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The Effect of Zein, Leucine, and Glutamic Acid on the Urinary Excretion of Kynurenine, 3-Hydroxykynurenine, and 3-Hydroxyanthranilic Acid in the Rat

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To the Graduate Council:

I am submitting herewith a thesis written by Florecita B. Acacio entitled "The Effect of Zein, Leucine, and Glutamic Acid on the Urinary Excretion of Kynurenine, 3-Hydroxykynurenine, and 3-Hydroxyanthranilic Acid in the Rat." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Jane R. Savage, Major Professor

We have read this thesis and recommend its acceptance:

Mary Nelle Traylor, Claire Gilbert

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

THE UNIVERSITY OF TENNESSEE
THE GRADUATE SCHOOL

ABSTRACT OF EDUCATIONAL RESEARCH STUDY COMPLETED

Author of Study Florecita B. Acacio Date March 3, 1966

Title of Study The Effect of Zein, Leucine, and Glutamic Acid on the Urinary Excretion of Kynurenine, 3-Hydroxykynurenine, and 3-Hydroxyanthranilic Acid in the Rat Course Number 501-2-3

Under direction of what department Nutrition Degree Master of Science

Abstract approved by Jane R. Savage (signature of major professor) Quarter Graduating 3/15/'66

Note: The student should consult with his major professor and follow his advice concerning the general format of the abstract. Additional pages, if required, should be 8 1/2 x 11 inches and of quality equivalent to that required in the case of the thesis. (Master's thesis - submit **one** single-spaced typed copy; doctoral dissertation - submit **two** double-spaced typed copies.)

The effect of the addition of zein, a mixture of all the indispensable amino acids simulating 1.71 per cent zein with or without leucine, or a mixture of all the dispensable amino acids simulating 1.71 per cent of zein with or without glutamic acid to a 6 per cent casein niacin-free diet on the urinary excretion of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in the rat was studied. Sixty male weanling rats of the Wistar strain, in groups of five, were fed the various experimental diets with or without a 0.1 per cent L-tryptophan supplement ad libitum for a period of two weeks. Forty-eight hour urine specimens were collected from each group of rats at the end of the experimental period and were pooled. The tryptophan metabolites were separated by ion-exchange chromatography and the concentration of each metabolite was determined by spectrophotometric method.

The various experimental diets supplemented with 0.1 per cent tryptophan did not affect the growth of rats. The addition of zein or a mixture of all the indispensable amino acids simulating zein to diets with limited tryptophan retarded the growth of rats which was improved significantly when a tryptophan supplement was added.

The addition of zein to the supplemented diet caused a decrease in the urinary excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid as compared to the basal values. In the rats fed diets containing a limited amount of tryptophan only the excretion of 3-hydroxyanthranilic acid was decreased. The observations that zein caused a decrease in the urinary excretion of the tryptophan metabolites and that no metabolite accumulated in excess suggests that there was no block in the metabolism of tryptophan but that less tryptophan on the whole was being metabolized.

The addition of a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent tryptophan depressed only the urinary excretion of 3-hydroxykynurenine. When leucine was removed from the amino acid mixture, the urinary excretion of 3-hydroxykynurenine increased to a value comparable to the basal value. The addition of a mixture of

all the indispensable amino acids to the unsupplemented diet increased the urinary excretion of kynurenine above the basal value but did not affect the urinary excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid. The removal of leucine from the mixture of indispensable amino acids markedly increased the urinary excretion of 3-hydroxykynurenine. These observations indicate that leucine, the indispensable amino acid found in zein in the largest amount, interferred with the conversion of tryptophan to 3-hydroxykynurenine.

The addition of a mixture of all the dispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent tryptophan increased the urinary excretions of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid. In the unsupplemented diets these increases were observed only in the excretions of kynurenine and 3-hydroxykynurenine. Removal of glutamic acid from the supplemented and unsupplemented mixtures resulted in decreases in the urinary excretion of 3-hydroxykynurenine when compared to the values excreted by rats fed the complete mixture of dispensable amino acids. The amounts of the metabolite excreted by the rats fed the amino acid mixture without glutamic acid supplemented and unsupplemented with tryptophan approximated those of the basal value. This suggests that glutamic acid, the dispensable amino acid found in zein in the greatest quantity, inhibited the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

March 2, 1966

To the Graduate Council:

I am submitting herewith a thesis written by Florecita B. Acacio entitled "The Effect of Zein, Leucine, and Glutamic Acid on the Urinary Excretion of Kynurenine, 3-Hydroxykynurenine, and 3-Hydroxyanthranilic Acid in the Rat." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Jane R. Savage
Major Professor

We have read this thesis and
recommend its acceptance:

Mary Nell Trayler
Claire Gilbert

Accepted for the Council:

Dean of the Graduate School

THE EFFECT OF ZEIN, LEUCINE, AND GLUTAMIC ACID ON THE URINARY EXCRETION OF KYNURENINE, 3-HYDROXYKYNURENINE, AND 3-HYDROXYANTHRANILIC ACID IN THE RAT

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Florecita B. Acacio

March 1966

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F. B. A.

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CHAPTER I

INTRODUCTION

The quest for the cause of pellagra which started in the early eighteenth century is surrounded with many fascinating theories. According to Harris (1)*, the many early theories advanced for the causation of pellagra may be classified into four groups: toxins, infections, food deficiencies of quantity and quality, and vitamin deficiencies. Maize consumption was associated with pellagra as early as 1735 by Casal but its relationship to the disease was not exactly understood at that time.

The first unreserved claim that pellagra results solely from the use of corn in the diet was made by Franzoni in 1807. Pellagra was defined as "a food poisoning generally due to the use of spoiled corn, having the characteristics of endemicity in the countries where spoiled corn is consumed as food, and seasons in which the corn crop is most seriously damaged" (2).

Goldberger *et al.* (3) in 1916 made the observation that the diet of pellagrous patients was unbalanced, being low in protein. Later work in 1924 showed that zein, the major protein in corn, was low in tryptophan. When it was finally established that pellagra was caused by a deficiency of niacin and its precursor tryptophan, a

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*Numbers in parentheses refer to similarly numbered entries in the Bibliography.

possible explanation offered for the association between diets high in corn and pellagra was the low tryptophan content of zein.

In 1945 Krehl et al. (4) made the observation that the addition of tryptophan-deficient proteins, such as gelatin and zein, to low-protein niacin-free diets resulted in the retardation of growth and the development of niacin-deficiency symptoms in rats. These symptoms were alleviated by the addition of tryptophan or niacin to the diets. The inhibition of growth which resulted from the inclusion of corn in the ration used by Krehl and his associates and the apparent dual role of niacin and tryptophan in counteracting this growth depression have resulted in considerable study on the mechanism of these relationships.

In a study by Goldsmith et al. (5) human subjects fed corn diets developed clinical signs of a niacin deficiency within 50 days, whereas subjects fed a wheat diet containing the same amounts of niacin and tryptophan as the corn diet did not show any niacin deficiency symptoms until they had been on the diet for 80 days or more. These studies suggested that some component of corn interferes with the utilization of tryptophan and/or niacin. It was theorized that the development of niacin-deficiency symptoms resulting from ingestion of corn diets might be due to an amino acid imbalance.

The ability of the rat to convert trytophan to niacin has made it difficult to resolve the question of whether the substances causing such imbalances affect tryptophan utilization, or niacin utilization or the pathway in the conversion of niacin from tryptophan.

In 1964 Coulter (6) studied the utilization of tryptophan for pyridine nucleotide synthesis in rats fed low-protein niacin-free diets containing either corn, zein, mixtures of the indispensable amino acids as found in zein with and without leucine, or a mixture of the dispensable amino acids as found in zein. The pyridine nucleotides are the metabolically-active form of niacin in the body. She found that zein, leucine, and one or more of the dispensable amino acids of zein interfered with the ability of the rat to synthesize pyridine nucleotides from tryptophan.

In 1965 Ellis (7) studied the effect of the stepwise addition of the dispensable amino acids of zein on the utilization of L-tryptophan for pyridine nucleotide synthesis in rats fed low-protein niacin-free diets. Her findings indicated that glutamic acid and possibly tyrosine inhibited the utilization of tryptophan.

The occurrence of certain intermediate products of tryptophan metabolism in the urine of mammals has provided important clues toward unraveling the metabolic pathway for the conversion of tryptophan to niacin. From the cumulative evidence obtained in studies with different organisms it appears that the initial steps in the major pathway of tryptophan metabolism are: tryptophan ⟶ formylkynurenine ⟶ kynurenine ⟶ 3-hydroxykynurenine ⟶ 3-hydroxyanthranilic acid (8). Recent studies have shown that 3-hydroxyanthranilic acid is further metabolized to niacin ribonucleotide (9). Many of the metabolites along the tryptophan-niacin pathway have been detected in urine of rats

and humans. Estimation of the quantities of these metabolites in the urine under various experimental conditions has been helpful in assessing the metabolic use of trytophan and in diagnosing certain diseases.

In view of these considerations, this study was designed to determine the effect of zein, leucine, and glutamic acid on the urinary excretion of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in the rat. An interference in any of the steps in the pathway of tryptophan metabolism (indicated above) would be expected to cause an increase in the urinary excretion of the specific metabolite formed in the preceding step. For example, an interference in the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid would be expected to cause an increase in the urinary excretion of 3-hydroxykynurenine.

CHAPTER II

REVIEW OF THE LITERATURE

Pellagra

In 1735, Casal described a disease which was prevalent in the province of Austria in Spain which he called "mal de rosa." One of its manifestations is still called "Casal's necklace." The disease which later became prevalent in Milan, Italy, was named by Frapoli as "pelle agra" or rough skin. It was from a contraction of this word that we now call the disease pellagra. Early observers of pellagra noted that the syndrome appeared to spread throughout southern Europe in conjunction with the use of maize in the diet. The disease was prevalent from the 1850's to the early 1900's in many parts of the world, especially in Italy, Spain, Egypt, Rumania, and in the southern United States (1).

Early symptoms include glossitis, stomatitis, insomnia, anorexia, weakness, irritability, abdominal pain, burning sensations in various parts of the body, numbness, forgetfulness, morbid fears, and vertigo. There are ill-defined disturbances of the alimentary tract, with changes of bowel function. In the advanced stages, pellagra can be diagnosed by the classical symptoms commonly termed as "three D's"--dermatitis, diarrhea, and dementia (10).

The Search for the Pellagra-Preventive Factor

Joseph Goldberger in 1914 began a series of epidemiological and experimental studies of pellagra. He and Wheeler (11) were able to produce pellagra in healthy humans fed a high carbohydrate diet. Two years later, Goldberger and his associates (3) established that the diet influenced both the prevention and cure of pellagra. In comparing the diets of pellagrous and non-pellagrous subjects, they found that individuals without pellagra had more animal protein in their diets than did those with pellagra.

Goldberger and Tanner (12) found that the improvement in the condition of pellagra patients due to the addition of meat and milk to their diet could not be explained entirely on the increase in the level of protein in the diet. This suggested that the amino acid mixture in the protein was an important factor in preventing pellagra and that some protein foods had a faulty or inadequate mixture of amino acids. They observed that the administration of cystine to the diets of the pellagrous patients led to improvement in skin lesions and that additions of cystine and tryptophan resulted in weight gain.

The theory of an "unbalanced diet, deficient in certain amino acids," as a cause of pellagra was later abandoned by Goldberger in favor of the hypothesis originally advanced by Funk that the disease was due to a Vitamin B deficiency (1). In 1923 Goldberger came to the conclusion that a specific deficiency of what he called the "pellagra-preventive" factor was the cause of pellagra. Goldberger *et al*. (13) found the pellagra-preventive factor to be present in dried yeast and

fresh beef, but not in butter.

The Relationship of Pellagra, Nicotinic Acid, Tryptophan, and Corn

Although nicotinic acid was known early to the organic chemist its importance in nutrition was not appreciated. Nicotinic acid was first prepared by Huber in 1817 and in 1911 was found by Funk to be present in rice polishings (14).

During the long search for the cause of pellagra, a close association between pellagra and maize consumption was emphasized repeatedly. It was thought that pellagra was due to some toxic or infectious factor in corn. The early theories about infections and toxins as a cause of pellagra were still widely accepted in the early 1900's when an endemic incidence of the disease was reported in this country.

Many experiments were conducted to study the transmissibility of pellagra. The first physician to carry out such experiments in the United States was E. L. McCafferty. In 1902 he tried to produce experimental pellagra among the inmates of a mental institution in Alabama. He swabbed the mouths of pellagrous patients and introduced the secretions and other materials obtained from the pellagrins into the mouths of healthy inmates. He also scraped the skins and sores of the extremeties of pellagrins and rubbed the material into scarified areas of the hands of healthy subjects. He was unable to transmit the disease. In 1916, Goldberger innoculated 16 normal individuals, including 13 physicians, with the blood of pellagrins. He also orally administered to them secretions from the mouth, nose and throat, urine, and

feces of pellagrins. Goldberger was also unsuccessful in his attempts to produce symptoms of pellagra in any of the volunteers (1).

In 1937, Elvehjem and his associates (15) isolated niacinamide* from liver concentrates. This compound cured black-tongue in dogs, the canine analogue of pellagra. Niacin was found by Sydenstricker et al. (16) to be effective in the treatment of pellagra in humans.

Dann (17) reported that studies in Wayne, North Carolina, showed the daily diet provided 7.5 mg. niacin and was low in corn. There were few cases of pellagra. In contrast, subjects fed Goldberger and Wheeler's experimental diet which had the same quantity of niacin but contained corn developed pellagra (18). The final explanation offered was that the effect of corn was not due to its low content of niacin but to its deficiency in the amino acid tryptophan.

In 1947 Krehl et al. (19) noted that when 2 parts by weight of corn meal or grits was added to 3 parts by weight of a low protein diet, a reduction in the growth rate of rats occurred. This effect could be prevented by the addition of 1 mg. niacin per 100 g. of ration or by raising the casein level of the diet from 15 to 20 per cent. This finding suggested that the growth depression was caused by an amino acid deficiency. Krehl et al. (4) showed that the addition of lysine to the diet had no effect on growth but the addition of 0.05 per

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*Both nicotinamide and nicotinic acid are active in the cure of pellagra, the latter being preferred in medical practice because it is better tolerated than the former. In order not to confuse these compounds with nicotine they have been given the names niacin and niacinamide.

cent tryptophan was as effective as the addition of niacin in improving growth. These findings confirmed the significance of tryptophan.

Further research findings indicated that tryptophan was a precursor of niacin and not simply a stimulant to the bacterial synthesis of niacin. Rosen et al. (20) found a significant decrease in the nicotinic acid excretion in the rat when the casein in the diet was replaced by the tryptophan-deficient protein, gelatin. A subsequent increase in the excretion of N'-methylnicotinamide, a urinary metabolite of niacin, with the addition of tryptophan to the diet provided additional evidence that tryptophan was a precursor of nicotinic acid. In 1947 Perlzweig et al. (21) observed a prompt and marked increase in the urinary excretion of nicotinic acid derivatives, chiefly the methylated form, in humans following the ingestion of 1 g. tryptophan.

These findings were further confirmed by Singal et al., (22) who measured the effect of tryptophan and nicotinic acid on the urinary excretion of nicotinic acid derivatives in rats fed corn and non-corn rations. There was an increased urinary excretion of nicotinic acid in rats receiving both rations supplemented with tryptophan but nicotinic acid supplements to the rations had no effect on the urinary excretion of nicotinic acid derivatives. Sarett and Goldsmith (23) found that the administration of tryptophan in large amounts to humans resulted in the cure of pellagra.

These studies all strongly suggested that pellagra was a deficiency disease due to a lack of the vitamin, niacin and also its

precursor, the amino acid tryptophan. Subsequent studies by many investigators have elucidated the pathway of the conversion of tryptophan to niacin.

Tryptophan Metabolism

Following the demonstration that tryptophan was a precursor of niacin, many extensive studies were conducted to determine how this compound was metabolized in various animals. Much knowledge has been derived from bacterial studies, the analysis of certain intermediate products of tryptophan metabolism in the urine of animals fed various diets, and studies with isotopically labeled compounds using intact animals.

The outcome of the different lines of investigation is that a number of pathways have been established. In the vertebrate organism, the two well-known pathways are the kynurenine pathway and the serotonin pathway as shown in Figure 1.

Tryptophan is the only amino acid containing an indole nucleus. It is an essential amino acid but it can be transaminated so that the keto acid, indole pyruvic acid, can replace tryptophan in a tryptophan deficient diet. The D-isomer will also support growth, but there is evidence that it is not metabolized in the same manner as the L-isomer (24, 25).

The kynurenine pathway for the metabolic breakdown of tryptophan begins with the conversion of the amino acid to kynurenine. This reaction which is catalyzed by the tryptophan pyrrolase enzyme system (TPO)

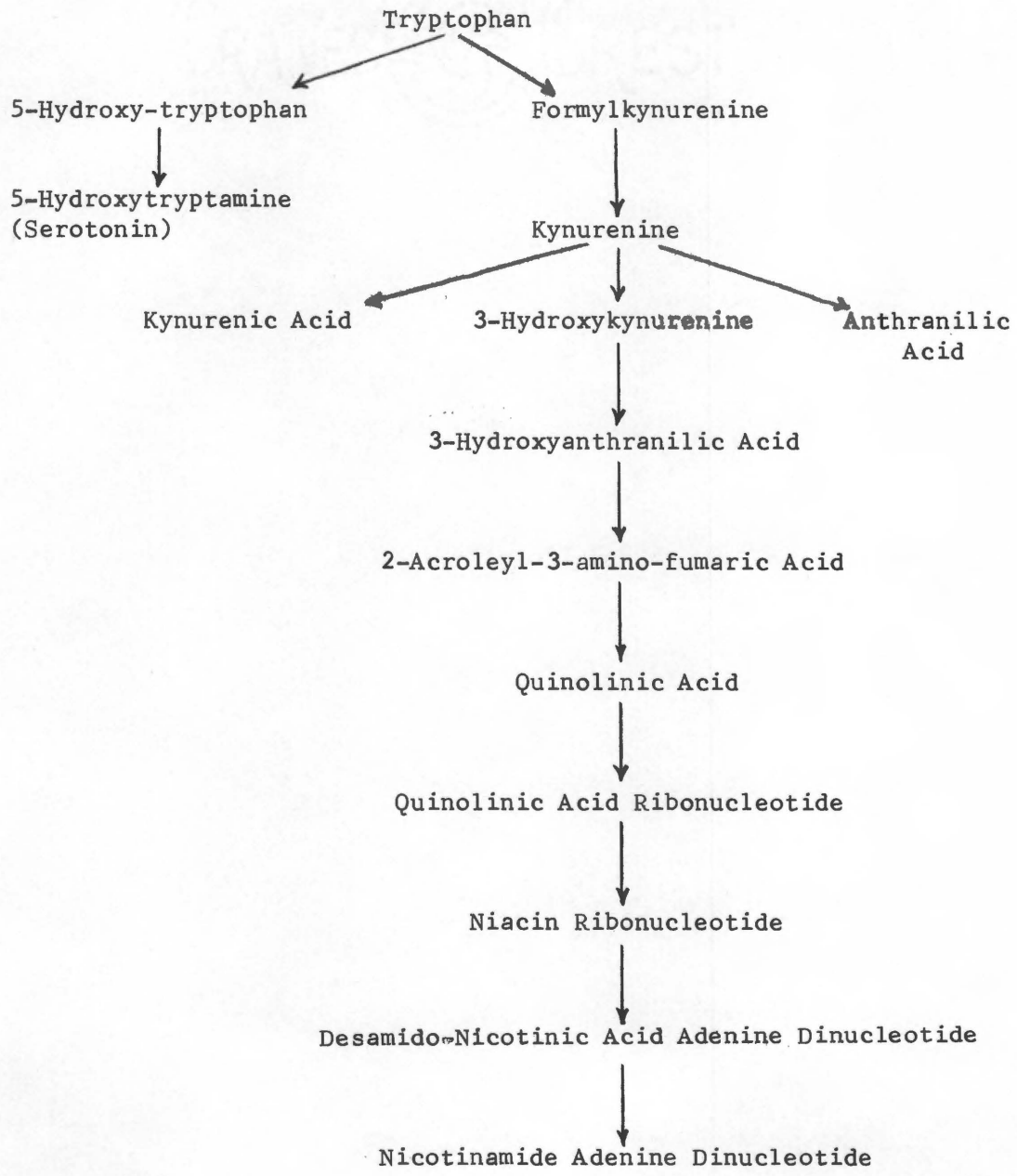

Figure 1. Abbreviated pathways and products of the metabolism of tryptophan.

involves a direct transfer of gaseous oxygen to tryptophan (26). Tanaka and Knox (27) have studied this enzyme system in rat liver and reported that it consists of two reactions. The first produces an opening of the pyrrole ring and oxidation of tryptophan to formylkynurenine. In the second step formylkynurenine is hydrolyzed by kynurenine formylase to kynurenine plus formate (Figure 2). Tryptophan pyrrolase is an iron porphyrin protein which is reduced to its active ferrous (Fe^{++}) form with H_2O_2 (8).

Kynurenine may be deaminated by transamination to the keto derivative, o-amino benzoylpyruvic acid which then loses water and undergoes spontaneous ring closure to form kynurenic acid (8). The major pathway for the further metabolism of kynurenine, however, involves its conversion to 3-hydroxykynurenine catalyzed by the enzyme kynurenine-3-hydroxylase. The hydroxylation occurs with molecular oxygen in a NADPH-* catalyzed reaction. This reaction is shown in Figure 3 (8). The importance of 3-hydroxykynurenine in the mammal was established when it was shown that it could replace nicotinic acid as a growth factor (28, 29).

The conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid is catalyzed by the enzyme kynureninase, which requires vitamin

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*The following abbreviations are used in this thesis: PRPP, 5-phosphoribosyl-1-pyrophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; desamido-NAD, desamido-nicotinic acid adenine dinucleotide; PP_i, inorganic pyrophosphate; AMP, adenosine monophosphate; NADH, reduced form of nicotinamide adenine dinucleotide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

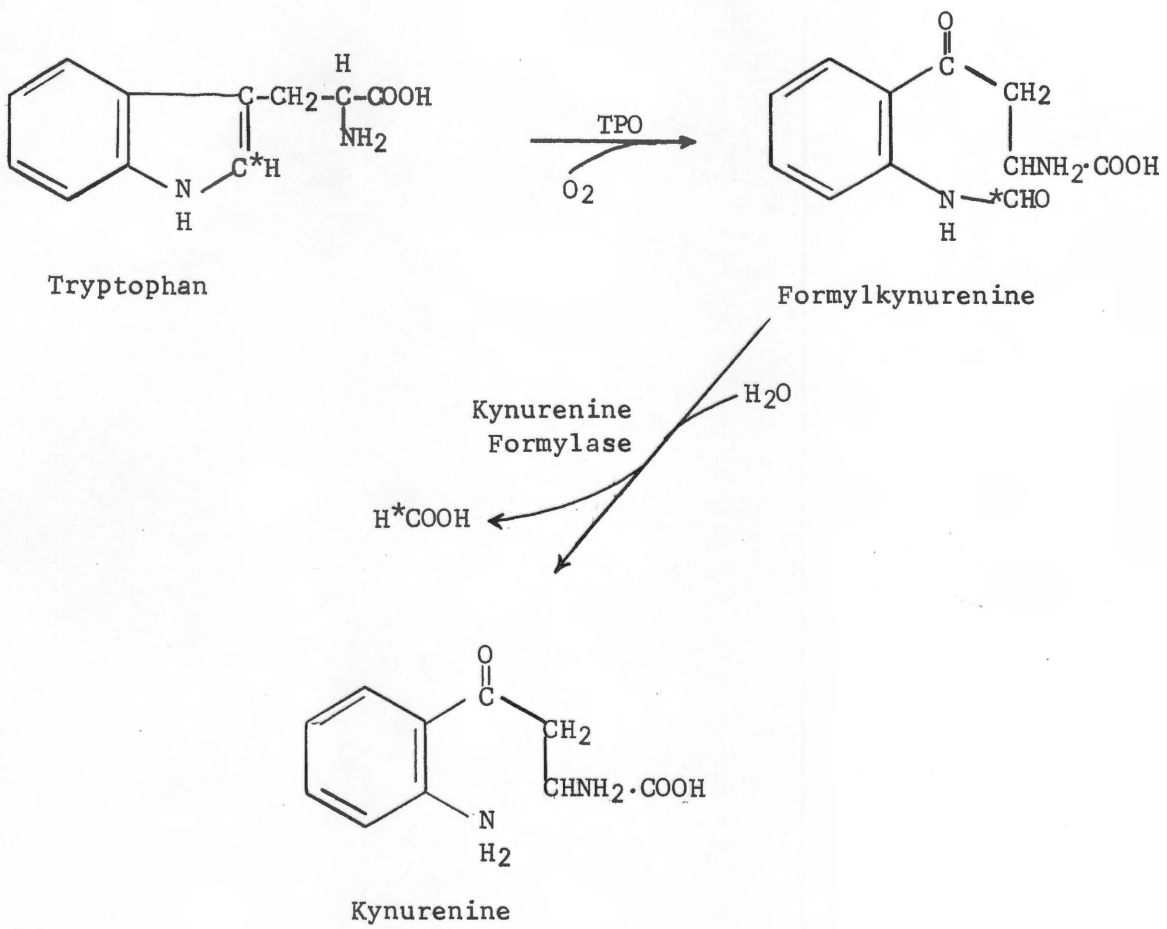

Figure 2. Conversion of tryptophan to kynurenine (8).

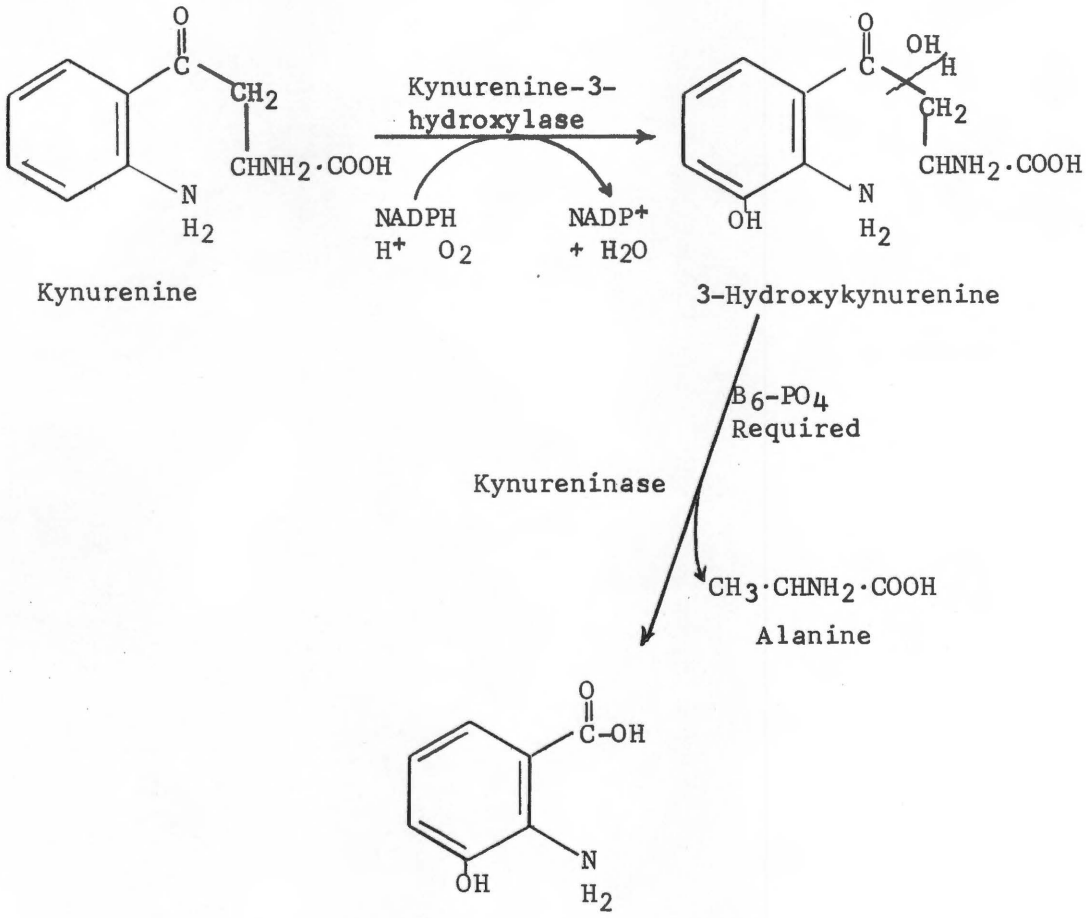

Figure 3. Conversion of kynurenine to 3-hydroxyanthranilic acid (8).

B_6 (pyridoxal phosphate) as a coenzyme (8). This reaction is also shown in Figure 3. Dalgleish et al. (30) and Knox (31) confirmed earlier studies which showed that the kynureninase activity in the liver of pyridoxine-deficient animals was greatly reduced and could be restored in vitro by the addition of pyridoxal phosphate to the diet. According to Knox (31) the removal of the alanyl side chain becomes the limiting step in the metabolism of tryptophan in pyridoxine deficiency and permits the accumulation of kynurenine, 3-hydroxykynurenine, and their conversion products.

Nicotinic acid functions as a constituent of two coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes operate as hydrogen and electron transfer agents by virtue of a reversible oxidation and reduction thus playing a vital role in metabolism. This function of niacin in metabolism explains its great importance in human nutrition and its requirement by many organisms including bacteria and yeast (32).

In 1949 Henderson et al. (33) found a marked increase in the amount of quinolinic acid in rat urine following the feeding of tryptophan which suggested that quinolinic acid was an intermediate in the conversion of tryptophan to NAD and that quinolinic acid was capable of replacing nicotinic acid in the diet of various animals. The mechanism of the conversion of 3-hydroxyanthranilic acid to NAD has only recently been elucidated by Nishizuka and Hayaishi (9). The diagram in Figure 4 presents the suggested pathway for the conversion of 3-hydroxyanthranilic acid to NAD.

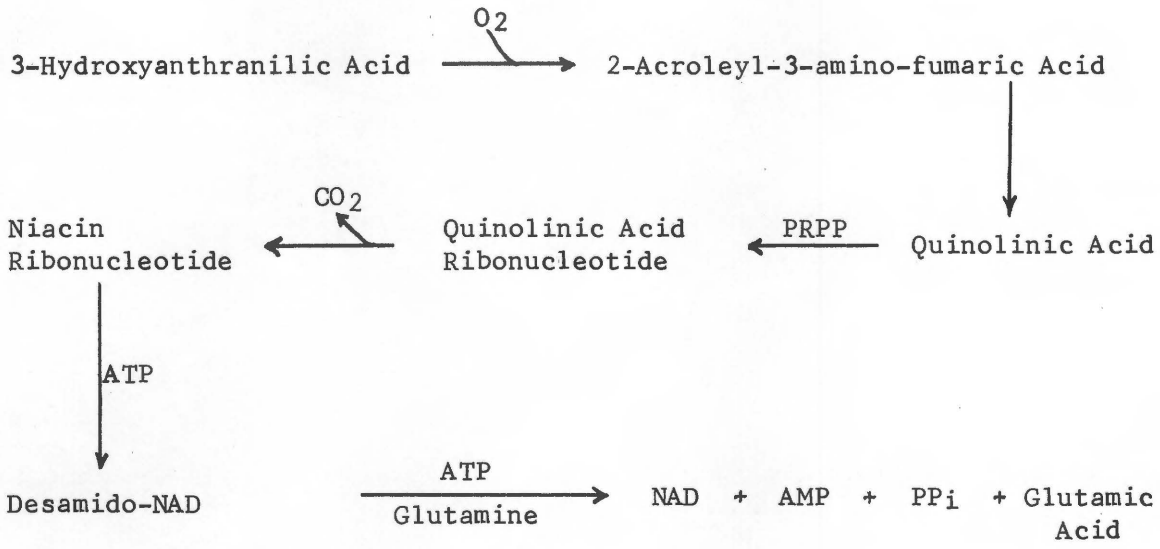

Figure 4. Suggested pathway for the conversion of 3-hydroxy-anthranilic acid to nicotinamide adenine dinucleotide (9).

Numerous investigations have been made to study the effect of tryptophan and niacin on the synthesis of pyridine nucleotide. Pyridine nucleotide is a term used to refer collectively to the two coenzymes NAD and NADH. Williams et al. (34) observed that tryptophan was probably more important than niacin in the formation of coenzymes. Niacin was found not to contribute at all to the synthesis of liver pyridine nucleotide in the absence of tryptophan.

Chaloupka et al. (35) studied the effect of tryptophan and niacin supplements on the blood concentration of pyridine nucleotides in rats depleted of niacin and tryptophan. The results obtained lend support to the findings of Williams and co-workers (34) that physiological levels of tryptophan support pyridine nucleotide synthesis more than does niacin.

Niacin is not excreted to any extent as free nicotinic acid. A small amount may occur in the urine as niacinamide or a nicotinuric acid, the glycine conjugate. By far the largest portion is excreted as methyl derivatives, viz, N'-methylnicotinamide and the 6-pyridone of N'-methylnicotinamide and the glycine conjugates of these methyl derivatives. This methylation is accomplished in the liver at the expense of labile methyl supply of the body. Methionine is the principal source of these methyl groups (8).

The Influence of Various Proteins and Amino Acids on Tryptophan-Nicotinic Acid Relationship

The influence of various proteins and amino acids on the tryptophan-niacin relationship has been of interest to many investigators.

Krehl et al. (18) were able to produce growth retardation and niacin-deficiency symptoms in rats fed low-protein niacin-deficient diets to which 40 per cent corn had been added. These effects were reversible with a dietary addition of tryptophan or niacin. Growth depression was not observed when other cereals like polished rice or rolled oats were added to the low-protein niacin-deficient diet.

Krehl et al. (4) demonstrated that a dietary source of niacin was not required by the rat, and that the dietary requirement of niacin varied with the character and quality of accompanying nutrients in the diet. The slight decrease in the growth rate of rats which occurred when corn grits was added to fibrin and egg albumin diets was attributed to a higher tryptophan content in these diets as compared to casein-corn diets.

The observation that rats grew well when fed non-corn low-casein diets containing only 108 mg. per cent tryptophan but failed to grow as well when fed corn diets containing even higher levels of tryptophan warranted further studies (36). Krehl et al. (37) studied the effect of adding zein to a 9 per cent casein ration on the growth rate of rats. It was observed that poor growth resulted unless nicotinic acid or tryptophan was added. The result of these studies strongly suggested that the adverse effect of corn in creating a nicotinic acid deficiency or in increasing the tryptophan requirement in rats was related to zein, the protein in corn, or to the amino acid distribution in the protein of corn.

The presence of some factors in corn which inhibit the conversion of tryptophan to niacin as observed by Krehl and co-workers (4, 19, 36, 37) was supported by results of a study on humans by Goldsmith et al. (5). It was observed that subjects fed wheat diets developed niacin-deficiency symptoms much more slowly than those fed corn diets, although the amounts of niacin and tryptophan were comparable in both diets. The findings from these studies have been ascribed to an amino acid imbalance in zein, the major protein in corn, which resulted in an increased tryptophan requirement (5, 38).

Briggs (39) found that feeding a 10 per cent gelatin diet to chicks caused a marked depression in the growth rate and produced symptoms suggestive of niacin deficiency which were alleviated by the addition of either niacin or tryptophan. Lyman and Elvehjem (40) in studying the effect of the amino acids in gelatin on rat growth, observed that the addition of cystine and/or methionine to the diet depressed growth.

Pearson et al. (41) observed marked growth inhibition in rats fed 8 or 9 per cent casein niacin-free diets with added DL-threonine. They further observed a decrease in urinary N'-methylnicotinamide, anthranilic acid, 3-hydroxyanthranilic acid, kynurenine, and quinolinic acid. These findings were interpreted to indicate that there was a reduced conversion of tryptophan to niacin induced by threonine.

Sauberlich and Salmon (42) made the observation that the tryptophan requirement of the rat was related to the amount of protein or

nitrogen found in the diet. When gelatin was added to the diet of rats, growth depression was observed which was prevented by tryptophan supplements, whereas niacin supplements had no effect. They offered the explanation that when the diet was adequate in niacin no sparing effect on tryptophan could be expected. Only when tryptophan was being utilized for niacin synthesis could added niacin be expected to have any effect.

Savage and Harper (43) investigated the effect of adding gelatin or mixtures of the indispensable and dispensable amino acids simulating the amino acid composition of gelatin on the growth and liver pyridine nucleotide concentrations of rats fed low-protein niacin-deficient diets. It was observed that a tryptophan supplement to the diet prevented the growth depression caused by the gelatin or the mixture of indispensable amino acids but not that due to the addition of the mixture of dispensable amino acids. A tryptophan supplement to the diet significantly increased the liver NAD-NADP of rats fed either the basal diet or the diet containing a mixture of the indispensable amino acids simulating gelatin, but did not increase the liver NAD-NADP of rats fed the diets containing gelatin or the mixture of the dispensable amino acids simulating gelatin. Both glycine and hydroxyproline were found to be the dispensable amino acids responsible for the growth depression and the decreased synthesis of liver NAD-NADP.

In 1960 Gopalan and Srikantia (44) investigated the relationship between leucine and pellagra. They observed that feeding a relative excess of 20 to 30 g. leucine to human subjects caused an increased

urinary N'-methylnicotinamide excretion. They suggested that the amino acid imbalance caused by excess leucine in the diet might result in the depletion of nicotinic acid of the tissues.

Truswell et al. (45) in repeating Gopalan and Srikantia's study failed to observe any change in the N'-methylnicotinamide urinary excretion when only 4 or 10 g. of L-leucine was added to adequate diets. This finding does not support the suggestion that relative excess leucine in the diet causes pellagra. However, the dietary levels of leucine used by Gopalan and Srikantia were higher than the amount used by Truswell and co-workers.

Results of studies by Belavady and associates (46) with human subjects suggested that leucine interferes with the synthesis of niacin from tryptophan by blocking the conversion of quinolinic acid to niacin ribonucleotide.

The effect of feeding excess leucine to young and adult rats has recently been reported by Raghuramulu et al. (47). The inclusion of leucine at a level of 1.5 per cent in the basal ration caused a significant increase in the excretion of N'-methylnicotinamide in adult rats. The increase in N'-methylnicotinamide excretion was less significant in young rats. Quinolinic acid excretion increased to a significant extent in the urine of both young and adult rats. The addition of isoleucine to the diet containing leucine counteracted the increase in the excretion of quinolinic acid and N'-methylnicotinamide in young rats.

Two studies at the University of Tennessee were designed to determine whether any of the amino acids in zein might be responsible for

inhibiting the utilization of tryptophan for NAD synthesis. Coulter (6) studied the ability of rats to utilize tryptophan for the synthesis of pyridine nucleotides when fed low-protein niacin-free diets supplemented with either corn, zein, mixtures of the indispensable amino acids as found in zein with and without leucine, or a mixture of the dispensable amino acids as found in zein. She found that rats fed diets in which leucine was omitted from the mixture of the indispensable amino acids showed a highly significant increase in the total liver pyridine nucleotide concentration. The total liver pyridine nucleotide concentrations of rats fed the basal diet, the complete mixture of indispensable amino acids, or the mixture of indispensable amino acids without leucine supplemented with tryptophan, were significantly higher than the values obtained for rats fed the same diets with limited tryptophan. The pyridine nucleotide concentration of rats fed tryptophan supplemented diets containing corn, zein, or the mixture of the dispensable amino acids were not significantly increased above the value obtained for rats fed limited amounts of tryptophan. The data indicated that one or more dispensable amino acids in zein as well as the amount of leucine in zein interfered with the utilization of tryptophan for pyridine nucleotide synthesis in rats.

Ellis (7) studied the effect of the stepwise addition of the dispensable amino acids of zein on the utilization of L-tryptophan for pyridine nucleotide synthesis in rats fed low-protein niacin-free diets. She found that the stepwise addition of these amino acids to the basal diet had no significant effect on the liver NAD concentration of rats

when the diets were not supplemented with tryptophan. In diets supplemented with tryptophan the addition of cystine and glycine caused a significant increase in the liver NAD concentration above the value obtained for rats fed the basal diet. The stepwise addition of aspartic acid, tyrosine, serine, alanine, and proline to diets supplemented with tryptophan did not produce any significant changes in the liver NAD concentration. But, when glutamic acid was added to complete the mixture of dispensable amino acids of zein, the liver NAD concentration was significantly lowered below the value obtained for the control rats. When the NAD concentration of rats fed similar diets with and without a tryptophan supplement were compared, higher NAD concentrations were observed in all groups of rats fed diets supplemented with tryptophan except the group fed diets to which glutamic acid had been added. There was no significant increase in NAD concentration when glutamic acid was present in the diet. These data indicated that glutamic acid inhibited the utilization of tryptophan.

Other Factors Which Influence the Tryptophan-Nicotinic Acid Relationship

The conversion of tryptophan to 3-hydroxyanthranilic acid is of interest in its dependence on other vitamins. The effect of some B complex vitamins on the conversion of tryptophan to niacin has been studied. The results of several studies indicated that thiamine, riboflavin, and pyridoxine are implicated in the metabolism of niacin and tryptophan. Dalgleish (48) has diagrammatically shown the stages in the tryptophan-niacin pathway where each of these vitamins are involved, Figure 5. The metabolism of tryptophan in animal tissues is known to be profoundly

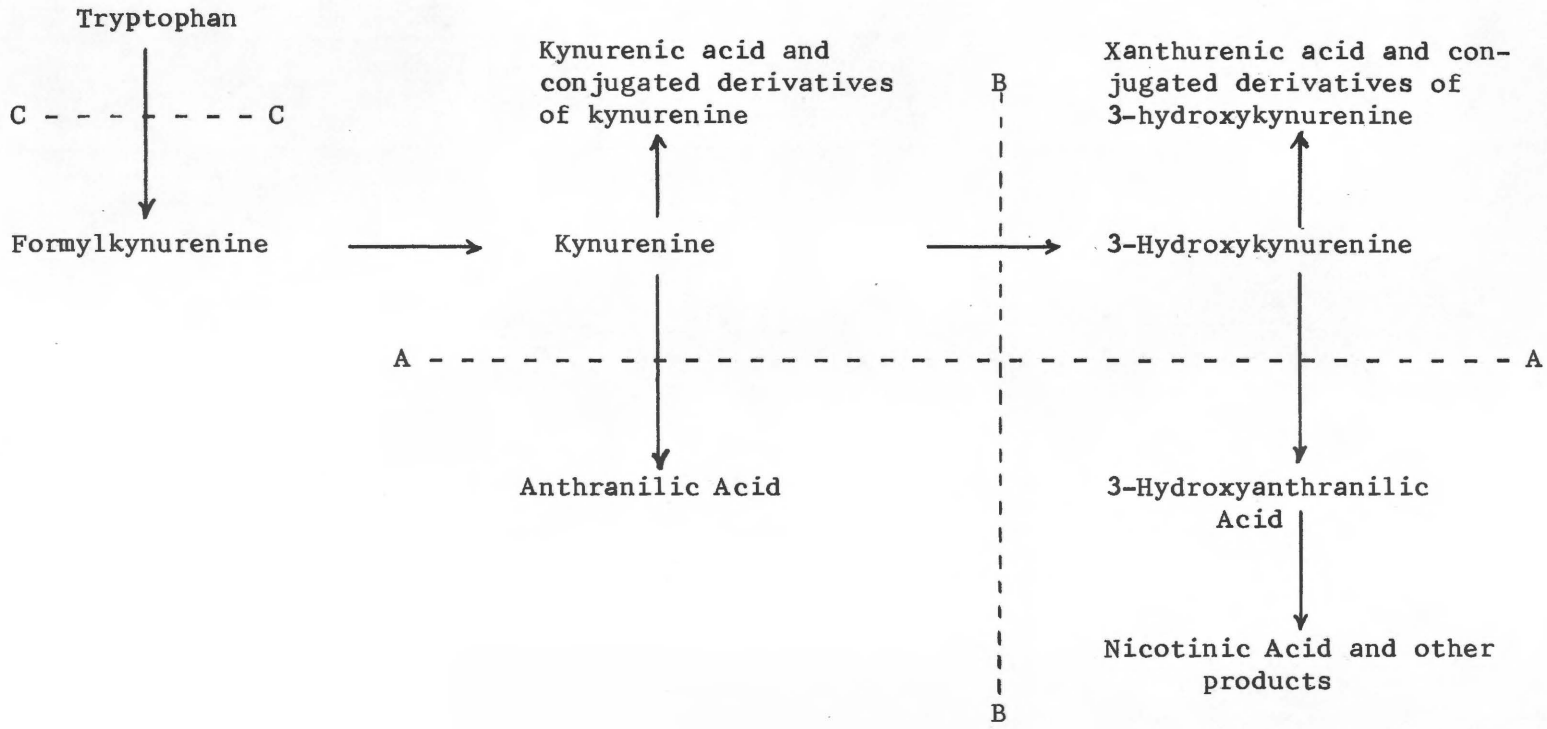

Figure 5. Relationship of other vitamins to the tryptophan-niacin conversion. Deficiencies of vitamins B_6, B_2, and B_1 cause blocks at A----A, B----B, and C----C, respectively (48).

altered by pyridoxine deficiency. It results in abnormal tryptophan metabolism in that the pyridoxal phosphate functions as a coenzyme in the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid. Thus, an interference in this conversion would result in the accumulation of kynurenine and 3-hydroxykynurenine. The excess 3-hydroxylkynurenine can be converted to xanthurenic acid (49).

Korbitz et al. (50) studied the effect of a vitamin B_6 deficiency on the ability of the rat to convert tryptophan to niacin. It was observed that pyridoxine deficient rats excreted abnormally large quantities of kynurenine, 3-hydroxykynurenine, acetylkynurenine and xanthurenic acid as compared with the control rats. These results are consistent with the view that the enzyme system concerned with the transamination of kynurenine to form kynurenic acid is distinct from the enzyme system which transaminates 3-hydroxykynurenine to form xanthurenic acid. It also suggests that there is an alternate pathway for the xanthurenic acid synthesis which remains active in pyridoxine deficiency.

Snyderman (51) observed that pyridoxine deprivation in human infants resulted in arrest in weight gain and failure to convert tryptophan to N'-methylnicotinamide.

A deficiency of thiamine or riboflavin interferes with the activity of tryptophan pyrrolase or kynurenine 3-hydroxylase, respectively (52). Various studies (53, 54, 55) demonstrated that in riboflavin deficiency, there is an increased excretion of kynurenine, anthranilic

acid, and kynurenic acid and its conjugates.

It was reported that animals submitted to a diet deprived of riboflavin, but with ascorbic acid added, responded normally to a large addition of DL-tryptophan, and showed no abnormal accumulation of anthranilic acid and xanthurenic acid in the urine. This finding was interpreted to indicate that the general state of the riboflavin deficient rats was improved by the administration of strong doses of ascorbic acid (48).

Brown et al. (56) studied the effect of other B vitamins in addition to pyridoxine on the excretion of tryptophan metabolites by normal pregnant women. Results indicated that the urinary levels of the tryptophan metabolites kynurenine, 3-hydroxykynurenine, and anthranilic acid were higher than values obtained for non-pregnant subjects. The levels of these metabolites could be lowered to levels similar to those found in non-pregnant subjects by the administration of pyridoxine. However, the excretion of N'-methyl-pyridone-5-carboxamide remained two or three times higher in pregnancy. Administration of other vitamins had no effect on the excretion of tryptophan metabolites. The pattern of urinary metabolites obtained suggests that other factors possibly endocrine changes in addition to vitamin B_6 nutrition may be involved in regulating tryptophan metabolism in pregnant women.

The observation that the inclusion of corn in the diet increases the need for niacin has been explained by some investigators to be caused by the unavailability of niacin in corn to living organisms since

it is in the "bound" form. In 1947 Pearson et al. (57) found that limed maize permitted more rapid growth of rats than did raw maize when added to a 9 per cent casein niacin-free diet. In 1956 Goldsmith et al. (58) demonstrated that lime treated corn and untreated corn gave comparable results in the production of niacin deficiency symptoms in man.

Kodicek (59) and Carpenter et al. (60) found that alkali treatment of maize increased the availability of nicotinic acid to the rat. It was concluded that the beneficial effect of alkali treated foodstuff in curing and preventing nicotinic acid deficiency in rats may be attributed solely to the release of nicotinic acid from a bound form and not to a correction of an amino acid imbalance, destruction of a toxic factor, or changes in the carbohydrate component of the treated samples.

In 1960 Chaudhuri et al. (61) studied the effect of feeding alkali-treated brans of wheat, barley, and rice and a purified preparation of bound nicotinic acid in weanling rats. It was found that alkali treatment cured tryptophan deficiency symptoms in the animals. They concluded that wheat, rice, and barley brans contain the nicotinic acid bound form which unless released is unavailable to the rat.

It has recently been reported that the alkali-labile bound form of niacin in cereal grains has been isolated and chemically characterized. According to Das and Guha (62) the bound form of niacin, "niacinogen," is composed of niacin, a chromophoric moiety or moities

and a peptide, the niacin being linked through an alkali-labile ester linkage.

Specie differences exist in the efficiency of the conversion of tryptophan to niacin. Man requires tryptophan in the L-form. The rat apparently can utilize the DL-form. Cat metabolism differs from that in other species at least from kynurenine onward as confirmed by Brown and Price (63).

CHAPTER III

EXPERIMENTAL PROCEDURE

Experimental Animals

Sixty male weanling albino rats of the Wistar strain, ranging in weight from 25 to 48 g., were obtained from the University of Tennessee colony for use in this study.

The animals were divided into 12 groups of five rats each so that the average weight of the twelve groups was about 177 g. All the animals were caged individually except during the period of urine collection at the end of the two-week experimental period. Distilled water and the various purified diets were fed *ad libitum*. The rats were weighed at the beginning and at the end of the study.

Urine Collection

Every rat at the end of the experimental period was placed in a metabolism cage and urine was collected for a 48-hour period. Urine adhering to the funnel was washed down into the collecting bottle with a small amount of 0.1 N. HCl. The urine from the five rats in each group was pooled into a clean 4 oz. bottle. Each pooled urine sample was centrifuged to remove any food particles present and the urine volume was measured. All the samples were frozen with added toluene until analyzed.

Diets

The basal diet used in this study was composed of (in per cent) vitamin-free casein, 6; fat-soluble vitamin mixture in corn oil, 5; choline, 0.15; DL-methionine, 0.3; niacin-free water-soluble vitamin mixture, 0.25; Hubbell, Mendell, and Wakeman salt mixture, 5; and sucrose, 83.3. The compositions of the fat-soluble and water-soluble vitamin mixtures are given in Tables I and II, respectively.

Various dietary treatments consisted of the addition of zein, a mixture of all the indispensable amino acids found in zein with and without leucine, and a mixture of all the dispensable amino acids found in zein with and without glutamic acid, as listed in Table III. The dispensable and indispensable amino acids were added in the quantities that would be present if zein had constituted 1.71 per cent of the diet as shown in Tables IV and V, respectively. The amino acid values for zein as given by Block and Weiss (64) were used to determine the amount of the various amino acids which would be present in a diet containing 1.71 per cent zein. It was determined by Coulter (6) in her study that 1.71 per cent zein is present in 40.0 g. corn. Krehl and co-workers (4) had shown that diets containing 40 per cent corn when mixed with a low-protein niacin-deficient ration produced niacin deficiency symptoms in rats.

Six groups of rats were fed the diets listed in Table III. Six other groups of rats were fed the diets listed in Table III supplemented with 0.1 per cent L-tryptophan. All additions to the basal diet were made at the expense of sucrose.

TABLE I

FAT-SOLUBLE VITAMIN MIXTURE

Component	Amount g.
Calciferol	0.27
α-DL-Tocopherol	12.75
Halibut Liver Oil	8.50
Mazola Corn Oil	616.00

Five grams of this fat-soluble mixture per 100 g. of diet provide 400 IU of vitamin A, 200 IU of vitamin D, and 10 mg. of vitamin E.

TABLE II

WATER-SOLUBLE VITAMIN MIXTURE

Vitamin	Amount
	g.
Thiamine	0.250
Riboflavin	0.250
Calcium Pantothenate	1.000
Pyridoxine HCl	0.130
Folic Acid	0.010
Menadione	0.030
Biotin	0.005
Vitamin B_{12} (1 g. triturate) 0.1 per cent with mannitol	0.001
Inositol	5.000
Ascorbic Acid	2.500

Sucrose was added to make 125 g. of vitamin mixture.

TABLE III

DIETARY TREATMENTS

Number	Diet
I	Basal Diet
II	Basal Diet + 1.71 Per Cent Zein
III	Basal Diet + A Mixture of All Indispensable Amino Acids Simulating 1.71 Per Cent of Zein
IV	Basal Diet + A Mixture of All Indispensable Amino Acids Simulating 1.71 Per Cent of Zein, Except Leucine
V	Basal Diet + A Mixture of All Dispensable Amino Acids Simulating 1.71 Per Cent of Zein
VI	Basal Diet + A Mixture of All Dispensable Amino Acids Simulating 1.71 Per Cent of Zein, Except Glutamic Acid

TABLE IV

MIXTURE OF DISPENSABLE AMINO ACIDS

Amino Acid	Amount[a] g./100 g. Diet
Glycine	0.006
Cystine	0.014
Aspartic Acid	0.050
Tyrosine	0.076
Serine	0.116
Alanine	0.150
Proline	0.150
Glutamic Acid	0.430

[a]Amounts of dispensable amino acids equivalent to that provided by a diet supplying 1.71 per cent zein.

TABLE V

MIXTURE OF INDISPENSABLE AMINO ACIDS

Amino Acid	Amount[a] g./100 g. Diet
Tryptophan	0.0015
Lysine (Lysine Monochloride 0.0019 g.)	0.0015
Histidine (Histidine Monochloride 0.0243 g.)	0.0180
Arginine (Arginine Hydrochloride 0.0293 g.)	0.0240
Methionine	0.0320
Valine	0.0390
Threonine	0.0410
Phenylalanine	0.0970
Isoleucine	0.1100
Leucine	0.3300

[a]Amounts of indispensable amino acids equivalent to that provided by a diet supplying 1.71 per cent zein.

Separation of the Urinary Tryptophan Metabolites by Ion-Exchange Chromatography

The three urinary tryptophan metabolites namely kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid were separated according to the methods used by Brown and Price (63) and Tompsett (65) with some modifications to suit the prevailing conditions in the laboratory.

Preparation of the chromatographic columns. A small plug of glass wool was packed into each of four chromatographic columns which had been made by sealing a glass tube, 15 cm. long with an outside diameter of 1.2 cm., to the base of a 125 ml. Erlenmeyer flask. Each column was fitted into a 500 ml. suction flask. Each of the four flasks was connected by means of rubber tubings and glass T-tubes to a single safety bottle which in turn was connected by means of rubber tubing to a side arm on a water faucet. The flow rate of the liquid through the columns could be regulated by adjusting the water faucet and suction to any column could be stopped by placing a clamp on the section of the rubber tubing between the glass T-tube and the particular column.

A slurry made of 3 g. of Dowex 50 H^+ (12 per cent cross-linkage, 200 to 400 mesh) resin* in 20 ml. of distilled water was carefully poured into the columns without suction to form a 7 cm. uniformly packed column. The columns were subsequently washed stepwise with

*Obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey.

80 ml. of 8 N. HCl, 200 ml. of distilled water, and 25 ml. of 0.1 N. HCl at a flow rate of 8 drops per minute. Fresh columns were prepared for each separation.

Separation of the metabolites. Two samples of urine representing 15 per cent of the pooled 48 hour urine collections were pipetted into 150 ml. beakers. The samples were diluted to 40 ml. with distilled water and acidified with a few drops of 12 N. HCl to a final pH of 1. To determine the per cent recovery of compounds from the columns 400 µg. of 3-hydroxykynurenine,* 400 µg. of kynurenine,** and 500 µg. of 3-hydroxyanthranilic acid*** were added to one of the urine samples. The samples were then applied to the prepared Dowex columns and allowed to pass through at a flow rate of 4 drops per minute. After the metabolites were adsorbed onto the Dowex resin, the columns were washed with the following concentrations of HCl at a flow rate of 8 drops per minute:

1. Eighty ml. of 2.4 N. HCl--Fraction I
2. Eighty ml. of 6 N. HCl--Fraction II

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*Prepared from a stock solution of 10 mg. 3-hydroxykynurenine dissolved in distilled water to 50 ml. (200 µg./ml.). The synthetic metabolite was obtained from the Calbiochem Research, Los Angeles 63, California.

**Prepared from a stock solution containing 100 mg. kynurenine dissolved in distilled water to 50 ml. (2000 µg./ml.). L-Kynurenine sulfate was obtained from the Mann Research Laboratories, Inc., New York.

***Prepared from a stock solution containing 12.5 mg. of 3-hydroxyanthranilic acid dissolved in 0.1 N. HCl to 50 ml. (250 µg./ml.). The synthetic metabolite was obtained from the same laboratory as L-kynurenine sulfate.

Fraction I contained 3-hydroxyanthranilic acid and Fraction II contained both 3-hydroxykynurenine and kynurenine. The volumes of the fractions collected were measured. The various fractions were refrigerated in 4 oz. plastic bottles until analyzed.

The separation of each of the metabolites in the different fractions was qualitatively determined by thin-layer chromatography according to the method of Diamanstein et al. (66). It was observed that Fraction I appeared to be free of 3-hydroxykynurenine and kynurenine and Fraction II appeared to be free of 3-hydroxyanthranilic acid.

Colorimetric Determination of the Metabolites

The method of Michael et al. (67) and Brown (68) was used to determine the concentration of 3-hydroxyanthranilic acid. Three ml. portions of Fraction I were pipetted into each of three matched colorimeter test tubes and subsequently cooled in an ice bath. By means of a B-D Cornwall Continuous Pipeting Outfit,* 0.2 ml. of freshly prepared sodium nitrite was added to tubes 2 and 3. Three minutes after mixing the nitrite 0.2 ml. of 10 per cent ammonium sulfamate** was added to all of the above tubes. The first tube was then made to the same volume as the others by the addition of 0.2 ml. of distilled water.

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*This pipeting outfit was used in adding sodium nitrite, ammonium sulfamate, and N-1-naphthylethylenediaminedihydrochloride in all of the determinations.

**Obtained from the LaMotte Chemical, Chester Town, Maryland.

All tubes were thoroughly mixed after the addition of each reagent by means of a Vortex Jr. Mixer. The tubes were allowed to warm to room temperature and the optical density was measured at 367 mμ in a Beckman Model B Spectrophotometer using the first tube as a blank. The optical densities of a series of standard solutions* containing 5, 10, 20, and 30 μg. of 3-hydroxyanthranilic acid were obtained under the same conditions of acidity and temperature as the fraction concerned. From the standard curve, the per cent recovery and the amount of the metabolite in μg. per 24 hour urine per rat were calculated.

The concentration of the tryptophan metabolite in the sample used for the spectrophotometric analysis was obtained from the standard curve and the concentration of the metabolite in 15 per cent of the pooled 48 hour urine was calculated according to the following formula:

$$\mu g.\ \text{metabolite/ml.}_{(u)} = \frac{\mu g.\ \text{metabolite/ml.}_{(f)} \times \text{ml.}_{(F)}}{\text{ml.}_{(f)}}$$

$$\mu g.\ \text{metabolite/rat/day} = \frac{\mu g./\text{ml.}_{(u)} \times \text{ml.}_{(U)}}{\text{ml.}_{(u)} \times 2 \times 5} \quad .$$

The per cent recoveries were calculated according to the formula below:

$$\%\ \text{recovery} = \frac{\mu g./\text{ml.}_{(uR)} - \mu g./\text{ml.}_{(uX)}}{\mu g._{(S)}} \times 100$$

where::

u = volume of urine applied to the columns (15 per cent)

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*A work solution was prepared with a concentration of 10 μg./ml. This was made by diluting 2 ml. of the stock solution (see footnote ***, page 37) to 50 ml. with 2.4 N. HCl.

U = total volume of urine pooled from 5 rats for 2 days

f = volume of fraction actually analyzed

F = total volume of fraction collected from the column

uR = recovery sample

uX = urine sample

S = concentration of standard added to the recovery sample.

The method of Brown (68) was used to determine the concentration of 3-hydroxykynurenine. Two-ml. portions of Fraction II were pipetted into each of three matched colorimeter tubes and subsequently cooled in an ice bath. To reduce the acid concentration of the samples 1.2 ml. of 9 N. NaOH was added into each tube and was thoroughly mixed. The samples were then treated in the same manner as in the analysis of 3-hydroxyanthranilic acid. Optical density was measured at 367 mμ in a Beckman Model B Spectrophotometer using the first tube as a blank. The optical densities of a series of standards* containing 2.4, 6, 9.6, and 12 μg. of 3-hydroxykynurenine were obtained under the same conditions of acidity and temperature as the fraction concerned. From the standard curve, the per cent recovery and the amount of the metabolite in μg. per 24 hour urine per rat were calculated using the previous formula.

Kynurenine was determined according to the method of Brown and Price (63). Two ml. portions of Fraction II were pippetted into each

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*A work solution with a concentration of 12 ug./ml. was used. This was prepared by diluting 3 ml. of the stock solution (see footnote *, page 37) to 50 ml. distilled water.

of three matched colorimeter tubes and cooled in an ice bath. To reduce the acid concentration the samples were treated with 1.2 ml. of 9 N. NaOH. Then 0.2 ml. of freshly prepared 0.25 per cent sodium nitrite was added to tubes 2 and 3 by means of a B-D Cornwall Continuous Pipeting Outfit. Three minutes after mixing the nitrite, 0.2 ml. of 10 per cent ammonium sulfamate was added to all of the tubes and followed two minutes later by 0.2 ml. of 0.25 per cent N-1-naphthylethylenediaminedihydrochloride.* The first tube was then made to the same volume as the others by the addition of 0.2 ml. distilled water. All tubes were thoroughly mixed after the addition of each reagent by means of a Vortex Jr. Mixer. After 3 hours, the optical density was measured at 550 mμ in a Beckman Model B Spectrophotometer using the first tube as a blank. The optical densities of a series of standards** containing 2, 6, 8, and 12 μg. kynurenine were obtained under the same conditions of acidity and temperature as the fraction analyzed. From the standard curve, the per cent recovery and the amount of the metabolite in μg. per 24 hour urine per rat were calculated using the previous formula.

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*Obtained from the Mann Research Laboratories, Inc., New York.

**A work solution with a concentration of 20 μg./ml. was used. This was prepared by diluting 1 ml. of the stock solution (see footnote **, page 37) to 100 ml. with 6 N. HCl.

CHAPTER IV

RESULTS AND DISCUSSION

The effect of adding zein, a mixture of all the indispensable amino acids simulating 1.71 per cent of zein with or without leucine, or a mixture of all the dispensable amino acids simulating 1.71 per cent zein with or without glutamic acid to a 6 per cent casein niacin-free diet with or without a supplement of 0.1 per cent tryptophan on the growth of rats is shown in Table VI. The various experimental diets did not affect the growth of rats fed tryptophan supplemented diets appreciably. The addition of zein, or a complete mixture of all the indispensable amino acids simulating zein to diets containing a limited amount of tryptophan retarded the growth of rats but not significantly. However, the addition of 0.1 per cent tryptophan to these diets improved the growth of the rats significantly ($0.01 > P > 0.001$).

The effect of adding zein, a mixture of all the indispensable amino acids simulating 1.71 per cent of zein with or without leucine, or a mixture of all the dispensable amino acids simulating 1.71 per cent zein with or without glutamic acid to a 6 per cent casein niacin-free diet supplemented with 0.1 per cent tryptophan on the urinary excretions of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in the rat is shown in Figure 6. The effect of the above mentioned dietary treatments on the urinary excretions of the different metabolites in rats fed diets with a limited amount of tryptophan is shown in Figure

TABLE VI

THE EFFECT OF ZEIN AND MIXTURES OF THE INDISPENSABLE AND DISPENSABLE AMINO ACIDS SIMULATING ZEIN WITH AND WITHOUT A 0.1 PER CENT L-TRYPTOPHAN SUPPLEMENT ON THE GROWTH OF RATS

Groups	Diets	No. of Animals	Without Added Tryptophan g./14 Days	No. of Animals	With 0.1 Per Cent L-Tryptophan g./14 Days
I	Basal (B)[a]	5	17 ± 3[b]	5	19 ± 2
II	B + Zein	5	13 ± 2	5	19 ± 1[c]
III	B + All the Indispensable Amino Acids in Zein	5	13 ± 1	5	21 ± 2[c]
IV	B + All the Indispensable Amino Acids in Zein, Except Leucine	5	18 ± 1	5	21 ± 2
V	B + All the Dispensable Amino Acids in Zein	5	18 ± 1	5	19 ± 1
VI	B + All the Dispensable Amino Acids in Zein, Except Glutamic Acid	5	15 ± 1	5	16 ± 1

[a]Six per cent casein niacin-free.

[b]Mean ± S. E.

[c]Significantly higher than corresponding diet without added tryptophan ($0.01 > P > 0.001$).

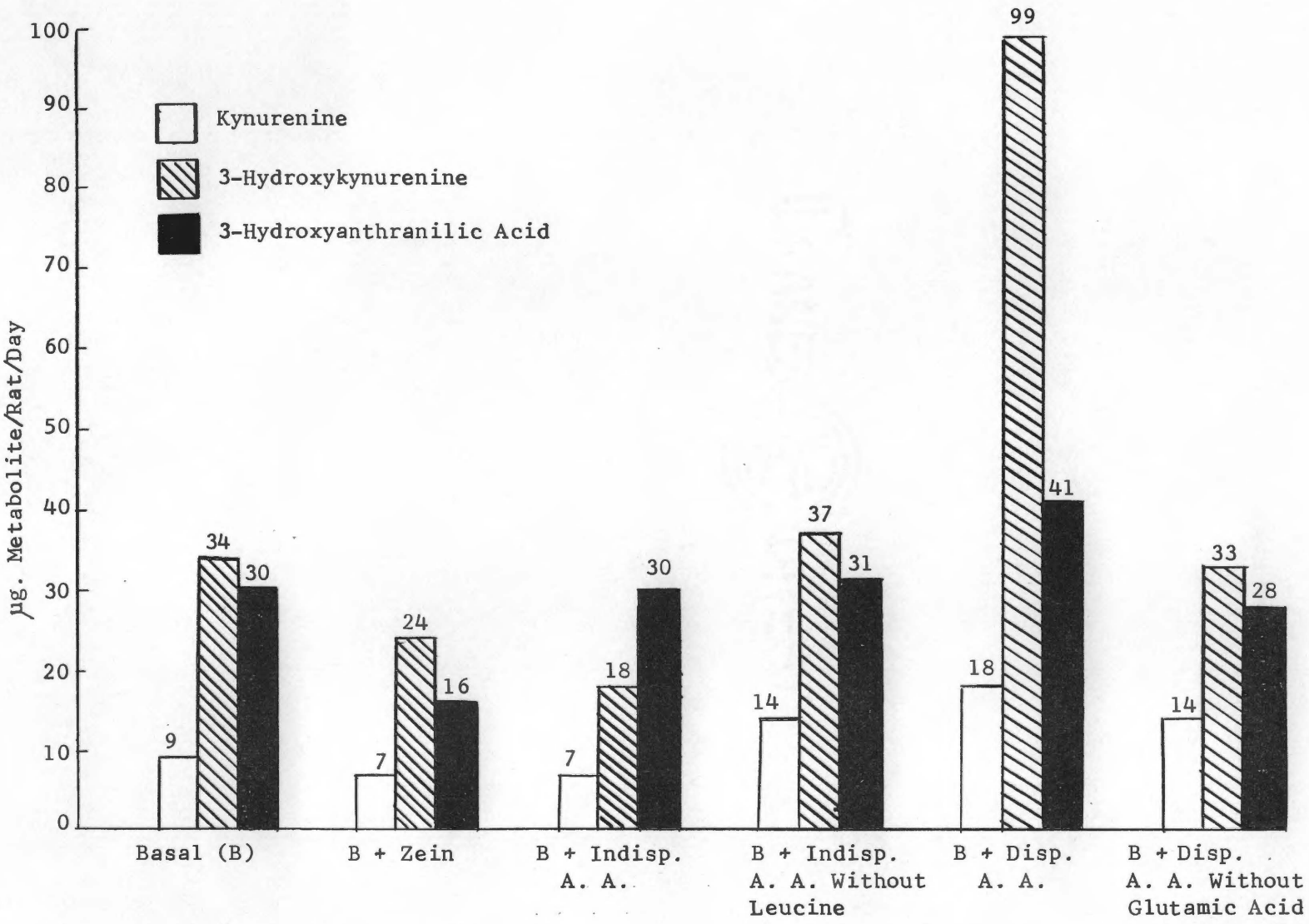

Figure 6. Urinary excretion of tryptophan metabolites in rats fed tryptophan supplemented diets.

7. Recovery samples for the tryptophan metabolites ranged from 87 to 112 per cent for kynurenine and 90 to 112 per cent for 3-hydroxyanthranilic acid. The greatest difficulty in the analyses was encountered in the spectrophotometric determination of 3-hydroxykynurenine. Only the determinations in which the recoveries ranged from 70 to 106 per cent were considered for this metabolite.

Excretion of Kynurenine

The addition of zein or a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent tryptophan (Figure 6) decreased slightly the urinary excretion of kynurenine by 2 µg. (22 per cent) when compared to the basal value. When a mixture of all the indispensable amino acids simulating 1.71 per cent of zein without leucine was added to the basal diet, the urinary excretion of kynurenine increased 5 µg. (55.5 per cent) above the value obtained for rats fed the basal diet. These findings might suggest that when leucine was not present in a mixture of the indispensable amino acids simulating zein some indispensable amino acids in zein interfered in the conversion of tryptophan to kynurenine. The addition of a mixture of all the dispensable amino acids simulating 1.71 per cent of zein to the basal diet increased the urinary excretion of kynurenine by 9 µg. (100 per cent) above the basal value. The removal of glutamic acid from the mixture of dispensable amino acids also caused an increase in the urinary excretion of kynurenine by 5 µg. (55 per cent) above the basal value, although the

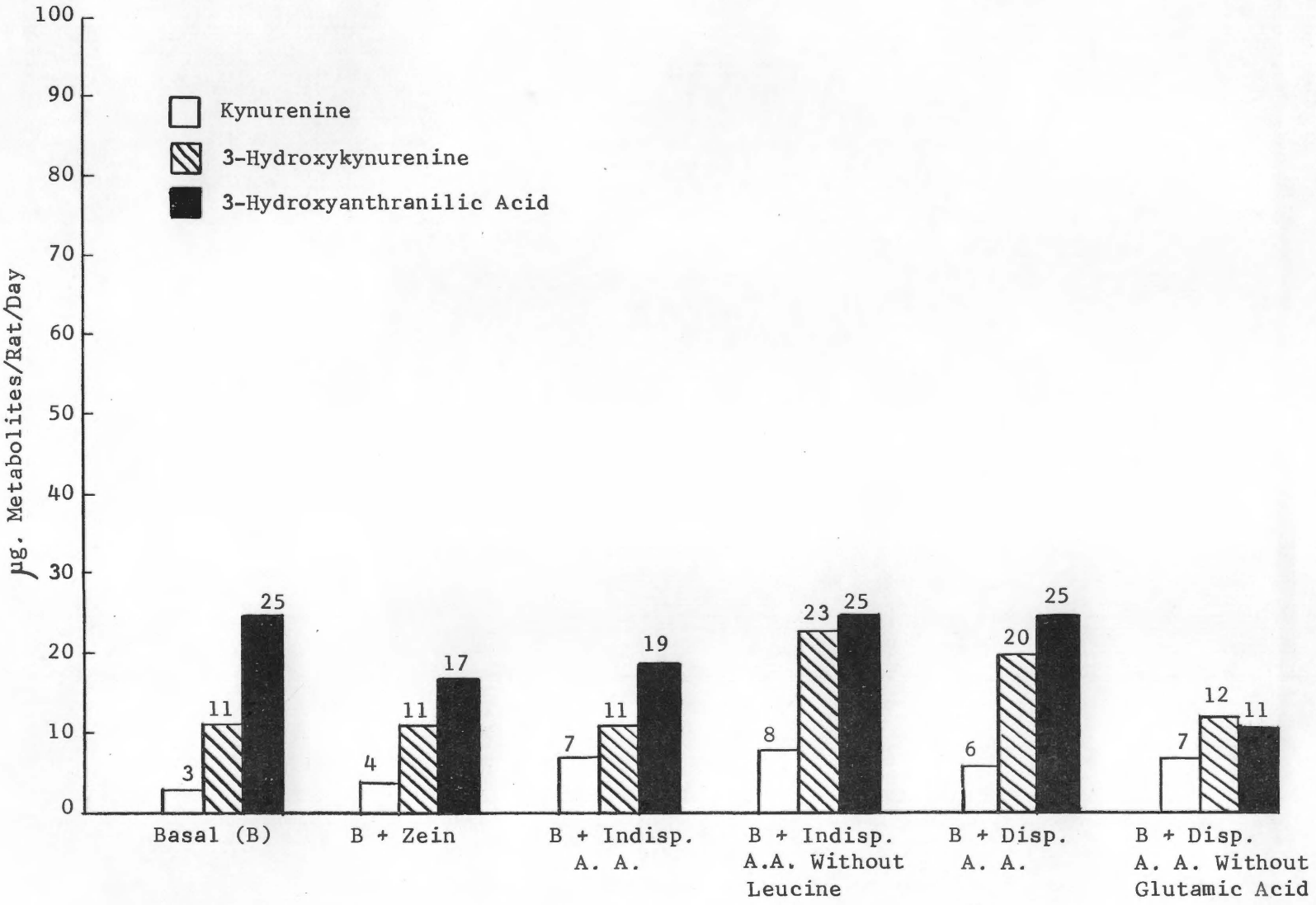

Figure 7. Urinary excretion of tryptophan metabolites in rats fed diets with limited tryptophan.

urinary kynurenine excretion was not markedly affected whether glutamic acid was present or not.

The urinary excretion of kynurenine in rats fed the various experimental diets without tryptophan supplement (Figure 7) showed a pattern similar to that observed for the urinary excretions of rats fed supplemented diets.

Excretion of 3-Hydroxykynurenine

The addition of zein or a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with tryptophan (Figure 6) decreased the urinary excretions of 3-hydroxykynurenine by 10 µg. (29.4 per cent) and 16 µg. (47 per cent), respectively. The decreased excretions of this metabolite suggest that some component of zein interfered with the conversion of tryptophan ⟶ kynurenine ⟶ 3-hydroxykynurenine, and that some indispensable amino acids in zein may act in the same manner. The addition of a mixture of all the indispensable amino acids simulating 1.71 per cent of zein without leucine to the basal diet resulted in a urinary excretion of 3-hydroxykynurenine comparable to the value obtained for rats fed the basal diet. The removal of leucine from the mixture of indispensable amino acids increased the 3-hydroxykynurenine excretion by 19 µg. above the value obtained for rats fed the complete mixture of indispensable amino acids. Since the urinary excretion of 3-hydroxykynurenine in rats fed a mixture of all the indispensable amino acids in zein decreased below the basal value and returned to a value comparable to the

basal value when leucine was removed from the mixture, it appears that leucine inhibited the conversion of tryptophan to 3-hydroxykynurenine. The addition of a mixture of the dispensable amino acids simulating 1.71 per cent of zein to the basal diet resulted in a marked increase of 65 µg. (191 per cent) in the urinary excretion of 3-hydroxykynurenine. The urinary excretion of this metabolite returned to a value comparable to the basal value when glutamic acid was removed from the amino acid mixture. This result suggests that glutamic acid inhibited the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

The urinary excretions of 3-hydroxykynurenine in rats fed the diets without tryptophan supplement (Figure 7) showed a pattern similar to that obtained for rats fed the tryptophan supplemented rations except for the rats fed a diet containing zein or a mixture of all the indispensable amino acids. Rats fed these diets did not show the decrease in urinary 3-hydroxykynurenine excretion which was observed in rats fed similar diets supplemented with tryptophan. The addition of a mixture of all the dispensable amino acids to diets containing a limited amount of tryptophan caused an increase in the urinary excretion of 3-hydroxykynurenine above the basal value. Removal of glutamic acid from the mixture decreased the urinary excretion of 3-hydroxykynurenine. The observations made on the urinary excretion of 3-hydroxykynurenine in rats fed diets with limited tryptophan support the finding that glutamic acid interfered with the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid in rats fed the diets with tryptophan supplement.

Excretion of 3-Hydroxyanthranilic Acid

The addition of zein to the basal diet supplemented with tryptophan (Figure 6) decreased the urinary excretion of 3-hydroxyanthranilic acid by 14 ug. (46.6 per cent) below the basal value. This may be interpreted to mean that a component of zein interfered somewhere in the metabolic pathway by which tryptophan was converted to 3-hydroxyanthranilic acid. When a mixture of all the indispensable amino acids simulating 1.71 per cent of zein was added to the basal diet, there was no change in the urinary excretion of 3-hydroxyanthranilic acid as compared to the basal value. This was true even when leucine was removed from the amino acid mixture. This suggests that none of the indispensable amino acids found in zein interfered in the conversion of tryptophan to 3-hydroxyanthranilic acid. When a mixture of the dispensable amino acids simulating 1.71 per cent of zein was added to the basal diet, there was an increase in the urinary excretion of 3-hydroxyanthranilic acid by 11 µg. (36.6 per cent). The removal of glutamic acid from the mixture of the dispensable amino acids resulted in an excretion of 3-hydroxyanthranilic acid equivalent to that obtained with the basal diet.

The urinary excretion of 3-hydroxyanthranilic acid in rats fed the experimental diets without tryptophan supplement (Figure 7) showed a similar pattern to those of rats fed supplemented diets except for the groups fed diets to which a mixture of all the indispensable amino acids and a mixture of all the dispensable amino acids were added. These groups excreted less 3-hydroxyanthranilic acid than the rats fed

the basal diets. The increase in the urinary excretion of 3-hydroxyanthranilic acid in the rats fed tryptophan supplemented diets to which was added a mixture of all the dispensable amino acids found in zein was not observed in the diets with limited tryptophan.

Effect of Zein on the Urinary Excretion of Tryptophan Metabolites

The addition of zein to the supplemented diets (Figure 6) caused a decrease in the excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid when compared to the basal values. In the diets containing a limited amount of tryptophan only the urinary excretion of 3-hydroxyanthranilic acid was decreased below the control value. Coulter (6) observed that the addition of zein to the basal diet regardless of the amount of tryptophan present markedly depressed the growth of rats. The growth depression caused by zein was attributed to the fact that zein is a poorly digested protein. She further made the observation that there was a decrease in the concentrations of liver NAD and NADH in rats fed diets to which zein was added regardless of the amount of tryptophan, however, these changes were not significant. The observations that zein caused a decrease in the urinary excretion of the tryptophan metabolites 3-hydroxykynurenine and 3-hydroxyanthranilic acid and that no metabolite accumulated in excess suggest that there was no block in the metabolism of tryptophan but that less tryptophan on the whole was being metabolized. One might suggest that less tryptophan was metabolized because zein was poorly digested.

Effect of a Mixture of all the Indispensable Amino Acids in Zein With or Without Leucine on the Urinary Excretion of Tryptophan Metabolites

The addition of a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent of tryptophan (Figure 6) depressed only the urinary excretion of 3-hydroxykynurenine. When leucine was removed from the mixture, the urinary excretion of 3-hydroxykynurenine increased to a value comparable to the basal value. The addition of a mixture of all the indispensable amino acids to the unsupplemented diet increased the urinary excretion of kynurenine above the basal value but did not affect the urinary excretions of kynurenine and 3-hydroxyanthranilic acid. The removal of leucine from the mixture of indispensable amino acids markedly increased the urinary excretion of 3-hydroxykynurenine.

These observations indicate that leucine, the indispensable amino acid found in zein in the largest amount, interfered with the conversion of tryptophan to 3-hydroxykynurenine. This would support the study of Coulter (6) who found that the addition of a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with tryptophan caused a significant increase in the liver concentrations of NAD and NADH of rats but that the removal of leucine from the mixture of indispensable amino acids further increased the liver concentrations of NAD and NADH. Her data indicated that leucine in some manner interfered with the ability of the rat to utilize tryptophan for NAD synthesis.

Effect of a Mixture of all the Dispensable Amino Acids in Zein With or Without Glutamic Acid on the Urinary Excretion of Tryptophan Metabolites

The addition of a mixture of all the dispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent L-tryptophan (Figure 6) increased the urinary excretions of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid above the basal values. In the unsupplemented diets (Figure 7) these increases were observed only in the excretions of kynurenine and 3-hydroxykynurenine. The removal of glutamic acid from the supplemented and unsupplemented amino acid mixtures resulted in decreases in the urinary excretion of 3-hydroxykynurenine when compared to values obtained for rats fed a complete mixture of the dispensable amino acids in zein. The urinary excretion of 3-hydroxykynurenine in rats fed a complete mixture of the dispensable amino acids except glutamic acid with or without a tryptophan supplement approximated the basal values. This suggests that glutamic acid, the dispensable amino acid found in the greatest quantity in zein, inhibited the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

With the marked increase in the urinary excretion of 3-hydroxykynurenine in rats fed a complete mixture of the dispensable amino acids simulating zein with or without tryptophan supplement, one would expect the urinary excretion of 3-hydroxyanthranilic acid to be below the basal value. However, it was found that the urinary excretion of 3-hydroxyanthranilic acid was slightly above the basal value in rats

fed supplemented diets and at basal value in the rats fed diets with limited tryptophan. When glutamic acid was removed from the mixture of dispensable amino acids supplemented with tryptophan, the urinary excretion of 3-hydroxyanthranilic acid was at basal value. The removal of glutamic acid from the mixture of dispensable amino acids with limited tryptophan resulted in a decrease in the urinary excretion of 3-hydroxyanthranilic acid to a value even lower than the basal value. These observations might suggest that glutamic acid interfered further down in the metabolism of 3-hydroxyanthranilic acid causing the metabolite to accumulate.

The removal of glutamic acid from both the supplemented and unsupplemented diets containing a mixture of dispensable amino acids caused a decrease in the urinary excretion of 3-hydroxykynurenine to basal values but not the urinary excretion of kynurenine which suggests that some dispensable amino acids other than glutamic acid interfered in the conversion of tryptophan to kynurenine. Ellis (7) observed a decrease in liver concentration of NADH and a slight increase in NAD concentration in rats when tyrosine was added to low-protein niacin-free diets containing cystine, glycine, and aspartic acid supplemented with tryptophan.

Coulter (6) observed a decrease in the liver NAD and NADH concentrations in rats fed diets to which a mixture of the dispensable amino acids in zein had been added, however, the changes were not significant. Ellis (7) in studying the effect of the stepwise addition of the dispensable amino acids of zein on the utilization of L-tryptophan for

pyridine nucleotide synthesis in rats fed low-protein niacin-free diets observed that there was no significant increase in the NAD concentration when glutamic acid was present in the diet. Her data indicated that glutamic acid inhibits the utilization of tryptophan for NAD synthesis.

In the conversion of tryptophan to niacin ribonucleotide, 3-hydroxykynurenine is converted to 3-hydroxyanthranilic acid. This reaction requires the vitamin B_6 coenzyme, pyridoxal phosphate (8). Glutamic acid also requires pyridoxal phosphate for its initial transamination to α-ketoglutaric acid, an intermediate in the Krebs cycle. The high levels of glutamic acid under the conditions of the present study may have interfered with the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid by competing for the available vitamin B_6.

The data obtained from this study indicates that leucine, glutamic acid, and probably some other dispensable amino acids found in zein interfered in the metabolism of tryptophan to NAD. Figure 8 shows the steps in metabolism of tryptophan where leucine and glutamic acid were found to interfere.

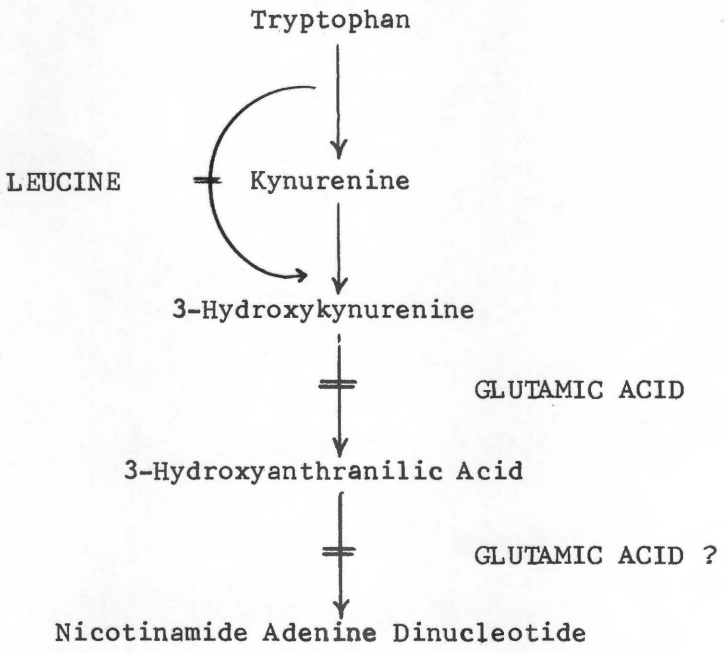

Figure 8. Steps in the metabolism of tryptophan where leucine and glutamic acid were found to interfere.

CHAPTER V

SUMMARY

The effect of adding zein, a mixture of all the indispensable amino acids simulating 1.71 per cent of zein with or without leucine, or a mixture of all the dispensable amino acids simulating 1.71 per cent of zein with or without glutamic acid to a 6 per cent casein niacin-free diet on the urinary excretions of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in the rat was studied. Sixty male weanling rats of the Wistar strain, in groups of five, were fed the various experimental diets with or without a 0.1 per cent L-tryptophan supplement ad libitum for a period of two weeks. Forty-eight hour urine specimens were collected from each group of rats at the end of the experimental period and were pooled. The tryptophan metabolites were separated by ion-exchange chromatography and the concentration of each metabolite was determined by spectrophotometric method.

The various experimental diets supplemented with 0.1 per cent tryptophan did not affect the growth of rats. The addition of zein or a mixture of all the indispensable amino acids simulating zein to diets with limited tryptophan retarded the growth of rats which was improved significantly when a tryptophan supplement was added.

The addition of zein to the supplemented diets caused a decrease in the amount of 3-hydroxykynurenine and 3-hydroxyanthranilic acid

when compared with the basal values. In the diets containing a limited amount of tryptophan only the excretion of 3-hydroxyanthranilic acid was decreased. The observations that zein caused a decrease in the urinary excretion of the tryptophan metabolites and that no metabolite accumulated in excess suggest that there was no block in the metabolism of tryptophan but that less tryptophan on the whole was being metabolized.

The addition of a mixture of all the indispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent tryptophan depressed only the urinary excretion of 3-hydroxykynurenine. When leucine was removed from the amino acid mixture, the urinary excretion of 3-hydroxykynurenine increased to a value comparable to the basal value. The addition of a mixture of all the indispensable amino acids to the unsupplemented diet increased the urinary excretion of kynurenine above the basal value but did not affect the urinary excretion of 3-hydroxykynurenine and 3-hydroxyanthranilic acid. The removal of leucine from the mixture of indispensable amino acids markedly increased the urinary excretion of 3-hydroxykynurenine. These observations indicate that leucine, the indispensable amino acid found in zein in the largest amount, interfered with the conversion of tryptophan to 3-hydroxykynurenine.

The addition of a mixture of all the dispensable amino acids simulating 1.71 per cent of zein to the basal diet supplemented with 0.1 per cent of tryptophan increased the urinary excretions of kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid. In

the unsupplemented diets these increases were observed only in the excretions of kynurenine and 3-hydroxykynurenine. Removal of glutamic acid from the supplemented and unsupplemented mixtures resulted in decreases in the urinary excretion of 3-hydroxykynurenine when compared to the values excreted by rats fed the complete mixture of dispensable amino acids. The amounts of the metabolite excreted by the rats fed the amino acid mixture without glutamic acid supplemented and unsupplemented with tryptophan approximated those of the basal value. This suggests that glutamic acid, the dispensable amino acid found in zein in the greatest quantity, inhibited the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid.

With the marked increase in the urinary excretion of 3-hydroxykynurenine in rats fed a complete mixture of the dispensable amino acids simulating zein with or without tryptophan supplement, one would expect the urinary excretion of 3-hydroxyanthranilic acid to be below the basal value. However, it was found that the urinary excretion of 3-hydroxyanthranilic acid was slightly above the basal value in rats fed supplemented diets and at basal value in the rats fed diets with limited tryptophan. When glutamic acid was removed from the mixture of dispensable amino acids supplemented with tryptophan, the urinary excretion of 3-hydroxyanthranilic acid was at basal value. The removal of glutamic acid from the mixture of dispensable amino acids with limited tryptophan resulted in a decrease in the urinary excretion of 3-hydroxyanthranilic acid to a value even lower than the basal value.

These observations might suggest that glutamic acid interfered further down in the metabolism of 3-hydroxyanthranilic acid causing the metabolite to accumulate.

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