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I am submitting herewith a thesis written by Yasmin Hashmy Beg entitled "The Effect of Leucine and Isoleucine Supplements to a Low Protein Diet on the Activity of Quinolinate Phosphoribosyl Transferase." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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Vice Chancellor
Graduate Studies and Research

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THE EFFECT OF LEUCINE AND ISOLEUCINE SUPPLEMENTS TO A LOW PROTEIN DIET ON THE ACTIVITY OF QUINOLINATE PHOSPHORIBOSYL TRANSFERASE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee

Yasmin Hashmy Beg

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ABSTRACT

Five groups of NLR strain, Wistar origin, SPF male rats were fed one of five experimental diets for twenty-eight days. Two diets contained 9 and 12 percent casein respectively. The third diet contained 9 percent casein plus 3 percent L-leucine. Two other diets contained 9 percent casein and 3 percent L-leucine plus 0.2 and 0.5 percent L-isoleucine respectively. Rats were decapitated after twenty-eight days and the liver from each rat was removed and analyzed for the activity of the enzyme quinolinate phosphoribosyl transferase.

Results indicated that L-leucine caused no significant difference in food consumption of the rats, but the weight gain for animals consuming the 9 percent casein plus 3 percent L-leucine diet was significantly lower than the rats fed the 9 percent casein diet. Addition of L-isoleucine to the diet counteracted the adverse effect of leucine on the weight gain of the animals. Animals fed 12 percent casein had significantly higher food consumption and weight gain as compared to the animals fed all the other diets.

The enzyme activity of quinolinate phosphoribosyl transferase was significantly lower in animals fed the 9 percent casein diet with leucine as compared to animals fed 9 percent casein diet. Addition of L-isoleucine counteracted the affect of L-leucine on the enzyme activity. Animals

fed 12 percent casein diet had a significantly higher value of enzyme activity as compared to animals fed all the other diets. There was a negative correlation between the ratio of leucine to isoleucine in the diet and the enzyme activity.

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CHAPTER I

INTRODUCTION

The traditional view of the pathogenesis of pellagra is that it is a nutritional deficiency disease caused by high consumption of maize, a staple which is deficient in tryptophan and in which the availability of nicotinic acid is poor. But pellagra also occurs in populations whose staple is jowar, a millet with a tryptophan content close to that of rice. One common feature between maize and jowar is the presence of large amounts of leucine in their proteins as compared to other cereals. It has been postulated that the large amount of leucine in jowar may play a role in the pathogenesis of pellagra.

Though some doubts were expressed about the link between a dietary excess of leucine and pellagra, other studies have provided conclusive evidence in support of this hypothesis. Further investigations suggested that it was the high level of leucine that caused pellagra by interfering with the conversion of tryptophan to nicotinamide adenine dinucleotide (NAD) (1, 2, 3, 4).

To understand the biochemical basis of leucine-induced changes in tryptophan-niacin metabolism, the effect of leucine

on enzymes of tryptophan-niacin metabolism was investigated by Ghafoorunissa and Rao (5). These studies showed that rats fed low protein diets with 3 and 5 percent levels of leucine had a significant decrease in the activity of quinolinate phosphoribosyl transferase and an increase in the activity of picolinate carboxylase, which would result in a decreased NAD formation from dietary tryptophan.

Isoleucine has been shown to counteract the increased excretion of quinolinic acid caused by feeding excessive amounts of leucine to young rats (4). Supplementation of jowar diets with isoleucine was shown to cure patients with pellagra, suggesting that the ratio of leucine to isoleucine in the diet may etiologically relate to pellagra (6). The purpose of the present study is to determine if feeding an excess of L-leucine (3 percent) in a low protein diet to rats will decrease the activity of liver quinolinate phosphoribosyl transferase, and if the addition of L-isoleucine to the diet will reverse the effect of leucine on the activity of this enzyme.

CHAPTER II

REVIEW OF LITERATURE

I. THE RELATIONSHIP OF TRYPTOPHAN, NICOTINIC ACID AND PELLAGRA

Pellagra has long been recognized as a nutritional deficiency disease affecting poor people subsisting on diets predominately based on maize. The identity of the pellagra-preventive factor remained a mystery until 1937 when Elvejhem et al. (7) isolated nicotinamide and successfully used it to cure canine black tongue, a disease comparable to human pellagra. In addition, nicotinic acid and nicotinamide were demonstrated to be equally effective in treating human pellagra (8, 9).

Nicotinic acid is β-pyridine carboxylic acid; its amide, nicotinamide, is the form found in the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). NAD and NADP function as coenzymes accepting hydrogen in the metabolic dehydrogenations of glycolysis, the Kreb cycle, and fat and amino acid metabolism (10).

In 1945, Krehl et al. (11) found that pellagra-like symptoms produced in rats by feeding niacin-deficient diets could be alleviated by feeding tryptophan. Studies which

showed elevated urinary excretion of nicotinic acid derivatives after the ingestion of tryptophan in rats and humans produced supportive evidence to this finding (12, 13). The conclusion was that tryptophan functioned as a precursor to nicotinic acid. In 1956 it was demonstrated that approximately 60 mg of dietary tryptophan are equivalent to 1 mg of nicotinamide in humans (14).

II. KYNURENINE PATHWAY OF TRYPTOPHAN METABOLISM

Once it had been determined that tryptophan could serve as a precursor of nicotinic acid, investigators began to trace tryptophan's metabolic pathway. The availability of radioactive ^{14}C provided a convenient tool to trace the intermediate products of tryptophan metabolism and subsequently studies proved conclusively that tryptophan can be converted to nicotinic acid (15, 16).

As a result of many studies, the kynurenine pathway, the pathway for metabolizing tryptophan to nicotinamide-containing compounds has been elucidated as shown in Figures 1 and 2. This preferred pathway of tryptophan metabolism provides endogenous niacin for the biosynthesis of NAD and NADP. Tryptophan is initially acted upon by tryptophan pyrrolase to yield formyl kynurenine (17). Tryptophan pyrrolase has been isolated from guinea pig, mouse, and rat liver and the distal one-sixth of the small intestine of the rabbit (17,

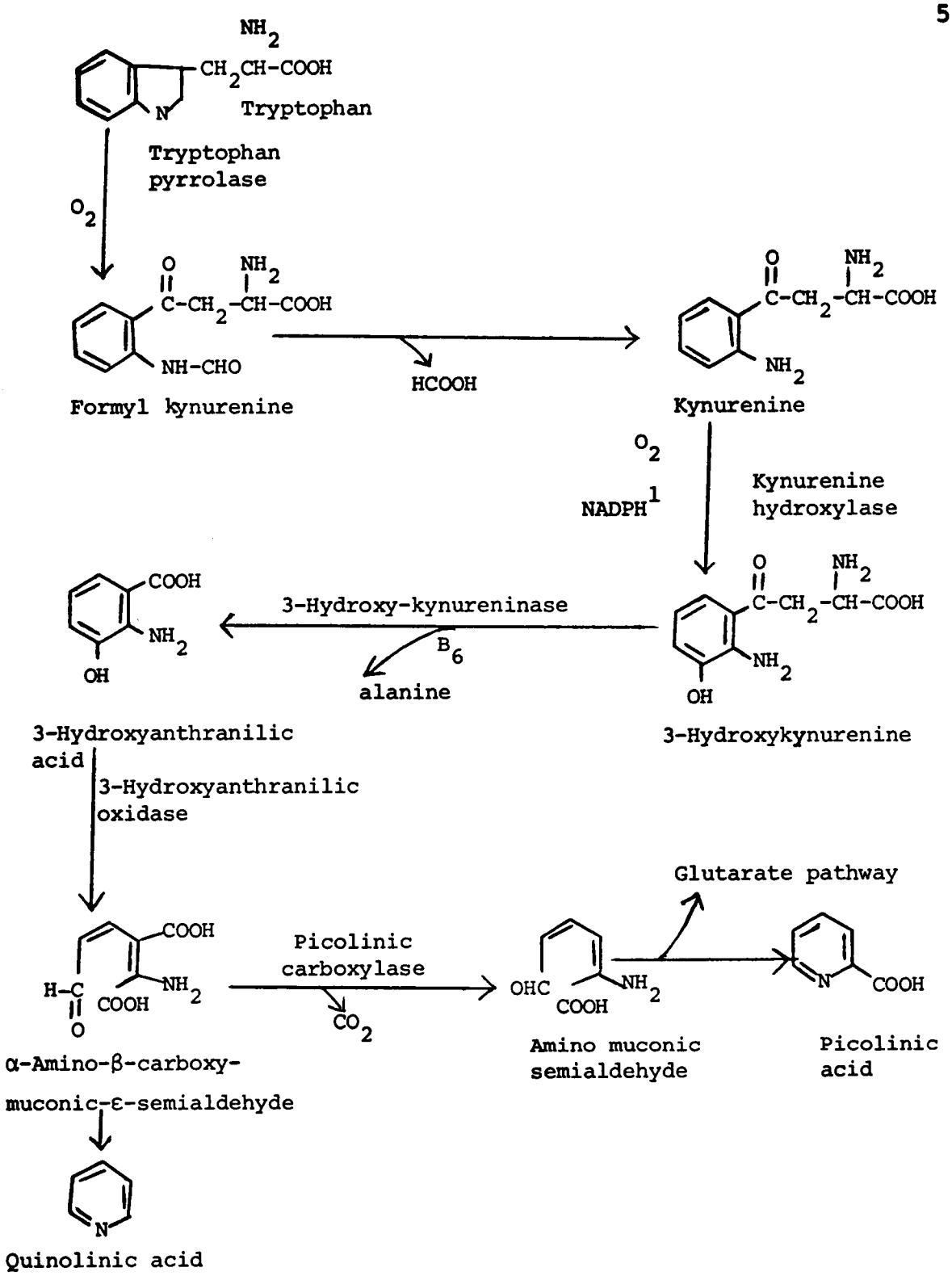


Figure 1. Conversion of tryptophan to quinolinic acid.

[1]Reduced nicotinamide adenine dinucleotide phosphate

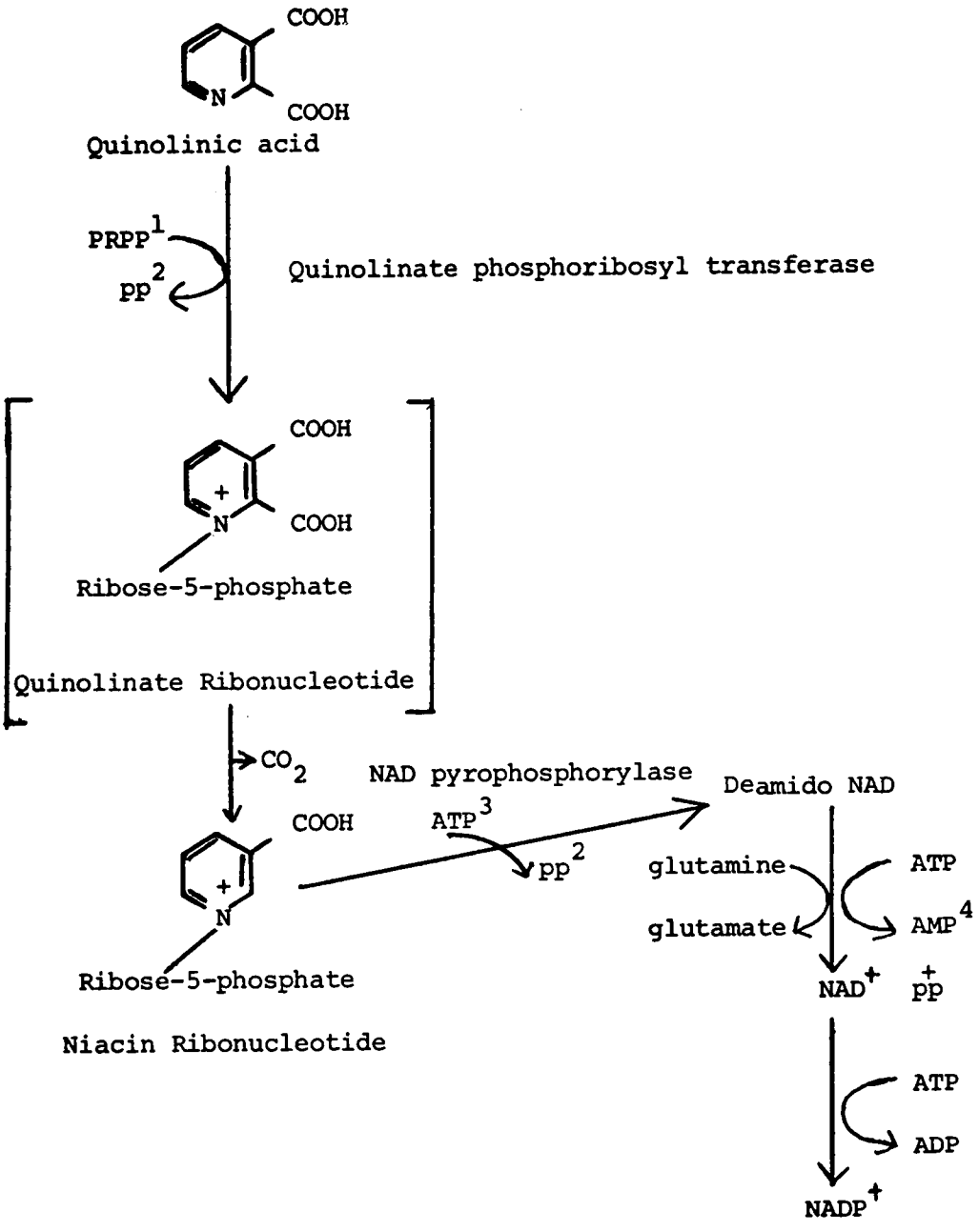


Figure 2. Conversion of quinolinic acid to NAD and NADP.

[1]5-phosphoribosyl-1-pyrophosphate

[2]Pyrophosphate

[3]Adenosine triphosphate

[4]Adenosine monophosphate

18, 19, 20) but it is generally considered a liver enzyme. Activity occurs over a pH range of 6.5—8.5 with an optimum reached around 7.0 (17).

The conversion of kynurenine to 3-hydroxy-kynurenine is catalyzed by the enzyme kynurenine hydroxylase. The hydroxylation, which occurs in the presence of molecular oxygen, also requires NADPH as a coenzyme (21, 22). The vitamin riboflavin is thought to be necessary for the activity of this enzyme (23). The enzyme 3-hydroxy-kynureninase catalyzes the cleavage of 3-hydroxy-kynurenine into alanine and 3-hydroxy-anthranilic acid with pyridoxal phosphate required as a coenzyme (24). Several studies have shown that the 3-hydroxy-kynureninase activity was greatly reduced in the liver of pyridoxine-deficient animals and could be restored by the addition of pyridoxal phosphate (24, 25).

The enzyme 3-hydroxy-anthranilic oxidase acts on 3-hydroxy-anthranilic acid to form an intermediate called α-amino-β-carboxy muconic-ε-semialdehyde. This intermediate may be converted spontaneously to quinolinic acid (26, 27), or it may be acted upon by picolinic carboxylase to form picolinic acid (28). Picolinic carboxylase is metabolically active over a pH range of 6.0—9.5 and is apparently independent of co-factors. The activity of the enzyme is inhibited by SH-inhibitors (29) and is increased in diabetic animals (30). Picolinic carboxylase shuttles the semialdehyde

intermediate into the glutarate pathway preventing it from being ultimately converted into NAD. If more α-amino-β-carboxymuconic-ε-semialdehyde enters the glutarate pathway, the production of NAD is reduced. This pathway produces glutaryl CoA which is converted into acetyl CoA and enters the Kreb cycle.

Direct cyclization of α-amino-β-carboxymuconic-ε-semialdehyde forms quinolinate, which was first suggested as part of the kynurenine pathway by Henderson et al. (31) after they discovered increased levels of quinolinate in the urine of rats fed a test load of tryptophan. The role of quinolinic acid in the metabolism of tryptophan remained obscure until Nishizuka and Hayashi (16) in 1963 proposed a pathway for the conversion of quinolinate to NADP. Figure 2 (p. 6) illustrates that suggested mechanism.

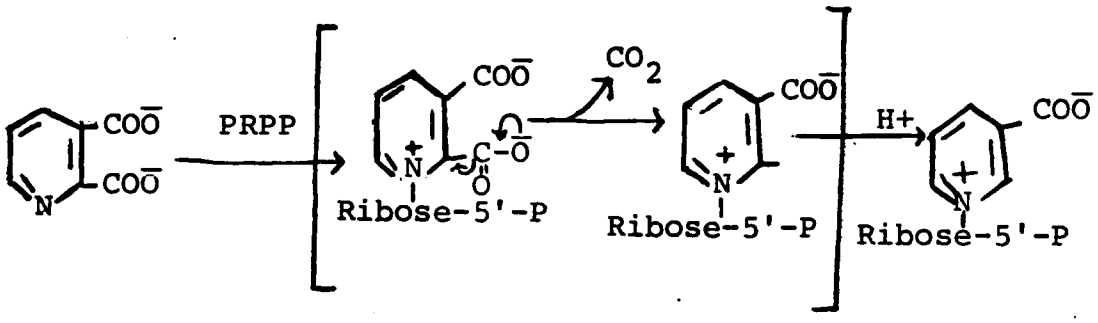
A. Role of the Enzyme Quinolinate Phosphoribosyl Transferase

Nishizuka and Hayashi showed that 3-hydroxy-anthranilic was converted to niacin ribonucleotide in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) by a soluble enzymic system present in rat and cat liver. The niacin ribonucleotide produced was identified by paper chromotography, paper electrophoresis, enzymically and by microbiological assay. Both quinolinic acid and α-amino-β-carboxymuconic-ε-semialdehyde were shown to be the intermediates in this

conversion (16). Consistent with previous findings of Preiss and Handler (32) niacin ribonucleotide was further converted to deamido-NAD in the presence of ATP and then amidated in the presence of ATP and glutamine to give NAD (Figure 2, p. 6). Direct evidence has been provided that the precursor of niacin ribonucleotide formation is quinolinic acid and that free niacin is not involved in the PRPP-dependent niacin ribonucleotide formation from quinolinic acid (33).

In order to elucidate the detailed mechanism of this reaction as well as the precise nature of the enzyme concerned, it was purified approximately 3,000 to 5,000 fold from beef liver powder. This enzyme was distinct from nicotinic acid mononucleotide pyrophosphrorylase. No evidence was obtained that more than one enzyme was required for this reaction or that quinolinic acid ribonucleotide occurred as a stable and dissociable intermediate in this conversion (34). Studies by Nokamura et al., and Gholson et al. (33, 34) strongly suggested that a single protein is responsible for both the condensation of quinolinic acid with PRPP and the decarboxylation to yield niacin ribonucleotide. The decarboxylation reaction, however, could be due to a nonenzymic reaction since the formation of quinolinic ribonucleotide makes the pyridine ring nitrogen a quaternary nitrogen. This positively charged nitrogen would raise the electronegativity

at position 2 in the ring (35), and could result in the elimination of CO_2 from the molecule as indicated below.



Quinolinate

Niacin ribonucleotide

Packman and Jakoby purified, crystallized, and studied the various properties of the enzyme (36, 37, 38) quinolinate phosphoribosyl transferase. The enzyme has a molecular weight of 165,000 and can be irreversibly dissociated into inactive protein units with molecular weights of 54,000. Unlike other reactions in which the enzymatic transfer of phosphoribosyl groups can occur, the kinetics of this reaction were consistent with involvement of a ternary complex of the enzyme, quinolinic acid and PRPP.

The enzyme was shown to be absolutely specific for quinolinic acid for neither nicotinic acid, nicotinamide, imidazol acetate, purines nor pyrimidines acted as a substrate for the enzyme even in the presence of ATP (39). The Michaelis constant (km values) for quinolinic acid and PRPP are 7.0×10^{-6} and 2.9×10^{-5}M, respectively. The reaction is irreversible and proceeds to completion under standard

conditions. The optimum pH for the reaction is about 6.5-7 with trisacetate and potassium phosphate buffer. Magnesium is absolutely required with a sharp optimum concentration of 5×10^{-5}M. At higher concentration Mg^{+2} inhibits the reaction. Most heavy metals are more or less inhibitory, for example, Hg^{+2} inhibits the enzyme almost completely at 10^{-4}M and Ni^{+2}, CO^{+2}, and Fe^{+3} inhibit the enzyme by 40-55 percent at 10^{-4}M. The enzyme does not require ATP and NAD inhibits the reaction. However, this is not thought to be a specific feedback inhibition but may be attributed to the Mg^{+2} binding capacity of NAD (39). Recently Ghafoorunissa and Rao reported an inverse relationship between the level of dietary protein and the activity of liver quinolinate phosphoribosyl transferase (40).

The enzyme has been found only in the liver and kidney of rats and mice and could not be detected in other tissues and organs so far tested, including the brain, lung, heart, spleen, intestine, skeletal muscle, and blood. In addition to its presence in several mammals, the enzyme was found in birds, fish, frogs, and certain microorganisms such as neurospora, E. coli, and pseudomonas (39).

III. BRANCHED-CHAIN AMINO ACID METABOLISM

The branched-chain amino acids leucine, isoleucine, and valine are metabolized by much the same mechanism. Catabolism

begins with transamination (involving α-ketoglutarate), yielding the analogous branched-chain α-keto acids. One enzyme probably catalyzes all of the three transaminase reactions in heart muscle but in liver there are thought to be three different enzymes, one for each reaction (41, 42, 43). The branched-chain α-keto acids are oxidatively decarboxylated to the coenzyme-A thio esters of the acids of one less carbon atom. The oxidative decarboxylations are irreversible reactions and are catalyzed by an α-keto acid dehydrogenase complex which contains coenzyme-A, NAD+, FAD, thiamin pyrophosphate, and lipoic acid as co-factors. In beef liver a dehydrogenase specific for α-ketoisovalerate (from valine) has been separated from another enzyme with double specificity towards α-ketoisocaproate (from leucine) and α-ketomethylvalerate (from isoleucine) (44, 45). By additional reactions, also remarkably similar in the three pathways, leucine yields two ketogenic fragments, acetoacetate and acetyl CoA. Isoleucine yields succinyl CoA and acetyl CoA; thus, it is both glucogenic and ketogenic whereas valine is oxidized to succinyl CoA, which is glucogenic (46). Figure 3 represents the pathway for the metabolism of leucine.

IV. LEUCINE-ISOLEUCINE ANTAGONISM

The depression in growth caused by the ingestion of a low protein diet with excessive amounts of naturally occurring

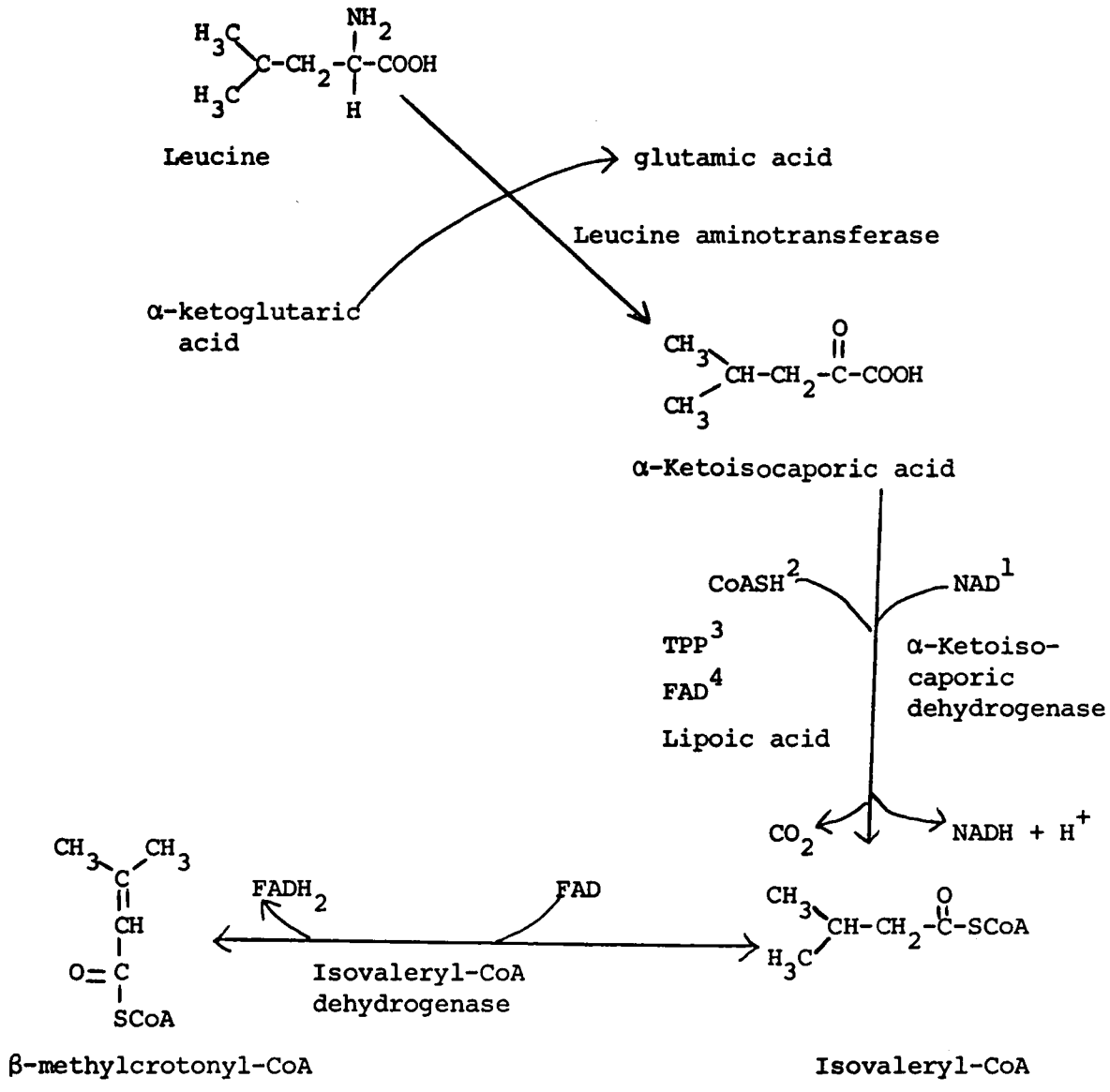


Figure 3. Leucine metabolism.

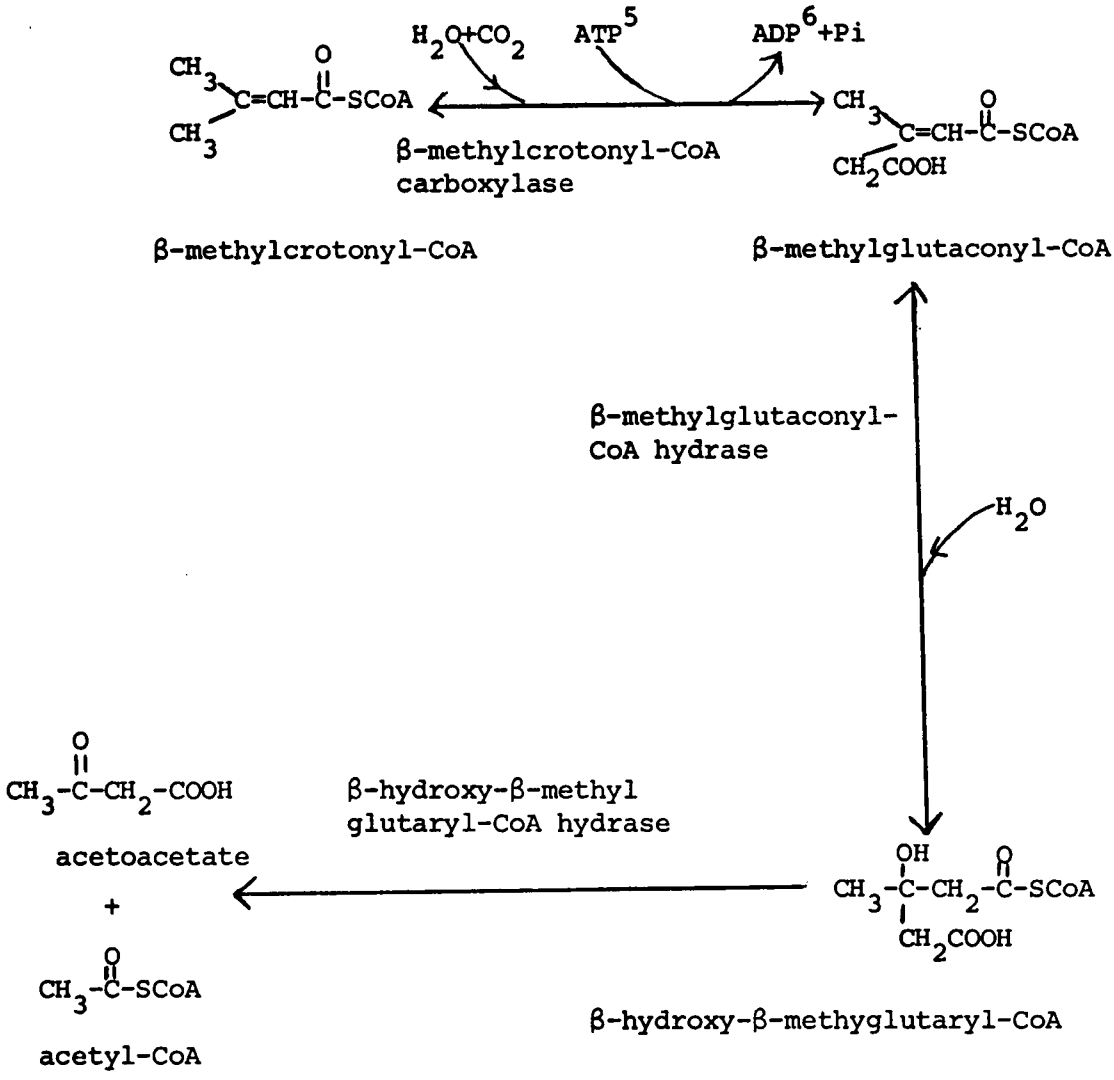


Figure 3 (continued)

[1]Nicotinamine adenine dinucleotide

[2]Coenzyme A

[3]Thiamin pyrophosphate

[4]Flavin adenine dinucleotide

[5]Adenosine triphosphate

[6]Adenosine diphosphate

amino acids and alleviated by supplements of structurally similar amino acids are attributed to amino acid antagonism (47). In 1954, Harper et al. (48) showed that the growth depression in rats caused by supplements of leucine to low-protein diets could be largely overcome by the concurrent addition of isoleucine. In view of the structural similarity of the two compounds, they postulated an antagonism between the two nutrients, whereby leucine "prevents the normal utilization of isoleucine." It thus appeared that an excessive amount of any one branched-chain amino acid can increase the requirement for the other two (49).

In a subsequent study by Spolter et al. (50), young rats were fed a 9 percent casein diet and test diets containing 3, 5, or 7 percent L-leucine added to the 9 percent casein diet. In each case, rats fed the test diets showed an initial weight loss and a lag period before growth resumed at a less-than-normal rate. The extent of the weight loss and the length of the lag were dependent on the amount of leucine in the diet; rats fed 3 percent leucine resumed growth after the third day, whereas rats fed 7 percent leucine did so only after the sixteenth day. The addition of 0.16 percent of L-isoleucine and 0.15 percent L-valine to a 5 percent L-leucine, 9 percent casein diet resulted in a growth rate of rats equal to that of the control rats, after a three-day lag. The growth retarding action of an excess of dietary leucine was also

demonstrated by feeding rats a diet in which the protein was replaced by an amino acid mixture, suggesting that the site of antagonism is not in some phase of protein digestion. No effect of excess dietary leucine on the overall nitrogen retention or on the rate of gastric emptying was observed.

Harper et al. suggested the following chain of events to explain the leucine-isoleucine antagonism:

> 1. Initial feeding of high leucine diets increase concentration ratios of leucine to isoleucine and to valine in several tissues.
> 2. These skewed ratios mitigate against the binding of isoleucine and valine to specific recognition sites within a central appetite-regulating apparatus; the failure to bind isoleucine and valine triggers the food avoidance behavior.
> 3. Either as a specific effect of high tissue concentration of leucine (or α-ketoisocaproic or more probably, as a consequence of diminished food intake, the branched-chain α-keto acid dehydrogenase activities are increased over a period of a few days.
> 4. α-ketoisocaproate particularly is increasingly oxidized, so that leucine, isoleucine and valine ratios return toward normal, permitting food intake to increase.
> 5. But increased dehydrogenase activities also result in increased oxidation of α-keto-β-methylvalerate and α-ketoisovalerate so that in the leucine-adapted rat eating inadequate quantities of protein, isoleucine and valine become growth limiting (49).

Thus, the addition of either isoleucine or valine to the diet would tend to prevent the levels of these amino acids from becoming limited.

V. RELATIONSHIP OF LEUCINE AND ISOLEUCINE TO TRYPTOPHAN METABOLISM AND PELLAGRA

Pellagra, though traditionally associated with consumption of maize, also occurs in populations whose staple is jowar (sorghum vulgare). One common feature between maize and jowar is the presence of relatively large amounts of leucine in their proteins as compared to other cereals. It was postulated that the excess of leucine in jowar might play a role in the pathogensis of pellagra. The effects of oral administration of leucine on the metabolism of tryptophan and nicotinic acid were, therefore, investigated in pellagrins and normal subjects by Gopalan et al. and Belvady et al. (1, 3). These studies showed that both in normal subjects and pellagrins, leucine administration brought about a significant increase in urinary excretion of quinolinic acid and N-methylnicotinamide. When a tryptophan load test was performed using these same subjects, the effects of leucine administration were more pronounced. Removal of the excess dietary leucine or supplementation with nicotinic acid alleviated this situation. Truswell et al. (51) studied the effect of a 10 gram leucine load on the urinary excretion of N-methylnicotinamide in both healthy humans and pellagrins. The results of this study did not support Gopalan's findings.

In agreement with the observations of Gopalan and Srikantia, Raghurmulu et al. (4) found that the urinary

excretion of quinolinic acid and N-methylnicotinamide increased in both young and adult rats when L-leucine was added at the 1.5 percent level to a 9 percent casein diet. These observations pointed to a possible role of leucine in the pathogensis of pellagra. To obtain direct experimental evidence the possibility of inducing black tongue in dogs was investigated by Belavady and Gopalan (2). They demonstrated that canine black tongue could be produced in dogs fed a diet containing 65 percent jowar. The development of a niacin deficiency was similar to that seen in dogs fed a corn diet. The study clearly demonstrated that classical black tongue can be induced in dogs by feeding jowar, which was not low in tryptophan and which contained fair amounts of nicotinic acid but was high in leucine.

Studies were next directed to elucidating the precise biochemical mechanism underlying the effect of leucine on nicotinic acid metabolism. The possibility that the effect of leucine may be mediated through changes in the circulating levels of tryptophan was investigated by Ghafoorunissa and Rao (52). Administration of 5 grams of L-leucine daily for five days to normal subjects belonging to a low-socioeconomic group was found to have no effect on plasma tryptophan levels. Their earlier studies had shown that the intestinal absorption of tryptophan was not affected by administration of leucine supplements (53).

Raghurmulu et al. (54) studied the effect of excess leucine on the synthesis of nicotinamide adenine dinucleotide in the erythrocyte. In both the controls and pellagrous subjects, the *in vitro* synthesis of these nucleotides was reduced after ingesting leucine.

Hankes et al. (55) studied the excretion of urinary tryptophan metabolites in pellagrins given a standard dose of 2 grams of L-tryptophan. They found that elevations of kynurenine, 3-hydroxy kynurenine, kynurenic acid, xanthurenic acid, and quinolinic acid were most common among their subjects. The investigators hypothesized that a lack of feedback control on tryptophan pyrrolase existed in pellagrins and that leucine prevented the synthesis of NAD from quinolinic acid.

All of these studies have been interpreted to suggest that an excess of leucine interferes with the kynurenine pathway resulting in the elevated excretion of metabolites of the pathway. To understand the biochemical basis of leucine-induced changes in tryptophan-niacin metabolites, the effect of dietary leucine on the enzymes involved in tryptophan-niacin metabolism were investigated by Ghafoorunissa et al. (5). They fed rats a basal 9 percent casein diet supplemented with 3 percent L-leucine and showed an increase in picolinic carboxylase and tryptophan pyrrolase activity and a decrease in quinolinate phosphoribosyl transferase

activity which infers that metabolites of the kynurenine pathway are shuttled off into the glutarate pathway via picolinic carboxylase. A decreased activity of quinolinate phosphoribosyl transferase may also explain the increased excretion of quinolinic acid observed in the presence of excess of leucine in the diet by Raghurmulu et al. (4). Ghafoorunissa's observation of the effect of leucine on the important enzymes of tryptophan metabolism can offer a biochemical explanation of the role of leucine in the etiology of pellagra. Increased picolinic carboxylase and decreased quinolinate phosphoribosyl transferase activity would result in decreased NAD formation from dietary tryptophan. Lowered NAD formation from tryptophan, particularly when the niacin concentration in the diet is marginal, would result in a state of conditioned niacin deficiency. Ghafoorunissa et al. concluded from this study that the rate limiting enzyme in the formation of NAD from tryptophan was quinolinate phosphoribosyl transferase.

Raghurmulu et al. (4) also studied the effect of addition of isoleucine on the urinary excretion of tryptophan-niacin metabolites in young and adult rats fed a diet with an excess of leucine. The addition of 0.2 percent isoleucine to a 9 percent casein diet containing 1.5 percent leucine significantly counteracted the increased excretion of

quinolinic acid and N-methynicotinamide caused by the excess of leucine in the diet.

Effect of isoleucine on 26 patients with pellagra was studied by Krishnaswamy and Gopalan (6). All patients were fed a jowar diet which provided about 2,000 kilocalories per day and 54 grams of protein of which 46 grams came from jowar. Sixteen patients received 5 grams of DL-isoleucine per day and were completely cured of pellagra in about fifteen days, but the ten patients fed the jowar diet only did not show any improvement. These findings further indicate that the ratio of leucine to isoleucine in the diet may be etiologically related to pellagra.

The present study was based on Ghafoorunissa and Rao's findings (5). The objectives of the study were to confirm the finding that an excess of leucine (3 percent) in a low protein diet decreased the activity of liver quinolinate phosphoribosyl transferase and to determine if the addition of L-isoleucine to the diet will reverse the effect of leucine on the activity of this enzyme.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

The effect of supplementing a low protein diet with leucine and leucine plus isoleucine on the activity of the enzyme quinolinate phosphoribosyl transferase was studied.

Male albino rats of the NLR strain,[1] Wistar origin, SPF were divided into five groups of six rats each in such a manner that average weights of the five groups did not vary more than one or two grams. All the animals were caged individually. Feed and distilled water were provided ad libitum throughout the experimental period.

Each rat was fed Diet I (described in next section) for three days to allow for adjustment to experimental conditions. Each group of rats was fed one of the experimental diets (described in next section) for a period of four weeks. The weight of each rat was recorded each week and records of feed consumption were kept. After being fed their respective diets for four weeks, the rats were decapitated and their livers removed immediately.

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[1]National Animal Company, P. O. Box 9502, O'Fallon, Missouri 63366.

II. EXPERIMENTAL DIETS

Five diets were used in this study. On a percent basis, Diet I consisted of vitamin-free casein, 9; fat-soluble vitamin mixture in corn oil, 0.5; corn oil, 4.5; choline, 0.15; water-soluble vitamin mixture, 0.25; Hubbell, Mendel and Wakeman salt mixture (56), 3; and sucrose, 82.6. Composition of the Diet II was the same as Diet I, but to make it more nearly comparable in nitrogen content to Diets III, IV, and V which contained 3 percent L-leucine, the casein level of the Diet II was 12 percent. Diet III was similar to Diet I except that it contained 3 percent L-leucine. Diets IV and V were similar to Diet III except that they contained 0.2 and 0.5 percent L-isoleucine respectively. The addition of amino acids and adjustments of casein levels were made at the expense of sucrose.

The leucine/isoleucine ratio in a 9 percent casein diet was calculated to be 1.9/1 and 6.9/1 in the diet containing casein plus L-leucine. The amount of isoleucine added to Diets IV and V made the leucine/isoleucine ratio in Diets IV and V 5.2/1 and 3.7/1.

Tables I and II summarize the components of water-soluble vitamin and fat-soluble vitamin mixtures respectively. Table III presents a brief summary of principal variation in components of the five diets.

III. DETERMINATION OF ENZYME ACTIVITY OF QUINOLINATE PHOSPHORIBOSYL TRANSFERASE

Each liver was homogenized in two vols of cold 0.14 M KCl in a glass teflon homogenizer. The homogenate was centrifuged[2] for twenty minutes at 20,000 x g at 4°. The supernatant fluid was treated for ten minutes with charcoal (15 g/100 ml of supernatant fluid). The charcoal was subsequently removed by centrifugation at 20,000 x g at 4° for fifteen minutes. The supernatant fluid was used for the assay of quinolinate phosphoribosyl transferase by the method of Ghafoorunissa and Rao (5). A part of this supernatant fluid was frozen for later analysis for protein.

The activity of the enzyme measured was expressed as nmoles of niacin ribonucleotide formed/hour/mg of protein. The complete incubation mixture contained 100 nmoles of quinolinic acid, 400 nmoles of PRPP, 50 μmoles of Tris-acetate buffer (pH 7), one μmole of $MgCl_2$ and 0.2 ml of

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[2]"Beta Fuge," Model A, Lourdes Instrument Corporation, Brooklyn, New York.

TABLE I

COMPONENTS OF NIACIN-FREE, WATER-SOLUBLE VITAMIN MIXTURE

Component	Amount (grams)
Thiamin HCl	0.250
Riboflavin	0.250
Calcium pantothenate	1.000
Pyridoxine HCl	1.130
Folic Acid	0.010
Menadione	0.030
Biotin	0.005
Vitamin B_{12} (1 g. triturate 0.1 percent mannitol)	0.001
Inositol	5.000
Ascorbic Acid	2.500
Sucrose	115.824

TABLE II

COMPONENTS OF FAT-SOLUBLE VITAMIN MIXTURE

Component	Amount[a] (grams)
Vitamin A acetate (1×10^6 units/g)	0.260
α-dl-tocopheryl acetate	7.000
Calciferol (40 units/μg)	0.003
Corn oil[b]	312.000

[a]Five grams of this mixture per 1,000 grams of diet were used.

[b]Mazola Corn Oil, Best Foods, CPC International Inc., Englewood Cliffs, N.J., 07632.

TABLE III

DIETARY TREATMENT GIVEN TO FIVE GROUPS OF RATS

Group	Diet I	Diet II	Diet III	Diet IV	Diet V
I	9% casein	---	---	---	---
II	---	12% casein	---	---	---
III	---	---	9% casein + 3% L-leucine	---	---
IV	---	---	---	9% casein + 3% L-leucine + 0.2% L-isoleucine	---
V	---	---	---	---	9% casein + 3% L-leucine + 0.5% L-isoleucine

supernatant fluid in a total volume of one ml. Incubation was carried out for forty-five minutes at 37°. The niacin ribonucleotide formed during incubation was hydrolysed to free niacin by heating for thirty minutes at 100° in one ml of 0.1 N NaOH. The samples were then neutralized to pH 6.8 and sufficient water was added to bring the volume of each sample to four ml. The niacin was estimated by a microbiological assay using Lactobacillus arabinosus[3] (A.T.C.C. 8014).

IV. MICROBIOLOGICAL METHOD FOR ESTIMATION OF NIACIN

Niacin was estimated by microbiological procedure as described by Freed (57) with Lactobacillus arabinosus as the test organism. Microbiological method is based on the observation that certain organisms require specific vitamins for growth. Using a basal medium complete in all respects except for the vitamin under test, growth responses of the organism are compared quantitatively in standard solutions and solutions containing unknown concentrations of the vitamin.

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[3]Pure culture obtained from "American Type Culture Collection," 12301 Parklawn Drive, Rockville, Md. 20852.

A. Reagents

1. Niacin stock standard solution: 50 mg of anhydrous niacin were dissolved in 500 ml of 50 percent ethyl alcohol.
2. Niacin working standard solution: one ml of standard niacin stock solution was diluted to one liter with water.
3. Agar medium for stock culture: 5.5 g of Lacto Bacilli MRS broth[4] were dissolved in 100 ml of distilled water and 2 g agar were added to it. The mixture was heated until the ingredients dissolved. Ten ml of the medium were dispensed into culture tubes which were plugged with foam rubber caps and autoclaved in a Barnstead sterilizer[5] at fifteen pounds of pressure for fifteen minutes. Tubes were cooled and stored in the refrigerator.
4. Basal Medium: 7.5 g of dehydrated niacin assay medium[4] were dissolved in 100 ml of distilled water.
5. Inoculum culture medium: one ml of niacin stock solution was added to 500 ml of Basal Medium. Five ml of this solution were transferred to a culture

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[4]Difco Laboratories, Detroit, Mich.

[5]Barnstead Still and Sterilizer Co., Boston, Mass.

tube to which 5 ml of distilled water were added and the tube was plugged with foam rubber cap and autoclaved at fifteen pounds pressure for ten minutes.

6. Isotonic saline solution: 0.9 g of NaCl were dissolved in 100 ml of distilled water. Ten ml quantities of this solution were transferred to culture tubes, plugged and autoclaved at fifteen pounds pressure for twenty minutes.

B. Procedure

Preparation of stock culture: stab cultures were prepared by transferring the cells from pure culture of Lactobacillus arabinosus to two agar stock culture tubes (reagent 3) aseptically. The tubes were incubated for about twenty-four hours at 37°. These tubes were stored in refrigerator under aseptic conditions not longer than one week before transferring to a new stab culture.

Preparation of Inoculum: cells were transferred from stock culture to sterile tube of inoculum culture medium and incubated for about eighteen hours at 37°. The inoculum was centrifuged and the supernatant fluid was decanted, cells were resuspended in 20 ml of sterile isotonic saline solution.

Preparation of standard solutions: to duplicate tubes 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 ml of niacin working standard solution (0 to 0.14 µg of niacin) were added. Sufficient distilled water was added to bring the volume in each tube to 5 ml. To each of these tubes 5 ml of basal medium were added and the tubes were plugged with foam rubber caps.

Preparation of assay solutions: to duplicate tubes 0.25 ml of the incubation mixture (quinolinic acid + $MgCl_2$ + Tris buffer + PRPP + supernatant fluid) were added and the volume in each tube was made to 5 ml by adding sufficient distilled water. To each of these tubes 5 ml of basal medium were added and the tubes were plugged.

Standard and assay solutions were autoclaved at fifteen pounds pressure for ten minutes. After allowing the tubes to cool to room temperature each tube was aseptically inoculated with one drop of inoculum. Tubes were incubated at 37° for approximately twenty-four hours. After incubation absorbance was measured using a Beckman B spectrophotometer at 640 nm. The absorbance values of the standard niacin solution were plotted against concentration of the niacin in the solution. From this standard curve the amount of niacin in assay tubes was calculated as follows:

$$\frac{\mu g \text{ of niacin formed/hr/0.25 ml of incubation mixture} \times 16}{\text{Mol. wt. of niacin} \times 10^{-9}} =$$

nmoles of niacin formed/hr/0.2 ml of supernatant fluid.

nmoles of niacin formed = nmoles of niacin ribonucleotide formed.

Activity of the enzyme is expressed as:

nmoles of niacin ribonucleotide formed/hr/mg of protein =

$$\frac{\text{nmoles niacin ribonucleotide formed/hr/0.2 ml of supernatant fluid}}{\text{mg of protein/0.2 ml of supernatant fluid}}$$

V. BIURET METHOD FOR PROTEIN DETERMINATION

Protein in the supernatant fluid was determined by a modification of the method of Shibko et al. (58) and the biuret reagent was used for color development (59). The biuret test depends on the formation of complex ions between copper and protein in an alkaline solution to give a violet color which can be measured spectrophotometrically.

A. Reagents

1. Bovine Serum Albumin standard (B.S.A.): 1 g of serum albumin was dissolved in 0.4 N NaOH and diluted to 100 ml. Working standards containing 1 to 10 mg B.S.A. per ml were prepared by diluting the stock standard with 0.4 N NaOH.

2. Biuret Reagent: 3 g $CuSO_4$ .5 H_2O, 9 g NaK $C_2H_4O_6$. $4H_2O$, 8 g NaOH, and 5 g KI were dissolved in water and diluted to one liter.
3. Perchloric acid solution (PCA) 0.6 percent (ω/ν): 5 ml of 70 percent PCA were diluted to one liter with 95 percent ethyl alcohol.

B. Procedure

For each sample one tenth ml of 70 percent PCA was added to one ml of supernatant fluid, mixed thoroughly and chilled in ice for fifteen minutes. The sample was then centrifuged at 800 x g for five minutes. The supernatant fluid was poured off and the precipitate washed with 3 ml of 0.6 percent PCA solution. This solution was centrifuged again and the precipitate washed with 3 ml of ether. After a third centrifugation, the supernatant fluid was discarded and the protein pellet was dissolved in 5 ml of 0.4 N NaOH. The samples stood overnight. To one ml of each of these protein solutions and to one ml of each standard solution were added 4 ml of biuret reagent. Then samples and standards were mixed using a mechanical mixer, incubated for thirty minutes in a 40° bath, allowed to cool, and the absorbance of each sample and standard was determined at 550 nm using a Beckman B spectrophotometer. The amount of protein in each sample was calculated as follows:

mg of protein ml of supernatant solution =

$$\frac{\text{concentration of standard} \times \text{absorbance of sample} \times 5}{\text{absorbance of standard.}}$$

VI. STATISTICAL ANALYSIS

The mean feed consumption and weight gain of each group of rats for the twenty-eight day experimental period were calculated. The standard error of these means were determined. The mean enzyme activity of quinolinate phosphoribosyl transferase and standard error of mean were calculated. Duncan's new multiple range test (60) was used to determine any significant difference in feed consumption, weight gain, and enzyme activities between the experimental groups. In the discussion ($p < 0.05$) level of significance was used to indicate significant difference between the means. A linear regression equation and correlation coefficient for the relationship between the ratio of leucine and isoleucine in the diet and the activity of quinolinate phosphoribosyl transferase were also calculated (60, 61).

CHAPTER IV

RESULTS AND DISCUSSION

I. WEIGHT GAIN AND FEED CONSUMPTION

The effect of the various dietary treatments on weight gain and feed consumption of rats are reported in Table IV. Rats fed the 9 percent casein diet (group I), 12 percent casein diet (group II), 9 percent + 3 percent L-leucine (group III), 9 percent casein + L-leucine + 0.2 percent L-isoleucine (group IV), and the 9 percent casein + 3 percent L-leucine + 0.5 percent L-isoleucine diet (group V) had weight gains of 54, 96, 39, 45, and 48 g per twenty-eight days respectively. The mean values for daily feed consumption were 10, 12, 9, 9, and 9 g for groups I, II, III, IV, and V respectively.

Feed consumption was not significantly different for rats in group I, III, IV, and V. However, the average amount of feed consumed by rats fed 12 percent casein diet (group II) was significantly higher ($p < 0.05$) than for the animals in groups I, III, IV, and V. Since there was no significant difference in feed consumption between rats fed 9 percent casein diet and 9 percent casein + 3 percent leucine, or 9 percent casein + 3 percent leucine and 0.2 or 0.5 percent

TABLE IV

THE EFFECT OF DIETARY TREATMENT ON THE WEIGHT GAIN AND FEED CONSUMPTION OF RATS

Group	Dietary Treatment	Weight Gain g/28 days	Feed Consumed g/day
I	9% casein	54 ± 6[a,b]	10 ± 0.6[a]
II	12% casein	96 ± 4[c]	12 ± 0.4[d]
III	9% casein + 3% L-leucine	39 ± 3	9 ± 0.5
IV	9% casein + 3% L-leucine + 0.2% L-isoleucine	45 ± 6	9 ± 0.6
V	9% casein + 3% L-leucine + 0.5% L-isoleucine	48 ± 4	9 ± 0.5

[a]Mean ± standard error mean.

[b]Significantly higher than value for rats in group III ($p < 0.05$).

[c]Significantly higher than values for rats in groups I, III, IV, V ($p < 0.05$).

[d]Significantly higher than values for rats in groups I, III, IV, V ($p < 0.05$).

isoleucine, it appears that high leucine content in the diet did not affect the feed consumption. Ghafoorunissa and Rao (5) reported that the presence of 1.5 or 3.0 percent leucine did not influence the food intake of rats added to a 9 percent casein diet.

Weight gain for the rats fed the 12 percent of casein diet (group II) was significantly higher than that of rats in groups I, III, IV, and V, which was in keeping with the higher feed consumed by this group. There was no significant difference in weight gain between rats of groups I, IV, and V, but the average weight gain per twenty-eight days of rats in group I was significantly higher than the rats in group III. The addition of 3 percent L-leucine to the casein diet (group III) retarded the growth of rats as compared to rats in group I which were fed the casein diet, and since there was no significant difference in weight gain between rats of groups I, IV, and V, the addition of 0.2 and 0.5 percent L-isoleucine to the diets containing L-leucine (groups IV and V) counteracts the growth retardation in rats caused by the addition of L-leucine to the diet. Increasing the amount of L-isoleucine from 0.2 to 0.5 percent in the diet had no significant effect on the weight gain. These results are consistent with those of Spolter et al. (50) who reported that 3, 5, and 7 percent L-leucine added to a 9 percent casein diet retarded the growth of the rats and that the growth retardation of

the rats fed a diet containing 5 percent L-leucine was almost completely overcome by the addition of 0.16 percent L-isoleucine and 0.15 percent L-valine.

However, Ghafoorunissa and Rao (5) did not find growth retardation in rats fed 1.5 and 3 percent L-leucine added to a 9 percent casein diet as compared to rats consuming the casein diet.

II. ENZYME ACTIVITY OF QUINOLINATE PHOSPHORIBOSYL TRANSFERASE

Mean values for the activity of quinolinate phosphoribosyl transferase in nmoles of niacin ribonucleotide formed/hr/mg of protein are reported in Table V. The nmoles of niacin ribonucleotide formed/hr/mg of protein were 1.67, 1.89, 1.00, 1.33, and 1.49 for rats in groups I through V respectively. The mean enzyme activity for rats fed the 12 percent casein diet (group II) was significantly higher than for rats fed any of the other diets. Also the value of enzyme activity for rats in group I was significantly higher than that for rats in groups III, IV, and V. Enzyme activities of rats in groups IV and V were significantly higher than those of rats in group III. There was no significant difference in enzyme activity between rats in Groups IV and V.

From the results of the present study it appears that 3 percent L-leucine added to 9 percent casein diet (group III) significantly decreased the activity of the liver quinolinate

TABLE V

THE EFFECT OF DIETARY TREATMENT ON THE ACTIVITY OF QUINOLINATE PHOSPHORIBOSYL TRANSFERASE IN RAT LIVER

Group	Dietary Treatment	Activity of Quinolinate Phosphoribosyl Transferase nmole Niacin Ribonucleotide/hr/mg Protein
I	9% casein	$1.67 \pm .06^{a,b}$
II	12% casein	$1.89 \pm .09^{c}$
III	9% casein + 3% L-leucine	$1.00 \pm .06^{d}$
IV	9% casein + 3% L-leucine + 0.2% L-isoleucine	$1.33 \pm .05^{e}$
V	9% casein + 3% L-leucine + 0.5% L-isoleucine	$1.49 \pm .03^{e}$

[a]Mean ± standard error of mean.

[b-e]Values with different superscripts are significantly different from each other ($p < 0.05$).

phosphoribosyl transferase in rats as compared to the activity of the enzyme found in the liver of rats fed 9 and 12 percent casein diets. This observation supports the hypothesis put forward by Ghafoorunissa and Rao (5) that in leucine fed animals quinolinate phosphoribosyl transferase activity is decreased, which would result in decreased NAD formation from dietary tryptophan. Ghafoorunissa et al. reported a significant decrease in the activity of the enzyme in rats fed a low-protein diet containing 3 percent L-leucine as compared to the rats fed a low protein diet. The values of the enzyme activity found in this study were somewhat higher than that of Ghafoorunissa et al. In the present study activities of the enzyme were 1.67 and 1.00 as compared to 1.10 and 0.80 nmole niacin ribonucleotide formed/hr/mg of protein reported by Ghafoorunissa for rats fed a 9 percent casein diet and a 9 percent casein plus 3 percent L-leucine respectively.

The addition of 0.2 and 0.5 percent L-isoleucine to the 9 percent casein L-leucine diet counteracted the inhibiting affect of leucine on the activity of the enzyme quinolinate phosphoribosyl transferase, since the activities of the enzyme for the rats in groups IV and V were significantly higher than that for rats in group III. But the addition of 0.2 and 0.5 percent isoleucine to the leucine diet did not bring the value of enzyme activity to the level of activity

found in rats of group I. Activity of the enzyme for rats in group I (9 percent casein diet) was significantly higher than for rats in groups IV and V. Increasing the amount of isoleucine from 0.2 to 0.5 percent in the diet increased the activity of the enzyme, but the increase in the activity was not significant.

Raghurmulu et al. (4) in a study of the effect of feeding excess leucine on the urinary excretion of tryptophan metabolites in rats, found that increased excretion of quinolinic acid and N-methylnicotinamide due to addition of 1.5 percent leucine to a 9 percent casein diet was counteracted by the addition of 0.2 percent isoleucine. It is possible to interpret from the observation of the present study that the increased excretion of quinolinic acid in Raghurmulu's study was due to leucine inhibition of the enzyme quinolinate phosphoribosyl transferase and the addition of 0.2 percent isoleucine which reversed the increased excretion of quinolinic acid was due to isoleucine counteracting the effect of leucine on the enzyme quinolinate phosphoribosyl transferase activity.

Krishnaswamy and Gopalan (6) studied the effect of isoleucine on patients suffering from pellagra and found that 5 grams of isoleucine, added daily to the jowar-containing diet, completely cured the pellagra in about fifteen days, while patients fed a jowar-containing diet only showed no

improvement. From the observations in the present study and the explanation put forward by Ghafoorunissa et al. (5) that leucine plays a role in the etiology of pellagra by inhibiting the activity of the enzyme quinolinate phosphoribosyl transferase and thereby decreasing NAD formation, it is possible that in the study carried out by Krishnaswamy and Gopalan, isoleucine added to the jowar diet with high leucine content was counteracting the inhibiting effect of leucine on the enzyme quinolinate phosphoribosyl transferase.

The ratio of leucine to isoleucine in the diets of rats in groups I to V was calculated to the $^{1.9}/_{1}$, $^{1.9}/_{1}$, $^{6.9}/_{1}$, $^{5.2}/_{1}$, $^{3.7}/_{1}$ respectively. An equation of the least square regression line was derived using individual values and the activities of the enzyme quinolinate phosphoribosyl transferase were plotted against the ratio of leucine to isoleucine in the various diets (Figure 4). A strong negative correlation was found between enzyme activity and the ratio of leucine to isoleucine, the activity of the enzyme increased as the ratio of leucine to isoleucine in the diet became smaller. The correlation coefficient was calculated to be -0.89.

The amount of protein in the diet seemed to affect the activity of the enzyme quinolinate phosphoribosyl transferase positively. The activity of the enzyme in rats fed the 12 percent casein diet was significantly higher than the activity

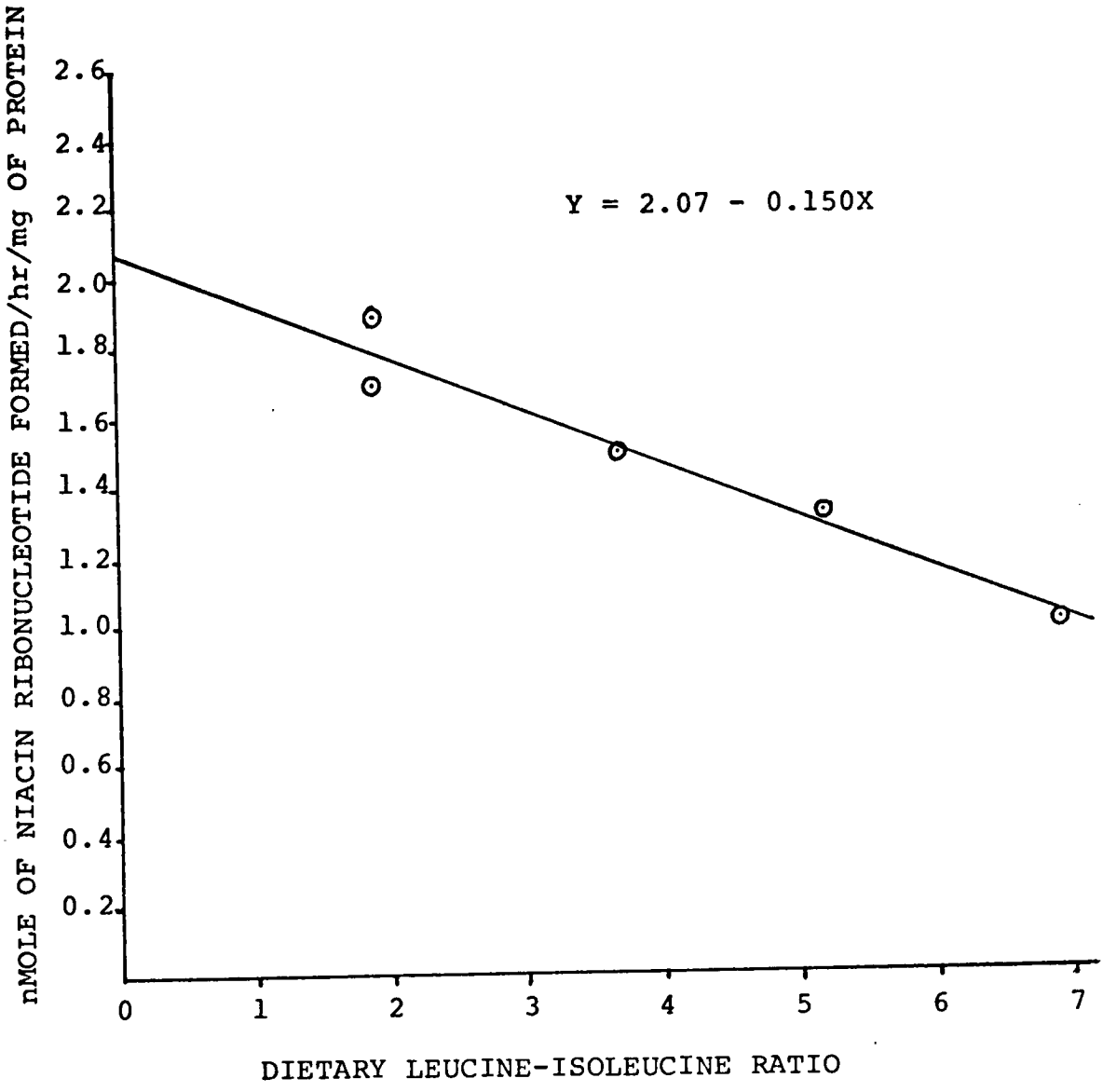

Figure 4. Relationship of dietary leucine-isoleucine ratio to the activity of the enzyme quinolinate phosphoribosyl transferase (r = -0.89).

for rats fed the 9 percent casein diet (1.89 vs. 1.67 nmoles niacin ribonucleotide formed/hr/mg of protein). However, Ghafoorunissa and Rao (40) showed an inverse relationship between the protein levels of the diet and the activity of liver quinolinate phosphoribosyl transferase. In their study rats were fed 2 and 18 percent casein diets and enzyme activities were found to be 2.30 and 1.15 nmoles of niacin ribonucleotide formed/hr/mg of protein. These diets used by Ghafoorunissa et al. had 10 mg of niacin/kg of diet. In the present study, no niacin was added to the diets.

CHAPTER V

SUMMARY

The effects of adding 3 percent L-leucine, 3 percent L-leucine plus 0.2 and 0.5 percent L-isoleucine to a niacin-free 9 percent casein diet on the feed consumption, weight gain, and activity of the enzyme quinolinate phosphoribosyl transferase in rats were studied and compared with data from rats fed niacin-free 9 and 12 percent casein diets. Male weanling rats of NLR strain, Wistar origin SPF, were fed one of the five experimental diets for a period of twenty-eight days. Animals were decapitated and the livers from each rat were removed immediately, homogenized in 0.14 M KCl and centriguged at 20,000 x g to produce a working supernatant fluid which was used for the assay of the activity of quinolinate phosphoribosyl transferase using a microbiological procedure.

The weight gains for rats fed the 12 percent casein diet were significantly higher ($p < 0.05$) than that for the rats fed the 9 percent casein diet and the rats fed the 9 percent casein diets supplemented with leucine and isoleucine. Rats fed the 9 percent casein diets had significantly higher weight gains than the rats fed the 9 percent casein plus 3 percent

L-leucine diet. No other significant differences in the weight gains were found.

Feed consumption was not significantly different for rats fed any of the diets except for those consuming the 12 percent casein diet. Rats fed this diet consumed significantly more feed ($p < 0.05$) than rats fed any other diet.

The activity of quinolinate phosphoribosyl transferase in rats fed the 12 percent casein diet was significantly higher ($p < 0.05$) than those for the rats fed the other diets. Addition of 3 percent L-leucine to the 9 percent casein diet significantly decreased the activity of the enzyme as compared to the values obtained for rats consuming the 9 percent casein diet. Inclusion of 0.2 and 0.5 percent L-isoleucine to 9 percent casein plus 3 percent L-leucine diet significantly increased the activity of the enzyme as compared to the activity found in rats fed 9 percent casein plus 3 percent L-leucine. Increasing the amount of isoleucine from 0.2 to 0.5 percent in casein plus L-leucine diet increased the activity of the enzyme but not significantly.

There was a linear relationship between the enzyme activity and the leucine-isoleucine ratio in the diet, the activity of the enzyme increased as the ratio of leucine to isoleucine in the diet became smaller. The correlation coefficient was calculated to be -0.89. The activity of the enzyme increased when the protein level in the diet was increased.

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