproteinization and to the brain tissue after the deproteinization.

Experimental

A. Experiment I: Phenylalanine Feeding

1. Growth of the Chicks

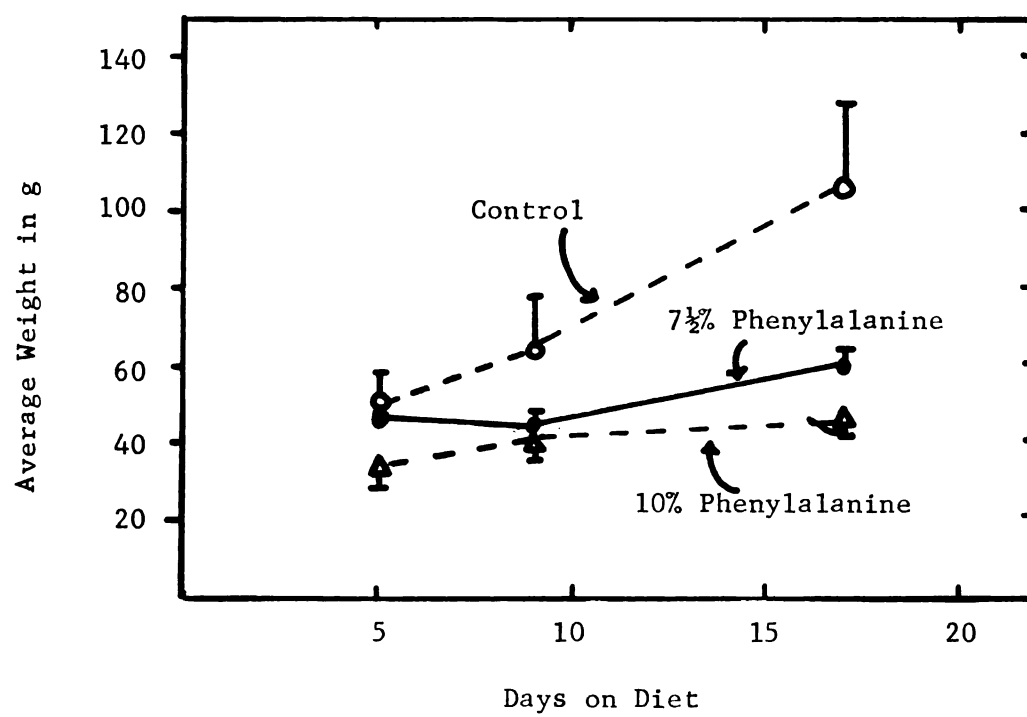
In the first experiment, male, 1 day old chicks were fed diets containing 7-1/2 and 10% L-phenylalanine for 17 days. The two experimental groups ate sparingly of the diet as reflected in Figure 2. Chicks on the phenylalanine diets gained little weight compared with the control chicks. The controls were fed ad libitum.

2. Analysis of Plasma

To better assess the chicks' nutritional state and the effectiveness of the diet in mimicing a PKU condition, the blood plasma was assayed for glucose, lactate, phenylalanine, tyrosine, and aromatic acids at two different times during the feeding period. Table 5 summarizes the results. Although food consumption was reduced, the phenylalanine fed chicks maintained normal blood glucose concentrations. Lactate, however, was decreased by 50% in the groups on the phenylalanine diets. Control lactate values are considerably lower for the 5 day dietary group than for the 17 day group. A trichloroacetic acid extraction procedure followed by an ether wash for removal of the acid had been used in determining the 5 day concentrations instead of the barium hydroxide-zinc sulfate method, suggesting partial extraction of lactic acid with the trichloroacetic acid. The difference

Figure 2: Growth rate of chicks fed diets containing
7-1/2 or 10% phenylalanine

Standard deviation is indicated.



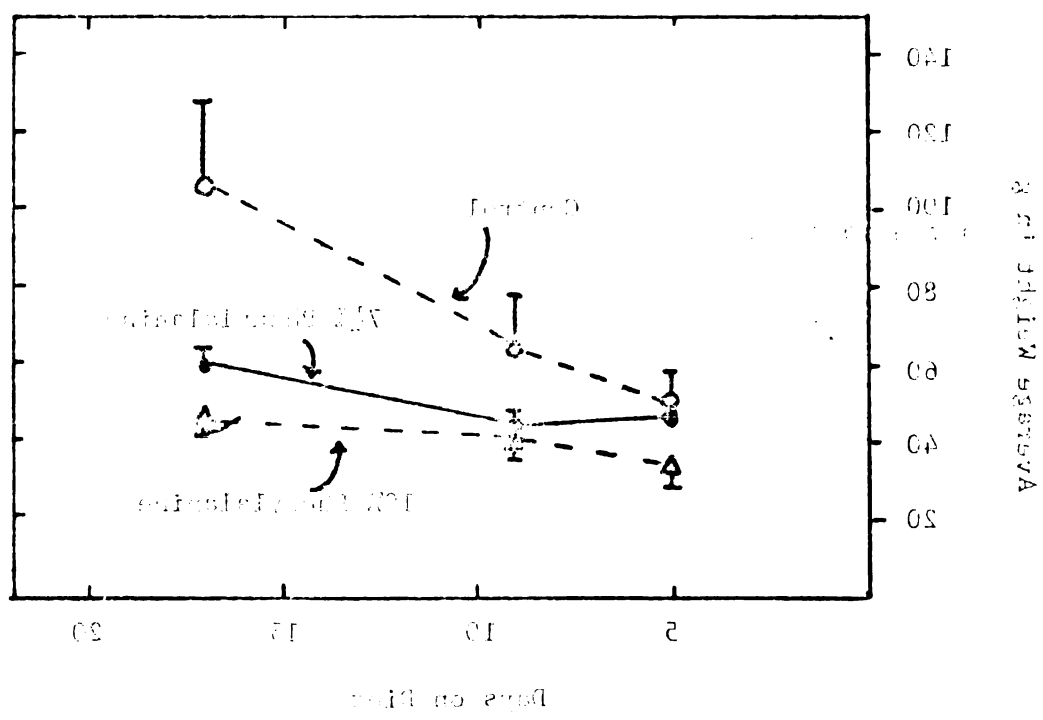


Table 5: Concentrations of Glucose, Lactate, Phenylalanine, Tyrosine, and the Aromatic Acids in Plasma from Phenylalanine Fed Chicks*

	Control ad libitum fed	7-1/2% phenylalanine	10% phenylalanine
	<u>5 day dietary group</u>		
Glucose	11.31 + 0.57	11.73 + 0.55	11.16
Lactate	1.24	0.52	0.36
Phenylalanine	0.17	2.40	3.44
Tyrosine	0.27	0.58	0.79
Phenyl lactic acid	none	not done	0.08 + 0.01
Phenylpyruvic acid	none	not done	0.07
p-OH phenylacetic acid	none	not done	detected
	<u>17 day dietary group</u>		
Glucose	12.81 + 1.51	12.58 + 1.01	13.66 + 2.06
Lactate	2.21	1.28	1.04
Phenylalanine	0.17	3.90	5.58
Tyrosine	0.26	1.97	1.89
Phenyl lactic acid	none	0.05	0.16 + 0.01
Phenylpyruvic acid	none	0.16	0.15
p-OH phenylacetic acid	none	0.10	detected

* Blood was taken by the drainage method and pooled from 2-3 chicks per group. Concentrations are expressed mM + standard deviation.

cannot be due to the ages of the animals as a value of 2.49 mM was determined in another experiment for blood lactate in 6 day old chicks.

The plasma phenylalanine and tyrosine concentrations increased with percentage of dietary phenylalanine and with time on the diet.

Of the aromatic acids surveyed by GLC (PAA, p- and o-OH PAA, PLA, PPA, and p-OH PPA) only p-OH PAA, PLA, and PPA were detected and quantitated. Their concentrations were extremely low.

Refer to Figure 3 for a standard chromatogram of phenylbutyrate (internal standard), PLA, PPA, and p-OH PAA and an experimental chromatogram of 7-1/2% phenylalanine, 17 day group, plasma. Three peaks are present corresponding to the standards. The p-OH PAA was quantified only in this sample and detected at considerably lower concentrations in the other samples. The 5 day, 7-1/2% sample, did not chromatograph well and there was insufficient plasma to repeat the determination. Although not shown, a programmed temperature run was made of control plasma extract and no peaks were apparent in the PLA-PPA area.

3. Analysis of Brain Glycolytic Metabolites and Adenine Nucleotides

The effect of high phenylalanine concentrations on energy metabolism in the brain was approached by studying the adenine nucleotide and glycolytic intermediate levels in the cerebral tissue of the chicks after 5 and 17 days of the 7-1/2 and 10% phenylalanine feedings (Figures 4a and 4b).

Figure 3: Gas chromatogram of aromatic acids extracted from plasma of chicks fed phenylalanine

A standard chromatogram of the trimethylsilyl derivatives of internal standard phenylbutyric acid (1), phenyllactic acid (2), p-OH phenylacetic acid (3), and phenylpyruvic acid (4) is represented. Also given is a chromatogram of an extract with corresponding peaks from 0.4 ml plasma from chicks fed 7-1/2% phenylalanine for 17 days. The arrow indicates a sensitivity increase of 4. A 6 foot 3% SE-30 column was used isothermally at a column temperature of 147°. Reagent used was pyridine (10) : hexamethyldisilazane (4) : trimethylchlorosilane (1).

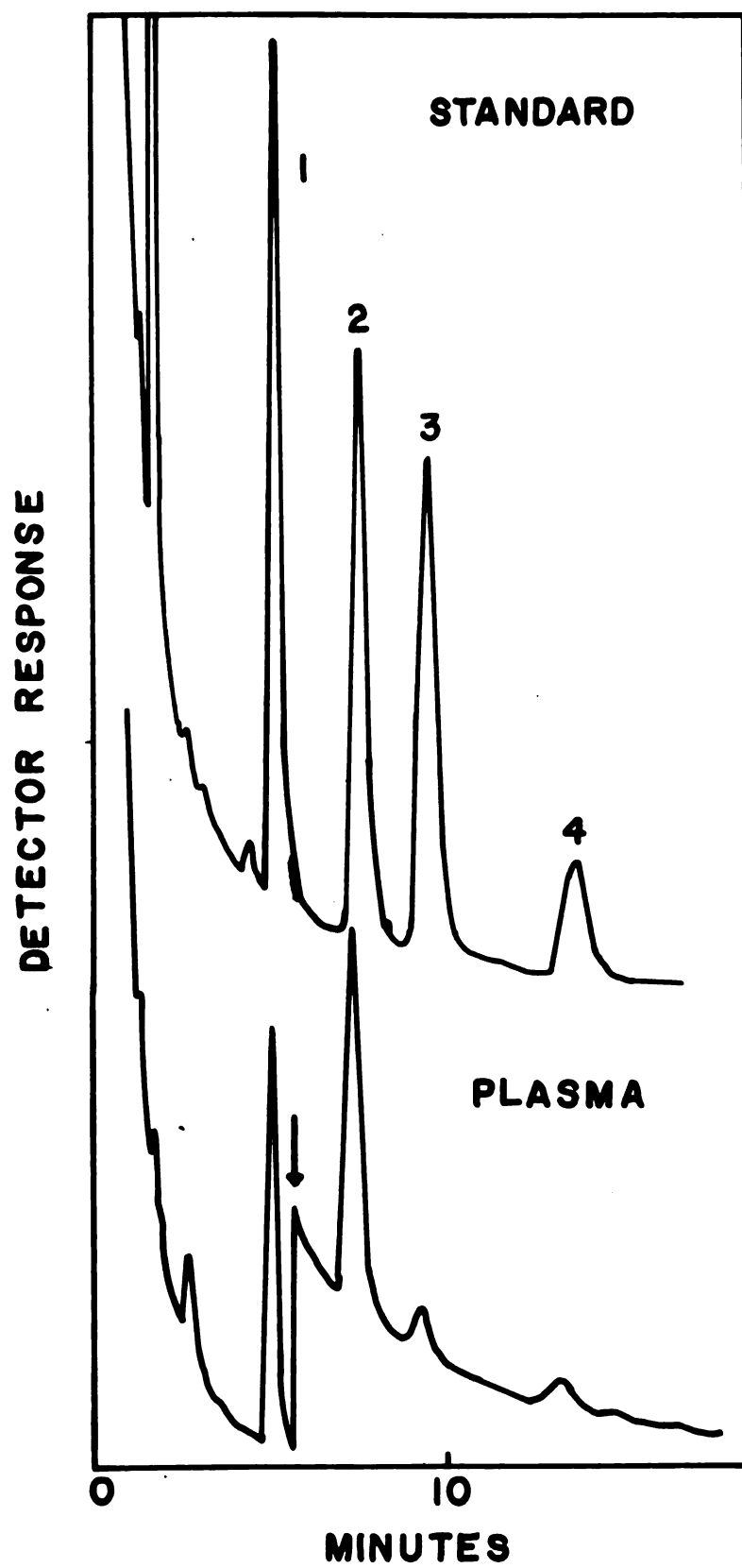
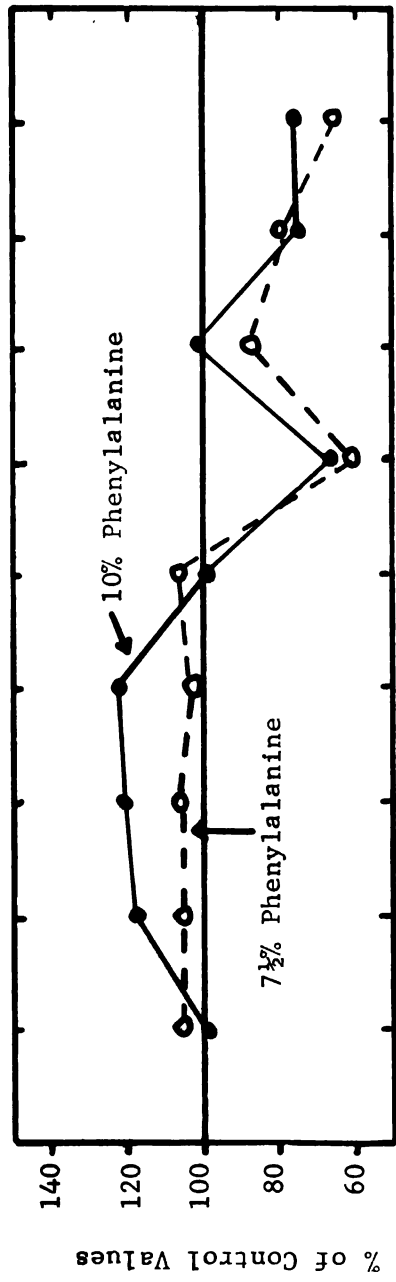


Figure 4a: Analysis of the adenine nucleotides and some glycolytic intermediates in the brains of chicks fed diets containing 7-1/2 and 10% phenylalanine

The values are expressed as percentage of control values. The control values are given below the metabolite in terms of μ moles per g of tissue $\pm$ standard deviation. The chicks were fed the experimental diets for 5 days and were about 6 days old at sacrifice. The controls were fed ad libitum. Each point represents the average of triplicate determinations on brain tissue pooled from 2 chicks. Statistical significance (p) for the 7-1/2% group is as follows: glucose and fructose diphosphate, less than 0.001; lactate, less than 0.02. Statistical significance (p) for the 10% group is as follows: glucose, less than 0.001; fructose diphosphate, less than 0.02; lactate and creatine phosphate, less than 0.05.



ATP	ADP	AMP	Cr-P	GLY	Glu	G-6-P	FdP	Lac
1770 ± 240	488 ± 68	196 ± 36	1770 ± 240	1470 ± 280	926 ± 66	47 ± 2	223 ± 8	2520 ± 230

$\Delta T_{\text{eff}}^{\text{eff}}$ 1100 F 920 F 880 F 850 F 820 F
 $\Delta T_{\text{eff}}^{\text{eff}}$ 1100 F 920 F 880 F 850 F 820 F

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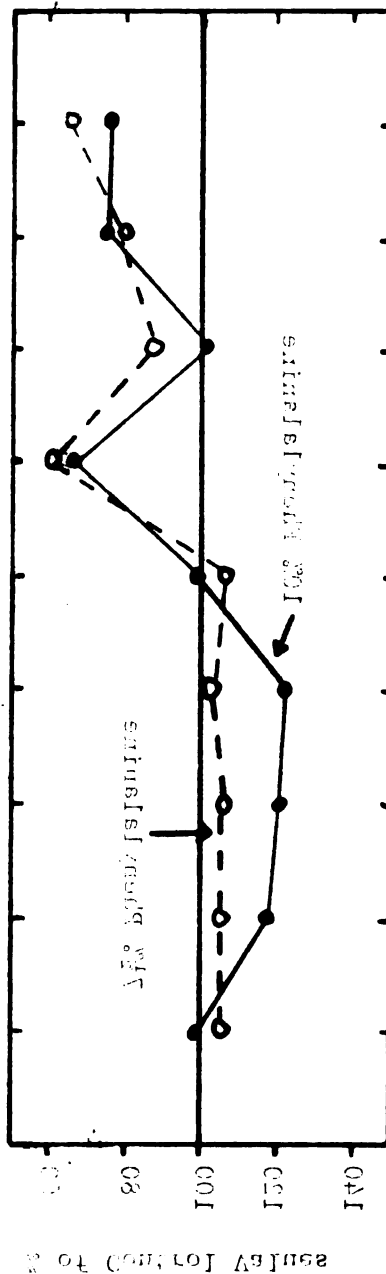
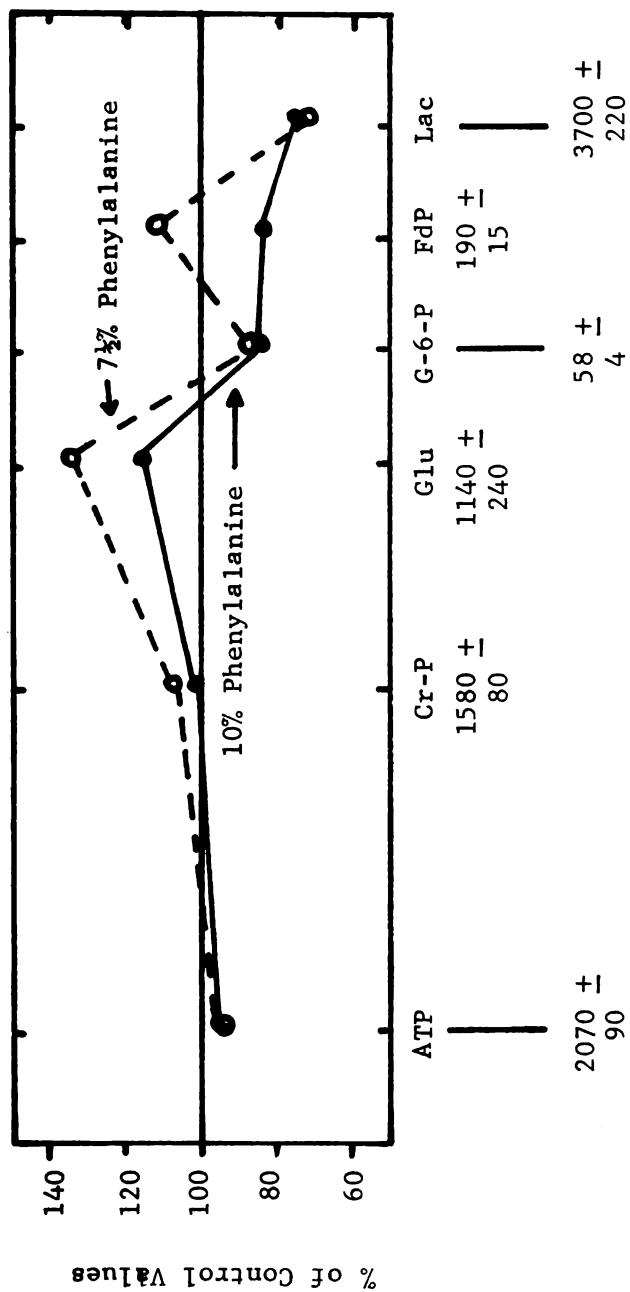
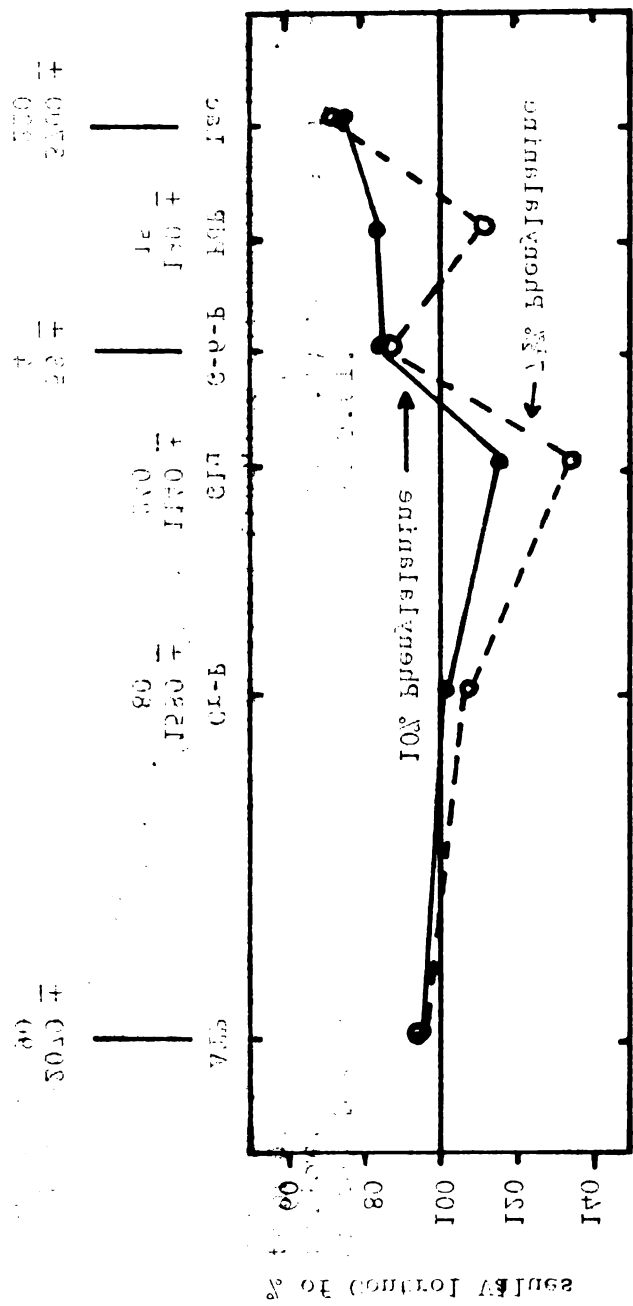


Figure 4b: Analysis of the adenine nucleotides and some glycolytic intermediates in the brains of chicks fed diets containing either 7-1/2 or 10% phenylalanine

The values are expressed as percentage of control values. The control values are given below the metabolite in terms of mmoles per g of tissue + standard deviation. The chicks were fed the experimental diets for 17 days and were 18 days at sacrifice. The controls were fed ad libitum. Each point represents the average of triplicate determinations of brain tissue pooled from 2 or 3 chicks. Statistical significance (p) for the 7-1/2% group is as follows: glucose and lactate, less than 0.01; fructose diphosphate, less than 0.05. Statistical significance (p) for the 10% group is the following: glucose-6-phosphate, less than 0.1; fructose diphosphate, less than 0.05; lactate, less than 0.01.





In those chicks on diet for 5 days, ATP, ADP, and AMP levels were not significantly different. Creatine phosphate was increased to 120% of the control in the 10% phenylalanine group and was unchanged in the 7-1/2% group. Brain glucose was decreased to 60 and 66% of control values for the 7-1/2 and 10% phenylalanine fed chicks, respectively. Fructose-1,6-diphosphate and lactate were also reduced in the experimental animals. Glucose-6-phosphate remained unchanged as did glycogen. The decreases observed in the metabolite levels for the 10% group generally seem to be less than those for the 7-1/2% group. In those chicks on diet for 17 days ATP levels remained the same; ADP and AMP were not assayed. Creatine phosphate was unchanged. Glucose levels for the 7-1/2 and 10% groups were no longer depressed but were 134 and 117% of control values. Fructose-1,6-diphosphate increased over the control in the 7-1/2% and decreased in the 10%. Glucose-6-phosphate was decreased significantly only in the 10% group. Lactate concentrations were decreased in both 7-1/2 and 10% chicks.

4. Analysis of Brain Aromatic Acids

A survey of aromatic acids in the brains of the phenylalanine fed chicks was attempted, but was unsuccessful. In the 5 day dietary group some material was detected by GLC at very low attenuations in the area of phenyllactate and p-OH phenylacetic acid, but the control background chromatogram displayed smaller amounts of corresponding peaks. In the 17 day group similar peaks were present and were highly unstable from one injection to the next. The standards did

not demonstrate such lability.

B. Experiment II: Phenylalanine Feeding

1. Analysis of Brain Phenylalanine and Tyrosine

Brain levels of phenylalanine and tyrosine were determined in a second phenylalanine feeding experiment. The chicks were fed 10% L-phenylalanine for 6 and 14 days. The values are presented in Table 6. The control tyrosine levels were determined by James Blosser employing an amino acid analyzer. Using the method previously described, we found no tyrosine in the control sample, although radioactivity was present in the material eluted from paper corresponding to the tyrosine position. The samples from the 6 day group fed phenylalanine were lost because of internal standard difficulties. Figure 5 is a gas chromatogram of standard phenylalanine and a control sample; Figure 6, a gas chromatogram of standard tyrosine and an experimental sample. Glutamate and stearate were used as internal standards for tyrosine. Stearate seemed to be more consistent; glutamate gave a small peak preceding the major peak, suggesting incomplete derivatization under our conditions. The first peak increased considerably in size when glutamate was directly butylated without prior methylation.

2. Analysis of Plasma

Table 7 summarizes the data on the plasma levels of phenylalanine and tyrosine. Concentrations were increased in the phenylalanine fed chicks.

Table 6: Brain Phenylalanine and Tyrosine Levels Resulting from Feeding a Diet Containing 10% Phenylalanine*

	Control	10% Phenylalanine
	<u>6 days on diet</u>	
Phenylalanine (mM)	0.041	0.986, 0.976
Tyrosine (mM)	0.044 ^a	-----
	<u>14 days on diet</u>	
Phenylalanine (mM)	0.046, 0.064	1.965, 2.910
Tyrosine (mM)	0.044 ^a	0.264, 0.182 ^b 0.152, 0.155 ^c

* Determinations were done in duplicates on tissue pooled from 6 or 8 chicks.

a Unpublished results by James Blosser determined on an amino acid analyzer.

b Calculated using glutamate as the internal standard.

c Calculated using stearate as the internal standard.

Table 7: Plasma Phenylalanine and Tyrosine Levels in Chicks Fed a Diet Containing 10% Phenylalanine, Experiment II*

	Control	10% Phenylalanine
<u>6 days on diet</u>		
Phenylalanine (mM)	0.122 $\pm$ 0.012	3.46 $\pm$ 0.19
Tyrosine (mM)	0.108	1.32 $\pm$ 0.04
<u>14 days on diet</u>		
Phenylalanine (mM)	0.147 $\pm$ 0.002	3.85 $\pm$ 0.78
Tyrosine (mM)	0.118 $\pm$ 0.038	2.17 $\pm$ 0.08

*Determinations were done in triplicate on pooled samples: 8-6 chicks per group. The controls were fed ad libitum. Values $\pm$ standard deviation

Figure 5: Gas chromatogram of phenylalanine from chick brain

A gas chromatogram of standard hydroxyproline and phenylalanine as the butyl ester and tri-fluoroacetamide derivatives is represented. Hydroxyproline was the internal standard chosen. Also given is a chromatogram of an extract with corresponding peaks from brain tissue from a control fed chick. A 6 foot 2% OV-17 column operated isothermally at 140° was used.

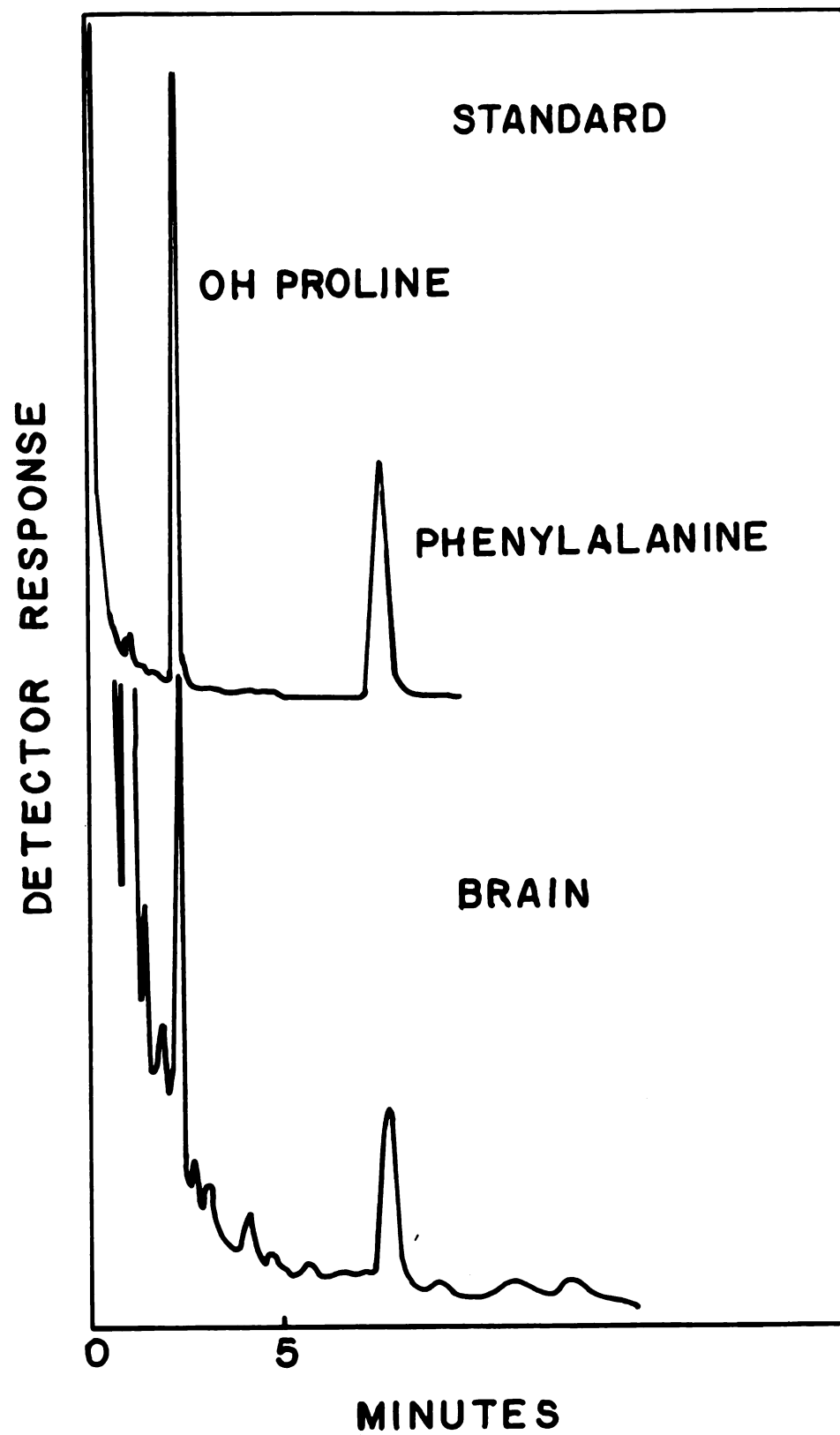
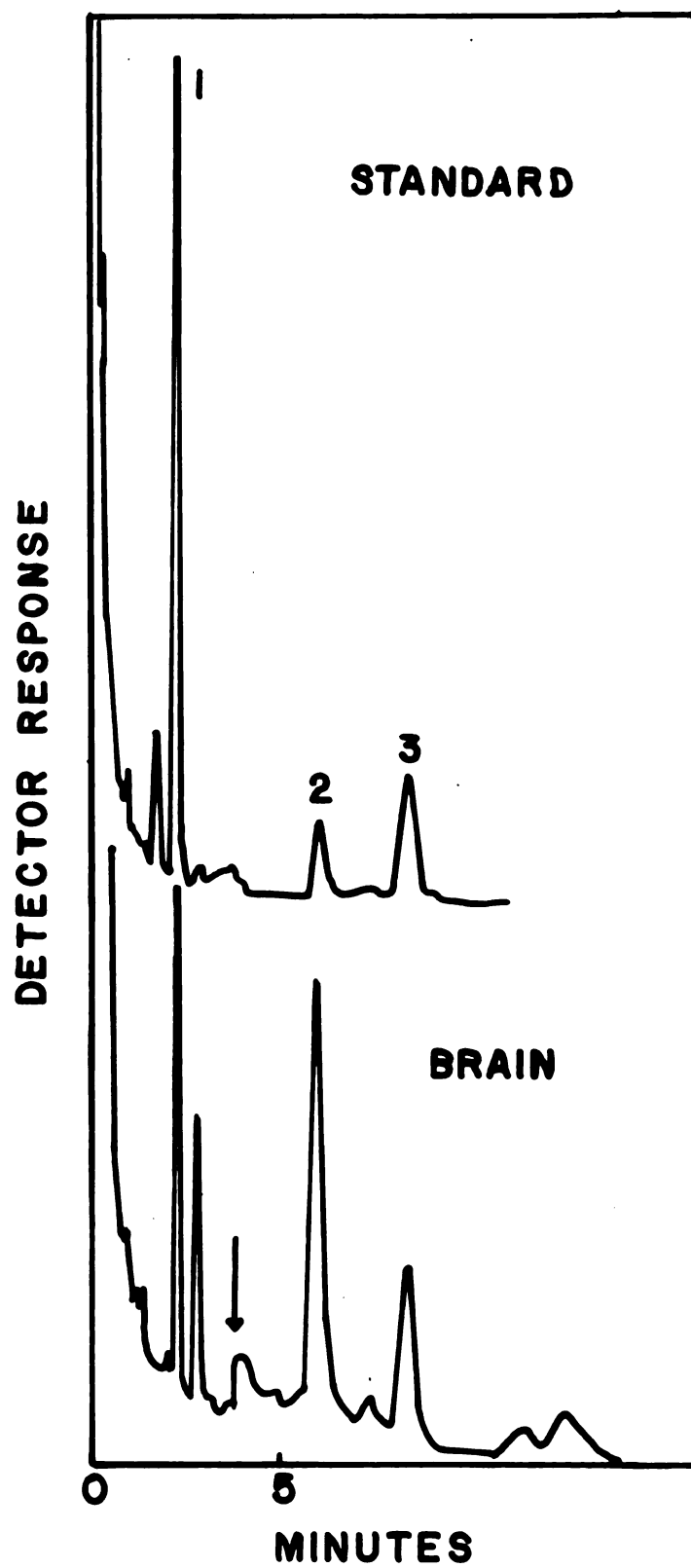


Figure 6: Gas chromatogram of tyrosine from chick brain

A gas chromatogram of standard glutamate (1), tyrosine (2), and stearic acid (3) as the butyl ester and trifluoroacetamide derivatives is represented. Glutamate and stearic acid were chosen as internal standards. Also given is a chromatogram of an extract with corresponding peaks from the brains of chicks fed 10% phenylalanine for 14 days. A 6 foot 2% OV-17 column operated isothermally at 185° was used. The arrow indicates a sensitivity increase of 2.



C. Experiment III: Phenylacetic Acid Feeding

1. Description of Toxic State

Phenylacetic acid has been detected predominantly in the conjugated form in the urine of phenylketonurics. Chicks were fed the free acid form of PAA at 5 and 10% levels; they displayed symptoms of toxicity after 4 or 5 days on either of the diets. Though death occurs, the chick's activity state is only slightly reduced; they undergo no convulsions. The chicks ate little of the diet. At time of sacrifice ad libitum fed controls, 6 days old, weighed on the average 57 g compared with 33 g for the PAA fed chicks.

2. Analysis of Plasma and Aromatic Acid Content of the Brain

The plasma was assayed for glucose, lactate, and phenylacetic acid. The results are summarized in Table 8. The 5% group was able to maintain normal glucose levels; however, a slight but significant hypoglycemia occurred in the 10% group. Lactate was reduced in both the 5 and 10% groups. Phenylacetic acid rose dramatically in the plasma, reaching a higher level in those chicks fed the 10% diet.

Analysis of brain aromatic acids (Table 9) showed that phenylacetic acid also accumulated in the brain. Figure 7 presents typical gas chromatograms of brain extracts from experimental tissue and control tissue, and of a standard of phenylacetic acid. A peak corresponding to standard PAA occurred in the experimental and was absent in the control. The chromatograms for plasma extracts were very similar to those for the brain.

Table 8: Plasma Concentrations of Glucose, Lactate, and Phenylacetic Acid from Chicks Fed Diets Containing Phenylacetic Acid*

	Control fed <u>ad</u> <u>libitum</u>	5% Phenyl- acetic acid	10% Phenyl- acetic acid
Glucose (mM)	12.77 $\pm$ 0.62	12.55 $\pm$ 1.17	10.38 $\pm$ 0.28**
Lactate (mM)	2.49 $\pm$ 0.32	1.11 $\pm$ 0.06**	1.15 $\pm$ 0.08**
Phenylacetic acid (mM)	none	3.68 $\pm$ 0.52	4.20, 5.07

* Blood was taken by drainage method and pooled, 4 to 6 chicks per group. Values $\pm$ standard deviation.

** P less than 0.01

Table 9: Brain Phenylacetic Acid Concentrations in Chicks Fed Phenylacetic Acid

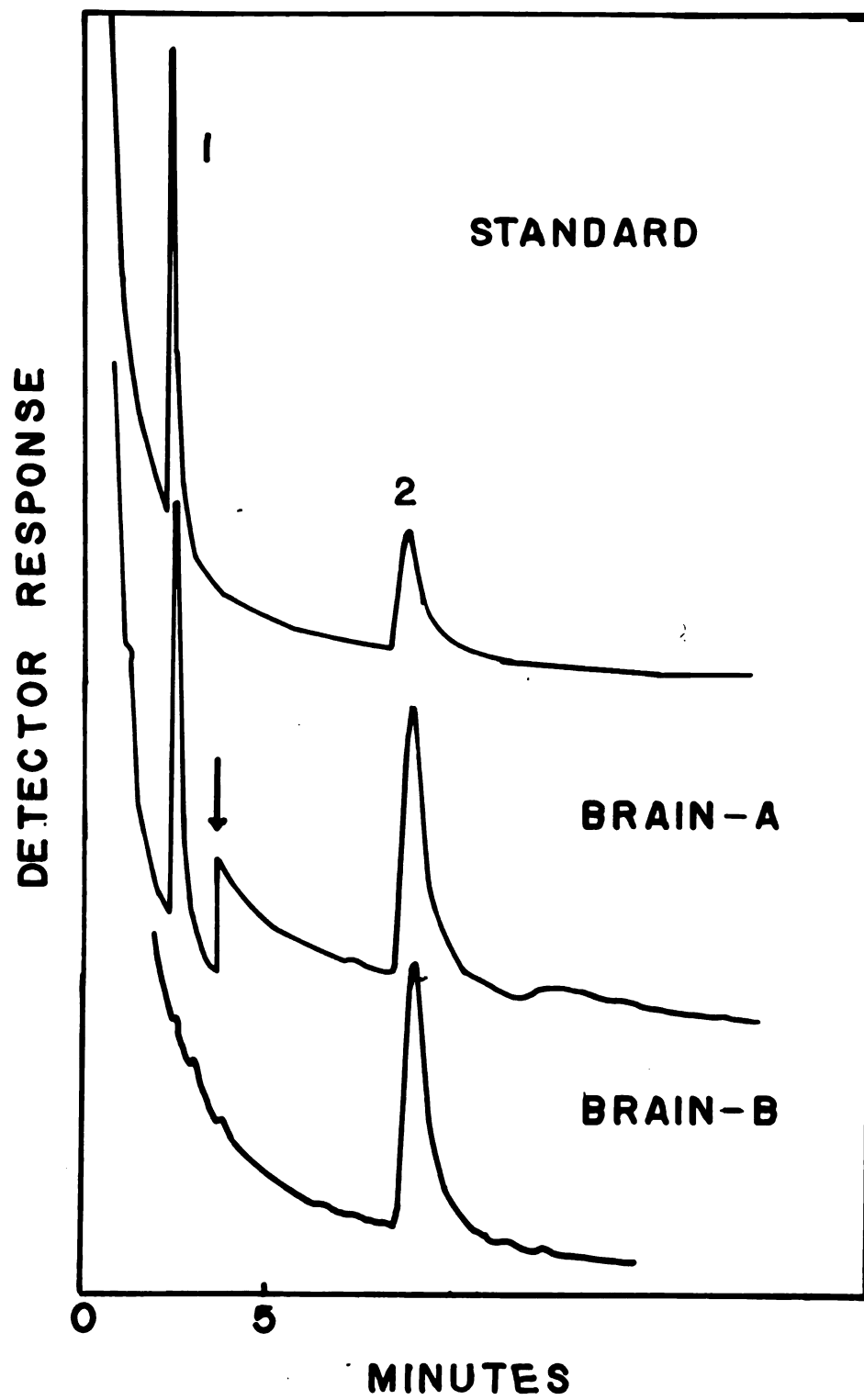
	Phenylacetic Acid (mM)
Control	None
5% Phenylacetic acid	1.97 $\pm$ 0.12*
10% Phenylacetic acid	1.83, 1.77

* $\pm$ standard deviation

Brains were pooled 6 per group.

Figure 7: Gas chromatogram of phenylacetic acid from the brains of chicks fed phenylacetic acid

A standard chromatogram of the trimethylsilyl derivatives of phenylacetic acid (1) and internal standard phenylbutyric acid (2) is represented. Chromatogram "brain - A" is characteristic of the extract from 200 mg brain tissue from chicks fed phenylacetic acid. The arrow indicates a sensitivity increase of 2. Chromatogram "brain - B" is characteristic of control brain tissue. A 6 foot 3% SE-30 column operated isothermally at a column temperature of 120° was used. BSA was used as the trimethylsilylation reagent.



3. Analysis of Brain Glycolytic Metabolites and Adenine Nucleotides

The adenine nucleotide and glycolytic intermediate levels for the 5 and 10% feeding conditions are given in Figure 8. The glycolytic pathway was significantly affected. Glucose, glucose-6-phosphate, fructose-1,6-diphosphate, and lactate decreased in both experimental groups, and the reduction was proportional to the PAA in the diets for all metabolites except glucose-6-phosphate. While glucose was reduced 40 and 70% of the control level, ATP and creatine phosphate concentrations were unchanged. ADP was lowered in the PAA fed; decreased AMP and glycogen in the PAA group were not significant at the 0.1 level.

D. Experiment IV: Phenylacetic Acid - Sodium Salt Feeding

1. Description of Toxic State

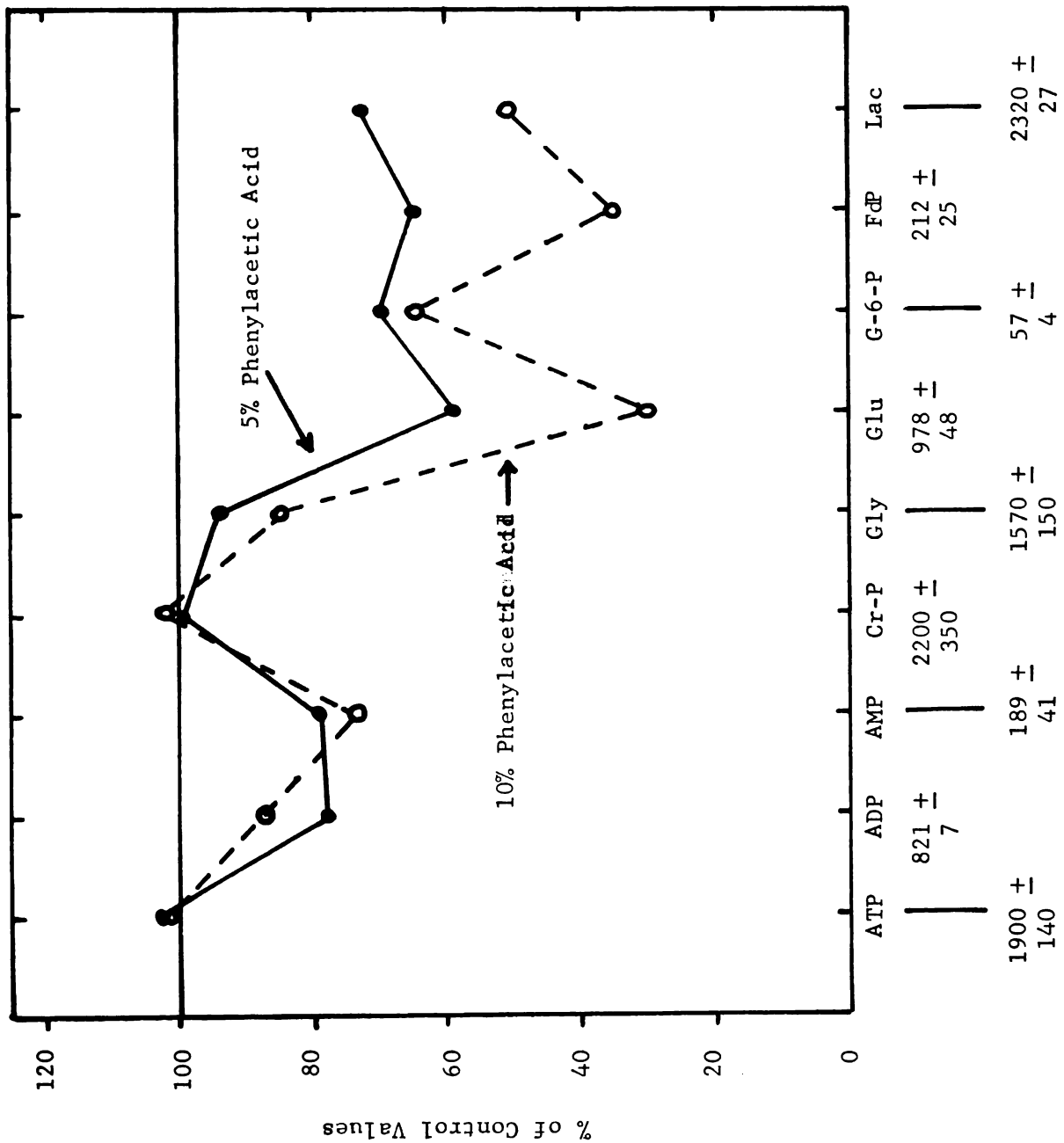
To eliminate any acidotic effects in the toxicity the chicks were fed a diet 10% in the sodium salt of phenylacetic acid; the 10% PAA salt diet is equivalent to less PAA than the 10% free acid diet. Also to assess the effect of starvation, the controls were fed the weight of food consumed by the experimental chicks the day before (paired-feeding technique). The toxicity developed in the chicks as before after about 4 days. The PAA chicks at 5 days of age weighed 33 g on the average and the "pair-fed" controls, 43 g. Ad libitum fed controls would have weighed 50 to 60 g.

2. Analysis of Brain Glycolytic Metabolites and ATP

Table 10 indicates that the changes observed previously in the brain glycolytic metabolites were independent

Figure 8: Analysis of adenine nucleotides and some glycolytic intermediates in brains from chicks fed diets containing 5 and 10% phenylacetic acid

Values for the determinations on the PAA fed chicks are expressed as percentage of control values. The control values are given below the specific metabolite and are expressed as $\mu\text{mole per g of tissue} \pm \text{standard deviation}$. Each point represents an average of 3 determinations done on tissue pooled from 3 to 5 chicks. The chicks were 6 days old. The controls were fed ad libitum. Statistical significance (p) of the values for the 5% group are summarized as follows: glucose and ADP, less than 0.001; glucose-6-phosphate, less than 0.01; fructose-1,6-diphosphate, less than 0.02; lactate, less than 0.05. Levels of significance for the 10% group are: glucose and fructose-1,6-diphosphate, less than 0.001; ADP and glucose-6-phosphate and lactate, less than 0.01.



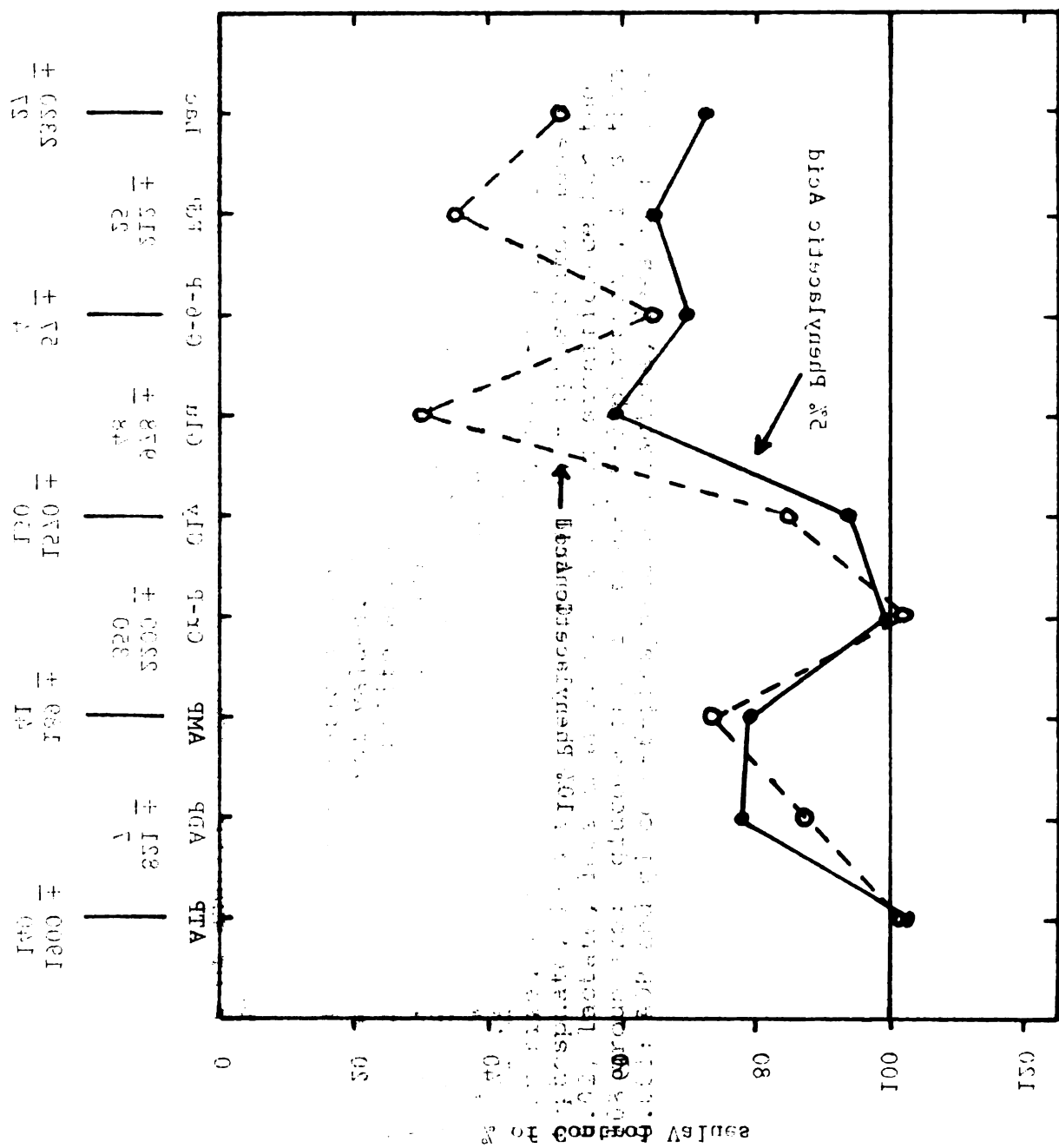


Table 10: Analysis of ATP and Some Glycolytic Intermediates
in Brains from Chicks Fed a 10% Phenylacetic
Acid - Sodium Salt Diet /

Metabolite	Control	10% Phenyl- acetic Acid	% Control
ATP	2000 $\pm$ 140	1850 $\pm$ 170	93
Creatine phosphate*	2450 $\pm$ 150	2210 $\pm$ 180	86
Glucose**	998 $\pm$ 189	449 $\pm$ 66	50
Fructose diphosphate***	210 $\pm$ 14	160 $\pm$ 25	76

/ The chicks were 5 days old and had been on diet for 4 days. The controls were fed a restricted diet. The values in terms of mumoles per g of tissue $\pm$ standard deviation represent an average of 3 or 6 determinations on brain tissue pooled from 6 chicks.

* P less than 0.02

** P less than 0.001

*** P less than 0.01

of acid or salt feeding. Glucose and fructose-1,6-diphosphate were again reduced. Creatine phosphate decreased by 14%, whereas in the first experiment with PAA feeding, it remained unchanged. ATP was the same in the experimental chick as in the control.

3. Analysis of Plasma

In Table 11 plasma glucose, lactate, uric acid, and phenylacetic acid levels are reported. The blood was taken at different times by different methods. When taken by the drainage method, the PAA plasma demonstrated 20% hypoglycemia. When the blood was removed by heart puncture, the glucose was reduced by 8% in one case and increased by 6% in another case compared with the control. Lactate determined in the plasma taken by the first method was reduced in the phenylacetic acid fed chicks. Uric acid was increased 160% of the control. Swanson (101) reported that the feeding of sodium benzoate to humans produced a decrease of uric acid in the urine and an increase in the blood. Benzoate is metabolized analogously to phenylacetic acid; it is conjugated with glycine to form hippuric acid in a reaction requiring CoASH and ATP.

4. Analysis of Aromatic Acids in Liver and Brain

The concentrations of phenylacetic acid in liver and brain tissue are presented in Table 12. Plasma and brain levels approximated those observed in the first experiment. The concentration in liver was similar to that in brain. No other aromatic acids were detected by GLC.

Table 11: The Concentration of Various Metabolites in Plasma of Chicks Fed Phenylacetic Acid - Sodium Salt Diet

Metabolite	Control $\neq$	10% Phenyl-acetic Acid	% Control
Glucose (a)	11.28 $\pm$ 0.43	8.97 $\pm$ 0.45	80*
(b)	10.64 $\pm$ 0.46	11.29 $\pm$ 0.23	106**
(b)	11.59 $\pm$ 0.39	10.62 $\pm$ 0.09	92*
Lactate (a)	1.68 $\pm$ 0.19	1.29 $\pm$ 0.26	77***
Uric acid (a)	0.56 $\pm$ 0.02	0.90 $\pm$ 0.03	161*
Phenylacetic acid (a)	none	3.32 $\pm$ 0.26	

$\neq$ Controls were fed a restricted diet.

a Blood was taken by drainage method and pooled, 6 chicks per group. Chicks were 5 days old. Values are expressed as mM $\pm$ standard deviation. They represent an average of triplicate determinations.

b Blood was taken by heart puncture and pooled, 3 animals per group. Chicks were 6 days old. Values are expressed as described for "a."

* p less than 0.001

** p less than 0.02

*** p less than 0.01

Table 12: Phenylacetic Acid Concentrations in Liver and Brain from Chicks Fed a 10% Phenylacetic Acid - Sodium Salt Diet*

<u>Liver</u>	<u>Brain</u>
2.39 mM $\pm$ 0.40	2.09 mM $\pm$ 0.27

* The values represent an average $\pm$ standard deviation of triplicate determinations on tissue pooled from 6 animals.

5. Analysis of Liver Glycolytic Metabolites and Adenine Nucleotides

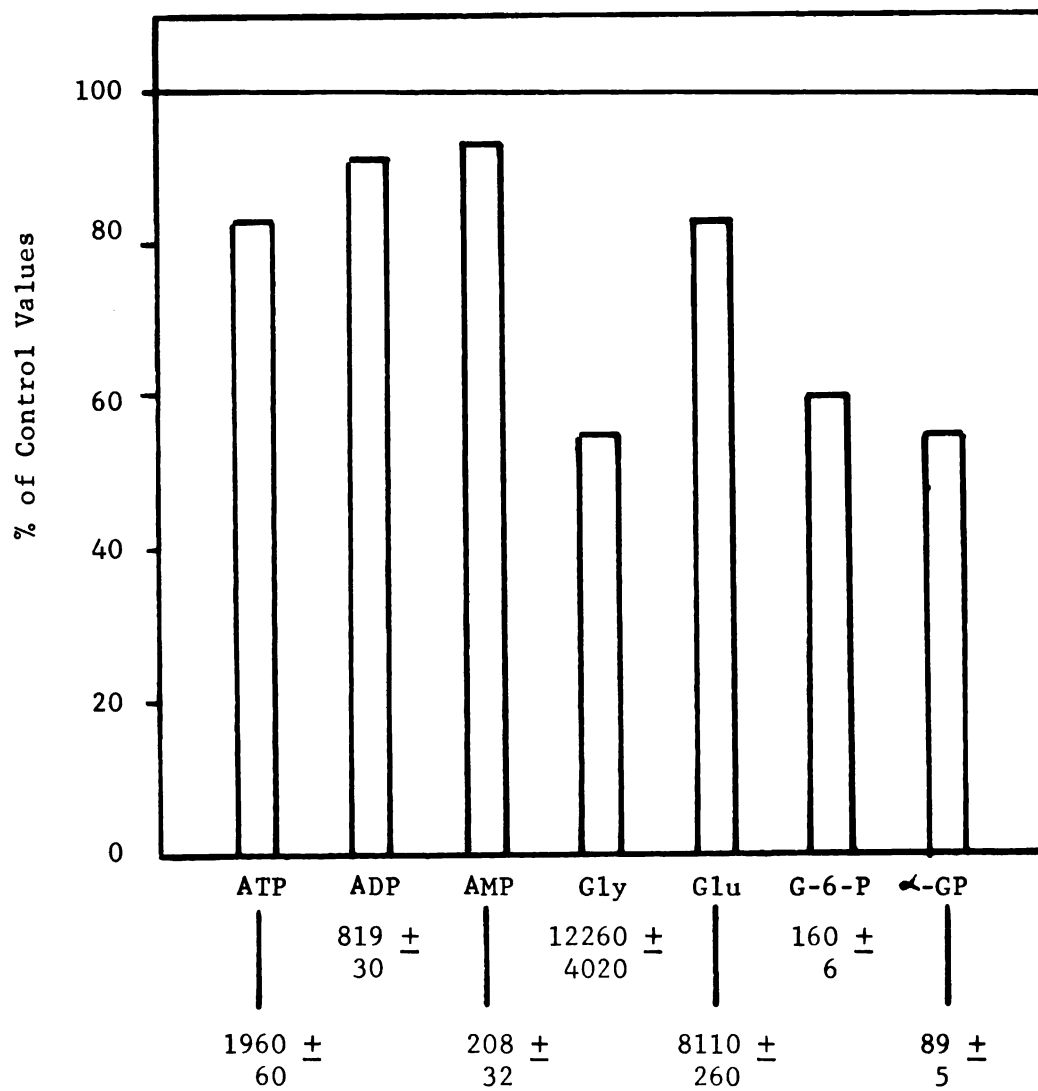
Liver glycolytic metabolism was also studied in the phenylacetic acid fed chicks since the only known reaction of the acid, conjugation, is confined to this organ and to the kidneys. Figure 9 presents the results from analyses on the livers from chicks fed the PAA - sodium salt diet for 5 days. ATP was significantly decreased; ADP and AMP levels were also reduced, but the high deaminase activity of the liver prevents attaching any significance to this. Liver glycogen in the phenylacetic fed animals was 55% of the control value. Glucose, glucose-6-phosphate, and L-alpha-glycerol phosphate were also decreased.

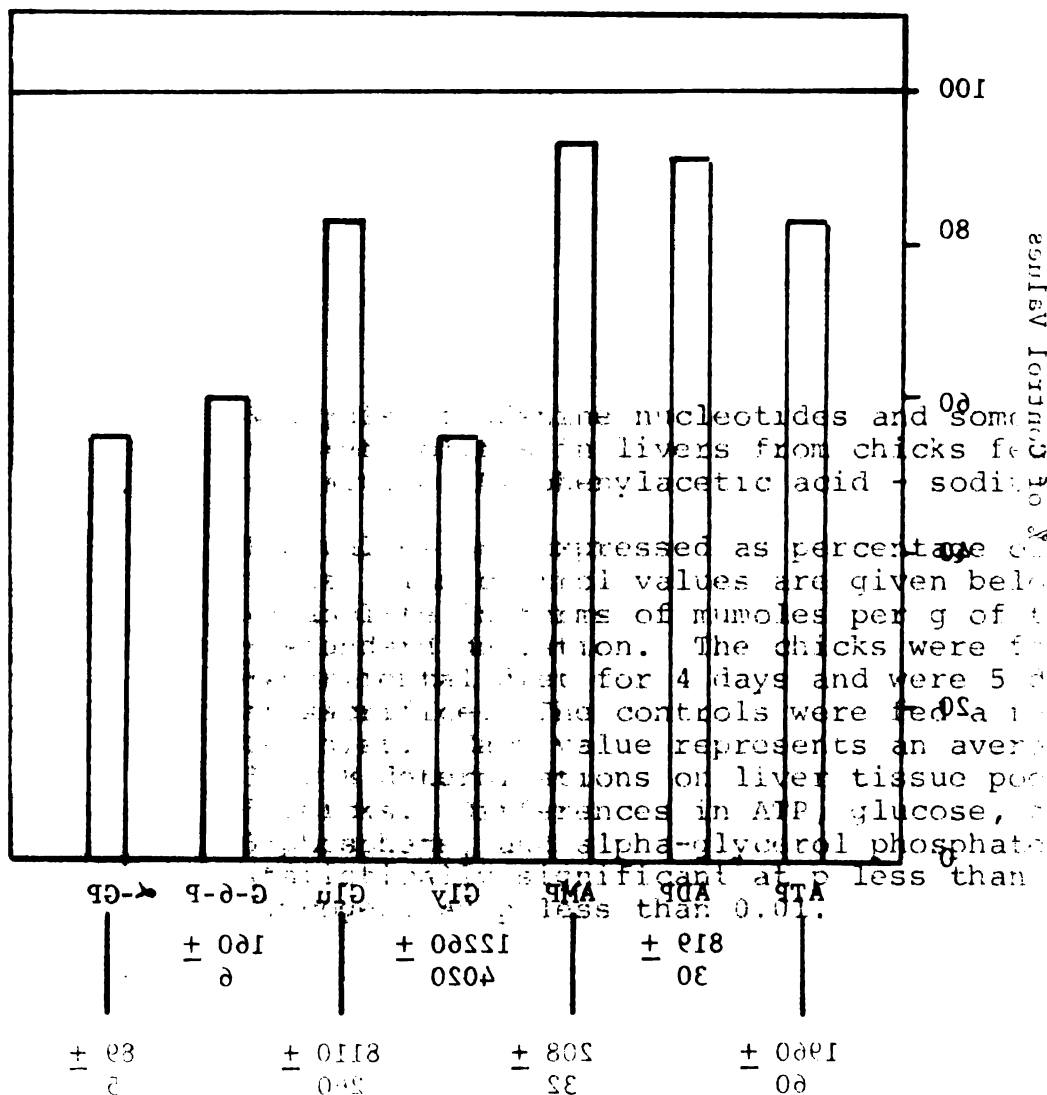
E. Liver Glycolytic Metabolites and ATP During Phenylalanine Feeding

Figure 10 illustrates some liver glycolytic intermediates in chicks fed the 10% L-phenylalanine diet for 6 (graph A) and 14 days (graph B). The tissue was taken from experiment II animals. The experimental chicks are compared with controls fed ad libitum. In the 6 day group, ATP increased; glycogen, glucose, glucose-6-phosphate, and alpha-glycerol phosphate decreased. The control values for the metabolites were higher than the previous control values in the phenylacetic acid study in which the control chicks were fed a restricted diet. The group fed for 14 days displayed normal liver ATP, decreased glucose, glucose-6-phosphate and alpha-glycerol phosphate. The glycogen value for the experimental group is questionable. Several samples were lost during hydrolysis and only one determination was made.

Figure 9: Analysis of adenine nucleotides and some glycolytic metabolites in livers from chicks fed a diet containing 10% phenylacetic acid - sodium salt

The values are expressed as percentage of controls. The control values are given below the metabolite in terms of mumoles per g of tissue $\pm$ standard deviation. The chicks were fed the experimental diet for 4 days and were 5 days old at sacrifice. The controls were fed a restricted diet. Each value represents an average of 3 or 8 determinations on liver tissue pooled from 6 chicks. Differences in ATP, glucose, glucose-6-phosphate, and alpha-glycerol phosphate were statistically significant at p less than 0.001; glycogen, at p less than 0.01.

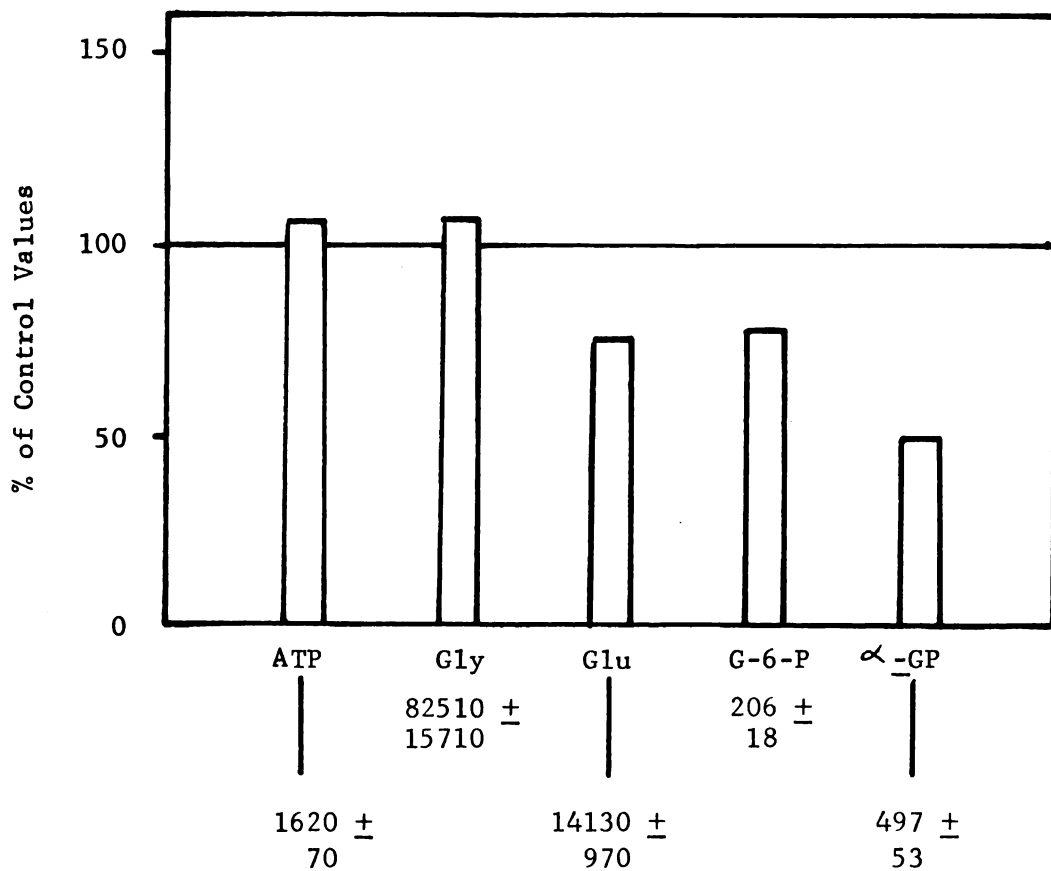
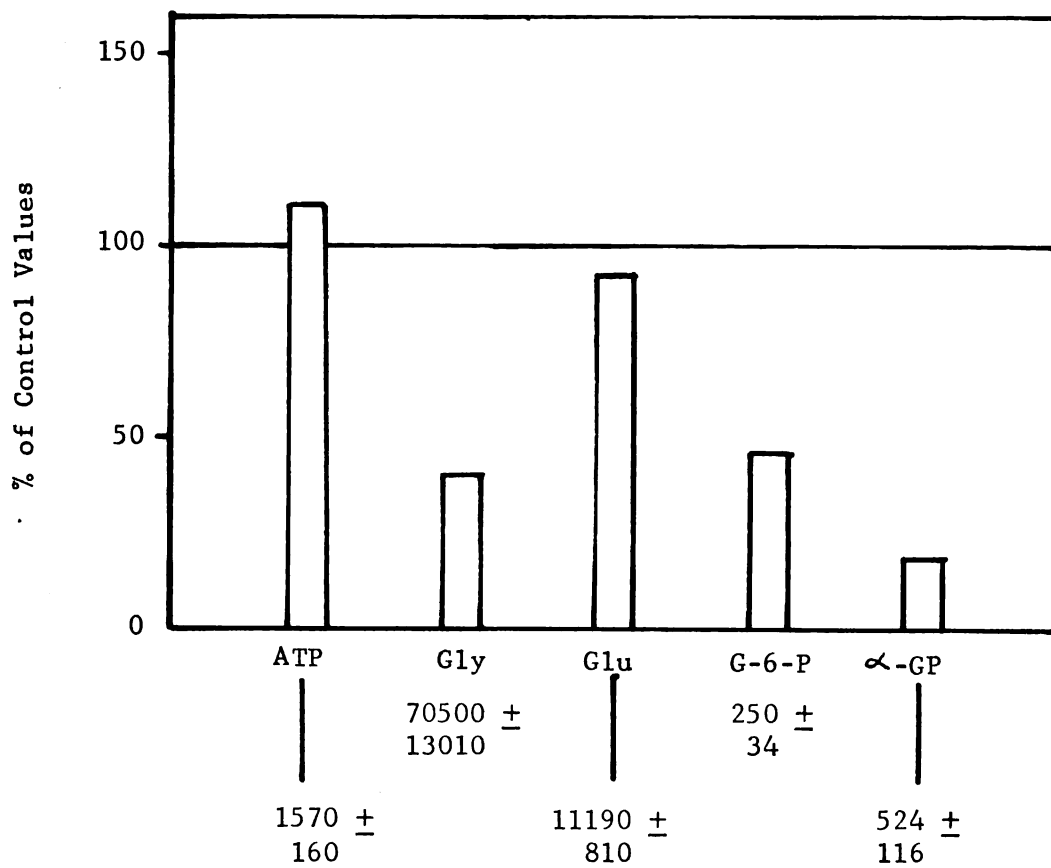


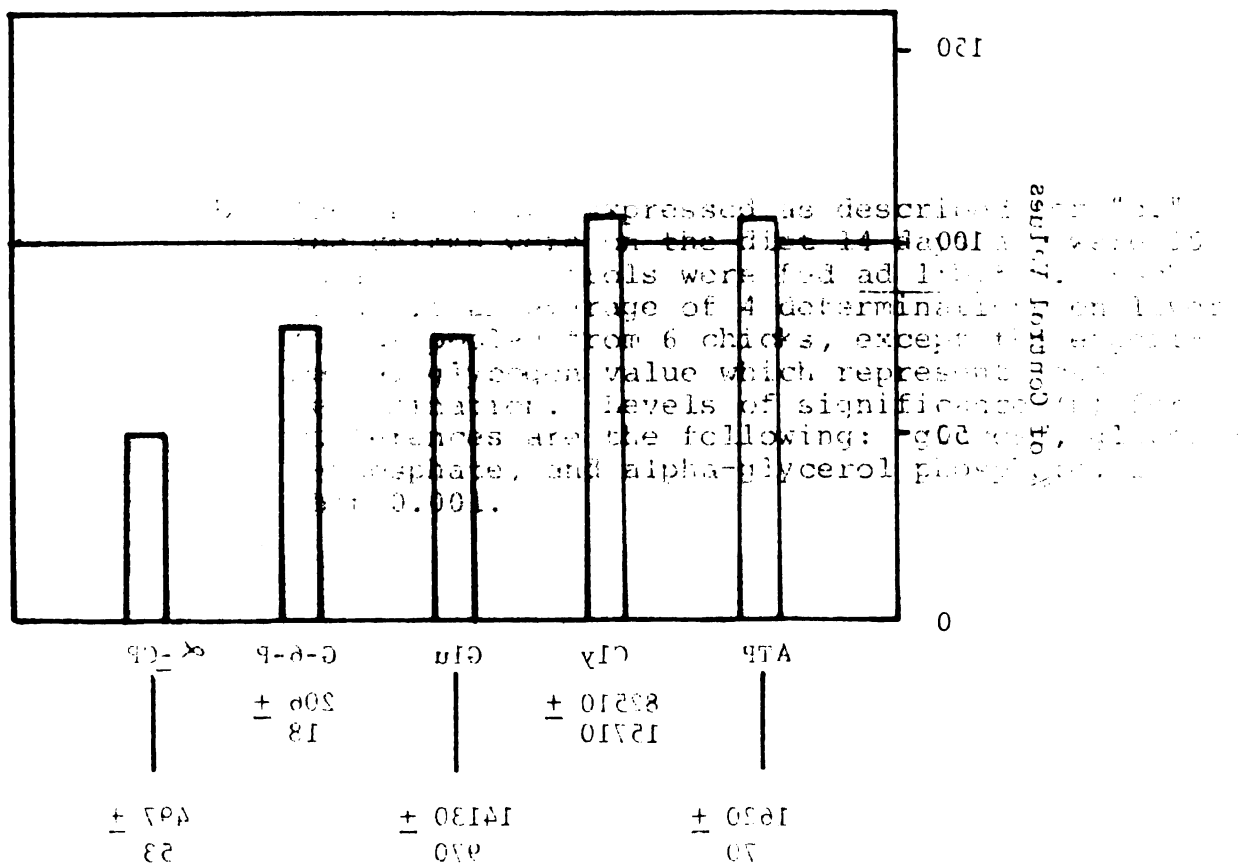
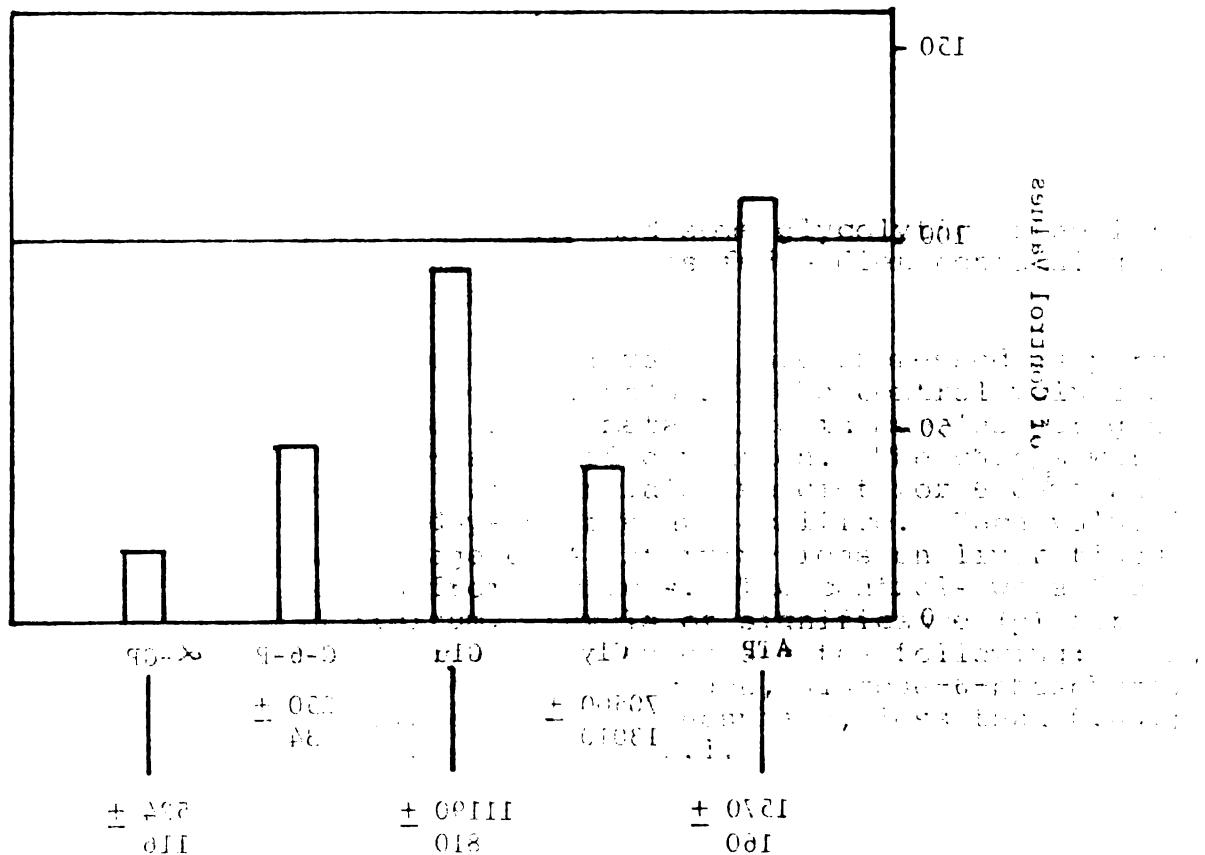


... nucleotides and some ...
 ... livers from chicks fed a diet ...
 ... phenylacetic acid - sodium salt ...
 ... expressed as percentage of control ...
 ... values are given below the ...
 ... of micromoles per g of tissue ...
 ... The chicks were fed the ...
 ... for 4 days and were 5 days old ...
 ... controls were fed a diet ...
 ... value represents an average of ...
 ... on liver tissue pool ...
 ... differences in ATP, glucose, ...
 ... and alpha-glycerol phosphate ...
 ... significant at p less than 0.01 ...
 ... less than 0.01.

Figure 10: Analysis of ATP and some glycolytic metabolites in livers from chicks fed a diet containing 10% phenylalanine

- a The experimental values are expressed as percentage of control values. The control values are given below the metabolite in mumoles per g of tissue + standard deviation. The chicks were fed the 10% phenylalanine diet for 6 days and were 7 days of age at sacrifice. Each value is an average of 4 determinations on liver tissue pooled from 8 chicks. The controls were fed ad libitum. Levels of significance (p) for metabolite differences are the following: ATP, less than 0.02; glycogen, glucose-6-phosphate, and alpha-glycerol phosphate, less than 0.001; glucose, less than 0.1.
- b The values are expressed as described for "a." The chicks were on the diet 14 days and were 15 days old. Controls were fed ad libitum. Each value is an average of 4 determinations on liver tissue pooled from 6 chicks, except the experimental glycogen value which represents one determination. Levels of significance (p) for differences are the following: glucose, glucose-6-phosphate, and alpha-glycerol phosphate, less than 0.001.





F. Experiment V: Phenylacetic Acid - Sodium Salt Injections

1. Description of Toxic State

To circumvent any effects of starvation on liver metabolism, we attempted to produce the PAA toxicity by intraperitoneal injection of the acid. The chicks had been fed a control diet, ad libitum. An injection of 1 mmole of sodium PAA caused the chicks to become depressed and sedated within 30 minutes.

2. Analysis of Plasma

Phenylacetic acid and uric acid accumulated in the blood to extremely high levels as indicated in Table 13, 30 and 60 minutes after the injection.

3. Liver ATP Determinations

Hepatic ATP levels at the two times are summarized in Table 14. No differences were observed.

G. Genetic Deficient (dld1) Mice Study

1. Analysis of Behavior and Plasma Glucose and Lactate

The dilute lethal mice were persistently stricken with tremors. They did not appear to undergo opisthotonic convulsions followed by relaxed periods. Because of their seizures, they were unable to nurse properly. Their caloric deficiency was reflected in the low plasma glucose and high lactate levels summarized in Table 15. The dld1 plasma glucose concentration was 38% of the dd control value, whereas lactate was 192% of the control.

2. Analysis of Brain Glycolytic Metabolites and Adenine Nucleotides

Figure 11 presents a profile of the brain glycolytic

Table 13: Plasma Uric Acid and Phenylacetic Acid Levels in Chicks Injected with Phenylacetic Acid - Sodium Salt*

	Control	Phenylacetic Acid Injected
Uric acid (mM)		
30 min post injection	0.339 $\pm$ 0.072	1.014 $\pm$ 0.206
60 min post injection	0.392 $\pm$ 0.041	1.381 $\pm$ 0.255
Phenylacetic acid (mM)		
30 min post injection	none	8.04 $\pm$ 4.12
60 min post injection	none	10.88 $\pm$ 0.66

* Three chicks per group were injected intraperitoneally with 1 mmole of the sodium salt of phenylacetic acid or 1 mmole of NaCl in a 0.3 ml volume. The chicks were 5 days of age. Determinations were done on individual samples. Values $\pm$ standard deviation.

Table 14: Hepatic ATP Levels in Chicks Injected with Phenylacetic Acid - Sodium Salt

	Control	Phenylacetic Acid Injected
30 min post injection	1.27 $\pm$ 0.26 mM	1.28 $\pm$ 0.19 mM
60 min post injection	1.28 $\pm$ 0.21 mM	1.34 $\pm$ 0.07 mM

Values $\pm$ standard deviation.

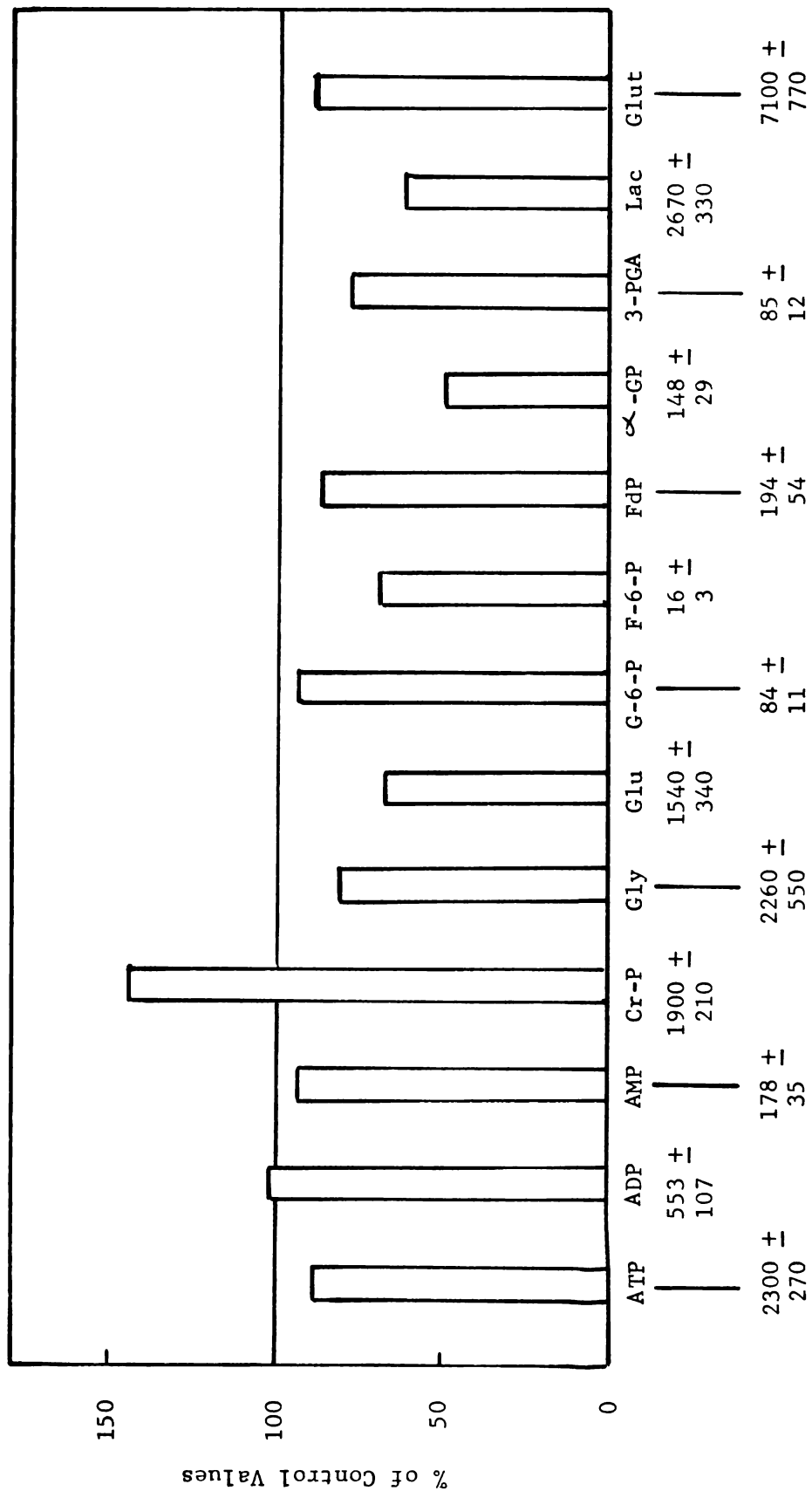
Table 15: Plasma Glucose and Lactate Concentrations in dd and dldl Mice*

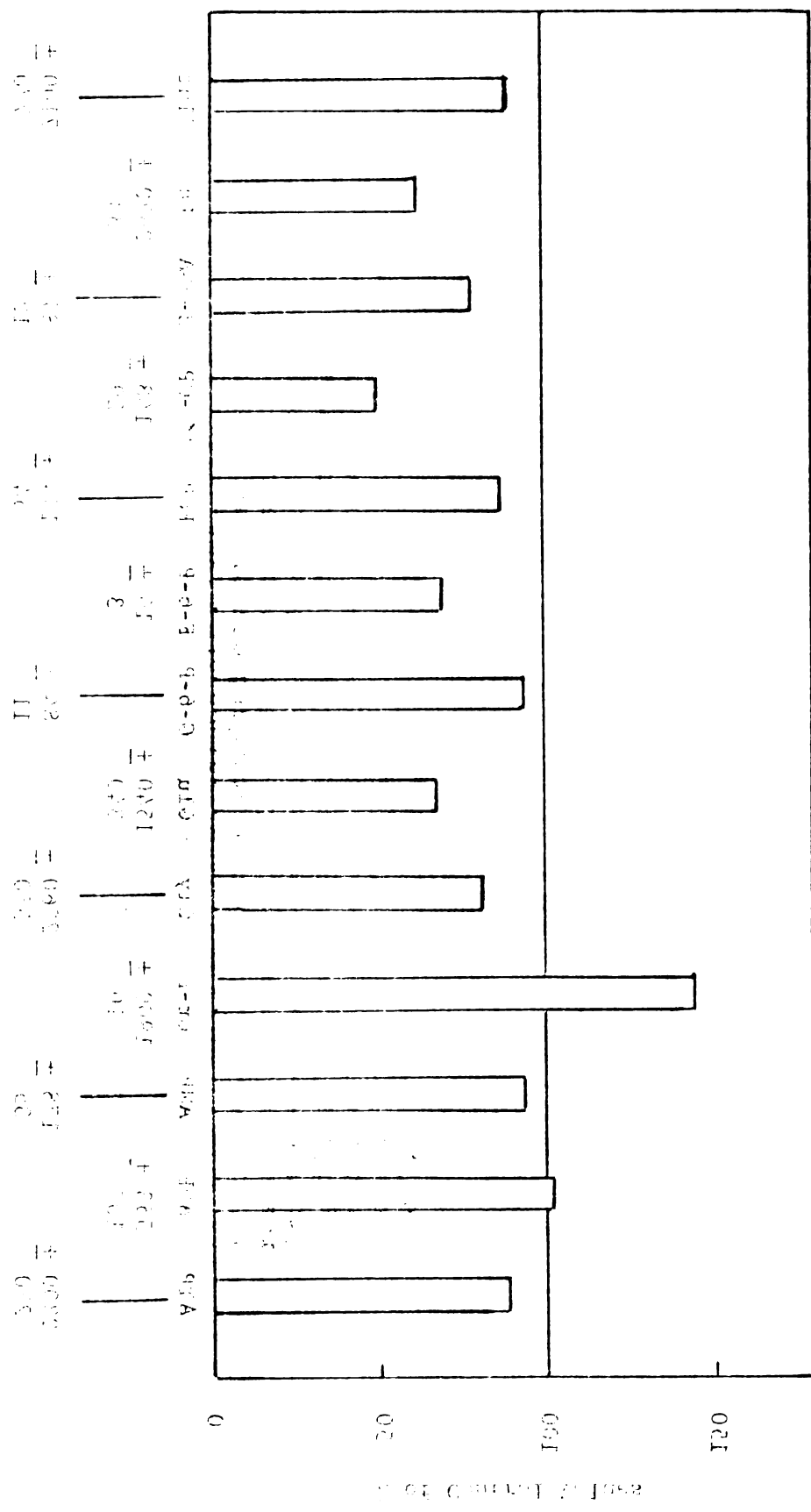
	Glucose	Lactate
Mother	5.15 mM	5.63 mM
dd young	5.88 mM	6.68 mM
dldl young	2.22 mM	12.80 mM

* Determinations were done on pooled plasma samples.

Figure 11: Analysis of adenine nucleotides and glycolytic intermediates in brains from dld1 mice

Values are expressed as percentage of control values determined from dd mice. The control values are given below the metabolite in μ moles per g tissue + standard deviations. The values represent averages of individual determinations on 7 to 10 brains. Fructose-6-phosphate and glutamate values are statistically significant at p less than 0.05; 3-phosphoglyceric acid and glucose, at p less than 0.02; creatine phosphate, at p less than 0.01; lactate and alpha-glycerol phosphate, at p less than 0.001.





intermediates and adenine nucleotides under the abnormal neurologic condition. ATP, ADP, and AMP were unchanged, although creatine phosphate was higher in the dld1 group. Glucose and fructose-6-phosphate were significantly lowered; glycogen, glucose-6-phosphate, and fructose-1,6-diphosphate, however, were only slightly decreased. The intermediates beyond fructose-1,6-diphosphate in the glycolytic pathway were more significantly affected. Alpha-glycerol phosphate was reduced by 50% in the dld1 mice; 3-phosphoglyceric acid, by 28%; lactate, by 39%. Glutamate concentrations were also reduced.

DISCUSSION

Energy Metabolism in the Brains of Phenylalanine Fed Chicks

Thus far the emphasis of the major investigations into the cause of mental retardation of phenylketonuria has been in the following areas: effects of phenylalanine or the aromatic acids on (1) cerebral protein synthesis; (2) neurologic amine metabolism; (3) cerebral lipid metabolism. The energy status of brain tissue under conditions of hyperphenylalanemia has received little attention. Since high energy phosphates function in the synthesis of macromolecules like proteins, lipids, DNA, and RNA and in active transport, especially that involved in nerve impulse transmission, any disturbance in energy production would be most detrimental to the brain.

The sensitivity of the adenine nucleotides to neural malfunctions is well substantiated. Decrease in brain ATP levels is associated with electroshock (102), drug (103), and hypoxia (104) induced convulsions. There is evidence in animals exposed to a low oxygen atmosphere, Metrazol, or hydroxylamine that the ATP reduction precedes the onset of the convulsion (105), indicating that the abnormal ATP level is not the result of the excited behavior. In the galactose toxic chick characterized by seizures preceeding death, ATP

hydrolysis is significant; however, the hydrolysis is observed to occur 1-1/2 days before the seizure onset (106).

Weber (59) has recently suggested a mechanism whereby ATP levels could be reduced in the brain of a phenylketonuric by demonstrating inhibition of human brain hexokinase by phenylpyruvic acid and inhibition of human and rat brain pyruvate kinase by L-phenylalanine. With a rat brain high speed supernatant fraction, Glazer and Weber (107) demonstrated phenylpyruvate competitive inhibition of lactate formation from glucose (K_i , 7.2 mM), glucose-6-phosphate (K_i , 10.7), and fructose-1,6-diphosphate (K_i , 10.7). Also phenylpyruvate potentiated ATP inhibition of glycolysis. These experiments indicate multiple inhibition sites, not exclusively at hexokinase. L-phenylalanine and phenylpyruvate also inhibited glycolysis in rat brain slices, and the inhibition was overcome with 10-20 mM glucose. The K_i for phenylalanine inhibition in fetal rat brain slices was 2.5 mM compared with 10 mM for the adult.

Under our high phenylalanine feeding conditions with the chick, no decreases in brain ATP or creatine phosphate concentrations indicative of an energy stress are observed. The reason for the higher ADP and AMP levels in the phenylalanine fed chicks is not apparent.

The decrease in brain glucose is of interest since the blood glucose is maintained under the conditions of high phenylalanine absorption. The decrease was apparently insufficient to jeopardize substrate availability for ATP synthesis. The phenylalanine accumulation in the blood

coupled to the low brain glucose, fructose diphosphate, and lactate might suggest a transport problem; for example, phenylalanine preventing adequate amounts of glucose from crossing the blood brain barrier. However, phenylalanine and glucose do not share the same transport system. Evidence for a carrier mediated transport of glucose across the blood brain barrier has been provided by LeFevre and Peters (108); whereas phenylalanine is concentrated in the brain by an active transport mechanism (109-111).

Equally interesting is the rise in brain glucose observed after about 2-1/2 weeks on the phenylalanine diet when the blood glucose concentration was normal. Simultaneously fructose diphosphate increased in the 7-1/2% group. The reduction in glucose-6-phosphate, fructose-1,6-diphosphate, and lactate in the 10% group may indicate the inhibition of glycolysis proposed by Weber for the rat system. But phenylpyruvate was not detected in chick brain. The phenylalanine concentration in chick brain approximated the K_i for lactate production in fetal rat brain. If glycolysis is blocked in the phenylalanine fed animal, then the increased glucose could be explained by substrate accumulation; however, a greater inhibition would be expected in the 10% group and the data shows a greater glucose accumulation in the 7-1/2% group. Thus, inhibition of glycolysis does not adequately explain our results.

Another consideration is the daily fluctuation in the glucose levels in the brain. Dr. Kozak (106) in this laboratory observed a range of values from 1 mM to 5 mM for brain

glucose concentrations in chicks 1 to 4 days of age fed the synthetic control diet used in these experiments. When he fed the chicks a commercial mash diet containing less cere-lulose (glucose), fluctuations also occurred, but to a lesser extent. It is not known if the glucose levels would display the same variability at a later age of the animal when the blood brain barrier was perhaps better formed.

Nutritional State and Liver Metabolism in Phenylalanine Fed Chicks

The chick on the phenylalanine diet was in a state of caloric deficiency as evidenced by the lack of proper weight gain. This slow development is characteristic of dietary manipulations where essential amino acids are fed in amounts greater than normal. The reduced lactate levels in the plasma suggest a sequestering or rapid utilization of the gluco-neogenic metabolite by the liver. Recently Allred and Roegrig (112) described hepatic gluconeogenesis in chicks fed the classical gluconeogenic diet of soybean oil. Under their conditions, the chicks' blood glucose was reduced only by 5 to 8%; yet liver glucose-6-phosphate decreased 56%; fructose-6-phosphate, 39%; alpha-glycerol phosphate, 41%. Glycogen and liver glucose were not measured. These changes are similar to those we observed under conditions of phenylalanine feeding. The phenylalanine fed chicks displayed less drastic reductions in glucose-6-phosphate and alpha-glycerol phosphate at 6 days than at 14 days, indicating increased caloric intake or possibly increased catabolism of phenylalanine to fumaric acid and acetoacetic acid.

Significance of Aromatic Acid Toxicity in Phenylketonuria

Knox (4) and Ambrose (85) reported serum phenylalanine values of 1.7 to 3.5 mM for untreated PKU patients. In treated patients the phenylalanine values are normal. In this investigation with the chick model, phenylalanine levels within the cited range for PKU patients were reached in the blood. Yet the aromatic acids present in the blood were few and at extremely low concentrations according to our method of analysis. The concentrations were below any of the K_i 's reported in the literature for dihydroxyphenylalanine decarboxylase (69), 5-OH tryptophan decarboxylase (66-67), hexokinase (59), etc. With the plasma concentration of the aromatic acids being low, it is doubtful that brain tissue levels would be significant, supported by our findings of extremely small amounts of the phenylalanine metabolites in the brain. Although recoveries of the keto acids and o-OH phenylacetic acid from blood and brain tissue were unreliable, I feel that any major accumulation, for example, 0.3 to 0.5 mM or greater, would have been detected by the procedure, especially since phenylacetic acid quantitation from the phenylacetic acid fed chicks was satisfactory.

No work has been reported on levels of aromatic acids in experimental PKU models since 1961, when Goldstein (113) conducted an experiment with rats injected intraperitoneally with phenylalanine; blood and brain tissue were examined for phenylalanine and aromatic acid content. He found that younger rats, 12-18 days old, were better able to metabolize phenylalanine along the alternative phenylacid pathways. The

following is a tabulation of some of the data he presented, included in this discussion for its comparison of blood and brain levels of the aromatic acids.

Table 16: Concentration of L-phenylalanine and Derivatives in Serum and Brain of Rats after Phenylalanine Loading (113)

Age (Days)	Dose g/Kg	Serum (mM)			Brain (mM)		
		PA	PPA	PLA	PA	PPA	PLA
12	0.75	10.55	0.72	1.63	3.33	trace	0.17
18	1.00	7.70	1.20	2.28	4.48	trace	0.33
18	0.50	5.58	0.27	0.66	1.94	trace	less than 0.03

It is interesting that although phenylpyruvate and phenyl-lactate do accumulate sufficiently in plasma, the brain level is 14% or less of the plasma level. Considerable quantities of the acids were excreted in the urine as the kidney has a low clearance for these compounds.

The potential for accumulation of the aromatic acids in the brains of phenylketonurics can be further evaluated by referring to the work of Jervis and Drejza (15). PKU patients were fed phenylalanine or phenylpyruvate, and their plasma was analyzed. Under the phenylalanine feeding, plasma phenylalanine rose to 4.1 mM; phenylpyruvate, to 0.14 mM; o-OH phenylacetic acid, to 0.044 mM. This latter acid was not detected in our chick model. When phenylpyruvate itself was fed, its concentration rose to 0.44 mM. Thus the forced

feeding of phenylpyruvate to PKU patients does not produce the elevation required by the K_i 's for hexokinase (59), dihydroxyphenylalanine decarboxylase (69), or tryptophan decarboxylase (66-67). The possibility that variable concentrations of the acid are localized in a particular area of the brain or of the cell must not be overlooked.

Phenylacetic Acid Toxicity

In phenylketonurics no phenylacetic acid, free or conjugated, has been found in the blood; the kidney seems to be particularly efficient in clearing these metabolites. Also in the chicks fed phenylalanine, phenylacetic acid wasn't detected in the blood. Excrement although not examined probably would have revealed high concentrations. On these grounds it is doubtful that this aromatic acid is the toxic agent in phenylketonuria. However, study of the toxicity for itself has uncovered some interesting points. No investigation into the animal's metabolic responses during the toxicity had previously been conducted.

Unfortunately, the chicks were repulsed by the diet whether in the acid or salt form at the 5 or 10% level; the salt diet is milder in its aromatic odor. The chicks entered a gluconeogenic, semi-starvation state similar to that of the phenylalanine fed chicks. Blood lactate and glucose were decreased. Feeding the controls a restricted diet corrected only partially for the starvation effects in the experimental animals. The control chicks consuming a restricted diet underwent gluconeogenesis since the hepatic glycolytic

metabolite levels were lower than in controls fed ad libitum; but the levels of hepatic glycogen, alpha-glycerol phosphate, etc. in the phenylacetic acid fed chicks were further reduced beyond the levels in the pair-fed controls. Since phenylacetic acid is useless as a carbohydrate energy source, a better control would have been a diet containing non-digestible cellulose equivalent to the amount of the phenylacetic acid employed.

Energy Metabolism in the Brains of Chicks Fed Phenylacetic Acid

In the brain the glycolytic intermediates were considerably more upset than the adenine nucleotides. Normal ATP and creatine phosphate levels were not surprising since any energy-requiring conjugation with ornithine would occur in the liver rather than in the brain. The decreased concentrations of ADP and AMP, however, are difficult to explain. The decreases in glucose, fructose diphosphate, and lactate inversely paralleled the dietary dose of phenylacetic acid. Brain glycogen tended to decrease, but not significantly. Glucose-6-phosphate decreased only slightly more in the 10% group than in the 5% group; the reduction was greater than that under phenylalanine feeding. This proportionality suggests phenylacetic acid interference with glucose transport into the brain. The fact that glucose levels can be decreased by 70% and elicit no reductions in ATP implies that either gluconeogenic amino acids such as glutamic, aspartic, or alanine provide energy via transamination and the citric

acid cycle and oxidative phosphorylation or that the brain has a tremendous safety margin to handle wide fluctuations in glucose before they become consequential. A comparison of control values from the ad libitum feeding (Figure 8) and restricted feeding (Table 10) indicates that although the restricted controls were undergoing some stage of gluconeogenesis, the brain was unaffected and able to function normally in its carbohydrate metabolism.

Liver Metabolism in Chicks Fed Phenylacetic Acid

The livers of the phenylacetic fed chicks displayed decreases in glycogen, glucose, glucose-6-phosphate, and alpha-glycerol phosphate greater than those observed in the livers of the phenylalanine fed chicks. Hepatic ATP values from control animals approximate 2 mM, 0.4 mM higher than the values calculated in the phenylalanine feeding experiment. The 2 mM value is considered more accurate since the brain to liver ATP ratio is 1 (114-115). The variability in these numbers arises from the method by which tissue samples were obtained. For realistic values, only the tissue directly between the frozen clamps should be taken, excluding marginal tissue. It should not be necessary to cut the sample in the clamps from the remaining liver in order to release it; rather the sample should break away because of its brittleness.

The depletion in the liver ATP in the phenylacetic acid fed chicks can be explained by increased conjugation activity. This is similar to the mechanism for fructose and

glycerol toxicity in rat liver (116-117) and galactose toxicity in chick brain (118), where ATP is consumed in phosphorylation reactions.

The accumulation of adenine nucleotide catabolites can serve as an indication of ATP hydrolysis. In the bird, uric acid is the major product of purine nucleotides. We observed large increases of this material in the blood of the chicks during the feeding and injection experiments; but it proved to be a poor monitor of adenine metabolism for two reasons. (1) Fasting is characterized by uric acid retention by the kidney (119). Comparing Table 11 with Table 13, uric acid plasma levels were 66% higher in restricted fed chicks than in ad libitum controls. (2) Quick (77) reported that uric acid excretion in the urine is depressed under phenylacetic acid feeding conditions. This could easily be reflected in uric acid retention in the blood. Swanson (101) noted that sodium benzoate fed to humans caused uric acid concentrations to decrease in the urine and to increase in the blood. The high phenylacetic acid concentrations in the kidney tubules could prevent excretion of other molecules by monopolizing the transport sites. Thus any observed plasma increase in uric acid could not be specific to adenine nucleotide catabolism.

The intraperitoneal injection of phenylacetic acid very efficiently produced toxic symptoms more easily defined than under dietary feeding conditions. A reduction in hepatic ATP in the experimental chicks was not observed; however, differences could easily have been obscured by the autolysis

reflected in the low control ATP levels.

Further Applications

Further applications of the discussed results include:

(1) The accumulation of phenylacetic acid in brain of 2 mM levels provides a model for investigating phenylacetic acid inhibition of brain amines and GABA synthesis in vivo.

(2) The intraperitoneal injection method resulted in a phenylacetic acid plasma concentration 5 times that of dietary feeding. Probably the brain level of the acid increased also, however this was not determined. By analogy phenylalanine could be injected using dosages greater than 1 mg/g tissue, the dose usually reported, to induce a visible toxicity. Thus cerebral energy metabolism may be affected only after higher phenylalanine or aromatic acid concentrations are reached than heretofore obtained. For example, in the work on galactosemia model systems in this laboratory, the rat tolerated the 40% galactose diet reasonably well; however, the chick was considerably more sensitive and developed a definite toxicity to the sugar. Galactose did not accumulate in rat brain to levels greater than 4 mM (120); whereas, in chick brain the levels were as high as 22 mM (106). Clearly the choice of model and conditions are most important.

(3) Similarly, the effect of the aromatic acids (phenyllactate and phenylpyruvate) on brain energy metabolism could be studied using the injection technique. Blood concentrations of specific acids could be elevated as shown with

phenylacetic acid.

Genetic Mice Study

The nutritional state of the dldl mice differed from the imbalances observed under the phenylalanine and phenylacetic acid feedings. Starvation was more acute with an accompanying hypoglycemia. Body tissues responded to the demand for more blood glucose by providing increased lactate; but the liver apparently was unable to produce glucose quickly enough to satisfy the needs.

The reduction in brain glucose and other glycolytic metabolites may be due to the decreased glucose availability and a subsequent influence on the Embden Meyerhof pathway. Of the intermediates determined for the brain alpha-glycerol phosphate was most markedly reduced. Since alpha-glycerol phosphate is an intermediate in phospholipid metabolism important in cerebral membrane formation like myelin, any alteration in its concentration is consequential. Thus a study of phospholipid turnover in these animals may be useful in evaluating factors leading to the rapid myelin degeneration observed by Kelton and Rauch (80).

It is surprising that brain ATP, ADP, and AMP levels are not altered during the abnormally great neural activity induced by the tremors. The reduction in glutamate levels indicates further that the glucose supply is inadequate, suggesting that ATP is maintained, in part, by glutamate entering the citric acid cycle at the alpha-ketoglutarate site. Our glutamate control levels are lower than those reported in

the literature (121): 4.26 mM, newborn; 11.5 mM, adult.

This may be due in part to the age variability in our samples or to the method of analysis.

Methodology

A. Determination of Particular Amino Acids by Gas Liquid Chromatography

The control values for phenylalanine approximate very closely the value determined by James Blosser for chick brain using an amino acid analyzer. The levels of phenylalanine and tyrosine reached in the experimental chicks are similar to those published for other investigations with the rat. The range for the elevated values is actually quite large depending on the method for inducing the hyperphenylalanemia. If an amino acid analyzer is not available and one is interested in only one or two amino acids that cannot be determined enzymatically, the method described here is recommended.

B. Determination of Aromatic Acids by Gas Liquid Chromatography

Previously no method for determination of aromatic acids in blood or tissue by GLC has been published. Goldstein's (113) method required gradient elution column chromatography and individual non-enzymatic organic quantitative analysis. The spectrophotometric method of LaDu and Michael (122) for phenylpyruvate required a plasma sample similar to that for GLC, but it was necessary to correct for interfering absorbances and it did not allow for simultaneous determination of any of the other acids. Advantages of determination of the phenyl-phenolic acids by GLC are specificity, speed, sensitivity

(0.1 to 0.5 ml plasma), and simultaneous determination of several acids.

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