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SOME NUTRITIONAL AND BIOCHEMICAL ASPECTS OF TYROSINE TOXICITY AND LYSINE AVAILABILITY

by

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SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
May, 1967

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Submitted to the Department of Nutrition and Food Science on April 24, 1967 in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

Abstract

Some Nutritional and Biochemical Aspects of Tyrosine Toxicity and Lysine Availability

by

Amal M. Boctor

This thesis consists of 2 parts. The first deals with tyrosine toxicity in rats while the second deals with the biological availability of lysine in proteins.

Tyrosine toxicity in rats is caused by feeding a low-protein high-tyrosine diet. It is manifested by external lesions as well as growth retardation, the extent of which depends on the tyrosine level in the diet (3 or 5%).

Addition of threonine, glycine, methionine, tryptophan and a mixture of leucine, isoleucine and valine alleviated the toxicity symptoms to varying degrees with threonine alleviating both the growth depression and the external lesions. Cortisol semisuccinate injection was without effect while cortisol acetate alleviated the external lesions and improved the weight of the animals. On the contrary, thyroxine injection greatly aggravated the condition of the rats fed the high tyrosine diet, however, addition of 0.08% thiouracil improved the condition of the animals to some extent. The beneficial treatments always resulted in lowered plasma tyrosine concentration.

Diurnal changes in the activities of tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase as well as plasma tyrosine concentration were observed in rats fed high tyrosine diet. In the control rats the enzyme activities showed also a diurnal rhythm.

High tyrosine feeding, with or without amino acid supplement, caused a marked increase in liver tyrosine transaminase activity the extent of which varied according to the treatment, however, p-hydroxyphenylpyruvate hydroxylase activity seemed not to be affected by high tyrosine alone. Thyroxine injection increased tyrosine transaminase activity in rats fed the high tyrosine diet, however, thiouracil treatment did not cause significant effect.

Daily cortisol acetate injection of rats fed the control diet increased tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase activities. This effect decreased progressively to the basal level by the end of a 15-day experimental period.

The treatment of animals fed the basal diet with or without 5% L-tyrosine tended to keep the ratio of the transaminase to the hydroxylase activities close to 1. Tyrosine feeding increased it to more than 1 and thyroxine administration increased it still further.

Animals fed the high tyrosine diet and injected daily with cortisol acetate for 5 or 10 days showed a higher capacity to oxidize a load of labelled tyrosine. On the other hand, rats fed the high tyrosine diet alone showed a gradual increase with time in their capacity to oxidize this load.

Feeding a 5% p-hydroxyphenylpyruvic acid diet proved not to be toxic to the rat in the same way as tyrosine as no pathological lesions were observed. Increasing tyrosine intake of the rat by force-feeding a high tyrosine diet supplied evidence that the condition is not an imbalance one but a true toxic condition.

The results obtained indicate that development of tyrosine toxicity is associated with high blood and tissue tyrosine concentrations and alleviation of the toxicity is accompanied by reduction of tyrosine concentration.

Availability of lysine in egg albumin, casein, beef muscle and wheat gluten was estimated by the dinitro fluorobenzene (FDNB), rat growth, carcass analysis and fecal analysis methods. A 25% wheat gluten diet proved to be a suitable basal diet for the growth assay. There was a good correlation between lysine consumed and weight gain, carcass nitrogen and protein efficiency ratio over a two-week feeding period. Effect of heat treatment in the presence of added glucose on the availability of lysine in egg albumin was studied. There was a decrease in available lysine with increasing increments of glucose as determined by the FDNB method. The FDNB-method gave high values for available lysine in heat-treated egg albumin and beef muscle proteins compared to values obtained biologically. The FDNB-method appears to be of limited value for measurement of availability of lysine in foods, heated in the presence of carbohydrates.

Thesis Supervisor: Alfred E. Harper
Professor of Biochemistry

ACKNOWLEDGEMENT

The author wishes to extend his deepest gratitude and appreciation to his supervisor Prof. A. E. Harper for his encouragement, guidance and critical suggestions throughout this work.

My thanks are due to Prof. Q. R. Rogers for his guidance and interest. My thanks are also due to Prof. N. J. Benevenga for his helpful suggestions.

The author wishes to thank his colleagues in M.I.T. and in the University of Wisconsin for their stimulating arguments.

PART I

SOME NUTRITIONAL AND BIOCHEMICAL ASPECTS OF TYROSINE TOXICITY

Introduction

Adverse effects resulting from the ingestion of disproportionate amounts of individual amino acids have been classified as imbalances, antagonisms or toxicities. These terms have been defined and discussed in some detail (Harper, 1956, 1958, 1964). The terms antagonism and toxicity have been applied particularly to adverse effects of excessive intakes of individual amino acids. Despite the fact that much work has been done on the gross effects of consumption of excessive amounts of amino acids and how to alleviate them the biochemical basis for these effects is not well understood.

The effects of disproportionate amounts of individual amino acids in diets have been studied for several years in this laboratory. Interest in this subject arose initially from observations that a high leucine intake depressed the growth rate of rats, apparently as the result of leucine, isoleucine and valine antagonism (Harper, Benton and Elvehjem, 1955). Subsequently effects of several other amino acids have been studied (Becker, 1961; Tannous, 1963). More recently the effects of excessive tyrosine intake have been examined. Alam (1966, a and b) showed that supplementing a high tyrosine diet with 1.25% of L-threonine could completely alleviate the toxicity caused by 3% of L-tyrosine in a low protein diet. This treatment resulted in a substantial decrease in plasma tyrosine concentration. This thesis represents a continuation of the studies of

tyrosine toxicity in an attempt to obtain more information about the nutritional and metabolic aspects of the toxicity and its alleviation.

Literature Review

Tyrosine occupies a prominent position in body metabolism as it is involved in the synthesis of some important hormones i.e. thyroxine and epinephrine as well as in protein synthesis.

Womack and Rose in 1934 studied the importance of tyrosine in the diet. They found that phenylalanine could be converted *in vivo* into tyrosine and that tyrosine was not an essential amino acid for the growth of the rat when sufficient phenylalanine was available. Definite proof of this was provided by Moss and Schoenheimer in 1940 who demonstrated that when deuterium labelled phenylalanine was given to rats deuterium appeared in both phenylalanine and tyrosine in body proteins. It was suggested then that the inclusion of tyrosine in the diet should lower phenylalanine requirement. Later Armstrong (1955) showed that in the complete absence of L-tyrosine, 1.2 g. of L-phenylalanine per 100 g. diet was the optimal amount to support growth and that 0.6 g. of L-phenylalanine was sufficient if 0.3-0.4 g. of L-tyrosine was added. This shows that tyrosine can become critical if phenylalanine is limiting in the diet.

Addition of a large amount of tyrosine to a low protein diet results in a syndrome which is known as tyrosine toxicity. It is manifested in the rat by external lesions which are accompanied by growth depression and in some cases death of the animal.

As early as 1932, Sullivan, Hess and Sebrell and Lillie (1932) studied the effects of feeding excessive amounts of

several different amino acids. They found that diets containing 5, 10 or 20% of tyrosine were toxic and even fatal when fed to rats. When 5% of cystine was added to a 5% tyrosine diet it resulted in a considerable decrease in the toxic action of tyrosine.

Martin and Heuber (1942) and Martin (1942) added 10% of tyrosine to an 18% casein diet and found it to have similar effects. They noticed that the first indication of tyrosine toxicity was a blanching of the paws which then became edematous and reddened. The animals assumed a hunched posture and walked painfully. The eyes became involved and a black exudate accumulated around them. They also found that during the blanching stage the blood pressure was high and then diminished thereafter. Decreasing the tyrosine level to 5% showed the same picture but rats survived longer and the development of the syndrome was delayed. Martin (1942) also found that the addition of 2% of ascorbic acid or 5% of yeast to a 10% tyrosine diet seemed to have no effect on the severity of the symptoms. Nurin et al. (1946) observed that the addition of 1% of phenylalanine and 1% of tyrosine to a basal ration containing 10% of casein produced a substantial growth retardation which was accompanied in about 50% of the cases by vascularization of the cornea and marked edematous swelling. Addition of 2% of phenylalanine and 2% of tyrosine completely inhibited growth and resulted in more severe external lesions. Interestingly enough, spontaneous healing was noted in rats fed all levels. On the other hand, the use of a 25% casein diet instead of the 10% one prevented the deleterious effects.

The growth depressing effect of an excessive intake of tyrosine was not only observed in rats but also in chicks. Feeding diets containing 4% of L-tyrosine was found to depress the growth rate of chicks. The growth depression was not influenced by addition of niacin (Anderson et al., 1951) or vitamin B_{12} (Hsu and Combs, 1952).

Schweizer in 1947 studied the effect on rats of feeding 1, 1.5, or 2% of tyrosine in a diet containing 12% of protein. He noticed that the degree of severity of the external symptoms was increased by increasing the tyrosine level in the diet. Similarly, the onset of symptoms was correspondingly earlier. When the dietary protein level was raised to 18%, he found that tyrosine had less effect on the rate of growth and duration of life. (An interesting observation was then made by Schweizer that older rats, both males and females, were less severely affected than young ones receiving equivalent quantities of tyrosine.) When the dietary protein level was increased to 20% and the level of tyrosine was 5%, Graham et al. (1950) observed no inhibition in the growth of the young rats. Sauberlich (1961) in his extensive studies on amino acid toxicities found that the addition of 5% of L-tyrosine to a 6% casein diet caused a severe growth depression as well as a decrease in the food intake of weanling rats. When he increased the dietary protein level up to 40% the depression in growth induced by feeding excess tyrosine was completely counteracted. Harper et al. (1966) studied the effect of adding 3% of L-tyrosine to 6% and 12% fibrin diets. The growth of rats fed

the first diet greatly depressed while with the latter diet there was only a slight effect. It appears then that increasing the dietary protein level can alleviate the growth retardation due to excessive tyrosine intakes.

Alam et al. (1966, a and b) found that as little as 1.25% threonine could completely alleviate the syndrome that developed due to ingestion of a 6% casein diet containing 3% of L-tyrosine. Feeding such a diet resulted in a several fold increase in tyrosine concentration in plasma, liver and muscle of the rat. Addition of threonine to this diet caused a substantial decrease in tyrosine concentration in the tissues studied.

It is clear then that when tyrosine toxicity is alleviated tissue tyrosine concentration is closer to the normal level. The beneficial effects of treatments that alleviate the toxicity may be mediated either through increased excretion of tyrosine or its immediate metabolites in urine or increased rate of catabolism or both.

The administration of tyrosine to scorbutic guinea pigs, scorbutic man and to premature infants has been found to result in a condition called tyrosyluria. It results in the excretion of p-hydroxyphenylpyruvic acid and p-hydroxyphenyllactic acid in the urine. The administration of ascorbic acid was found to abolish the appearance of such metabolites in the urine. Painter and Zilva (1947) found that the amount of p-hydroxyphenylpyruvic acid excreted in the urine was dependent upon the relative intakes of tyrosine and of ascorbic acid.

Knox and associates (1963) studied the effect of feeding excess tyrosine to rats which are able to synthesize ascorbic acid. When 5% of L-tyrosine was added to an 18% protein diet excessive excretion of p-hydroxyphenylpyruvic acid in urine was observed. This is unlikely to be the result of ascorbic acid deficiency as the administration (Martin, 1942) of 2% of ascorbic acid did not alleviate the toxicity symptoms. Later Knox and associates (1964) showed that groups of rats fed 5% of tyrosine for one to two weeks excreted large amounts of p-hydroxyphenylpyruvic acid provided that these rats were under 42 days of age. Addition of ascorbic acid to the diet did not cause any remarkable effect. Age was a critical factor since no excretion of p-hydroxyphenylpyruvic acid occurred if the tyrosine feeding was delayed even for one week after weaning.

Tyrosine metabolism and inborn errors of tyrosine metabolism:

Figure I represents the main pathway of tyrosine degradation in mammals. An enzymatic defect at any step may result in mental alteration or physiological malfunction and other specific clinical symptoms depending on the step that is defective. These were classified as inborn errors of metabolism.

Alkaptonuria has been known to exist since the sixteenth century, when Scribonius described a school student who excreted black urine. Garrod was the first to suggest that the syndrome was the result of a chemical defect. In an interesting article (1908) Garrod discussed these conditions of malfunction in metabolism. He said, "They apparently result from a failure

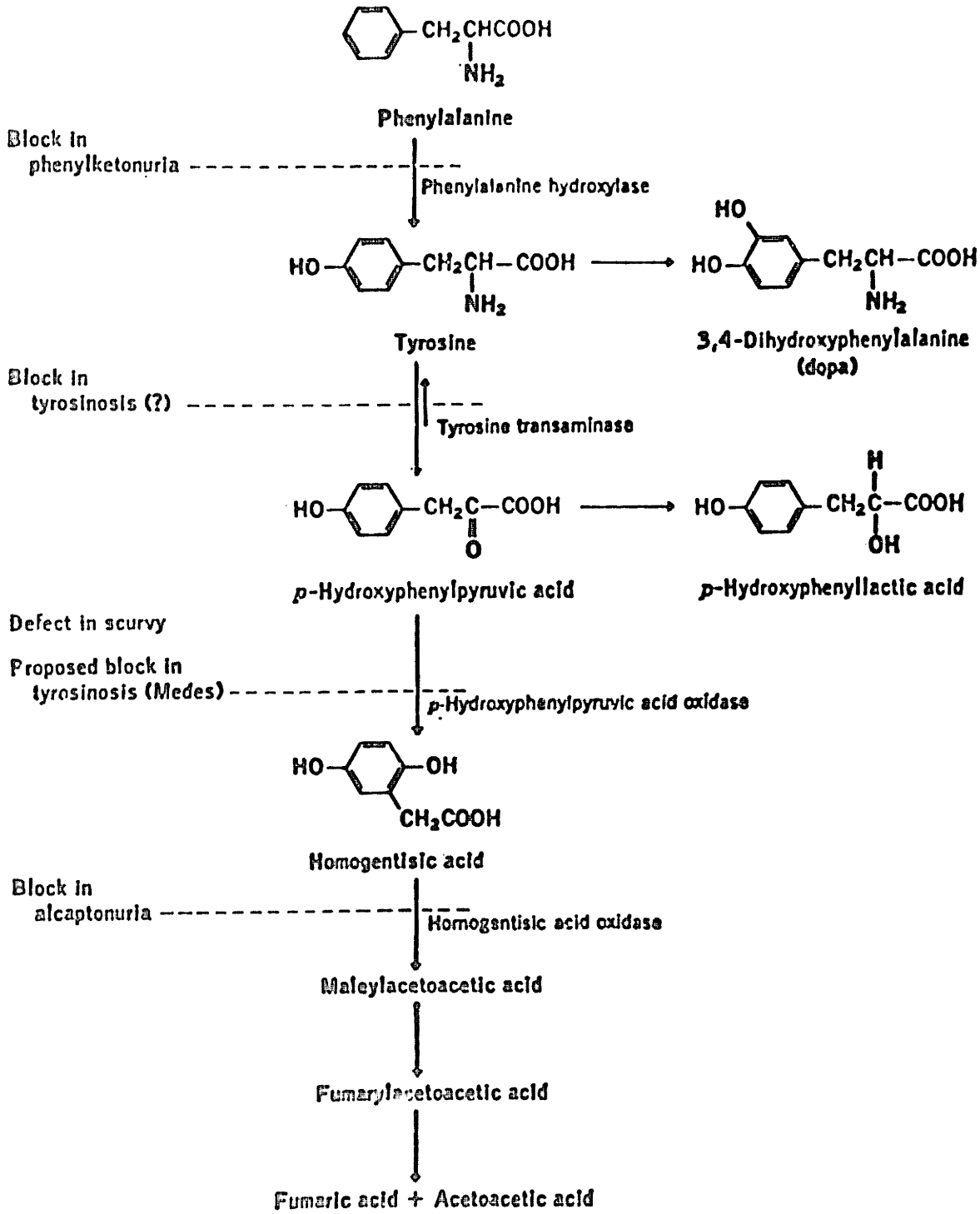


Fig. I: Metabolic blocks in oxidation of phenylalanine and tyrosine (from LaDu, 1960)

of some step or other in the series of chemical changes which constitute metabolism and are in this respect most nearly analogous to what is known as malfunction by defect." Garrod viewed the accumulation of homogentisic acid in alkaptonuria as evidence that this substance was a normal intermediate in the metabolism of tyrosine. He correctly attributed its accumulation to a failure of homogentisic acid oxidation. Almost half a century after Garrod made his original observation LaDu et al. in 1958 demonstrated the absence of homogentisic acid oxidase from an alkaptonuric patient.

Medes studied the first and only reported case of tyrosinosis in humans, in 1932. She studied the pattern of urinary metabolites after placing her patient on various dietary regimens. She found that p-hydroxyphenylpyruvic acid was excreted at all times even during fasting or on a low protein low-tyrosine diet. When tyrosine was introduced into the diet at doses of approximately three grams daily, tyrosine appeared in the urine that was excreted by the patient. Medes mentioned in this respect that Abderhalden in 1912 ingested 150 g. tyrosine, which is a tremendous amount, without recovering any in the urine. When the patient was given a dose of 8 g. of homogentisic acid, that acid was not recovered in the urine. Such experiments furnished convincing evidence that a block occurred after the formation of p-hydroxyphenylpyruvic acid and that although the patient was unable to oxidize tyrosine, he could oxidize homogentisic acid.

Excretion of aromatic acid derivatives also occurred in some other patients. Fölling in 1934 discovered that in certain cases of mental disturbance phenylpyruvic acid was excreted in urine. Such cases were sometimes named after Fölling or were called phenylpyruvic acid oligophrenia but later the name phenylketonuria was coined by Penrose and Quastel (1937). The latter studied the effects of feeding phenylalanine and tyrosine to phenylketonuric patients. They found that tyrosine was probably normally metabolized and that phenylpyruvic acid and phenylalanine were not. They claimed that the error in metabolism was to be found in a block at the stage of phenylpyruvic acid as the patients were unable to break down the benzene ring.

In 1940, Jervis et al. studied the effect of feeding 5 g. of phenylalanine, phenylpyruvic acid or phenyllactic acid in a high protein diet in 16 phenylketonuric patients, who had fasted for 16 hours. Phenylpyruvic acid and phenyllactic acid while increasing the phenylpyruvic acid output in the urine, failed to induce a rise of the ketonic acids in the blood but caused instead an appreciable increase of blood phenylalanine. This led the authors to suggest that the patients were able to aminate the hydroxy acid and the keto acid and that the phenylalanine thus formed was eventually deaminated in the kidney to phenylpyruvic acid. Later Jervis (1947) demonstrated that in the fasting normal rabbit and human subject, the ingestion of phenylalanine was followed by an increase in plasma tyrosine concentration while phenylketonuric patients showed no increase.

The presence of phenylalanine and absence of phenylpyruvic acid in the blood of patients together with the ability of these patients to aminate phenylpyruvic acid cannot agree with the claim offered earlier by Penrose and Quastel. Jervis concluded, then, that these patients were unable to bring about the hydroxylation of phenylalanine and that the biochemical error consisted of a block in the conversion of phenylalanine to tyrosine.

Udenfriend and Cooper (1952) were the first to demonstrate that the conversion of phenylalanine to tyrosine was catalyzed by an enzyme system, which was found in mammalian liver. This provided enzymatic evidence why tyrosine in contrast to phenylalanine is not an essential amino acid (Womack and Rose, 1934) as it can be synthesized from phenylalanine provided that the latter is in excess. Jervis (1953) then made use of the enzyme assay and demonstrated the lack of this enzyme in phenylketonuric patients.

As a matter of fact the great interest in metabolic diseases of tyrosine metabolism resulted in the establishment of the scheme for the main pathway for phenylalanine and tyrosine catabolism. Later the pathway of tyrosine oxidation in human liver was found to be similar to that of other animals (Lerner, 1949; Knox and Knox, 1951).

Some aspects of enzymatic degradation of tyrosine:

Tyrosine is normally degraded to fumarate and acetoacetate and for this reason it is considered to be ketogenic as well as

glucogenic. My discussion will be limited to the first few steps of enzymatic catabolism of tyrosine.

Tyrosine-α-ketoglutarate transaminase (L-tyrosine:2 oxoglutarate amino transferase, EC 2.6.1.5) is the enzyme which catalyzes the first reaction in the sequence disposing of excess tyrosine with the formation of p-hydroxyphenylpyruvic acid. Lin and Knox (1958) studied the distribution of tyrosine transaminase activity in the rat. They found that the activity was mainly confined to the liver as compared to the kidney and heart. Such distribution pattern was also recognized in the dog (Canellakis and Cohen, 1956). Then later it was found that the activity was mainly confined to the cytoplasm with some in the microsomal fraction (Kenney, 1962; Litwack et al., 1963). In this connection an interesting observation was made by Bellamy in 1966 that when liver slices were incubated with tyrosine and α-ketoglutarate the tissue transaminase had fallen by about 60% after 3 hours of incubation and the enzyme was then found in the incubation mixture, i.e. the enzyme diffused into the incubation medium.

This enzyme has been of great interest since its activity can be induced by hormones as well as substrate. Many enzymes appear to be induced in response to changes in the normal environment of a system.

It has been found that injection of tyrosine (Kenney and Flora, 1961, Lin and Knox, 195) increased the enzyme level in the intact animal several fold within a few hours after injection. Also, injection of similar amounts of L-phenylalanine

and glutamic acid produced a definite increase about one-third as great as tyrosine did in intact rats. Interestingly enough, adrenalectomy did not alter the level of the enzyme when tyrosine was injected into these animals. On the other hand hydrocortisone injection has been shown to be a sufficient stimulus to elevate the enzyme level in both intact and adrenalectomized rats (Rosen and Milholland, 1963). Cortisol injection has been also found effective in inducing hepatic activity of tyrosine transaminase in guinea pigs, chicks, horned toads and painted turtles (Chow and Cohen, 1964).

Attempts to demonstrate a direct effect of cortisol on the level of tyrosine transaminase activity in liver slices incubated in tissue culture medium have been unsuccessful (Goldstein, Stella and Knox, 1962). This led Goldstein and others (1962) to study the effect of cortisol in isolated perfused rat liver. They found that cortisol in the perfusion medium caused a threefold increase in enzyme activity. A more striking increase in activity was obtained when both tyrosine and cortisol were added in the perfusion medium (Bernabei and Sereni, 1964).

Kenney and Flora (1961) tried to elucidate the mechanism of such enzyme induction. They showed that induction of tyrosine transaminase was not a specific result of substrate per se. Of the materials found effective as inducers in intact rats only hydrocortisone was active in absence of functional adrenals. Thus adrenal steroid was required and induction by tyrosine seemed to be secondary to stimulation of adrenocortical hormone secretion. They also showed that the effect of a small

dose of hydrocortisone was potentiated by celite and tryptophan as evidenced by increased hepatic concentration of the steroid. However, Knox et al. in 1964 and Knox (1964) showed that tyrosine feeding elevated tyrosine transaminase activity equally in young adrenalectomized as well as intact rats, but the response was slower in adrenalectomized rats. It is of interest that feeding tyrosine to older rats did not raise the transaminase level in their livers. Therefore they concluded that the elevation of the transaminase activity was a substrate-type of induction not dependent upon the adrenal gland and occurred after tyrosine was fed for several days to rats under two months of age.

Experiments to determine whether enzyme induction represents an acceleration of rate of enzyme synthesis or a decrease in the rate of turnover of the enzyme have been devised. Lin and Knox (1958) measured the rate of loss of tyrosine transaminase activity after the activity had stopped increasing. Kenney(1962) used an immunochemical assay to measure the amount of radioactivity incorporated into liver enzyme protein at different time intervals after injecting cortisol and the labelled amino acid. Both laboratories came to the same conclusion that the half life of this enzyme was between 3 and 4 hours. However, Kenney has reported recently (1967) that the half life is 1.4 hours when using improved immunological technique. The rate of enzyme synthesis increased during cortisol induction.

Rivlin in 1963 showed that thyroxine injection tends to modify the induction of tyrosine transaminase due to cortisol

injection, in that higher doses of cortisol were required in order to induce tyrosine transaminase in rats made hyperthyroid by daily injection of thyroxine.

The effect of the coenzyme administration was studied by Greengard and Gordon (1963). They found a three fold increase in tyrosine transaminase activity 4 hours after injecting two doses each of 100 mg. of pyridoxine. Puromycin administration did not affect the normal enzyme level but had an inhibitory effect on the pyridoxine induced rise. On the other hand actinomycin administration was without effect on the pyridoxine induced rise. However the hormonal induction of this enzyme was inhibited by both agents (Greengard, Smith and Acs, 1963). This finding suggested that the administration of pyridoxine stimulated the rate of apoenzyme synthesis. Also that the hormonal induction may depend on an increase in the amount of certain RNA species whereas cofactor induction might result in a faster rate of synthesis of enzyme by the already present synthetic machinery.

The enzyme activity has also been found to be influenced by dietary protein level (Litwack et al., 1959; Al-Neggar and Litwack, 1961; Potter et al., 1966). Also the enzyme showed a diurnal rhythm with a maximum around midnight (Potter et al., 1966). Sereni and others (1959) studied the development of tyrosine transaminase in rat liver. They found that the enzyme activity was very low during fetal life. By 12 hours after birth, the activity increased strikingly up to double that of the adult, then it gradually decreased. On the other hand

adrenalectomy at birth caused a marked inhibition of this development which could be reversed by cortisol injection.

Besides the fact that tyrosine transaminase can be induced by substrate and cortisol, it was found (Litwack, 1962) that DL-methionine, nucleic acids, heparin as well as other mixtures of agents increased the enzymic activity. However as these compounds were injected in pharmaceutical doses the response may have been the result of a stress effect which stimulated glucocorticoid hormone production. Similarly Rosen and Milholland (1963) found that tyrosine transaminase can be induced by tryptophan and some of its derivatives in the intact rat as well as the adrenalectomized rat but cortisol alone induced a much greater response. The induction by tryptophan was also found to be inhibited by ethionine and puromycin which indicated that synthesis of the enzyme was blocked.

Non-specific stress such as starvation for 48 hours has been found to induce tyrosine transaminase not only in the intact rat but also in adrenalectomized ones. However, the reason for this kind of adaptation is not clear (Goswami and Chatagner, 1966). The need for energy in starvation can be met by tissue protein catabolism. Thus the increase in catabolic enzymes may represent an adaptive response contributing toward maintenance of homeostasis. This may be true because when glucose was administered to the rat at the onset of starvation, the increase in enzyme activity was considerably less. On the other hand the stress of 30 minutes on a reciprocating shaker provoked an increase in adrenal cortical hormone secretion in

the adult rat (Geller et.al.,1964). However, tyrosine transami- activity did not change. Further (Schapiro et.al, 1964) it was noticed that the enhancement of the transaminase activity following the administration of cortisol was markedly suppressed by simultaneous exposure to this stress. Later (Schapiro et.al., 1966) it was found that the infant rat showed increased enzyme activity due to stress. It is of interest to mention that the stressed adrenalectomized adult rats showed markedly decreased enzyme activity while adrenalectomized infant rats showed no change in enzyme activity when stressed. It was then suggested that stress activated a mechanism mediated by the pituitary which opposed the elevation in enzyme activity brought about by adrenal cortical hormones.

It is clear then that tyrosine transaminase can be an influential step in the overall oxidation of tyrosine in the liver. However, its effectiveness may be influenced by the relative rates of the subsequent steps of tyrosine degradation. Knox et.al (1964) have studied the next enzyme, i.e. p-hydroxyphenylpyruvate hydroxylase (p-hydroxyphenylpyruvate ascorbate:oxygen oxireductase EC 1.14.2.2) activity in the liver of rats with tyrosyluria. They emphasized the importance of the ratio of tyrosine transaminase to the hydroxylase, as the steady state concentration of p-hydroxyphenylpyruvate will be controlled by the rates of its formation and removal by these two reactions. The hydroxylase, however, has not been studied as extensively as tyrosine transaminase. p-hydroxyphenylpyruvic acid was found to inhibit the activity of

hyroxylase in scorbutic guinea pigs and dogs (Zannoni and LaDu, 1959). This could be prevented by ascorbic acid or reduced 2,6 dichlorophenolindophenol. Zannoni and LaDu (1960) further found the activity of p-hydroxyphenylpyruvate hydroxylase in normal animals to be essentially the same whether or not p-hydroxyphenylpyruvic acid had been administered. Also the level of p-hydroxyphenylpyruvate hydroxylase in the untreated scorbutic animals was essentially the same as that in the normal. The ability of ascorbic acid to maintain normal tyrosine metabolism in vivo appeared to be mediated by its capacity to protect p-hydroxyphenylpyruvate hydroxylase from inhibition.

Lin and Knox (1958) found a parallel increase in tyrosine transaminase and homogentisic acid oxidase in response to cortisol injection. However p-hydroxyphenylpyruvate hydroxylase activity did not change under their conditions. It seems that any disturbance in the relative rates of the enzyme members of tyrosine degradation can limit the rate of oxidation of tyrosine and its metabolites.

SECTION I

Effect of different amino acids and cortisol on rats fed 3% L-tyrosine

(In collaboration with S. Q. Alam)

Introduction

As mentioned before there have been many attempts to alleviate tyrosine toxicity in rats, e.g. addition of 1.25% of L-threonine to a diet containing 3% of L-tyrosine alleviated tyrosine toxicity (Alam et al., 1966). Cystine (Sullivan et al., 1932) was found to partially prevent the growth depression and pathological lesions. However, the way in which tyrosine toxicity is alleviated is not known. Addition of threonine caused a substantial decrease in plasma tyrosine concentration so the question arises to whether other agents that exert some protective effect also lower the concentration of tyrosine in body fluids. Also, since tyrosine-α-ketoglutarate transaminase, the enzyme catalyzing the initial step in tyrosine degradation, is elevated in rats subjected to various dietary and hormonal treatments, the possibility exists of a relation between this enzyme activity and the occurrence of tyrosine toxicity in rats fed a high tyrosine diet. Hydrocortisone injection can result in more than a 4-fold increase in tyrosine-α-ketoglutarate transaminase activity within a short period of time (Lin and Knox, 1958; Goldstein et al., 1962) and other treatments such as celite injection, that initiates stress reaction, also cause elevation in the activity of this enzyme (Kenney and Flora, 1961). Munro and associates (1963, 1965) have reported that feeding certain amino acids stimulated corticosterone production. If the amino acids that alleviated tyrosine

toxicity acted similarly this might enhance tyrosine catabolism and thereby lowered the plasma tyrosine concentration.

As part of a study of the basis for the alleviation of tyrosine toxicity we have assessed the effects of injections of cortisol and celite (which acts as a non-specific stress) and several amino acid supplements on growth, adrenal weight and on the signs of toxicity of rats fed a high tyrosine diet. Plasma tyrosine concentration and liver tyrosine-α-ketoglutarate transaminase activity were estimated at different time intervals in rats receiving the various amino acid supplements.

Experimental

Animals: Young male albino rats from Holtzman Company or from Sprague-Dawley Inc. were individually housed in suspended cages and kept at a temperature of 23°C. Food and water were given ad libitum. These conditions were followed throughout the investigations reported unless it is otherwise indicated.

Diets: All of the diets were fed as agar-gels containing about 50% of moisture. The composition of the basal 6% casein diet is shown in tables I and II. To prepare the agar gel diets all the ingredients except the vitamin mixture and choline were mixed together in a mixer. The agar was dissolved in an amount of boiling distilled water equal to the solid ingredients. The agar solution was then added and mixing was continued until the temperature of the mixture was about 60°C. The vitamins and choline were then added and mixing was continued for a while. The diet was then poured into a polyethylene tray and left to gel at room temperature and then stored at 4°C throughout the experiments. This procedure was followed in subsequent experiments.

Injections and rating of symptoms: Cortisol (hydrocortisone acetate) was purchased from Mahedy Company, Cambridge, Massachusetts. Celite 545 was a product of Johns-Manville Company, California. Celite and cortisol were suspended in physiological saline (0.85% sodium chloride). Intraperitoneal

TABLE 1

Compcsition of the basal diet (Agar-gel)

Casein	6 %
L-methionine	0.2%
Mineral mixture (Harper's)	4.0%
Vitamin mixture*	0.5%
Choline (50% solution)	0.4%
Corn oil	5.0%
Agar	2.5%
Sucrose	40.7%
Dextrine	40.7%
	100.00%

*Vitamin mixture was prepared to provide per 100 g dry diet: Vitamin A, 400 USP units; Vitamin D_2, 40 USP units; thiamine-HCl, 0.5 mg; riboflavin, 0.5 mg; niacinamide, 2.5 mg; D-calcium pantothenate, 2.0 mg; pyridoxine-HCl, 0.5 mg; folic acid, 0.5 mg; Vitamin K (menadione), 0.05 mg; d-biotin, 0.02 mg; Vitamin B_{12}, 0.003 mg; ascorbic acid, 5.0 mg; and vitamin E acetate, 10 mg.

TABLE 2

Composition of the Salt Mixture

Compound	% Compound in salts	Elements	% Elements in salts mix
$CaCO_3$	29.29	Ca	11.83
$CaHPO_4 \cdot 2H_2O$	0.43	P	7.88
KH_2PO_4	34.31	Ca/P	1.50
NaCl	25.06	K	9.86
$MgSO_4 \cdot 7H_2O$	9.98	Na	9.86
$Fe(C_6H_5O_7) \cdot 6H_2O$	0.623	Cl	15.20
$CuSO_4$	0.156	Mg	0.98
$MnSO_4 \cdot 2H_2O$	0.121	Fe	0.098
$ZnCl_2$	0.020	Cu	0.039
KI	0.0005	Mn	0.039
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0025	Zn	0.008
$Na_2SeO_3 \cdot 5H_2O$	0.0015	I	0.00038
		Mo	0.00010
	99.995	Se	0.00005

Add 5 gm to 100 gm diet.

injections of 0.3 ml volume containing the amounts indicated in the tables were given daily between 2 and 5 p.m.

The procedure for rating the symptoms consisted of giving a score of zero to 10 depending on the degree of severity of the external symptoms.

Plasma tyrosine determination: Rats were anesthetized with ether and the blood was drawn by heart puncture. The red blood cells were removed by centrifugation at low speed (2000 rpm).

In the experiments on the effect of different amino acids on tyrosine toxicity, plasma tyrosine concentration was determined after 2, 10 and 21 days of feeding. The method used was that adopted by Udenfriend and Cooper (1952). It is as follows:

To 1 ml of the plasma are added 3.0 ml of distilled water and 1 ml of 30% trichloroacetic acid; the precipitate is removed by centrifugation. To 2 ml of the supernate are added 1 ml of nitrosonaphthol (0.1% 1 nitroso-2 naphthol in 95% alcohol) and 1 ml of nitric acid reagent (1 : 5 nitric acid containing 0.5 mg/ml of sodium nitrite) in a glass stoppered centrifuge tube. The stoppered tube is left in a water bath at 55°C for 30 minutes and then cooled. 10 ml of ethylene dichloride are added and the excess nitrosonaphthol is extracted by shaking the tube which is then centrifuged. The color density of the supernatant aqueous solution is then read at 450mu in a spectrophotometer. Standards as well as blanks are treated similarly.

In the experiments on the effects of cortisol and celite on tyrosine toxicity, plasma tyrosine concentration was determined together with the other amino acids using a Technicon automatic amino acid analyser. The procedure was as follows:

Pooled plasma was analyzed by mixing equal volumes from each plasma sample. It was then mixed with 5 volumes of 1% picric acid. The precipitated proteins were then removed by centrifugation. The protein-free picric acid supernate was then added to a Dowex 2-X 8 column prepared according to the method of Stein and Moore (1954). The solution was then passed through the column and the walls of the tube were washed down with 5, 5 ml portions of 0.02 N hydrochloric acid. The combined effluent was concentrated to dryness using a rotary vacuum evaporator. The residue was then taken into a known volume of distilled water. An aliquot was then applied to the column of the amino acid analyzer. Values were calculated as micromoles of amino acid per 100 ml plasma.

Assay procedure of liver tyrosine-α-ketoglutarate transaminase activity:

Livers were removed and kept frozen at -20°C. On the day of assay liver samples were homogenized in 0.14 M potassium chloride solution containing 0.005 N sodium hydroxide.

A colorimetric procedure was adopted which was described by Canellakis and Cohen (1956) and modified by Rosen and others (1963). A buffer-substrate reagent was prepared by mixing 12 ml of 0.1 M α-ketoglutaric acid (adjusted to pH 7.4), 10 ml of

0.2 M sodium phosphate buffer (pH 7.4), 4.0 ml of 2.8 M pyridoxal phosphate, 0.4 ml of 0.5 M diethyldithiocarbamate and 50 ml of 0.25 M sucrose. 0.3 ml of 10% tissue homogenate was added to 1.9 ml of the buffer-substrate reagent. Individual blanks were prepared for each sample by adding 0.3 ml of 100% trichloroacetic acid (W/V) solution to the buffer-substrate reagent plus tissue homogenate before incubation. The standard was prepared by adding 150 µg (0.3 ml) of a freshly prepared aqueous solution of p-hydroxyphenylpyruvic acid to 1.9 ml of the buffer-substrate reagent. All samples, blanks and standards, in duplicate, were pre-incubated in a metabolic shaker for 3 minutes at 38°C. 0.6 ml of 0.01 M L-tyrosine was then added to each and the incubation was continued for 10 minutes after which the reaction was stopped by adding 0.3 ml of the trichloroacetic acid solution. The solutions were then centrifuged. To a 1 ml aliquot, 2 ml of the color reagent were added. The color reagent was prepared immediately before use. It is composed of (per sample) 0.6 ml of 3% ammonium molybdate in 5 N hydrochloric acid, 1.0 ml of 1% potassium dihydrogen phosphate and 1.6 ml of water. The color was left to develop for 1 hour at room temperature and then read in a Zeiss spectrophotometer at 850 mµ.

Results

Effect of cortisol and celite on tyrosine toxicity:

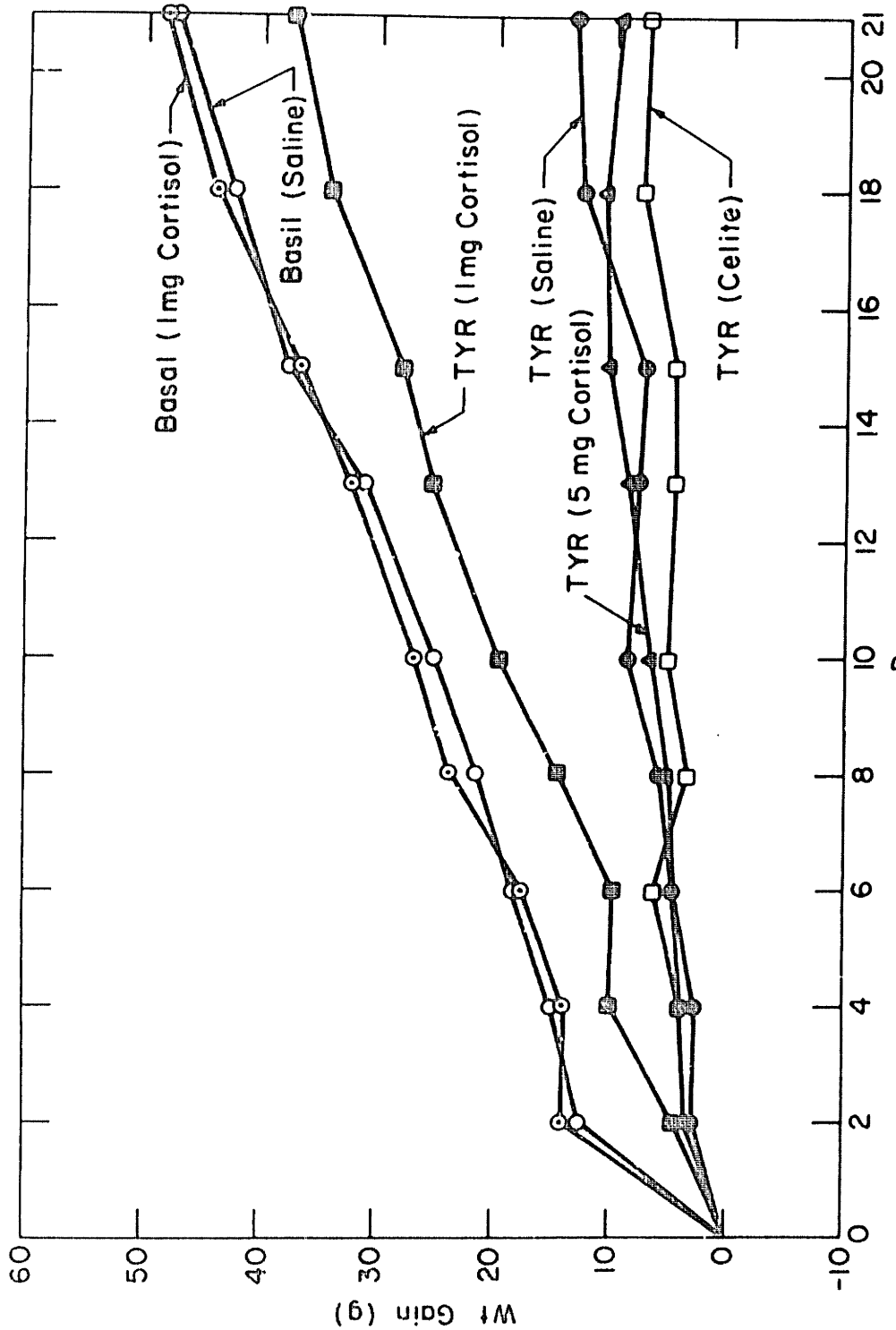
The effects of daily injections of cortisol or celite on the growth of rats fed the high tyrosine diet are shown in figure 1. Although 1 mg cortisol per day had no effect on the weight gain of the control group, it improved that of the group fed the high tyrosine diet. On the other hand, injections of 6 mg per day of celite or 5 mg per day of cortisol did not improve the growth of rats fed the high tyrosine diet at all; however, both of these treatments prevented the development of pathologic lesions (table 3).

Ingestion of the high tyrosine diet with or without celite injection increased adrenal weight (table 3); cortisol injection prevented the increase in adrenal weight.

The plasma tyrosine concentration of the rats fed the high tyrosine diet decreased substantially when they were injected daily with celite or 5 mg of cortisol. However, daily injections of 1 mg of cortisol did not decrease plasma tyrosine concentration much.

Effects of amino acid supplements on growth and food intake:

All rats consuming the high tyrosine diet grew slowly and developed eye and paw lesions within one week (table 4). Threonine was the only amino acid that alleviated both the growth depression and the pathologic lesions. The supplement of 1.25% of L-threonine also increased markedly the food intake



Effect of Cortisol and Celite on the Growth of Rats Fed 3% L-Tyrosine

FIGURE I

TABLE 3

Effects of cortisol and celite on the severity of eye and paw lesions of rats fed 3% L-tyrosine and on adrenal weight after 24 days

Additions to 6% casein basal diet	Treatment	Eye and Paw Lesions			Adrenal Weight	Plasma Tyrosine[2]
		Incidence	severity			
			eye	paw	mg/100 g body wt.	µmole/100ml
None	Saline	0	0	0	17 ± 1 (5)[1]	5
None	Cortisol (1 mg)	0	0	0	15 (2)	6
3% L-tyrosine	Saline	5	2.0	7.0	29 ± 2 (4)	302
3% L-tyrosine	Cortisol (1 mg)	3	3.7	2.6	18 ± 3 (3)	283
3% L-tyrosine	Cortisol (5 mg)	0	0	0	17 ± 2 (4)	63
3% L-tyrosine	Celite (6 mg)	0	0	0	33 ± 1 (4)	34

Five rats/group from Holtzman Co. with initial wt. 75-80 g were used. Cortisol and celite were injected intraperitoneally in 0.3 ml saline each day, the control rats were injected with 0.3 ml saline/day.

[1]Standard error of the mean. Number of observations in parentheses.

[2]Plasma of 5 rats was pooled.

TABLE 4

Effect of dietary additions of several amino acids on weight-gain and food intake of rats fed excess of tyrosine

Additions to 6% casein basal diet		Av. Wt. gain	Av. Food intake	Av. adrenal wt.
L-tyr.	Other amino acids			
%		gm/3 wk.[1]	gm/day	mg/100 gm[1] body wt.
-	-	47 ± 2	8.6	16 ± 1
3.0	-	12 ± 1	5.3	31 ± 2
3.0	1.25% L-thr	38 ± 2	7.0	21 ± 1
-	2.5% gly	30 ± 4	6.2	20 ± 2
3.0	2.5% gly	12 ± 2	4.6	29 ± 1
3.0	1.0% L-met	18 ± 3	6.0	32 ± 2
3.0	5.0% L-leu 0.2% L-ile 0.2% L-val	16 ± 2	5.1	30 ± 1
3.0	1.0% L-trp	14 ± 2	5.0	29 ± 2

[1] ± Standard error of the mean of rats/group.

of rats fed the high-tyrosine diet. Of the rats receiving the other amino acid supplements only one in the methionine-supplemented group exhibited signs of toxicity but weight gain and food intake were not appreciably improved by any of these supplements.

Adrenal weight and amino acid supplements:

The adrenal weights recorded after 3 weeks are also shown in Table 4. Adrenals damaged during removal were not included in calculating the averages. Adrenals of rats fed the diet containing 3% of L-tyrosine were much larger than those of the controls. Threonine was the only amino acid that substantially decreased the adrenal weight of rats receiving 3% of L-tyrosine.

Effects of different amino acid supplements on plasma tyrosine concentration and liver tyrosine-α-ketoglutarate transaminase of rats fed high tyrosine diet:

Plasma tyrosine concentration of the rats fed the diet containing 3% of L-tyrosine was greatly elevated at 2 days, 10 days and 3 weeks (Table 5). The addition of the various amino acids to the high tyrosine diet resulted in a lowering of plasma tyrosine concentration and this effect persisted throughout the experiment. It seems unlikely that this was due to low tyrosine intake as food intakes of all groups except for that receiving tyrosine and glycine were as high as or higher than that of the high tyrosine group.

Within 2 days there was a marked increase in liver tyrosine-α-ketoglutarate transaminase activity of rats receiving tyrosine

TABLE 5

Effect of different amino acids on liver Tyr α-K.G. transaminase and plasma Tyr concentration of rats fed 3% L-Tyr

Diet	2 days		10 days		21 days	
	[1]Plasma Tyr	[2]Enz. activity	[1]Plasma Tyr	[2]Enz. activity	[1]Plasma Tyr	[2]Enz. activity
9% casein + 0.2% L-Met(1)	2.5	44.0 ± 2.7	4.2	44.3 ± 2.4	4.6	47.2 ± 6
(1) + 3.0% L-Tyr(2)	359.0	84.4 ± 10.8[3]	471.0	135.6 ± 29.3[3]	164.8	78.8 ± 8.8[3]
(2) + 1.25% L-Thr	200.0	90.6 ± 28.8	130.0	56.9 ± 28.2	31.0	79.9 ± 22.7
(1) + 2.5% Gly	3.3	122.8 ± 24.0[3]	21	94.6 ± 15.3[3]	1.8	90.9 ± 5.1[3]
(2) + 2.5% Gly	76.5	129.9 ± 5.3[3]	73.7	125.3 ± 12.7[3]	26.3	153.4 ± 19.1[3]
(2) + 1% L-Met	154.0	100.4 ± 24.7	291.0	95.6 ± 32.5	102.6	120.5 ± 25.2[3]
(2) 5.0% L-Leu + 0.2% L-isol + 0.2% L-Val	26.0	82.7 ± 9[3]	90.1	137.5 ± 24.1[3]	61.9	103.5 ± 17.6[3]
(2) + 1.0% DL-Trp	72.0	121.4 ± 22.4[3]	65.8	86.8 ± 17	70.0	150.5 ± 39.8

[1]Average of 3-4 rats/group μm Tyr/100 ml plasma.

[2]μm pH PP/g liver/hr with standard error of the mean.

[3]Significantly different from the control at a 5% level.

alone or with the various amino acid supplements (table 5). However, the degree of increase varied from period to period. The enzyme activity of the rats fed the high tyrosine diet rose then fell at the end of 3 weeks, whereas the values for the tyrosine-glycine and tyrosine-tryptophan groups were highest at the end of the 3-week feeding period. At 21 days the values for groups receiving the various supplements other than threonine tended to be above that for the high tyrosine group.

Discussion

Tyrosine transaminase, the catalyst for the first reaction on the main pathway of tyrosine catabolism, is elevated in rats injected with cortisol or celite (Lin and Knox, 1958; Kenney and Flora, 1961), so this might be expected to enhance tyrosine catabolism. Plasma tyrosine concentration was only moderately elevated and pathologic lesions did not develop in rats fed the high tyrosine diet and injected daily with 5 mg of cortisol or 6 mg of celite (which is considered to stimulate endogenous corticosterone production). Injection of 1 mg of cortisol was not effective in preventing the lesions but it did stimulate growth. In relation to this, Rivlin (1963) found that a dose of 1 mg of cortisol did not induce liver tyrosine-α-ketoglutarate transaminase in hyperthyroid rats although a 5 mg dose did. Since tyrosine is a thyroxine precursor the possibility that a high tyrosine diet stimulates thyroxine production should be examined.

Munro and associates (1963, 1965) have reported that plasma corticosterone increases if rats are fed a meal containing 1 g of either methionine or leucine and that prolonged feeding of these two amino acids increases adrenal corticosterone concentration. Neither celite nor the amino acid supplements increased adrenal weight above the value for the high tyrosine group, yet, like cortisol, all of these supplements prevented the rise in plasma tyrosine concentration and the development of pathologic lesions. It is possible that these supplements act as an

additional stress which enhances corticosterone production or release even though they do not cause further enlargement of the adrenal glands.

The activity of tyrosine transaminase was elevated in all groups fed high tyrosine diets, as would be expected from the fact that the activity of this enzyme increases in rats injected with cortisol or fed large amounts of tyrosine. However there is obviously no direct relationship between plasma tyrosine concentration and the activity of the transaminase, even though values for transaminase activity for groups receiving amino acid supplements other than threonine tend to be above that for the high tyrosine group at 2 and at 21 days. Knox et al. (1964) have reported that the severity of tyrosyluria in rats fed a high tyrosine diet depends not only upon the activity of tyrosine transaminase but also upon the relative activities of tyrosine-α-ketoglutarate transaminase and p-hydroxy-phenylpyruvate hydroxylase.

All of the dietary additions of amino acids that prevented the development of pathologic lesions in rats fed these high tyrosine diets also prevented the usual rise in plasma tyrosine concentration. In fact there is quite a close relationship between plasma tyrosine concentration and the occurrence of signs of tyrosine toxicity. High plasma tyrosine was associated with the occurrence of severe signs in rats fed the 3% tyrosine diet; 1 mg of cortisol did not alleviate the signs nor lower plasma tyrosine concentration; 1% of L-methionine which was the least effective amino acid supplement caused the least lowering of

plasma tyrosine concentration; all of the treatments that prevented the appearance of toxicity signs lowered plasma tyrosine concentration markedly. Also, plasma tyrosine decreased substantially by the 21st day in the group fed the high tyrosine diet and this coincides well with the time of regression of signs in rats fed this diet. There was no obvious relationship between tyrosine intake and plasma tyrosine concentration as several of the groups with low plasma tyrosine concentrations consumed more tyrosine than the high tyrosine control group.

The beneficial effects of cortisol injections and amino acid supplements may be mediated through either increased urinary excretion of tyrosine or its metabolites or increased rates of tyrosine oxidation.

SECTION II

Effect of different amino acids on some urinary components of rats fed 3% L-tyrosine diet

Introduction

Addition of certain amino acids to the low protein diet containing 3% L-tyrosine alleviated - partially or completely - the tyrosine toxicity syndrome. Increased excretion of tyrosine and its metabolites could contribute toward the lowering of plasma tyrosine concentration and, hence, toward the beneficial effect that resulted from the addition of these amino acids.

In order to determine whether this occurred, total phenols and homogentisic acid excretion were measured in urine from rats fed several of the high tyrosine diets used in the study.

Experimental

Animals: Young Sprague-Dawley male albino rats with initial weights ranging between 45 to 50 g. were fed purina laboratory chow for 2 days.

Diets and animal feeding: The experimental diets were prepared in the agar gel form as described before (p.34). Amino acid supplements were added at the expense of the sucrose-dextrin mixture. These diets contained in addition to 3% L-tyrosine: 1.25% L-threonine, 1% L-methionine or 2.5% glycine. Animals were divided into groups of 4 rats each and housed individually in stainless steel metabolic cages so that urine could be collected separately from feces with minimum contamination. Urine was collected for the first 2 days of the experimental period from the rats fed the control diet. Later urine was collected from all groups for 2-day periods; on the 6th and 7th; 12th and 13th; and 21st and 22nd days. Urine was collected in 4 N hydrochloric acid and made up to 100 mL volume so that the final hydrochloric acid concentration was about 0.5 N. Samples were kept in the refrigerator until they were analyzed. Food and water were provided ad libitum.

Determination of total phenol:

The method used was that used by Bernhart and Zilliken (1959). It consisted of:

10 ml samples of urine from each of the rats in a group were pooled and hydrolyzed by heating for one hour in a boiling

water bath. To 1 ml of the urine hydrolysate were added 10 ml of water, 1 ml of phenol reagent and 5 ml of saturated sodium carbonate solution. The mixture was left for half an hour at room temperature and the color that developed was read in a spectrophotometer at 625 mμ.

P-hydroxyphenylpyruvic acid solutions of different concentrations were used as working standards and treated in the same way as the urine samples. Values are expressed per 100 g body weight.

Determination of homogentisic acid:

The method used was that used by Neuberger (1947) which involved the microiodine titration. Two 10 ml aliquots of the pooled urine were pipetted in two Erlenmeyer flasks and another 10 ml aliquot was placed in a beaker. The pH of the urine sample in the beaker was adjusted to 7.6 using saturated sodium bicarbonate solution, equivalent volumes of which were added to the two flasks. One ml of 4% potassium iodide solution was then added followed by 1 ml of a 2% solution of soluble starch. The mixture was then titrated with 0.05 N iodine solution (6.5 g iodine sublimated per one litre) until a definitely blue color developed which persisted for 45 seconds. Five ml of 7 N sulphuric acid were then added cautiously to avoid sudden effervescence. After 3 minutes the liberated iodine was titrated with 0.01 N sodium thiosulphate (2.5 g/L) until the blue color disappeared, then back-titrated with iodine solution until a faint blue color was attained. One ml of the thiosulphate was equivalent to

0.84 mg. homogentisic acid. Values are expressed per 100 g. body weight.

Results

Effect of amino acid supplement on growth and symptoms:

To permit comparison between the results obtained here and those obtained in other experiments, food intake and weight gain of the different groups are reported in table 1. Ingestion of a diet containing 3% of L-tyrosine caused a severe growth depression and the appearance of paw and eye lesions characteristic of tyrosine toxicity; one rat died on the 20th day of the experiment. The condition was completely alleviated by a supplement of 1.25% L-threonine. Glycine was effective again in alleviating the pathological lesions but it did not improve growth appreciably. Two out of 4 rats fed the methionine supplemented, high tyrosine diet showed toxic symptoms, however, the onset was after about two weeks as compared with 8 days for the group fed 3% of L-tyrosine. Food intake was depressed by all the high tyrosine diets except for that containing the supplement of threonine. The rate of growth of the latter group was greater than that of the control.

Effect of different amino acids on total urinary phenol excretion:

In the first two days of the experimental period the control group excreted 10 mg. of phenol per 100 g. body weight. Feeding tyrosine caused an increased excretion of total phenols in urine (table 2). The value for the methionine-supplemented group was highest on the 6th and 7th day period. On the 12th and 13th day period total phenol excretion by rats fed tyrosine with or

Table 1

Effect of some amino acid supplements on weight gains and food intakes of rats fed 3% L-Tyr.

Group No.	Diet	Dry food intake g/rat/2W	Weight gain g/2W
1	Basal (6% casein + 0.2 L-Met.)	155.5	35.3
2	Basal + 3% L-Tyrosine	105.0	11.8
3	No.2 + 1.25% L-Thr.	144.0	42.8
4	No.2 + 1.0% L-Met.	126.8	17.2
5	No.2 + 2.5% Gly.	118.5	16.5

Table 2

Effect of different amino acid supplements on body weight,[1] tyrosine intake,[2] and total phenol excretion[3] of rats fed 3% L-Tyr.[4]

Diet	6th and 7th day			12th and 13th day			21st and 22nd day		
	Av. Wt. of rats	Tyr. Intake	Total Phenols	Av. Wt. of rats	Tyr. Intake	Total Phenols	Av. Wt. of rats	Tyr. Intake	Total Phenols
6% Casein (+ 0.2% L-Met.)	70.1	50	11	82.9	59	7	95.8	48	8
" + 3% L-Tyr.	63.6	555	79	66.5	436	221	72.1	517	154
" + 3% L-Tyr. + 1.25% L-Thr.	70.1	636	73	83.4	530	37	100	508	51
" + 3% L-Tyr. + 1% L-Met.	62.3	650	157	72.0	528	243	77	505	69
" + 3% L-Tyr. + 2.5% Gly.	65.1	502	42	70.7	481	55	76	568	63

[1]Average g. body weight of 4 rats during the 2 days collection period.

[2]mg. tyrosine consumed/100 g body weight in the 2 days collection period.

[3]mg. equivalent to p-hydroxyphenylpyruvic acid excreted in urine/100 g. body weight.

[4]Average body weight of 4 rats at start being 60.6. 7 mg total phenol were excreted per rat in the 1st and 2nd day period.

without methionine increased substantially. On the other hand, urinary total phenol excretion by groups fed 1.25% L-threonine or 2.5% glycine did not increase; in fact, excretion by the threonine-supplemented group, which had the highest tyrosine intake, actually decreased.

By the end of the experiment, there was a drop in the total phenol excretion of rats fed the 3% L-tyrosine diet but the amount was still higher than that of any of the other groups. The methionine-supplemented group showed a marked decrease in total phenol excretion during the last period.

Effect of different amino acids on urinary homogentisic acid excretion:

Feeding tyrosine alone or together with the other amino acids for one week did not cause a rise in urinary homogentisic acid excretion above the value for the control group (Table 3). During the 12th and 13th day period homogentisic acid excretion by rats fed threonine and glycine supplements increased slightly, however, those fed tyrosine alone or together with methionine did not show any marked change. By the end of the three weeks addition of 1.25% L-threonine or 2.5% glycine to the high tyrosine diet resulted in greatly increased homogentisic acid excretion.

In between the periods of collection of urine the collecting flasks were left without acid. After the second collection period 3 urine samples of 3 rats fed the glycine

TABLE 3

Effect of different amino acids on urinary homogentisic acid excretion[1] of rats fed 3% L-Tyr.

Diet	6th and 7th day	12th and 13th day	21st and 22nd day
6% casein (+0.2% L-Met.)	2.4	2.1	2.0
" + 3% L-Tyr.	2.4	3.2	2.9
" + 3% L-Tyr. + 1.25% L-Thr.	2.4	5.0	28.8
" + 3% L-Tyr. + 1% L-Met.	2.6	3.5	5.7
" + 3% L-Tyr. + 2.5% Gly.	3.2	7.1	22.9

[1] mg./100g. body weight excreted during the 2 days collection period.

supplement were dark and 3 urine samples of rats fed the threonine supplement showed a similar color but only one sample from rats fed the methionine-tyrosine diet showed a similar change.

Discussion

It is clear that excess tyrosine feeding resulted in increased total phenol excretion in the urine at all periods especially the 12th and 13th day period where it amounted to almost 50% of the total tyrosine consumed. Methionine was the only amino acid which, when added to the 3% tyrosine diet, was found to cause increased total phenol excretion above that for the rats fed the unsupplemented high tyrosine diet; however, the tyrosine intake was high too. In spite of the increased total phenol excretion in urine, 2 out of 4 rats receiving methionine showed external lesions. On the other hand, in spite of the higher tyrosine consumption by the rats fed the threonine and the glycine supplements they excreted less phenols than the rats fed tyrosine alone.

The development of alcaptonuria in rats fed high tyrosine together with 1.25% L-threonine or 2.5% glycine is of great interest. As a matter of fact the amounts of homogentisic acid excreted during the last period represents a significant fraction of the total phenols excreted during this period. Threonine supplementation alleviated the external symptoms as well as counteracted the growth depressing effect of tyrosine while glycine supplementation alleviated only the external lesions. Increased homogentisic acid excretion in the urine of the group treated with glycine and threonine may indicate that the previous enzymatic steps are rapid under these conditions and that further oxidation of homogentisic acid then becomes limiting. This view

is favored by the previous finding that the addition of glycine to the 3% L-tyrosine diet has resulted in a substantial increase in tyrosine-α-ketoglutarate transaminase activity which was accompanied by a substantial decrease in plasma tyrosine concentration. In this connection the effects of glycine on the enzyme activity and the plasma tyrosine concentration were more pronounced than those of threonine supplementation. However, the improvement of growth as the result of supplementation with threonine, the limiting amino acid in this diet (Harper, 1959), would result in greater utilization of tyrosine for tissue synthesis, in larger organ size and probably therefore in a greater total capacity of the rat to metabolize tyrosine.

SECTION III

Diurnal changes in food intake, tyrosine-α-ketoglutarate transaminase and p-hydroxyphenylpyruvate hydroxylase activities of rats fed 5% L-tyrosine

Introduction

Liver tyrosine transaminase activity has been found to change with dietary protein level (Al-Nejjar and Litwack, 1961; Potter et al., 1966), age (Sereni et al., 1959) and sex (Lin et al., 1959). Immobilization of rats by tying their feet resulted in 4-fold increase in this enzyme activity within 12 hours after the stress was initiated (Hänninen and Hartiala, 1967). Activity of p-hydroxyphenylpyruvate hydroxylase has also been shown to be influenced by dietary protein level (Al-Nejjar and Litwack, 1961), age (Goswami and Knox, 1961) and the administration of an inhibitor diethyldithiocarbamate (LaDu and Zannoni, 1958).

It was thought that the activities of these enzymes might be different at different time intervals of the day and that the results might be influenced by the time at which animals were sacrificed for enzyme determinations. Such changes might result in, or may be a result of, changes in food intake (or tyrosine consumption) or plasma tyrosine concentration. Some experiments were therefore done to determine whether diurnal changes in the activities of these enzymes and in food intake occurred in animals fed the high tyrosine diet used during these investigations.

Experimental

Rats weighing 55-60 g were fed the control diet (6% casein + 0.2% L-methionine) or a high tyrosine diet (5% L-tyrosine) for a period of time. Animals were then divided into 8 groups of 4 rats each; each 4 groups were fed one dietary regimen. Food intake for each group was recorded each 3 hours over a 24 hour period. The lights in the animal laboratory were on from 8 a.m. to 8 p.m. daily. Groups of rats were killed at 6-hour intervals over a 24-hour period. Livers were removed and kept at -20°C until analyzed for the enzyme p-hydroxyphenylpyruvate hydroxylase. Other groups were kept on the experimental diets for 7 days and then killed at 6-hour intervals over a 24-hour period. Livers were treated as mentioned above. Blood was taken by heart puncture and plasma amino acid concentrations were estimated using the Technicon amino acid analyzer as described before (P. 38).

Assay of tyrosine-α-ketoglutarate transaminase:

The procedure which was described earlier (P.38) was followed for this assay (Lin et al., 1958).

Assay of p-hydroxyphenylpyruvate hydroxylase:

The method used was the same as that adopted by Lin et al. (1958). Rat livers were homogenized with 4 volumes of a solution containing 0.005 N sodium hydroxide and 0.14 M potassium chloride. The homogenate was centrifuged for 45 minutes at 25,000 X g. The

Experimental

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Assay of tyrosine-α-ketoglutarate transaminase:

The procedure which was described earlier (P. 38) was followed for this assay.

Assay of p-hydroxyphenylpyruvate hydroxylase:

The method used was the same as that adopted by Lin et al. (1958). Rat livers were homogenized with 4 volumes of a solution containing 0.005 N sodium hydroxide and 0.14 M potassium chloride. The homogenate was centrifuged for 45 minutes at 25,000 X g. The

clear supernatent was decanted and used for enzyme assay. The substrate mixture was prepared by mixing 0.7 ml of water, 1.7 ml of 1 M borate solution of pH 8.0 and 0.3 ml of p-hydroxyphenylpyruvic acid solution (18 mg/50 ml borate solution). 0.7 ml of the enzyme homogenate was mixed with 0.7 ml of pyridoxal-5-phosphate solution (6.25 mg/25 ml). The enzyme preparation as well as the substrate mixture were then incubated separately in a water bath adjusted to 30°C for 5 minutes. Then 0.3 ml of the enzyme mixture was added to the substrate mixture. The rate of disappearance of p-hydroxyphenylpyruvic acid was followed spectrophotometrically at 310 mμ using a Gilford recording spectrophotometer.

Different enzyme concentrations were tested and the values were reproduceable up to 0.5 O.D./4 minutes. All samples were run in duplicate together with blanks.

Results

Diurnal rhythm in food intake:

Group food intake patterns of rats fed the experimental diets are shown in Figure I. Group food intake was preferred as these rats were small and the food intake will be relatively small especially over a short period of time. Each bar represents the average of 4 groups of 4 rats each. The control animals ate over the 24-hour period with distinct peaks at about 6-12 p.m. and 3-9 a.m. (Fig. I-a). Rats fed the high tyrosine diet had a more or less distinct peak of food intake at about the same times as the controls.

Figure II shows daily food intake of rats fed the experimental diets over a period of 2 weeks. The control animals showed a gradual increase in food intake by time, while animals fed the high tyrosine diet showed a more or less constant daily food intake.

Diurnal changes in enzyme activities and plasma tyrosine concentration:

Table 1 shows the changes in tyrosine transaminase and plasma tyrosine concentration at 9 a.m., 3 p.m., 9 p.m. and 3 a.m. in rats fed the experimental diets for 7 days. From Figure III-a it is clear that the plasma tyrosine concentration of the control animals did not change over the experimental period. However, tyrosine transaminase did change and showed a

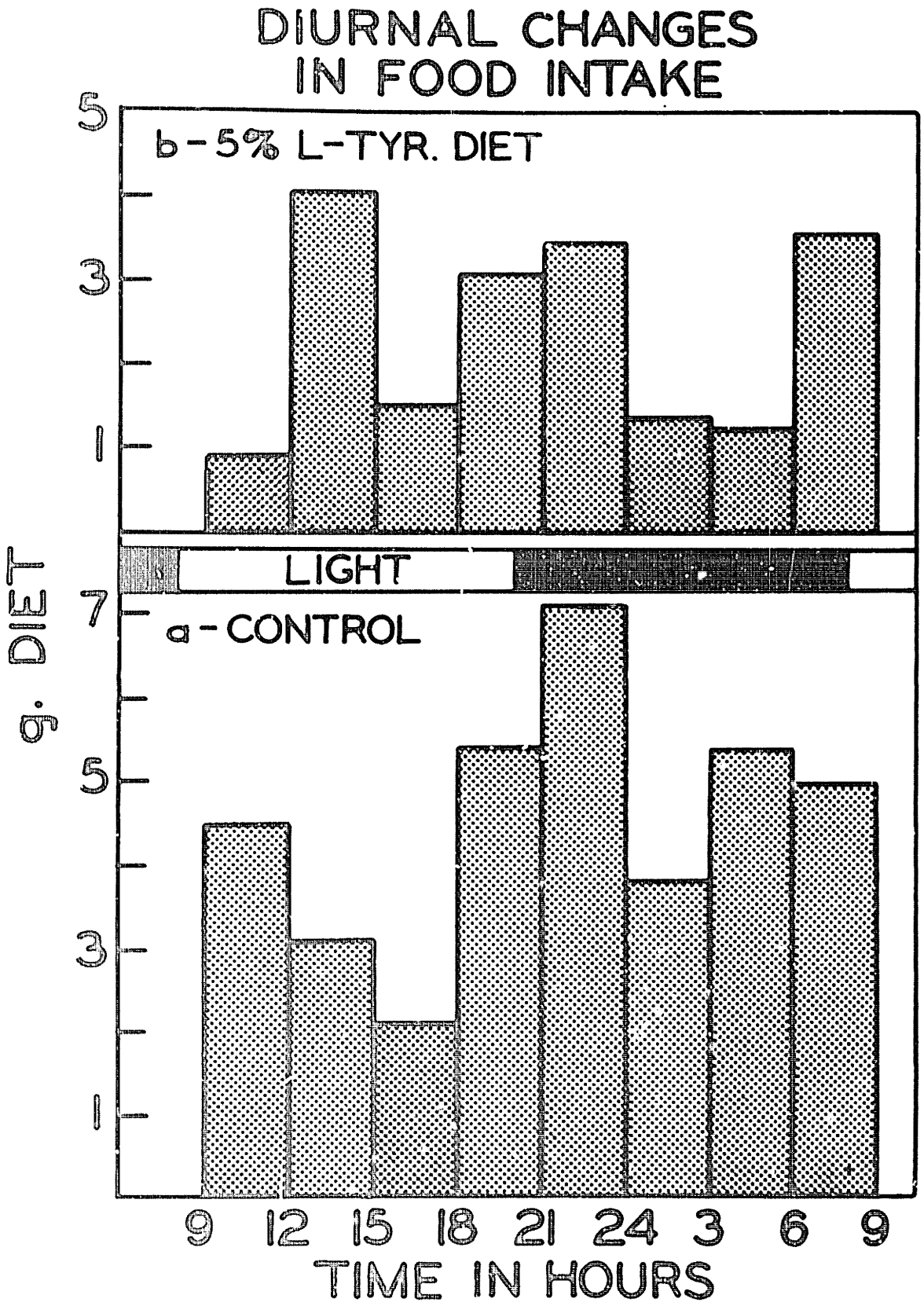

DIURNAL CHANGES
IN FOOD INTAKE
b-5% L-TYR. DIET
LIGHT
a-CONTROL
g. DIET
5
3
1
7
5
3
1
9 12 15 18 21 24 3 6 9
TIME IN HOURS

FIGURE I

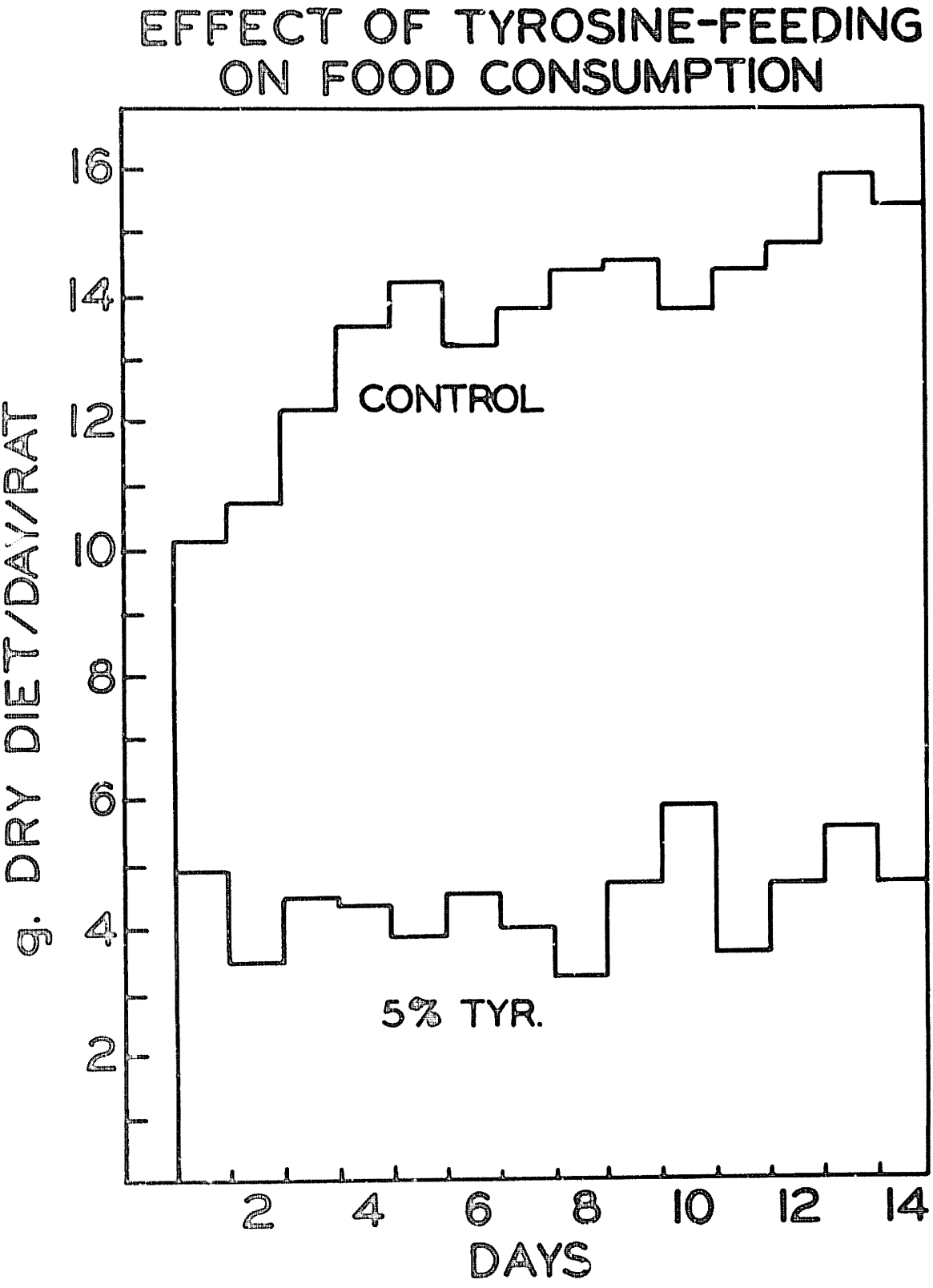

EFFECT OF TYROSINE-FEEDING
ON FOOD CONSUMPTION
g. DRY DIET/DAY/RAT
16
14
12
10
8
6
4
2
CONTROL
5% TYR.
2
4
6
8
10
12
14
DAYS

FIGURE II

TABLE 1

Diurnal changes of liver tyrosine-α-ketoglutarate transaminase and plasma Tyr concentration of rats fed 5% L-tyrosine[1]

Diet	Time			
	9 a.m.	3 p.m.	9 p.m.	3 a.m.
6% Casein (+0.2 L-Met)				
Plasma Tyr[2]	18	19.2	20.4	18.5
Enz. activity[3]	72.4 ± 13.6	56.2 ± 7.4	108.3 ± 16.7	89.2 ± 9.4
5% L-Tyr				
Plasma Tyr	275	246	254	306
Enz. activity	359 ± 44	261 ± 33	222 ± 29	278 ± 35

[1] 7 days on the experimental diets

[2] μmole/100 ml pooled plasma from 4 rats

[3] μm p-hydroxyphenylpyruvic acid formed/g liver/hour

maximum at 9 p.m. which was about twice the value found at 3 p.m. On the other hand, rats fed the high tyrosine diet showed maximum tyrosine transaminase activity around 9 a.m. with a minimum at 9 p.m. Also these rats showed a highly elevated plasma tyrosine concentration (Figure III-a). Table 2 shows the change in hepatic p-hydroxyphenylpyruvate hydroxylase with time. The control animals showed maximum activity at 9 p.m. similar to the pattern for tyrosine transaminase. However, in rats fed the high tyrosine diet the peak in enzyme activity occurred at 3 p.m. (Figure III-b). Thus the maxima for the two groups were 6 hours apart.

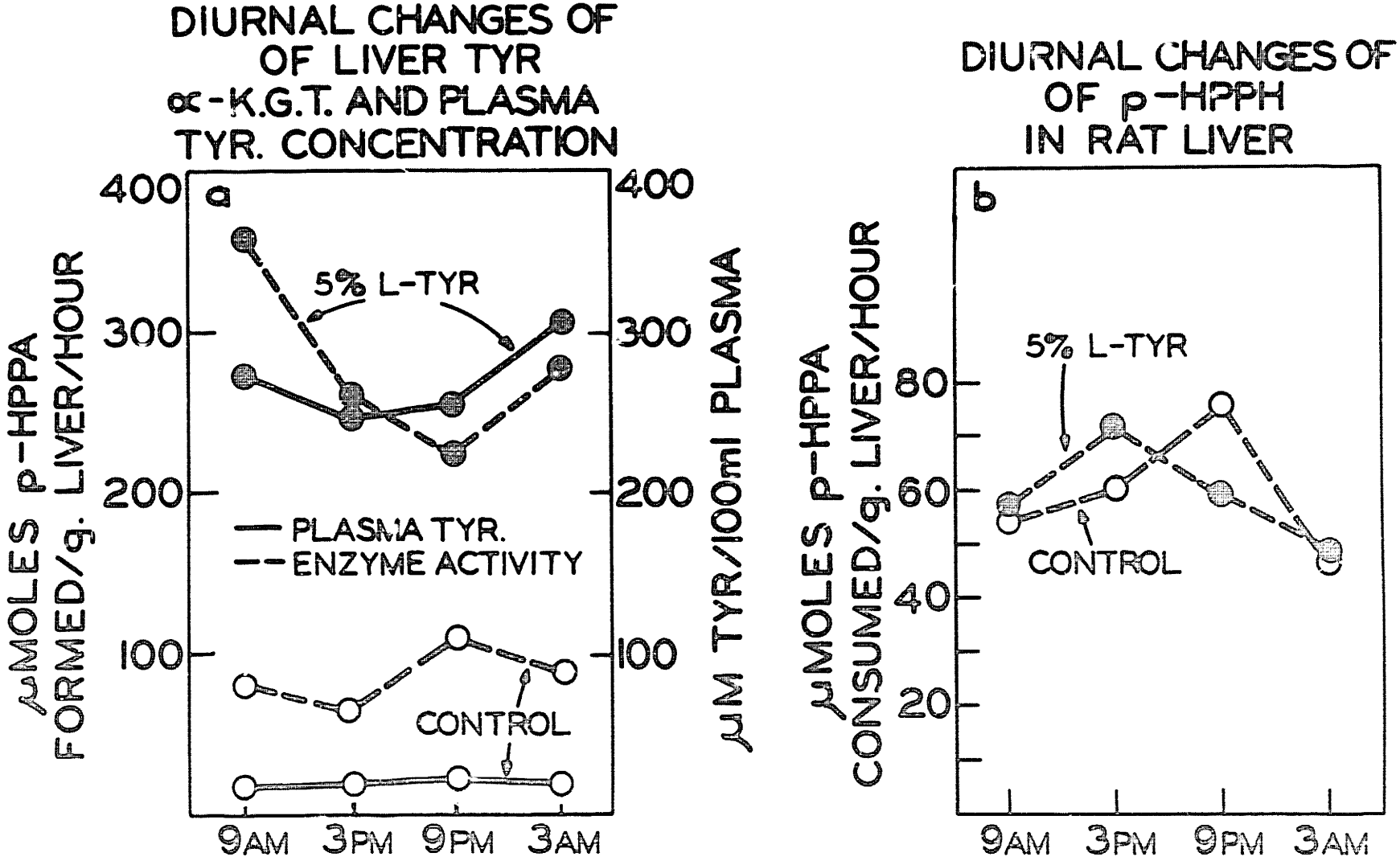

DIURNAL CHANGES OF
OF LIVER TYR
α-K.G.T. AND PLASMA
TYR. CONCENTRATION
a
400
300
200
100
5% L-TYR
PLASMA TYR.
ENZYME ACTIVITY
CONTROL
μMOLES p-HPPA
FORMED/g. LIVER/HOUR
μM TYR/100ml PLASMA
9AM
3PM
9PM
3AM
DIURNAL CHANGES OF
OF p-HPPH
IN RAT LIVER
b
80
60
40
20
5% L-TYR
CONTROL
μMOLES p-HPPA
CONSUMED/g. LIVER/HOUR
9AM
3PM
9PM
3AM

FIGURE III

TABLE 2

Diurnal changes of liver p-hydroxyphenylpyruvate hydroxylase activity of rats fed 5% L-tyrosine[1]

Diet	Enzyme activity[2]			
	9 a.m.	3 p.m.	9 p.m.	3 a.m.
6% Casein (+ 2% L-Met)	53.8 ± 3.3	60.8 ± 6.1	76.4 ± 5.3	47.3 ± 3.2
6% Casein (+ ") + 5% L-Tyr	57.6 ± 4.5	72.1 ± 3.3	60.2 ± 4.2	48 ± 2.5

[1] 5 days on the experimental diets

[2] µmole p-hydroxyphenylpyruvic acid consumed/g liver/hour ± standard error of the mean of 4 rats.

Discussion

It is evident that the addition of 5% of L-tyrosine to the 6% casein diet depressed the food intake of the rats. Also diurnal rhythms in food intake (Figure I) seemed to be exhibited by the controls and by groups fed the high tyrosine diet. Both ate a substantial amount of their daily food intake during the dark cycle. This agrees generally with results obtained by Siegel (1961) who observed that the maximum food intake of rats occurred between 7 and 10 p.m.

Animals fed the control diet showed a gradual increase in food intake throughout the 14-day experimental period (Figure II). This is to be expected as these rats were growing. However, food intake of animals fed the high tyrosine diet was more or less constant over the two-week period. They consumed much less food than the control rats during the same period.

In rats fed high tyrosine diet for 7 days tyrosine transaminase activity was elevated at all time intervals tested (Figure III-a). Variations among the individual values within a group was large. This can be attributed to the fact that the condition of the individual animals within each group varied even though an effort was made to separate them into more or less equal groups on the basis of pathological lesions. Liver tyrosine transaminase activity was maximal at around 9 p.m. while plasma tyrosine concentration remained constant in rats fed the basal diet. As mentioned earlier, this enzyme activity could be

increased to five times its basal level after 5 hours of cortisol injection. Also it has been found (McCarthy et al., 1960) that rats kept on a light schedule from 8 a.m. to 8 p.m. (similar to ours) showed highest concentration of plasma corticosterone around 2-3 p.m. A pattern of corticosterone secretion of this type might contribute to the high value for tyrosine transaminase of the control rats around 9 p.m. (Figure III-a).

On the other hand, addition of 5% of L-tyrosine to the control diet caused a displacement in the maximum activity of tyrosine transaminase to about 9 a.m. Administration of excessive amounts of certain amino acids increases the plasma corticosterone level (Munro et al., 1963, 1965), also our previous data suggest a similar effect. From Figure I-b there is an indication of a gradual build up of food intake to a maximum near midnight which was reflected by a peak in plasma tyrosine concentration around 3 a.m. (Figure III-a). If such a high concentration can act to further increase plasma corticosterone level through stimulation of the adrenal gland, this might explain the shift and appearance of the peak of tyrosine transaminase at 9 a.m.

A high tyrosine intake seemed to affect the activity of the enzyme p-hydroxyphenylpyruvate hydroxylase very little (Figure III-b), yet a diurnal rhythm was observed with maximum enzyme activity exhibited by the animals fed the high tyrosine diet at 3 p.m. as compared to 9 p.m. for the control animals.

In subsequent studies the animals were sacrificed for enzyme determinations around 9 a.m. when the differences between groups approached the maxima.

SECTION IV

EFFECTS OF THYROXINE ON TYROSINE TOXICITY

Introduction

Schweizer (1947) observed that the administration of thyroxine increased the loss in weight of rats fed a high tyrosine diet and the frequency with which they developed pathological lesions. Thiouracil administration (0.1% of the diet) increased the survival time of rats fed the high tyrosine diet. He also reported that inclusion of 1.0, 1.5 or 2.0% tyrosine in the diet did not modify the oxygen consumption of rats. Since these observations that thyroxine influenced the development of tyrosine toxicity were reported, no studies have been done to explain why thyroxine aggravates the pathological lesions of the rats fed a high tyrosine diet.

In 1956 Litwack found that tyrosine transaminase activity of rat liver was inhibited by the addition of thyroxine in concentrations as low as 5×10^{-5} to the incubation mixture. This observation was later investigated in more detail (Litwack, 1957). It was found that the inclusion of 1% of thyroid powder in a 30% protein diet inhibited the growth of the animals as well as tyrosine transaminase activity. Thyroxine administration has been shown to suppress the activity of a number of other pyridoxal phosphate-dependent enzymes including serine dehydratase, threonine dehydratase (Howarth, 1957) and cysteine desulfurase (Chatagner *et al.*, 1962). The effect of thyroxine administration may be mediated through direct inhibition of the apoenzyme and also indirectly by decreasing the degree of saturation of the apoenzyme with the coenzyme. It was found that thyroxine

administration significantly lowered the level of hepatic pyridoxal phosphate and that thyroidectomy resulted in a significant increase in the level of this coenzyme (Labouesse et al., 1960).

Most of these studies have been done over short periods of time. A long term study was later conducted by Litwack et al. in 1964. They studied the effect of including thyroid powder in the diet on liver tyrosine transaminase in the rat. During the first three days they observed a progressive decrease in the activity of this enzyme to almost half its basal value. This was followed by a rapid increase to above the normal level after 7 days on such diet. On the other hand when they measured the activity of p-hydroxyphenylpyruvate hydroxylase they found it to decrease progressively until it reached about 25% of the basal level by the end of the 7-day experimental period.

The interrelationship between thyroxine and the adrenals has been studied by many authors. The administration of thyroxine results in a progressive increase in adrenal weight (Walloch and Reinek, 1949) and the zona fasciculata of the adrenal cortex hypertrophies (Dean and Greep, 1947). Chronic administration of triiodothyronine to male and female rats also induced hypertrophy of the adrenal cortex as indicated by marked increases in glucocorticoid concentration of plasma and adrenal as well as hypertrophy of the gland (D'Angelo and Grodin, 1964). On the other hand, administration of thiouracil either in the food or in the drinking water for various periods of time

resulting in involution of the adrenal gland to less than half its size (Bauman and Marine, 1945; Zarrow and Money, 1954). Adrenal hypertrophy apparently required the presence of an intact pituitary since it did not occur in hypophysectomized animals (Freedman and Gordon, 1950).

The correlation between protein intake and thyroid activity has been studied by Klimek et al. (1958). They found that a protein deficient diet diminished iodine uptake by the rat thyroid. Simon et al. (1962) found that the addition of tyrosine to a protein-deficient diet resulted in a marked increase in iodine uptake by the thyroid gland. Thyroid hypofunction induced by protein deficiency may arise, at least partly, as a consequence of tyrosine deficiency since the administration of tyrosine increased although it did not normalize iodine uptake by the gland.

Thyrotoxic patients show elevated levels of plasma tyrosine (Levine et al., 1962). Oral tyrosine tolerance has been studied by Melmon et al. (1964). When a load of tyrosine was fed to a hyperthyroid patient and plasma levels of amino acids were then measured at hourly intervals, plasma tyrosine concentration was elevated several times above the level found in euthyroid individuals. These studies were later extended by Rivlin et al. (1965) who found that hyperthyroidism was characterized by elevated fasting levels of plasma tyrosine after an orally administered load of tyrosine. In hypothyroid subjects both the fasting level and the level after an oral tyrosine load were reduced below values found in euthyroid patients.

From this brief review it appears that thyroxine might affect tyrosine metabolism especially when tyrosine intake is high. Thiouracil is a potent inhibitor of thyroxine formation in the thyroid gland (Franklin et al., 1944). It also alters periferal utilization of thyroxine (Stasilli et al., 1960). It was found to increase the deiodination from 3- or 5- position of thyroxine as well as its biliary excretion (Flock and Bollman, 1962). Inhibition of thyroxine synthesis leads to hypothyroidism.

Administration of thiouracil has some growth depressing effect. This was earlier ascribed to thyroxine deficiency (Leathem and Seeley, 1947). Paik and Cohen (1961) suggested that the abnormal uracil supplied by thiouracil administration may affect protein synthesis through the production of "non physiological" ribonucleic acid molecules. Yatvin et al. (1964) studied the mechanism of action of thiouracil on liver protein biosynthesis. They found that RNA/DNA ratio and the incorporation of labelled methionine into liver protein were unchanged by addition of 0.5% thiouracil to the control diet. This occurred in spite of increases in total liver weight and protein content. It appears that thiouracil exerts its effect by decreasing protein catabolism while anabolism in the liver is normal.

The following investigations were conducted to examine the effect - if there is any - of thyroxine on tyrosine metabolism in rats fed a high tyrosine diet. The growth, the development of the external lesions, liver tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase activities as well as plasma

amino acid concentrations were studied in relation to thyroxine status of the animal. An attempt was also made to see whether pyridoxine might have an effect on tyrosine metabolism in relation to thyroxine administration.

Experimental

Groups of rats of the same specifications as described earlier (P.34) were used throughout this investigation. The experimental diets were fed in agar gel form as described before (P. 34). In a preliminary experiment the effect of two different levels of thiouracil (0.05 and 0.08%) on tyrosine toxicity in rats fed a 6% casein diet supplemented with 0.2% of L-methionine and 3% of L-tyrosine was studied. Later the effect of thyroxine on rats fed 5% L-tyrosine diets was examined. Tyrosine transaminase was determined in this experiment using the technique of Rosen and Milholland (1963) as described before (P.38). In another experiment the effect of thyroxine injection on liver p-hydroxyphenylpyruvate hydroxylase activity was studied under the experimental conditions mentioned in the respective table. The method used was that of Lin et al. (1958) as described before (P. 65). Tyrosine transaminase activity was also measured using the enol-borate method (Lin et al., 1958) described below. In the study of the effect of pyridoxine on tyrosine toxicity, 1 g of pyridoxine was added per kg dry diet. In most of the experiments reported here, plasma amino acid concentrations were analyzed as described before (P. 38).

Assay of liver tyrosine-α-ketoglutarate transaminase:

Livers were removed and kept frozen at -20°C. On the day of the assay, liver samples were homogenized in 0.14 M potassium chloride solution containing 0.005 N sodium hydroxide. The

homogenate was centrifuged for 45 minutes at 25,000 X g. The clear supernate was decanted and used for enzyme assay. The assay medium consisted of (per tube): 2.1 ml of a solution containing 1 mg of L-tyrosine/ml in 1 M borate buffer of pH 8.0; 0.5 ml of α-ketoglutaric acid solution (160 μmole/ml and adjusted to pH 8.0); 0.1 ml diethyldithiocarbamate solution (22.5 mg/ml); 0.15 ml of pyridoxal phosphate solution (6.25 mg/25 ml potassium chloride solution of 0.14 M concentration); and 0.15 ml of the liver homogenate supernatent. The enzyme-coenzyme mixture as well as the substrate medium were preincubated separately for 5 minutes at 30°C and then mixed together. The rate of appearance of p-hydroxyphenylpyruvic acid was followed by the change in absorption at 310 mμ over a 12 minute period using a Gilford spectrophotometer.

Thyroxine (L-thyroxine-sodium pentahydrate) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. It was dissolved in 0.005 N sodium hydroxide to give solutions containing either 25 μg or 5 μg per 0.2 ml. It was injected intraperitoneally and the control animals were injected similarly with 0.2 ml of the solvent solution.

Weight gain was recorded and group food intake was corrected for the amount spilled in all experiments.

Results

Effect of thiouracil on rats fed 3% L-tyrosine:

It is clear from Fig. I that 0.05 or 0.08% of thiouracil were equally effective in improving the growth of animals fed the diet containing 3% of L-tyrosine. Daily injection of the control animals with 25 µg of thyroxine caused weight loss during the first week of the feeding period then growth improved. The addition of thiouracil improved the food intake of rats fed the high tyrosine diet (Table 1) but despite this, two rats out of six from each group developed paw lesions. However, the lesions appeared later than those of animals fed the 3% of L-tyrosine diet without thiouracil. Thiouracil feeding lowered the food intake of rats fed the control diet while thyroxine injection did not.

Effect of thiouracil on rats fed 5% L-tyrosine for two weeks:

Ingestion of a diet containing 5% of L-tyrosine caused marked body weight loss (Fig. II) and daily injection of 5 µg of thyroxine further increased the rate of body weight loss. Addition of 0.08% of thiouracil prevented weight loss of animals fed the high tyrosine diet. This treatment lowered the rate of growth of the control rats as well as their food intake (Table 2). However, external lesions developed but in a mild form. Thyroxine injection of rats fed the high tyrosine diet aggravated the toxicity symptoms and 3 rats out of 6 died during the experimental period, while 2 out of 6 rats that were fed the

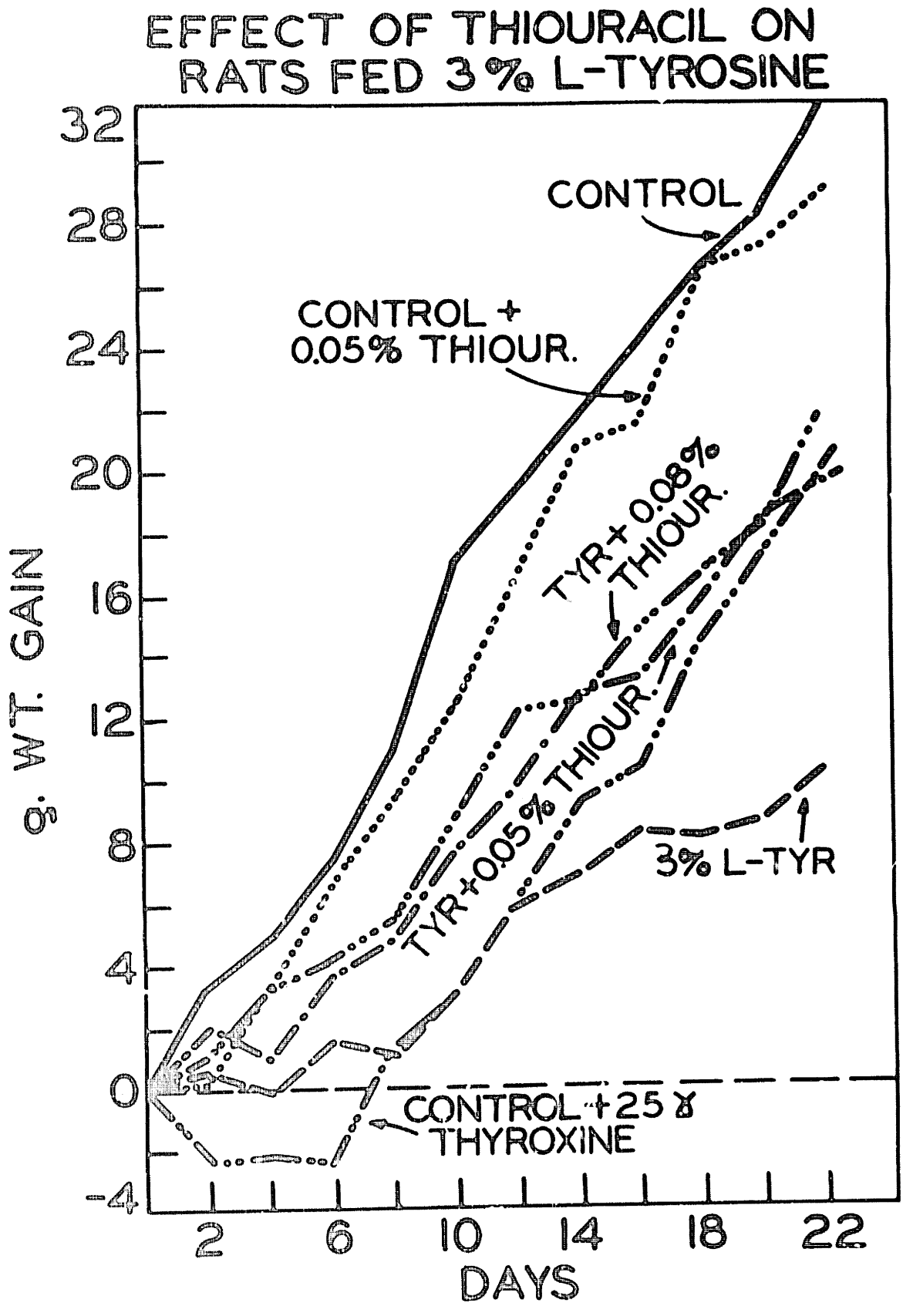

EFFECT OF THIOURACIL ON
RATS FED 3% L-TYROSINE
g. WT. GAIN
32
28
24
20
16
12
8
4
0
-4
2
6
10
14
18
22
DAYS
CONTROL
CONTROL + 0.05% THIOUR.
TYR + 0.08% THIOUR.
TYR + 0.05% THIOUR.
3% L-TYR
CONTROL + 25 γ THYROXINE

FIGURE I

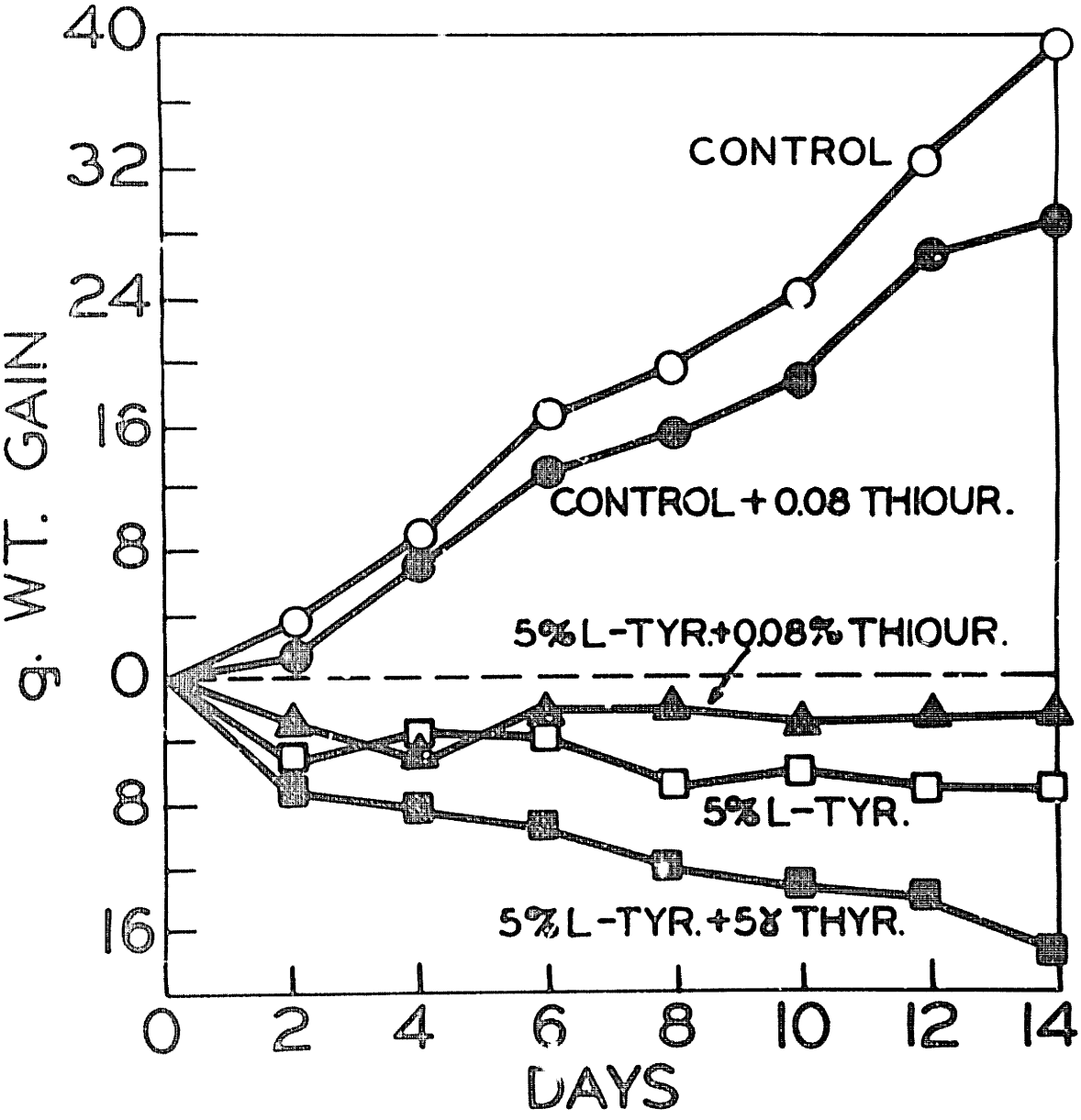
TABLE 1

Effect of thiouracil on rats[1] fed 3% L-tyrosine diets for 22 days

Diet	Treatment	Food intake	Weight gain	Symptoms
		g/rat/22 day		
6% casein + 0.2% L-Met	-	157	34 ± 4	0 out of 6
"	0.05% Thiouracil	133	29 ± 4	0 out of 6
"	Thyroxine[2]	159	20 ± 5	0 out of 6
6% casein + 3% L-Tyr	-	92	10 ± 2	3 out of 6
"	0.05% Thiouracil	110	20 ± 2	2 out of 6
"	0.08% Thiouracil	107	22 ± 2	2 out of 6

[1]Average of 6 rats per group ± standard error of the mean (Holtzman rats).

[2]25 γ L-thyroxine injected daily intraperitoneally.



ROLE OF THYROXINE ON GROWTH OF RATS FED 5% L-TYR.

FIGURE II

TABLE 2

Effect of thyroxine on rats fed 5% L-Tyr for 2 weeks

Diet	Food Intake	Weight gain [4]	Survivals	Plasma Tyr [3]	T.T. [1,2]
	g/rat/2 W				
6% Casein (c)	157	29.6 ± 2.4	6/6	5.1	233 ± 13.7
C + 0.08% Thiouracile	110	28.5 ± 2.3	6/6	6.2	215 ± 13.5
C + 5% L-Tyr (T)	55	- 7.0 ± 1.7	4/6	262	1275 ± 23.1
T + 0.08% Thiouracile	54	2.3 ± 0.6	6/6	236	930 ± 22.7
T + Thyroxine	54	-17.3 ± 1.5	3/6	528	2825 ± 13.2

[1]Tyrosine-α-ketoglutarate transaminase activity as µm p-hydroxyphenyl-pyruvate formed /hour/100 g. body weight (by the method of Rosen and Milholland, 1963).

[2]Standard error of the mean is given as percentage of the average enzyme activity.

[3]µmole/100 ml pooled plasma from the respective number of rats.

[4]Average weight gain of rats ± standard error of the mean (Sprague-Dawley rats).

high tyrosine diet alone died. On the other hand all animals fed 5% of L-tyrosine diet with 0.08% of thiouracil survived until the end of the 2-week feeding period.

Ingestion of the high tyrosine diet caused about a 50-fold increase in plasma tyrosine concentration; this was slightly lowered by the dietary addition of thiouracil (Table 3). In contrast daily injection of rats fed the 5% L-tyrosine diet with 5 µg of thyroxine increased plasma tyrosine concentration more than two fold resulting in more than a 100 fold increase as compared to that of rats fed the basal diet. Thiouracil feeding of the control animals resulted in decrease in plasma threonine, phenylalanine and glutamic acid concentrations and an increase in alanine concentration (Table 3). High tyrosine feeding caused a marked increase in most of the plasma amino acids while the inclusion of thiouracil in such a diet caused a marked decrease in lysine compared to the values for the controls with or without tyrosine. Thyroxine injection of the rats fed the high tyrosine diet caused a marked decrease in plasma serine, glycine, alanine and glutamic acid concentrations. In general there was a marked decrease in total dispensable amino acids and a slight increase in the total indispensable amino acids measured, however, there was a marked decrease in plasma threonine concentration as compared to the high tyrosine feeding alone.

Ingestion of the diet containing 5% of L-tyrosine for two weeks resulted in about a 5-fold increase in liver tyrosine transaminase activity. On the other hand thyroxine injection of rats receiving a high tyrosine diet resulted in an activity more

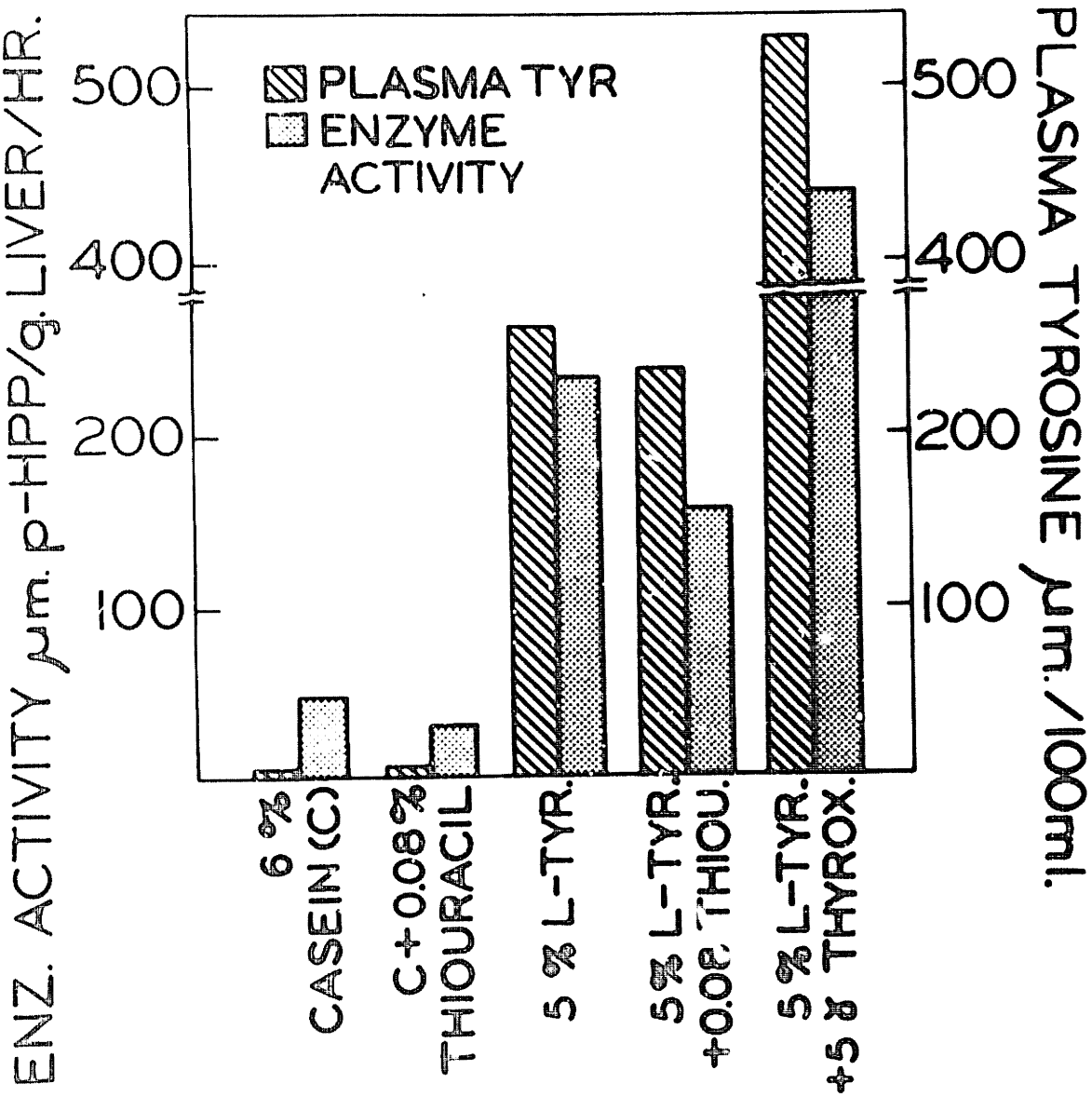
TABLE 3

Effect of thyroxine on plasma amino concentration[1] of rats fed 5% L-Tyr for 2 weeks

Amino acid	Treatment				
	Control	Control + 0.08% Thiouracil	+ 5% L-Tyr	+ 5% L-Tyr + 0.08% Thiouracil	+ 5% L-Tyr + 5 γ Thyroxine
Thr	16.3	7.8	31.2	37.1	17.3
Ser	43.9	46.4	63.5	69.1	28.5
Glu	23.2	12.9	31.2	32.2	17.0
Gly	28.4	55.0	43.4	62.0	17.4
Al	51.9	75.0	83.0	103.3	28.9
Val	11.6	11.6	17.5	13.8	16.4
Isol	7.5	5.7	5.8	8.1	5.7
Leu	8.6	8.5	9.4	13.0	9.4
Tyr	5.1	6.2	261.8	236.0	528.1
Phe	9.4	5.9	11.0	8.8	9.7
Lys	40.3	44.6	46.0	27.9	49.7
Hist	10.3	10.2	7.9	9.5	6.6
Total dispensable amino acids[2]	147.4	189.3	221	266	91.8
Total indispensable amino acids	104.0	94.3	97.6	81.1	114.8

[1] μ moles/100 ml pooled plasma

[2] minus tyrosine value.



ROLE OF THYROXINE AS AFFECTING LIVER TYR. α-K.G. TRANSAMINASE ACTIVITY OF RATS FED 5% L-TYR.

FIGURE III

than double that of the saline injected animals fed the high tyrosine diet. The total activity was more than 10 times the control value. Thiouracil did not cause a change in tyrosine transaminase activity of the rats fed the control diet. Also thiouracil did not cause a significant change in tyrosine transaminase of rats fed the high tyrosine diet.

Effect of thyroxine on rats fed 5% L-tyrosine for 9 days:

This experiment was designed to study the effect of daily injection of thyroxine on liver tyrosine transaminase activity in relation to p-hydroxyphenylpyruvate hydroxylase activity of rats fed the 5% L-tyrosine diet for 2 weeks. However, the experiment was terminated at 9 days, as 2 out of 5 rats injected with thyroxine and fed the high tyrosine diet had died by the end of the first week. Daily thyroxine injection of the rats fed the control diet resulted in decreased liver weight but was without effect on the liver weight of rats fed the high tyrosine diet (Table 4).

Thyroxine injection of the control rats resulted in a slight increase in liver tyrosine transaminase activity. However there was a several fold increase in the latter enzyme activity in rats fed the high tyrosine diet and injected with thyroxine. Thyroxine injection tended to decrease p-hydroxyphenylpyruvate hydroxylase activity of rats fed the high tyrosine diet or the control diet. Expressing the enzyme activity per gram of fresh liver or per 100 g body weight gave the same relations. Thyroxine injection resulted in a marked increase in

TABLE 4

Effect of thyroxine[1] on rats fed 5% L-Tyr for 9 days

Diet	Treatment	Survivals	Enz. activity/g.liver[2]		T.T./pHPPH	Enz.act./100 g.B.wt.[2]		T.T./pHPPH
			T.T.	pHPPH		T.T.	pHPPH	
6% Casein (c)	saline	5/5	21.4 ± 25.7	51.8 ± 8.1	0.41	108 ± 24.1	268 ± 12.7	0.40
"	Thyroxine	5/5	30.9 ± 5.5	43.2 ± 7.8	0.72	128 ± 4.3	181 ± 9.4	0.71
C + 5% L-Tyr	saline	4/5	123.0 ± 23.0	69.6 ± 17.8	1.77	625 ± 29.0	352 ± 14.7	1.78
"	Thyroxine	3/5	170 ± 20.0	53.7 ± 21.7	3.17	941 ± 28.9	298 ± 29.2	3.16

[1] 5γ L-thyroxine daily intraperitoneally.

[2] Both tyrosine-α-ketoglutarate transaminase activity (T.T.) and p-hydroxyphenyl pyruvate hydroxylase (pHPPH) activities were determined by methods of Lin et al. (1958). Activities are expressed as μmole pHPPA formed or consumed per hour respectively. Standard error of the mean is given as percentage of the mean.

the ratio of activities of tyrosine transaminase to the hydroxylase in both the control animals and the animals fed the high tyrosine diet.

Animals fed the high tyrosine diet with or without thyroxine treatment resulted in a generalized decrease in plasma amino acid concentrations (except tyrosine and phenylalanine (Table 5). However, daily injection of the animals fed the control diet did not cause any substantial increase or decrease in plasma amino acid concentrations. Plasma tyrosine values for rats fed the high tyrosine diet with or without thyroxine injection were almost the same.

Effect of pyridoxine on tyrosine toxicity:

Daily injection of the control animals with 5 µg of thyroxine resulted in growth depression during the first two days of the experiment, then the rate of growth paralleled that of the saline injected controls (Fig. IV). On the other hand the addition of 1 g pyridoxine per Kg of diet did not affect the rate of growth at all. However, thyroxine injection of the group fed the diet high in pyridoxine caused a marked growth depression as well as depression in food intake (Table 6). Thyroxine injection of the rats fed the high tyrosine diet aggravated the condition of the animals as shown before, yet excess pyridoxine together with thyroxine treatment aggravated the condition still more. Two rats in this group and one of the animals fed high tyrosine alone died by the end of the first week.

TABLE 5

Effect of thyroxine on plasma amino acid concentration[1] of rats fed 5% L-tyrosine for 9 days

Amino acid	Treatment			
	Control (C)	C + Thyroxine[2]	5% L-Tyr	5% L-Tyr + Thyroxine[2]
Ser	49.6	45.6	28.6	32.4
Glu	14.1	16.5	7.0	7.0
Gly	24.0	17.3	18.3	13.5
Ala	84.1	90.9	36.3	35.5
Val	16.4	16.6	10.7	8.9
Iso	8.8	9.5	4.4	5.1
Leu	10.1	11.3	7.1	5.5
Tyr	4.9	5.5	160.0	164.0
Phe	4.9	6.9	5.0	5.3
Lys	61.1	74.8	46.6	37.6
His	9.9	12.9	6.4	8.1
Arg	9.6	10.6	8.2	7.6
Total dispensable amino acids[3]	171.8	170.3	90.2	89.4
Total indispensable amino acids	120.8	142.6	88.4	78.1

[1] µmole/100 ml plasma. Plasma are pooled from the corresponding rats survived as shown in Table 4.

[2] 5 γ L-thyroxine /0.2 ml injected daily intraperitoneally; other animals were injected with 0.2 ml saline

[3] minus tyrosine value.

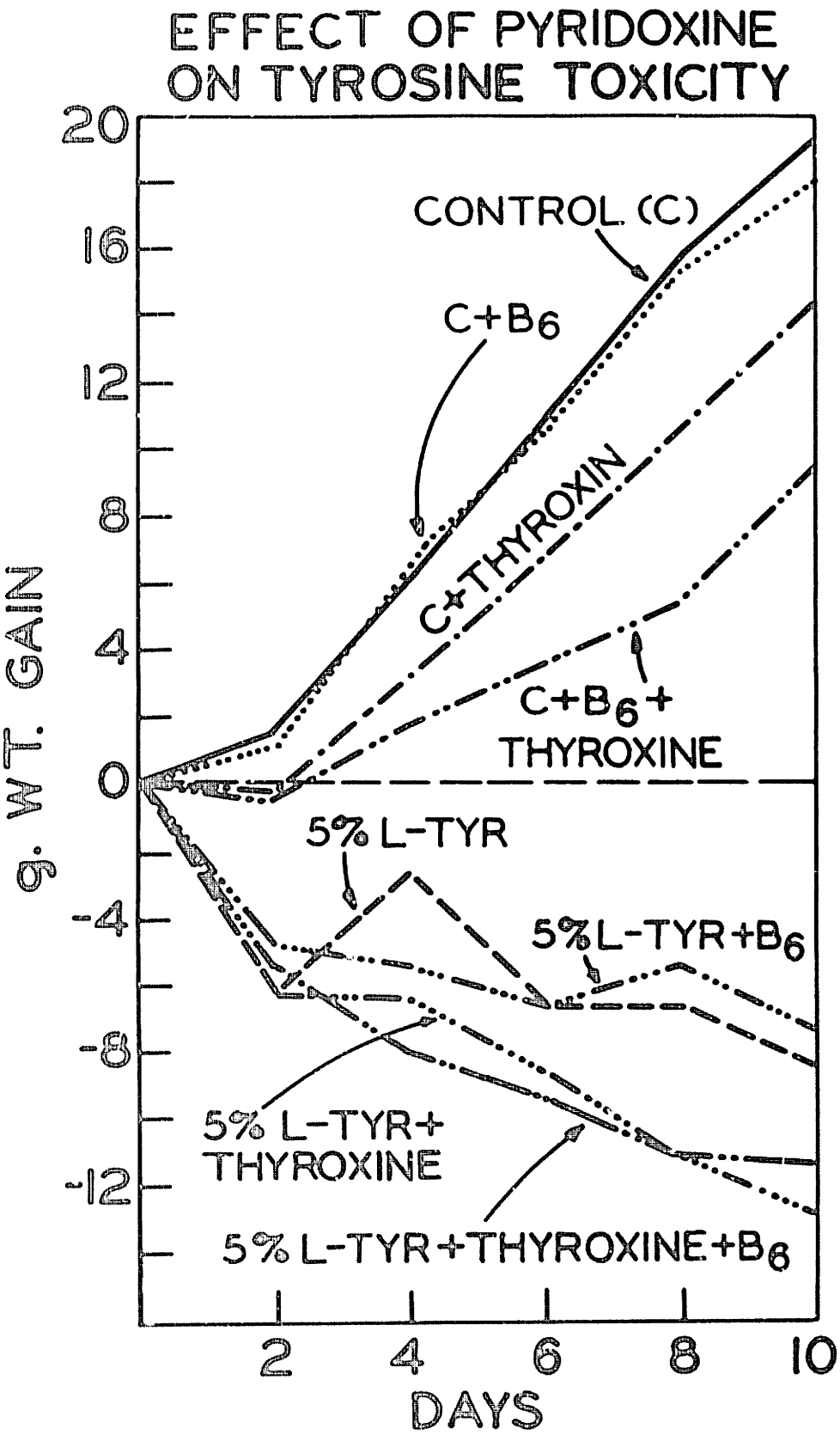

EFFECT OF PYRIDOXINE
ON TYROSINE TOXICITY
20
16
12
8
4
0
-4
-8
-12
g. WT. GAIN
CONTROL (C)
C+B6
C+THYROXIN
C+B6+
THYROXINE
5% L-TYR
5%L-TYR+B6
5% L-TYR+
THYROXINE
5% L-TYR+THYROXINE+B6
2
4
6
8
10
DAYS

FIGURE IV

TABLE 6

Effect of pyridoxine[1] on rats fed 5% L-tyrosine and injected with thyroxine[2] for 10 days

Diet	Treatment	Food intake	Weight gain[3]	Survivals
		g/10 d		
6% casein (+ 02% L-Met)	saline	82.0	19.2	5/5
"	saline, B_6	79.8	18.0	5/5
"	L-thyroxine	86.1	14.4	5/5
"	" , B_6	68.8	9.4	5/5
6% casein + 5% L-Tyr	saline	39.8	- 8.5	4/5
"	saline, B_6	42.6	- 7.3	5/5
"	thyroxine	42.5	-13.0	5/5
"	" , B_6	38.1	-11.4	3/5

[1]Pyridoxine was added to the diet as 1 g/Kg diet.

[2]5 γ L-thyroxine /0.2 ml injected daily intraperitoneally, other rats were injected with 0.2 ml saline.

[3]Average of 5 rats or less per group (Sprague-Dawley rats).

Discussion

It is obvious that administration of thyroxine to rats fed diets high in tyrosine aggravated the toxicity symptoms, i.e., greater body weight loss, earlier onset of more severe pathological lesions, and decrease in the survival time of the rat. On the other hand, the addition of thiouracil to the high tyrosine diet improved the rate of growth of the rats fed 3% or 5% L-tyrosine diets (Figures I and II). Also no rats receiving thiouracil died during the experiments and the external lesions were mild. The addition of 0.08% thiouracil to the control diet depressed growth and food intake. Leathem and Seeley (1947) found that the addition of 0.5% thiouracil to a diet fed to young rats depressed the growth of the animals. They interpreted their growth findings as due to a voluntary reduction of food intake subsequent to a lowered metabolic efficiency induced by thyroxine deficiency.

Addition of excess pyridoxine to the high tyrosine diet did not affect the development of the toxicity symptoms to any extent. However, when thyroxine and excess pyridoxine were administered together to either the control animals or the animals fed the high tyrosine diet growth and food intake were depressed.

Thyroid status of the rats, i.e., hypo- or hyperthyroid, clearly influenced plasma tyrosine concentration as well as the concentrations of some other amino acids especially in rats fed high tyrosine diet. Rats fed the high tyrosine diet and injected

with thyroxine for 9 days had a plasma tyrosine concentration as high as the non-treated rats fed the high tyrosine diet (Table 5). However, in the 14-day experiment there was a marked increase in plasma tyrosine concentration of rats injected with thyroxine and fed the high tyrosine diet (Table 3). The difference in response may be due to the condition of the animals deteriorating between 9 and 14 days, i.e., severe body weight loss as well as aggravation of the external lesions. Also, the total dispensable plasma amino acids of rats fed the high tyrosine diet and injected with thyroxine for 9 or 14 days were much less than those of the animals fed the high tyrosine diet alone. Thyroxine is well known to increase the metabolic rate of animals, so this together with decreased food intake might lead to increased utilization of the dispensable amino acids as a source of energy. In favor of this view is the increased plasma dispensable amino acids of rats fed thiouracil with or without tyrosine (Table 3).

The comparatively low plasma tyrosine concentration of the rats injected with thyroxine and fed the high tyrosine diet for 9 days (Table 5) (as compared with the respective value at 14 days, Table 3) was accompanied by comparatively low tyrosine transaminase activity (as compared with the respective value at 14 days). Animals fed the high tyrosine diet alone showed the same picture with respect to the relative concentrations of enzyme activity and plasma tyrosine concentration. Such changes in plasma should reflect corresponding changes in the amino acid pools in the liver which can exert a regulatory role in enzyme induction.

One might expect high enzyme activity to be accompanied by lowered plasma amino acid level or high tyrosine level with low enzyme activity. It has been observed that thyrotoxicosis in humans (Levine et al., 1962) as well as in rats (Rivlin and Levine, 1963; Rivlin and Kaufman, 1965) results in increased plasma tyrosine concentration. Rivlin and Levine (1963) have shown that thyroxine administration to rats for 4 weeks increased the activity of tyrosine transaminase by 40% and that thyroidectomy reduced it by 50%. Later Rivlin and Kaufman (1965) found that when rats were made hyperthyroid by thyroxine administration the volume of distribution of tyrosine was reduced when compared to that of euthyroid rats. However, the basis for this change was not established. This was the explanation given by these authors to explain the increased plasma tyrosine concentration in thyrotoxicosis. Another possibility is that because of the increased tyrosine transaminase activity as well as the probable high plasma p-hydroxyphenylpyruvic acid with high tyrosine feeding, the latter could be transaminated back to tyrosine and hence increase the plasma tyrosine concentration under thyroxine treatment.

The enzyme activity values varied widely because of differences in the condition of the animals, especially those fed the high tyrosine diet with or without thyroxine injection. Also the number of rats was small because some died during the experiment. Thus the standard error of the mean is quite large, however, the trends among the groups is quite clear.

From table 4 one can notice that thyroxine injection decreased the activity of p-hydroxyphenylpyruvate hydroxylase in liver whether on a per gram liver basis or per 100 g. body weight basis. Under these circumstances it is more meaningful to express the data per unit body weight as this represents the physiological capacity of the animal to metabolize tyrosine. It seems then that the p-hydroxyphenylpyruvate hydroxylase step may become limiting in the catabolism of excess tyrosine. This is in agreement with the observations made by Litwack (1964) who found that feeding a diet containing 2% thyroid powder to rats decreased the activity of p-hydroxyphenylpyruvate hydroxylase severely.

Such limitation in tyrosine catabolism will result in a gradual buildup of tyrosine in plasma and possibly other tissues. It was observed during these investigations that whenever plasma tyrosine concentration was high the tyrosine transaminase activity was high, however, there is not a direct relationship between the two parameters. An explanation can be proposed that the high tyrosine level may act to induce the tyrosine transaminase enzyme in two ways (either separately or collectively). Firstly, it may act to increase adrenocortical hormone production. Munro et al. (1963, 1965) have shown that the ingestion of leucine, methionine and glycine can result in an increased output of corticostrone by the adrenals. This may induce tyrosine transaminase as described earlier. Secondly: a direct tyrosine effect on the enzyme, i.e., substrate-type induction, which is quite possible especially under our experimental

conditions as these rats were less than two months old, the age at which substrate induction of this enzyme can be most readily shown (c.f. Knox et al., 1964). Another possibility is that thyroxine administration may stimulate adrenal output and hence induce more the tyrosine transaminase (D'Angelo and Grodin, 1964).

Investigation of the plasma corticosterone level as well as the excretion of its metabolites in urine under these experimental conditions will be needed to clarify this problem.

It seems that the deleterious effect due to thyroxine injection of rats fed the high tyrosine diet might be mediated through increased tyrosine concentration in plasmas and probably in other tissues as well.

SECTION V

EFFECT OF FORCE-FEEDING A HIGH TYROSINE DIET AND SUBSTITUTING p-HYDROXYPHENYLPYRUVIC ACID FOR TYROSINE IN A HIGH TYROSINE DIET

Introduction

In the previous experiments the food intake of rats fed a low protein diet (6% casein + 0.2% L-methionine) was always depressed when the tyrosine content of the diet was greatly increased. The growth depression of the rats fed low protein diets in which there is an amino acid imbalance is accompanied by low food intake; however, when their food intake is stimulated by injecting them with insulin they grow well (Kumta and Harper, 1961). Klein et al. (1962) also induced higher voluntary food intake and growth of rats fed an imbalanced diet by exposing them to a cold environment. It was thought that excess tyrosine may increase the requirement for a specific amino acid especially when it was found that threonine supplementation alleviated the symptoms of tyrosine toxicity (Alam et al., 1966-a) and the question arose as to whether or not the toxicity might be alleviated if the food intake of rats fed a high tyrosine diet were increased.

In the experiments described in this section the effect of force-feeding rats a high tyrosine diet was examined. Also the effect of replacing tyrosine in the high tyrosine diet with p-hydroxyphenylpyruvic acid was studied. Growth, food intake, plasma amino acid concentrations as well as the activities of tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase were measured.

Experimental

Effect of substituting p-hydroxyphenylpyruvic acid for tyrosine in the diet:

Rats (see P. 34 for specifications) were fed the basal diet (6% casein + 0.2% L-methionine) for 2 days. They were then divided into 3 groups of 4 rats each and fed the basal, the 5% L-tyrosine or the 5% p-hydroxyphenylpyruvic acid diet for either 10 or 14 days. Weights were recorded and group food intakes were corrected for spillage.

Effect of force-feeding rats 5% L-tyrosine diet:

In this experiment, the procedure used by Sidransky and Farber (1958) was followed. The basal diet was made in liquid form to contain 0.8 g dry diet per ml. Rats 50-55 g similar to those used before were given this diet by stomach tube for 2 days as a period of adjustment. They were given 6 ml of this diet in three meals at 9 a.m., 3 p.m. and 9 p.m. Animals were then divided into 2 groups of 7 rats each. They were fed the basal diet or the 5% L-tyrosine diet in liquid form at the concentration mentioned but the dose was divided into 2 equal meals, one at 9 a.m. and another at 9 p.m. The first day, both groups were fed 7 ml of the liquid diet per rat per day. This was increased on the 2nd and 3rd day to 8 ml. On the 4th-6th days, the dose was increased to 9 ml and on the 7th and 8th days the dose given was 9.5 ml per day. Concurrently a group of 5 rats was fed the high tyrosine diet ad libitum so it was possible

to compare the growth of this group with that of the force fed group.

At the end of each experimental period the rats were killed and blood was obtained by heart puncture. Plasma amino acid concentrations were measured using the Technicon amino acid analyzer. Liver tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase activities were measured using the methods described before (P. 65 , P. 83).

Results

Effect of feeding p-hydroxyphenylpyruvic acid:

Animals fed the 5% L-tyrosine diet ad libitum lost weight during the two-week experimental period. They showed typical toxicity symptoms and one rat died on the 9th day. On the other hand, animals fed the diet containing 5% p-hydroxyphenylpyruvic acid instead of 5% L-tyrosine grew slowly (Fig. I) and not one developed external lesions. All looked active and healthy. Food intake of rats fed the high tyrosine diet was depressed and that of the animals fed 5% p-hydroxyphenylpyruvic acid was also depressed but not as much (Table 1).

Plasma tyrosine concentration was increased 34 fold due to high tyrosine feeding. However, feeding 5% p-hydroxyphenyl-pyruvic acid also resulted in increased plasma tyrosine level but to a lesser degree (about 11 fold increase) as shown in Table 1.

When the previous experiment was repeated for 10 days exactly the same growth-pattern was observed (Fig. I-b).

High tyrosine feeding caused about a 3-fold increase in tyrosine transaminase activity (Table 2). Feeding 5% p-hydroxyphenylpyruvic acid resulted in enzyme activity about twice that of the controls. The latter values, when calculated per 100 g body weight, were significantly different from those for the control group at the 5% and 1% levels respectively. p-hydroxyphenylpyruvate hydroxylase activity was not affected by any treatment when calculated per 100 g body weight. However,

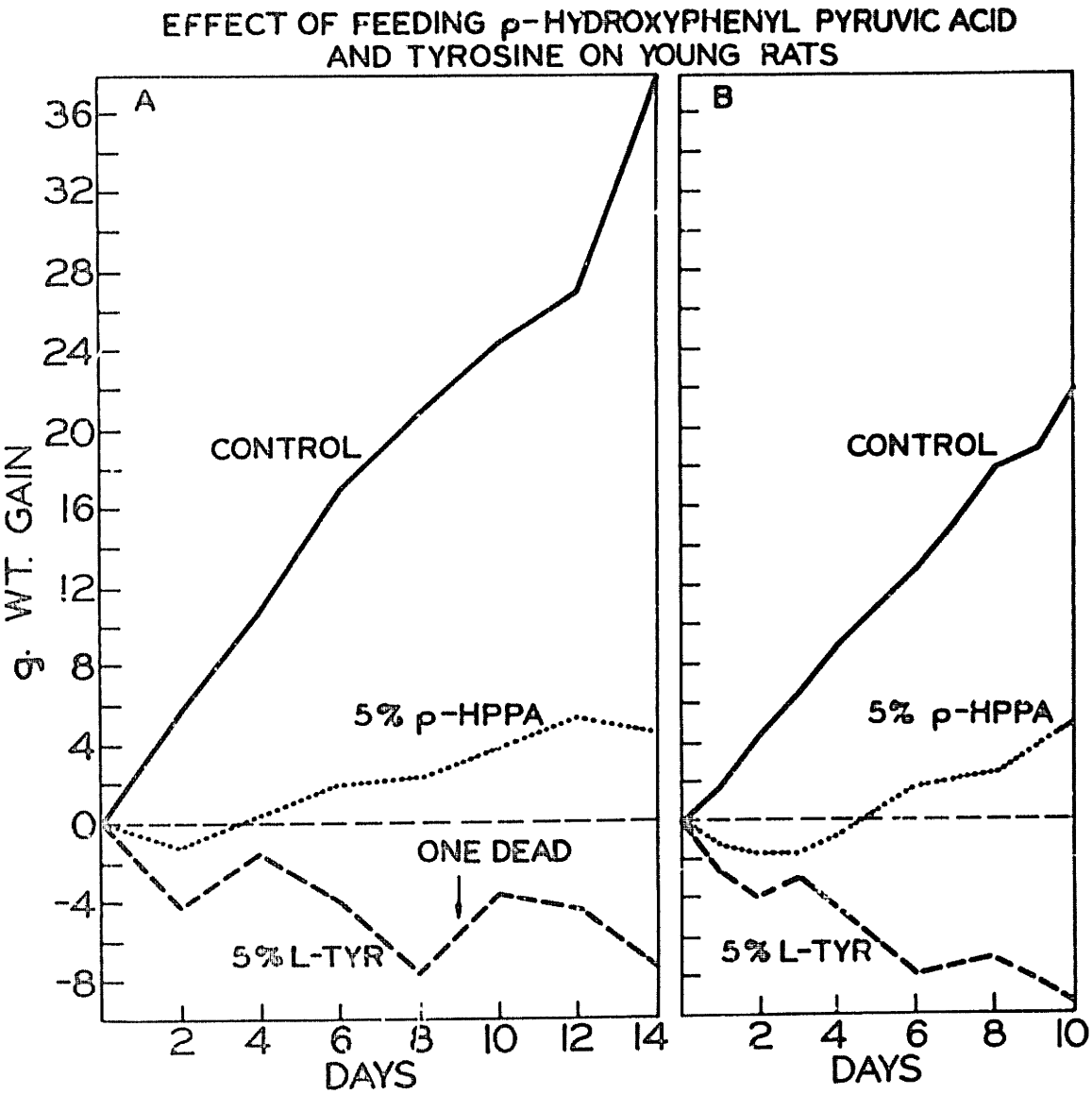

EFFECT OF FEEDING p-HYDROXYPHENYL PYRUVIC ACID
AND TYROSINE ON YOUNG RATS
A
B
g. WT. GAIN
36
32
28
24
20
16
12
8
4
0
-4
-8
CONTROL
5% p-HPPA
ONE DEAD
5% L-TYR
2 4 6 8 10 12 14
DAYS
CONTROL
5% p-HPPA
5% L-TYR
2 4 6 8 10
DAYS

FIGURE I

TABLE 1

Effect of feeding[1] 5% L-tyrosine or 5% p-hydroxyphenyl-pyruvic acid (p-HPPA) on young rats (Two Weeks)

Diet	Symptoms	Food consumed	Weight gain[2]	Plasma Tyr
		g	g	µmole/ 100 ml
Basal	0/4	135.2	37.8 ± 3	6.0
Basal + 5% L-Tyr	4/4	59.5	-7.4 ± 1	207.9
Basal + 5% p-HPPA	0/4	79.0	4.5 ± 1	66.6

[1]Two weeks' period.

[2]Average/rat ± standard error of the mean.

TABLE 2

Effect of feeding 5% p-hydroxyphenyl pyruvic acid (p-HPPA) or 5% L-tyrosine on young rats (10 days)

	Food intake g/rat/10 days	Weight gain g/rat/10 days	Liver weight g/100 g B.Wt.	Enz. activity/g. liver		Enz. act./100 g.B.wt.	
				T.T.[2]	p-HPPH [3]	T.T.[2]	p-HPPH [3]
Control	88.4	22 ± 3	5.91 ± 0.4	25.8 ± 2.9	56.3 ± 7.7	152 ± 17	333 ± 35
5% p-HPPA	52.9	5.2 ± 1	3.78 ± 0.2	65.7 ± 7.2	76.0 ± 4.4	248 ± 37	287 ± 26
5% L-Tyr	39.4	-9.2 ± 1	5.97 ± 0.1	74.9 ± 5.3	48.4 ± 7.3	447 ± 18	289 ± 38

[1]Average/rat ± standard error of the mean.

[2]Tyrosine-α-ketoglutarate transaminase activity as μmole p-hydroxyphenylpyruvic acid formed per hour.

[3]p-hydroxyphenylpyruvate hydroxylase activity as μ mole p-hydroxyphenylpyruvic acid consumed per hour.

when this enzyme activity was calculated as units per g liver the value for the rats fed the keto acid was significantly different from that for the controls as well as from that for the high-tyrosine-fed group (at the level of 5% and 2% respectively).

From Table 3 it is also clear that not only the feeding of the high tyrosine diet caused a marked increase in plasma tyrosine level, but also the feeding of 5% p-hydroxyphenylpyruvic acid caused an increase in this amino acid concentration. The increase for the latter group was much less than that for the high tyrosine group. High tyrosine feeding for 10 days caused a lowering in concentration of most of the amino acids especially glutamic acid, alanine, the branched-chain amino acids, phenylalanine and histidine. Also the keto acid feeding resulted in a decrease in the concentration of the branched chain amino acids while the rest of the amino acids (except tyrosine) were slightly higher or lower than the corresponding values for the animals fed the control diet.

Effect of force-feeding the 5% L-tyrosine diet:

Fig. II shows how the type of feeding affects the response of rats. Ad-libitum feeding of the high tyrosine diet caused a severe body weight loss while tube-feeding this diet supported some weight gain but much less than that for the control. Two out of 7 rats tube-fed the high tyrosine diet died, not because of tube feeding, as they survived for hours after being fed. No rats tube-fed the basal diet died during the 8-day

TABLE 3

Effect of feeding 5% p-hydroxyphenyl pyruvic acid (p-HPPA) or 5% L-Tyr for 10 days on plasma amino acids

Amino acid	Plasma a.a. concn. µmole/100 ml[1]		
	Control	5% L-Tyr	5% p-HPPA
Ser	35.2	24.0	39.9
Glu	12.0	8.1	11.5
Gly	18.1	15.0	21.7
Al	99.8	53.8	82.9
Val	16.6	9.9	10.9
Iso	7.0	3.9	5.1
Leu	12.5	7.1	8.4
Tyr	8.7	170.0	113.0
Phe	7.9	3.9	6.5
Lys	56.7	45.9	64.1
His	12.3	5.7	13.1
Arg	9.6	8.3	10.0

[1]Pooled plasma from 4 rats/group.

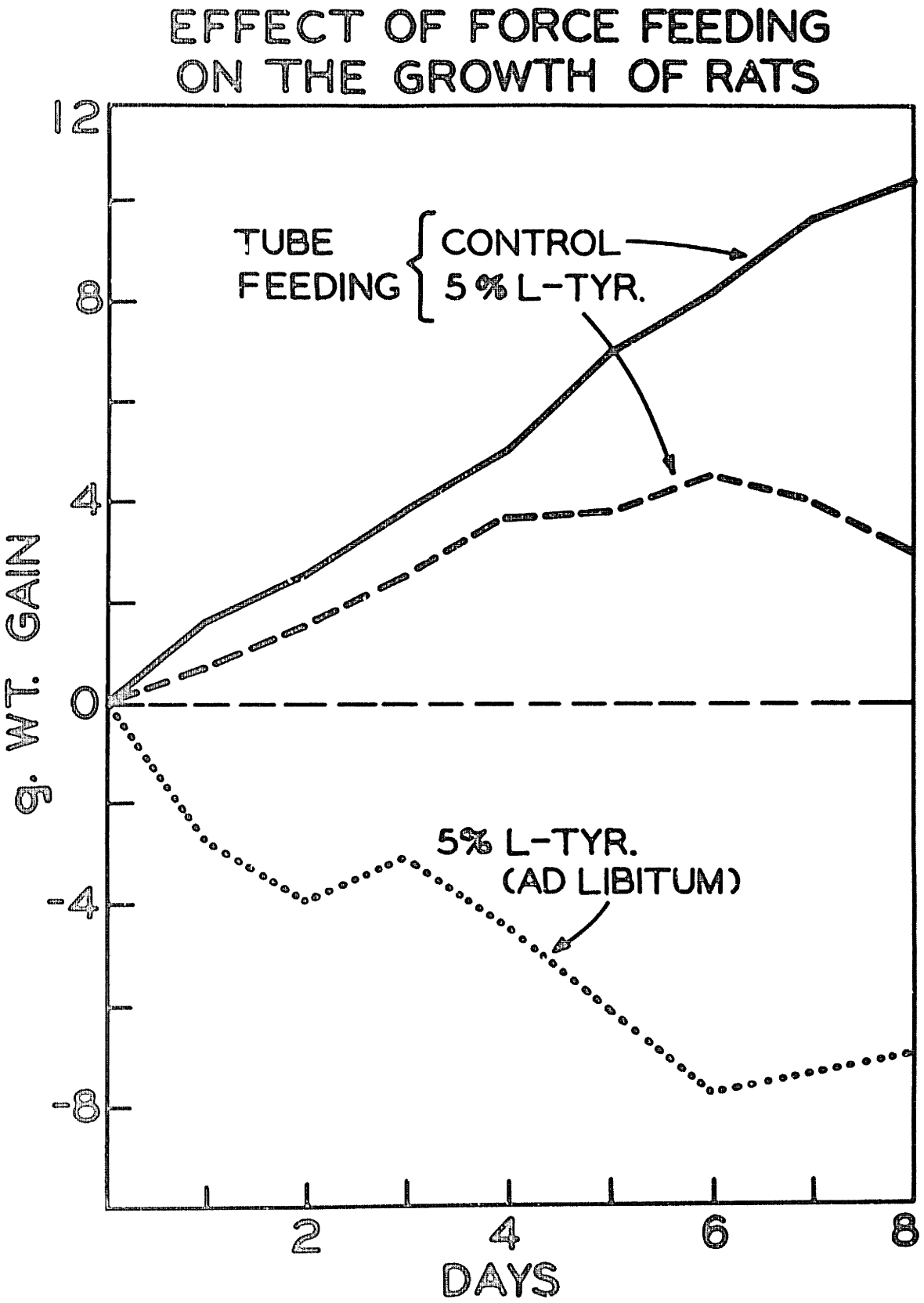

EFFECT OF FORCE FEEDING
ON THE GROWTH OF RATS
12
8
4
0
-4
-8
g. WT. GAIN
TUBE FEEDING
CONTROL
5% L-TYR.
5% L-TYR.
(AD LIBITUM)
2
4
6
8
DAYS

FIGURE II

experimental period. The deaths of the animals tube-fed the high tyrosine diet were sudden, i.e., mild symptoms appeared first and within 24 hours the animals died.

Force-feeding the high tyrosine diet caused a 4-fold increase in tyrosine transaminase activity (Table 4). However, p-hydroxyphenylpyruvate hydroxylase activity was not significantly affected.

TABLE 4

Effect of force feeding 5% L-tyrosine for 8 days on young rats

	Weight gain	Liver weight g/10 g B. wt.	Enz. activity/g liver		Enz. act./100g B. wt.	
			T.T.[3]	p-HPPH[4]	T.T.[3]	p-HPPH[4]
Control[1]	10.4 ± 1	4.39 ± 0.1	20.2 ± 2.3	75.7 ± 13.9	88 ± 9.3	332 ± 54
5% L-Tyr[2]	3.1 ± [illegible]	4.71 ± 0.7	85.2 ± 15	60.1 ± 7.6	400 ± 67	281 ± 32

[1]Average of 6 rats ± standard error of the mean.

[2]Average of 4 rats ± standard error of the mean.

[3]Tyrosine-α-ketoglutarate transaminase activity as μmole p-hydroxyphenylpyruvic acid formed per hour.

[4]p-hydroxyphenylpyruvate hydroxylase activity as μmole p-hydroxyphenylpyruvic acid consumed per hour.

Discussion

P-hydroxylphenylpyruvic acid is the product of the transamination reaction between tyrosine and α-ketoglutaric acid which is catalyzed by the enzyme tyrosine transaminase. The observations that excessive tyrosine intake always resulted in substantial increases in this enzyme activity, led to the idea that p-hydroxyphenylpyruvic acid might be the toxic agent in rats fed high tyrosine diets. These experiments illustrate quite clearly that this is not the case. It is true that there was growth depression when the keto acid was fed yet external lesions were not observed at all.

There are two aspects of the enzyme activities reported here depending on the way the data are presented. Under these experimental conditions it is preferable to consider the data per 100 g body weight rather than per g liver, as the former represents the total capacity of the animal and should be related to the performance of the animal as a whole. Feeding the keto acid resulted in a significant difference in tyrosine transaminase as compared to feeding the basal diet or the high tyrosine diet, while p-hydroxyphenylpyruvate hydroxylase activity was not significantly different from values for the other two groups. Liver tyrosine transaminase was significantly increased in rats fed the keto acid when compared to the controls, while it was not significantly different from that of the animals fed the high tyrosine diet. This would imply then that the marked decrease in liver weight of rats fed the keto acid was due to shrinkage

or a mere concentration of the liver cells with respect to p-hydroxyphenylpyruvate hydroxylase activity. (As a matter of fact, when Zannoni and LaDu (1960) injected the keto acid intraperitoneally in guinea pigs they observed no change in this enzyme activity at one hour after injection). Also it would imply that there was an increase in tyrosine transaminase activity (de-novo) after excessive tyrosine/keto acid intake.

The tyrosine transaminase reaction is a reversible one and with increased p-hydroxyphenylpyruvic acid in the body fluids this would probably result in the formation of tyrosine. This may explain the increased plasma tyrosine concentration of rats fed the keto acid. Increased plasma tyrosine level should in turn induce the tyrosine transaminase in a manner as suggested before and may explain the significant increase in tyrosine transaminase of rats fed the excess keto acid whether the activity is calculated per g liver or per 100 g body weight.

The results obtained in the force-feeding experiment provide good evidence that we are dealing with a toxicity condition and not with an imbalance or deficiency. The sudden deaths of the animals tube-fed the high tyrosine diet in spite of the improved weight gain as compared with the group fed ad libitum illustrates clearly that the severe body weight loss is not solely due to depressed voluntary food intake. Also the animals force-fed the high tyrosine diet still showed a biochemical picture similar to that under ad-libitum feeding with respect to the enzyme levels in the liver.

SECTION VI

ROLE OF CORTISOL IN TYROSINE TOXICITY

Introduction

Shewell and Long (1956) classified animals, on the basis of their response to adrenal glucocorticoid treatment, into two groups according to their ability to maintain body weight and γ-globulin. Cortisone-sensitive animals, e.g. mice, rat and rabbit, lost weight and plasma γ-globulin decreased when they were treated with cortisone while cortisone-insensitive animals, e.g. monkey and guinea pig, did not show these effects.

Treatment with glucocorticoids evokes rapid induction of tryptophan pyrrolase and tyrosine transaminase (Goldstein et al., 1962; Rosen and Milholland, 1963) as well as some other gluconeogenic enzymes e.g. Glucose-6-phosphatase and fructose 1-6 diphosphatase (Webber et al., 1965). Very recently, Rosen and Milholland (1967) reported that marked increases in amino acid uptake preceded the induction of tyrosine transaminase. They suggested that changes in amino acid pools in liver may act to regulate the enzyme activity in the liver. On the other hand, Kostyo and Redmond (1966) showed that the addition of corticosterone to the incubation mixture suppressed the uptake of amino acids by muscle tissue thus interfering with the synthesis of proteins. They postulated that such inhibition will decrease the production of labile protein elements necessary to maintain the functional integrity of the membrane process. So glucocorticoids - in general - are catabolic in muscle and anabolic in liver.

It had been found earlier that cortisol injection of rats fed a diet containing 3% of L-tyrosine alleviated the pathological lesions due to excessive tyrosine intake. The following investigation was undertaken in an attempt to learn how cortisol exerted its beneficial effect.

Experimental

Holtzman albino male rats weighing 50-55 g were fed the basal diet for 2 days. They were then divided into groups of 5 rats each and fed the basal diet or the 5% L-tyrosine diet in the agar-gel form as described before (p.34). In one experiment rats were injected intraperitoneally with 4 mg of hydrocortisone semisuccinate-sodium salt (from Mann Research Laboratories, N.Y.) in 0.2 ml physiological saline or the physiological saline alone between 4-5 p.m. daily. In another experiment the animals were injected with cortisol acetate (Hydrocortone acetate, Merck Sharp and Dohme, West Point, Pa.) in 0.2 ml physiological saline in the same manner as described above. Corresponding control groups i.e. fed either the basal diet or the high tyrosine diet were injected with 0.2 ml physiological saline only. Body weight gain was recorded either daily or each other day and group food intake was corrected for the amount of food spilled.

At different time intervals (5, 10 and 15 days) groups of rats were killed under anesthesia; blood was obtained by heart puncture and livers were removed and kept at -20°C until assayed for enzyme activity. Tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase activities were measured according to the methods of Lin et al. (1958) which were described before (p. 65,83).

Oxidation studies of labelled tyrosine:

Six glass metabolic cages were available in our laboratory so it was only possible to run two rats for each treatment simultaneously, i.e., 2 rats fed the control diet, 2 rats fed the 5% L-tyrosine diet and 2 rats fed the high tyrosine diet but injected with 2 mg of hydrocortisone acetate. Two groups of male albino rats were obtained with one group received 2 days after the other. When received, both were the same age and weighed 50-55 g. They were all kept on the basal diet (6% casein + 0.2% L-methionine) for 3 days and were then divided into groups of 3 rats each and assigned to each treatment at random. The test diets were prepared in the agar-gel form. The control animals as well as those fed the high tyrosine diet were injected intraperitoneally with 0.2 ml physiological saline at 4-5 p.m. each day. Another group fed the high tyrosine diet was similarly injected with 2 mg hydrocortisone acetate.

On the night before the day the oxidation study was carried out, food was withdrawn at 12 o'clock midnight. At 9 a.m. in the following morning, the control animals, the animals fed the high tyrosine diet with or without cortisol injection, were each tube-fed the same amount of diet which consisted of 1.0 g L-tyrosine, 1.5 g of the basal diet (powdered form), 2.5 ml water and 6.5 uc L-tyrosine-1-C^{14} of specific activity 19.1 millicuries per millimole, i.e., 0.95 mg per 100 uc (obtained from New England Nuclear, Boston, Mass.). Immediately after the diets had been intubated the animals receiving the basal and those receiving the high tyrosine diets were injected with 0.2

ml physiological saline. The third group of rats receiving the high tyrosine diet and regularly injected with cortisol was injected with 2 mg of hydrocortisone acetate. Rats were then housed individually in the glass metabolic cages. Dry carbon-dioxide-free air was drawn through the cages at a rate of about 500 cc per minute. Water was provided in the metabolic cages. Urine and feces were collected separately.

Carbon dioxide was collected at hourly intervals for the first 8 hours in 15 ml of a mixture of ethanolamine and methyl-cellosolve (1:2,V/V)(Jeffay and Alvarez, 1961). Thereafter carbon dioxide was collected for two periods of 2 hours each and then for 2 periods of 6 hours each using 25 ml and 60 ml of the trapping mixture respectively.

At the end of the 24-hour experimental period, rats were removed from cages and anesthetized with ether. The gastro-intestinal tract was removed, the contents were washed out and combined with the fecal mass excreted during the experimental period. The combined mass was then homogenized and made into 100 ml volume.

Measurement of radioactivity:

A Packard Tri-Carb liquid scintillation spectrometer model 2211 was used throughout this investigation. The automatic external standardization technique was used to calculate the efficiency of counting for each sample and the samples were corrected for background.

Radioactivity in carbon dioxide expired was measured by transferring 3 ml of the 2-hydroxyethyl ammonium N-(2-hydroxyethyl) carbamate methyl cellosolve to a counting vial. 15 ml of an organic scintillator composed of: 2 volumes, toluene; 1 volume, methylcellosolve, and 5.5 g/L 2,5 diphenyloxazol (PPO) (Jeffay and Alvarez, 1961) was then added.

Radioactivity in urine:

The collected urine was made to 100 ml volume, centrifuged and 2 ml aliquot was placed in a counting vial. 15 ml of an aqueous scintillator solution composed of: 1 volume, xylene; 3 volumes dioxane; 3 volumes, methyl cellosolve; 10 g/L PPO: 0.5 g/L of 1,4 bis-2(5 phenyloxazol) benzene (POPOP) and 8.0% naphthalene (Bruno and Christian, 1961) was added.

Radioactivity in feces and gastrointestinal tract homogenate:

4 ml of the homogenate were placed in a 100 ml round bottom flask and evaporated to dryness at 40°C using a flash evaporator. The residue was oxidized with Van Slyke oxidizing agent composed of: 50 g, chromium trioxide; 5 g, potassium iodate; 167 ml, phosphoric acid 85%, and 334 ml of 20% fuming sulfuric acid. The technique followed was that described by Jeffay and Alvarez (1961). 15 ml of the oxidizing mixture were used for each determination. The carbon dioxide formed was trapped in 50 ml of ethanolamine-methylcellosolve mixture (1:2, V/V). 3 ml of solution were counted by the same procedure described for measuring radioactivity in carbon dioxide.

Liver and Plasma tyrosine concentration at 0 time and 3 hours after tube-feeding the tyrosine load:

It was of interest to know the tyrosine concentration in plasma in relation to that in liver of animals at the time when they were given the tyrosine load as well as 3 hours later. The experimental conditions were almost the same as mentioned under "Oxidation studies. . .". Paired groups of 4 rats each were fed the control diet (6% casein + 0.2% L-methionine) and injected with either 0.2 ml of the saline solution or with 0.2 ml saline solution that contained 2 mg cortisol acetate. Rats were kept under treatment for 5 days. They were also fasted for 9 hours (12 midnight until 9 a.m.). At 9 a.m. (zero time) representative groups were tube-fed the tyrosine load and treated the same way as mentioned before. The rest of the animals (zero time) were killed; blood was drawn by heart puncture and liver was removed and kept frozen at -20°C. Rats that were tube-fed were killed after 3 hours and treated similarly to those at zero time.

25% liver homogenate was prepared from each liver sample. Equal volumes of these homogenates were pooled and proteins were precipitated by the addition of 15% sulfosalicylic acid (0.25 ml acid to every 1 ml homogenate). Similarly plasma proteins were precipitated by the same procedure. The protein precipitates were removed by centrifugation at about 25,000 xg for 30 minutes. Amino acids in the supernates were determined by the Technicon amino acid analyzer.

Results

Figure I shows that the daily injection of rats fed the diet high in tyrosine, with cortisol semisuccinate did not alter the rate of body weight loss. Also the rats showed external pathological lesions characteristic of tyrosine toxicity. When the control animals were injected with the semisuccinate, the rate of growth was depressed. This form of cortisol did not affect the activity of either tyrosine transaminase or of p-hydroxyphenylpyruvate hydroxylase to any considerable extent (Table 1).

The daily injection of rats fed the high tyrosine diet with cortisol acetate alleviated almost completely the external lesions except that 2 rats showed mild symptoms by the 13th day of the experimental period. Also the severe body weight loss was substantially prevented (Fig. II) Cortisol acetate injection arrested the growth of rats fed the control diet. This was accompanied by a depressed food intake. In contrast, cortisol acetate injection improved the food intake of the rats fed the high tyrosine diet (Table 2).

Table 3 shows that cortisol injection increased the liver weight of the controls as well as of the rats fed the high tyrosine diet. The injection of rats fed the control diet with hydrocortisone acetate for 5 days caused more than a 3-fold increase in liver tyrosine transaminase activity. On the other hand, such treatment did not further elevate the enzyme activity of rats fed the high tyrosine diet for 5 days (Table 4).

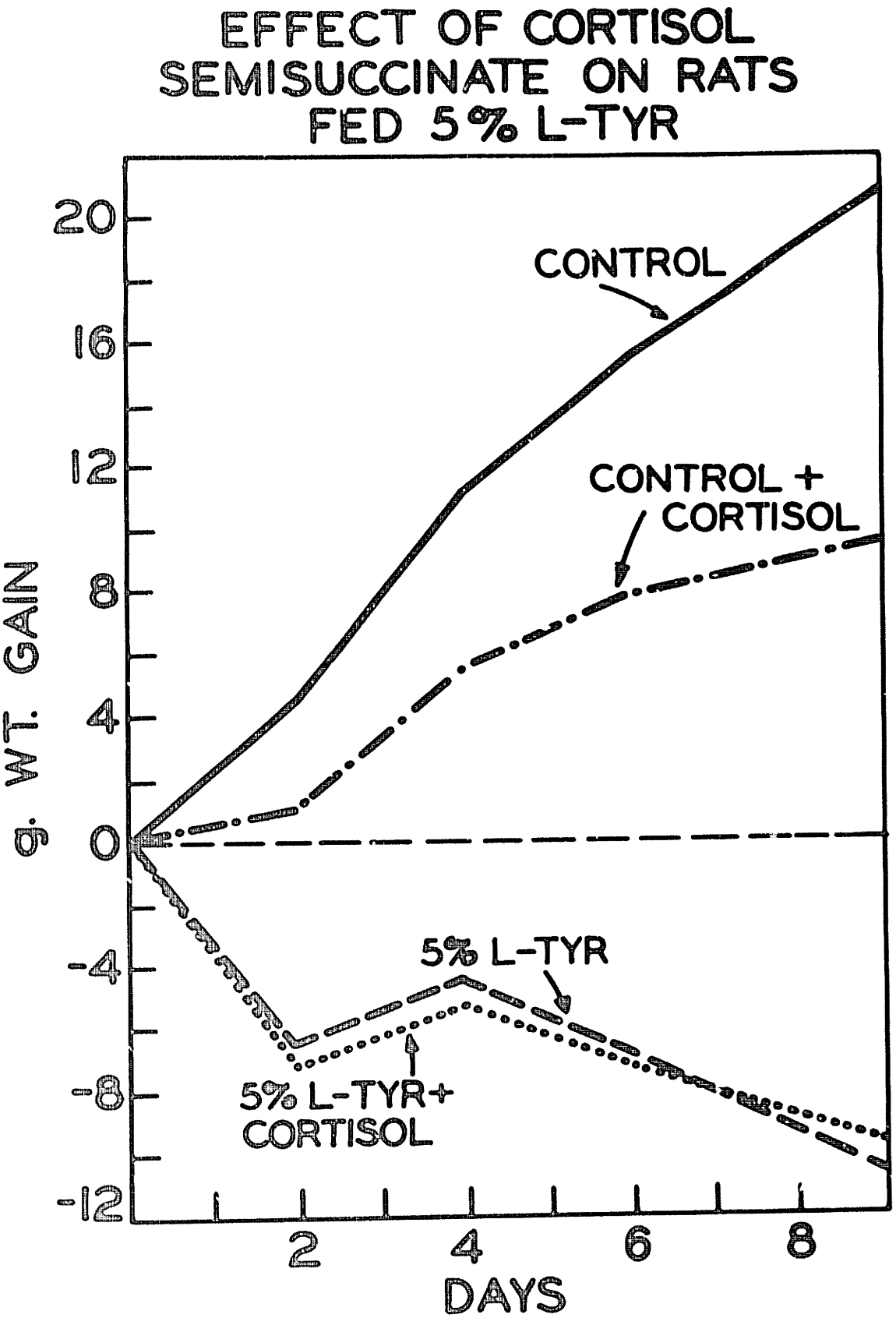

EFFECT OF CORTISOL SEMISUCCINATE ON RATS FED 5% L-TYR
20
16
12
8
4
0
-4
-8
-12
g. WT. GAIN
CONTROL
CONTROL + CORTISOL
5% L-TYR
5% L-TYR+ CORTISOL
2
4
6
8
DAYS

FIGURE I

TABLE 1

Effect of cortisol semisuccinate on liver enzyme activities of rats fed 5% L-tyrosine for 9 days

Diet	Treatment	Enz. activity/g liver		Enz. activity/100 g B. wt.	
		T.T.	p-HPPH[3]	T.T.	p-HPPH
Basal	saline	21.4 ± 6.4	51.8 ± 4.2	109 ± 25.6	268 ± 34.5
"	cortisol[1]	26.0 ± 4.0	57.5 ± 6.0	105 ± 17.7	234 ± 30.8
Basal + 5% L-Tyr.	saline	123.0 ± 23.0	69.6 ± 17.8	625 ± 29.0	352 ± 14.7
" + "	cortisol	117.9 ± 17.6	68.1 ± 4.5	614 ± 96	350 ± 20.4

[1]4 mg cortisol semisuccinate daily intraperitoneally.

[2,3]Tyrosine-α-ketoglutarate transaminase (T.T.) and p-hydroxyphenylpyruvate hydroxylase (p-HPPH) activities as μmole p-hydroxyphenylpyruvic acid formed or consumed per hour ± standard error of the mean.

EFFECT OF CORTISOL ACETATE ON GROWTH OF RATS FED 5% L-TYR

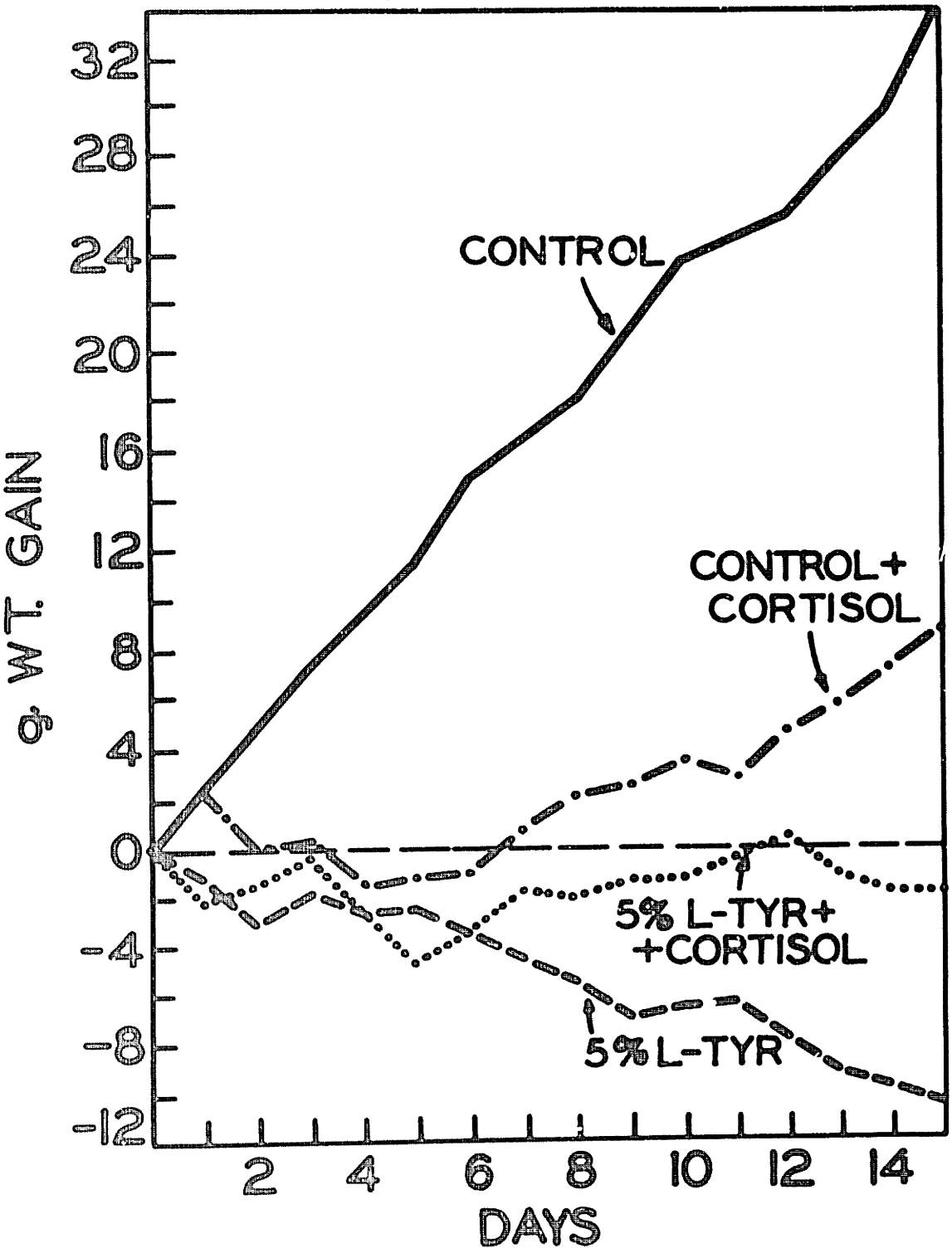


FIGURE II

TABLE 2

Effect of cortisol acetate[1] on weight gain and food intake of rats fed 5% L-tyrosine for 15 days

Diet	Treatment	Food Intake	Weight gain[2]
		g/rat	
Basal	Saline	150	34.4 ± 1.6
"	Cortisol	128	9.2 ± 0.6
Basal + 5% L-Tyr	Saline	59	-10.5 ± 3.1
" + "	Cortisol	84	- 1.5 ± 0.9

[1]Cortisol acetate was injected daily intraperitoneally in 3 mg dose for the first 5 days and then in 2 mg-dose for the rest of the experimental period.

[2]Average per rat ± standard error of the mean.

TABLE 3

Effect of cortisol acetate on liver-weights of rats fed 5% L-tyrosine

Diets	Treatment	Liver weight[2]		
		5 days	10 days	15 days
Basal	Saline	5.03 ± 0.10	5.84 ± 0.50	6.01 ± 0.37
"	Cortisol[1]	6.47 ± 0.26	6.48 ± 0.20	6.63 ± 0.39
Basal + 5% L-Tyr.	Saline	4.96 ± 0.25	5.19 ± 0.26	5.50 ± 0.59
"	Cortisol	6.29 ± 0.4	6.82 ± 0.65	5.86 ± 0.29

[1]Cortisol acetate was injected daily intraperitoneally in 3 mg-dose for the first 5 days and then in 2 mg-dose for the rest of the experimental period.

[2]g liver per 100 g body weight ± standard error of the mean.

TABLE 4

Effect of cortisol acetate on liver enzymes[1] of rats fed 5% L-tyrosine

Diet	Treatment	5 days		T.T./p-HPPH	10 days		T.T./p-HPPH	15 days		T.T./p-HPPH
		T.T.	p-HPPH		T.T.	p-HPPH		T.T.	p-HPPH	
Basal	Saline	119± 22.4	424±68.8	0.28	169±42.8	320±20.6	0.53	176±28.4	493±26.9	0.36
Basal	Cortisol[2]	405± 47.1	587±35.4	0.69	247±25	492±51.3	0.50	168±12.1	456±60.8	0.37
Basal + 5% L-Tyr	Saline	445± 72.2	353±27.6	1.26	624±42.3	344±76.8	1.81	686±183	443±28.6	1.55
" + "	Cortisol	423±100.6	439±69.7	0.96	475±28.9	464±82.3	1.02	327±58.4	393±28.2	0.83

[1]Tyrosine-α-ketoglutarate transaminase (T.T.) and p-hydroxyphenylpyruvate hydroxylase (p-HPPH) activities are expressed as μmoles p-hydroxyphenylpyruvic acid formed or consumed per hour per 100 g body weight ± standard error of the mean.

[2]Cortisol acetate was injected daily intraperitoneally in 3 mg dose for the first 5 days and then in 2 mg dose for the rest of the experimental period.

However, tyrosine feeding alone further increased this enzyme activity by the 10th day and the activity continued to increase until the 15th day of the experimental period. On the other hand, liver tyrosine transaminase activity of rats fed the high tyrosine diet and injected with cortisol acetate was not markedly increased at 10 days as compared to the value obtained at 5 days of the experimental period and this enzyme activity showed a marked drop by the end of the experimental period (Fig. 3).

Similarly daily injection of rats fed the control diet with cortisol acetate for 5 days resulted in a marked increase in liver p-hydroxyphenylpyruvate hydroxylase activity. The activity decreased progressively at 10 and 15 days (Fig. 3). Liver p-hydroxyphenylpyruvate hydroxylase activity of rats fed the control diet showed a marked drop by the 10th day and then returned to above the basal level by the end of the experiment. High tyrosine feeding caused no change in this enzyme activity by 5 days. This level was maintained until the 10th day and then markedly increased around the 15th day of the experimental period. On the other hand, cortisol acetate injection of rats fed the high tyrosine diet caused an elevated enzyme activity as compared to that of the saline-injected animals. This elevated p-hydroxyphenylpyruvate hydroxylase activity was more or less maintained during the rest of the experiment (Fig. 3).

The ratio of tyrosine transaminase activity to the activity of p-hydroxyphenylpyruvate hydroxylase was highly increased by

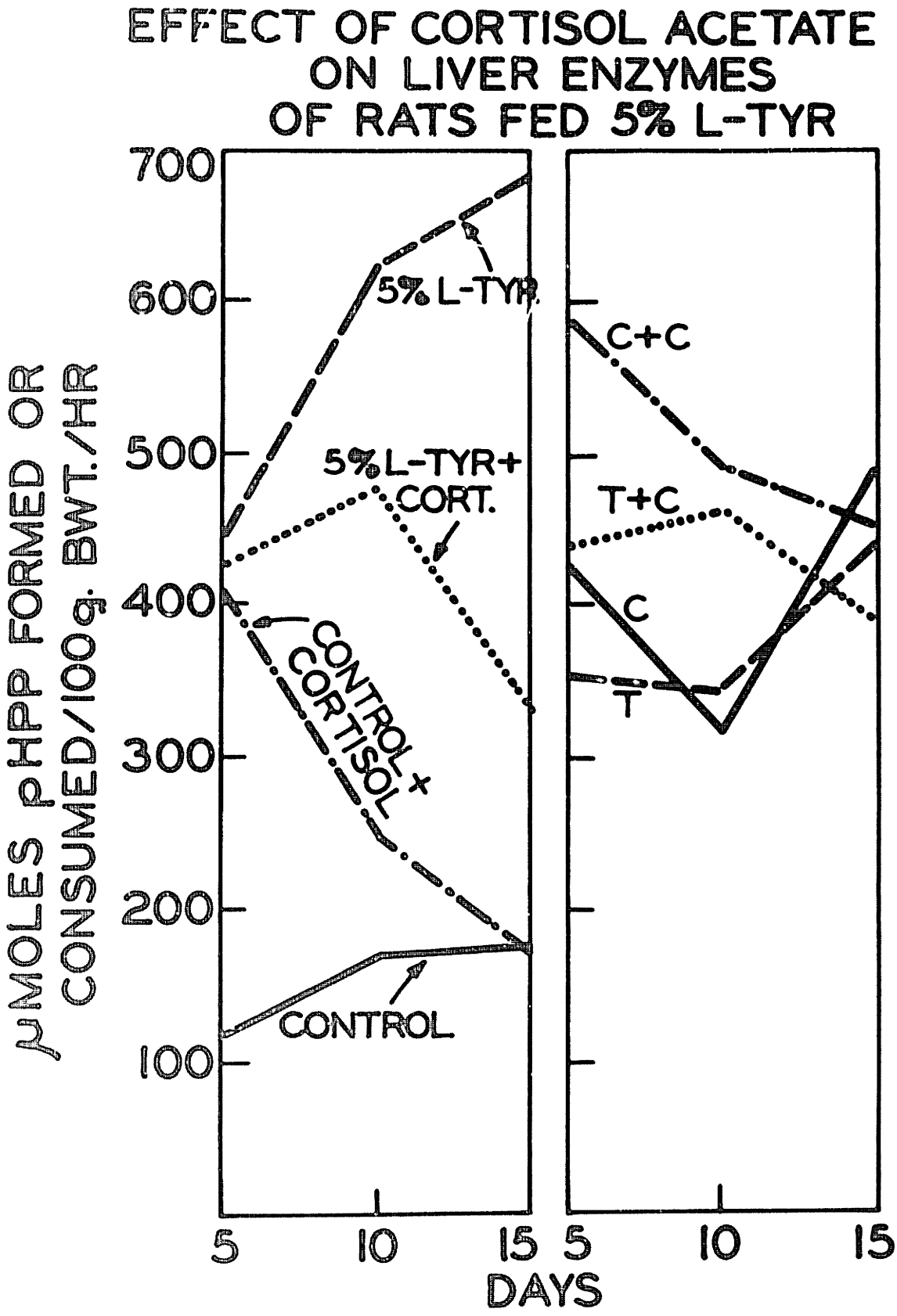

EFFECT OF CORTISOL ACETATE
ON LIVER ENZYMES
OF RATS FED 5% L-TYR
μ MOLES p HPP FORMED OR
CONSUMED/100g. BWT./HR
700
600
500
400
300
200
100
5% L-TYR.
5% L-TYR+
CORT.
CONTROL +
CORTISOL
CONTROL
C+C
T+C
C
T
5
10
15
5
10
15
DAYS

FIGURE III

tyrosine feeding. However, hydrocortisone acetate injection of rats fed the high tyrosine diet tended to maintain the value close to 1 (Table 4).

Figure IV (a-c) shows effects of different treatments on plasma tyrosine concentration. High tyrosine feeding caused a substantial increase in plasma tyrosine concentration at 5 and 15 days yet it was somewhat low at 10 days. On the other hand, cortisol injection of rats fed the high tyrosine diet greatly decreased plasma tyrosine concentration at 5 days but it increased by 10 days and remained unchanged at 15 days. The 15-day value was much below that for the uninjected group. Also cortisol injection caused a marked increase in serine and a marked decrease in lysine and glycine concentration of rats fed the basal or the high tyrosine diets for 5 days (Fig. IV-a). The drug caused a marked decrease in plasma alanine leucine and lysine concentrations of rats fed the basal diet for 10 days. At 15 days cortisol injection of animals fed the basal diet caused a decrease in plasma lysine, histidine and arginine concentrations. However, tyrosine feeding alone caused a decrease in plasma alanine concentration at the 3 periods tested and at 15 days it increased levels of lysine, histidine, arginine, serine and glycine.

Oxidation study:

Table 5 shows the pattern of radioactivity in the carbon dioxide expired by rats over 24 hours after the administration of the tyrosine load that was labelled with L-tyrosine-1-C^{14}. Rats fed the experimental diet for either 5 or 10 days before

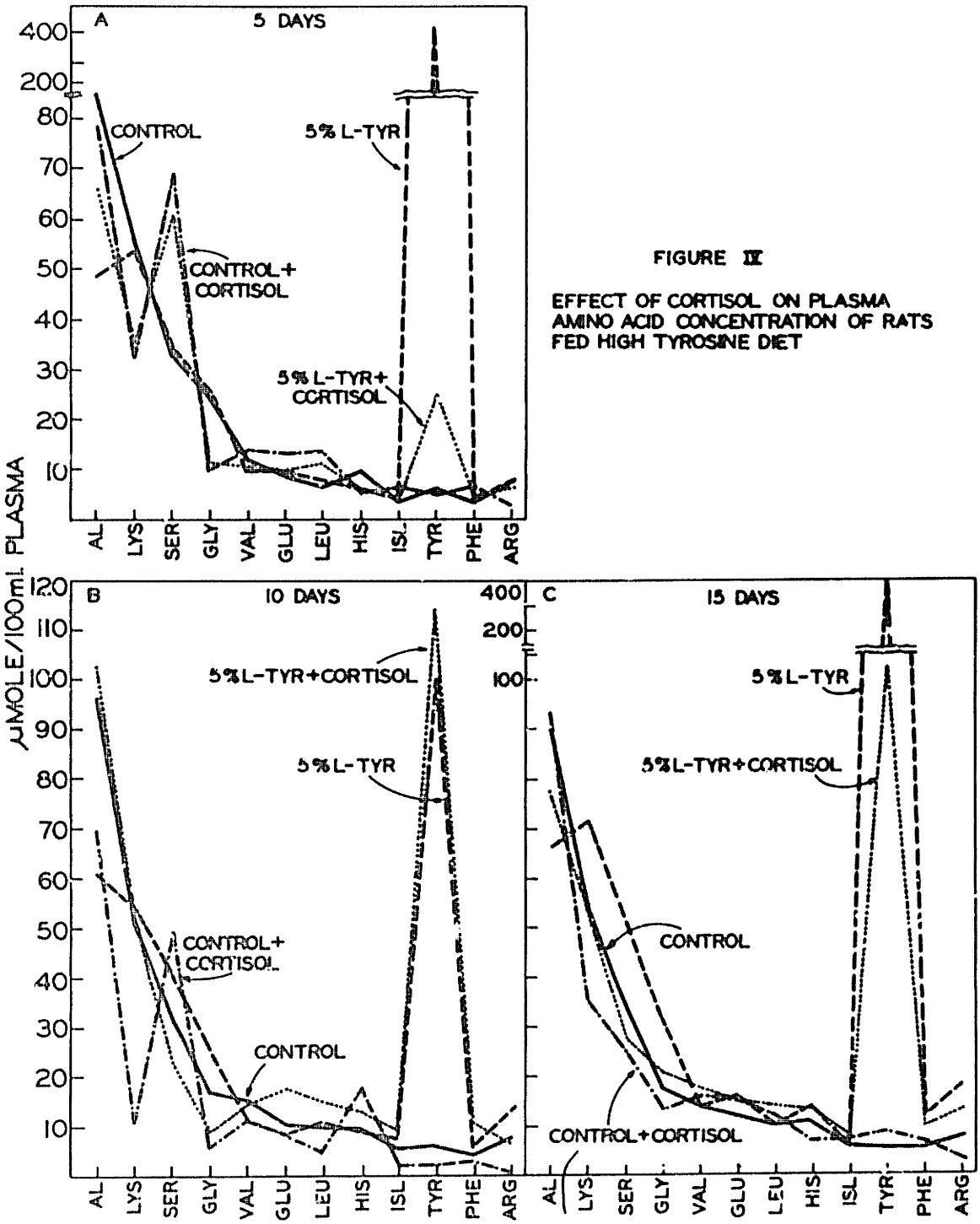


FIGURE II

EFFECT OF CORTISOL ON PLASMA AMINO ACID CONCENTRATION OF RATS FED HIGH TYROSINE DIET

TABLE V

Effect of cortisol injection on the pattern[1] of $^{14}CO_2$ expired by rats fed high tyrosine diets

Time	5 days			10 days		
	Control	5% L-Tyr.	5% L-Tyr. + cortisol	Control	5% L-Tyr.	5% L-Tyr + Cortisol
1st hour	4.7 ± 0.85	6.54 ± 0.94	9.7 ± 1.34	6.7 ± 0.18	10.6 ± 0.76	10.0 ± 0.98
2nd hour	7.2 ± 0.98	7.9 ± 0.28	12.4 ± 1.17	9.2 ± 0.88	11.7 ± 1.28	15 ± 0.65
3rd hour	6.8 ± 1.23	6.7 ± 0.94	12.2 ± 1.66	9.3 ± 0.76	9.7 ± 1.07	13 ± 0.68
4th hour	5.7 ± 1.19	5.6 ± 0.96	10.2 ± 1.24	6.2 ± 0.5	8.8 ± 1.11	10.6 ± 1.12
5th hour	4.0 ± 1.1	4.2 ± 1.01	9.1 ± 0.57	4.1 ± 0.41	7.4 ± 1.22	7.9 ± 0.94
6th hour	3.0 ± 0.84	4.32 ± 0.88	8.8 ± 0.64	2.9 ± 0.3	7.1 ± 1.30	6.4 ± 0.92
7th hour	2.4 ± 0.56	3.9 ± 0.7	6.9 ± 0.58	2.1 ± 0.2	6.1 ± 1.20	5.2 ± 0.76
8th hour	1.2 ± 0.39	3.66 ± 0.74	5.4 ± 0.93	1.2 ± 0.1	4.2 ± 0.68	4.1 ± 0.44
9th-10th hr.	3.1 ± 0.48	6.33 ± 1.36	6.5 ± 2.24	2.5 ± 0.37	5.5 ± 0.84	5.3 ± 0.81
11th-12th "	2.4 ± 0.37	5.04 ± 1.0	2.5 ± 0.95	2.7 ± 0.14	3.5 ± 0.88	3.6 ± 0.73
13th-18th "	5.0 ± 0.84	9.4 ± 0.52	2.7 ± 0.38	6.60 ± 1.18	3.00± 0.52	2.5 ± 0.34
19th-24th "	3.6 ± 1.15	4.4 ± 1.04	1.4 ± 0.15	6.4 ± 1.32	1.45± -	1.5 ± 0.16

[1]% of the absorbed dose expired/period of time ± standard error of the mean per rat.

the day of the experiment showed a peak at more or less the same time, i.e. between 2-3 hours after the administration of the dose (Fig. V-b and Fig. VI-b). Animals fed the 5% tyrosine diet and injected with cortisol showed the highest oxidation rate. The values for the tyrosine-fed rats were closer to the values for the controls than to those for rats injected with cortisol for 5 days (Fig. V-a). (The difference between the value for the controls and that for rats fed the high tyrosine diet but not receiving cortisol was not significant.) However, rats fed the control diet for 10 days (Fig. VI-b) showed a higher rate of oxidation than those fed this diet for 5 days presumably due to the greater weight of the older rats (Table 6). Similarly the rats fed the high tyrosine diet for 10 days showed a marked increase in the rate of oxidation of tyrosine above that for rats fed this diet for only 5 days. The animals fed the high tyrosine diet and injected with cortisol maintained their high rate of oxidation during the two periods but the difference between this group and the one injected with saline was not significant at 10 days. The difference between the value for the animals fed the basal diet and that for the animals fed the high tyrosine diet but not receiving cortisol was significant.

The amounts of radioactivity excreted in feces and retained in the gastrointestinal tract were not greatly different for the different treatments at the two periods. Correction for this did not result in the values calculated as a percentage of the dose (Table 7) being appreciably different from those calculated as a percentage of the absorbed dose (Table 6).

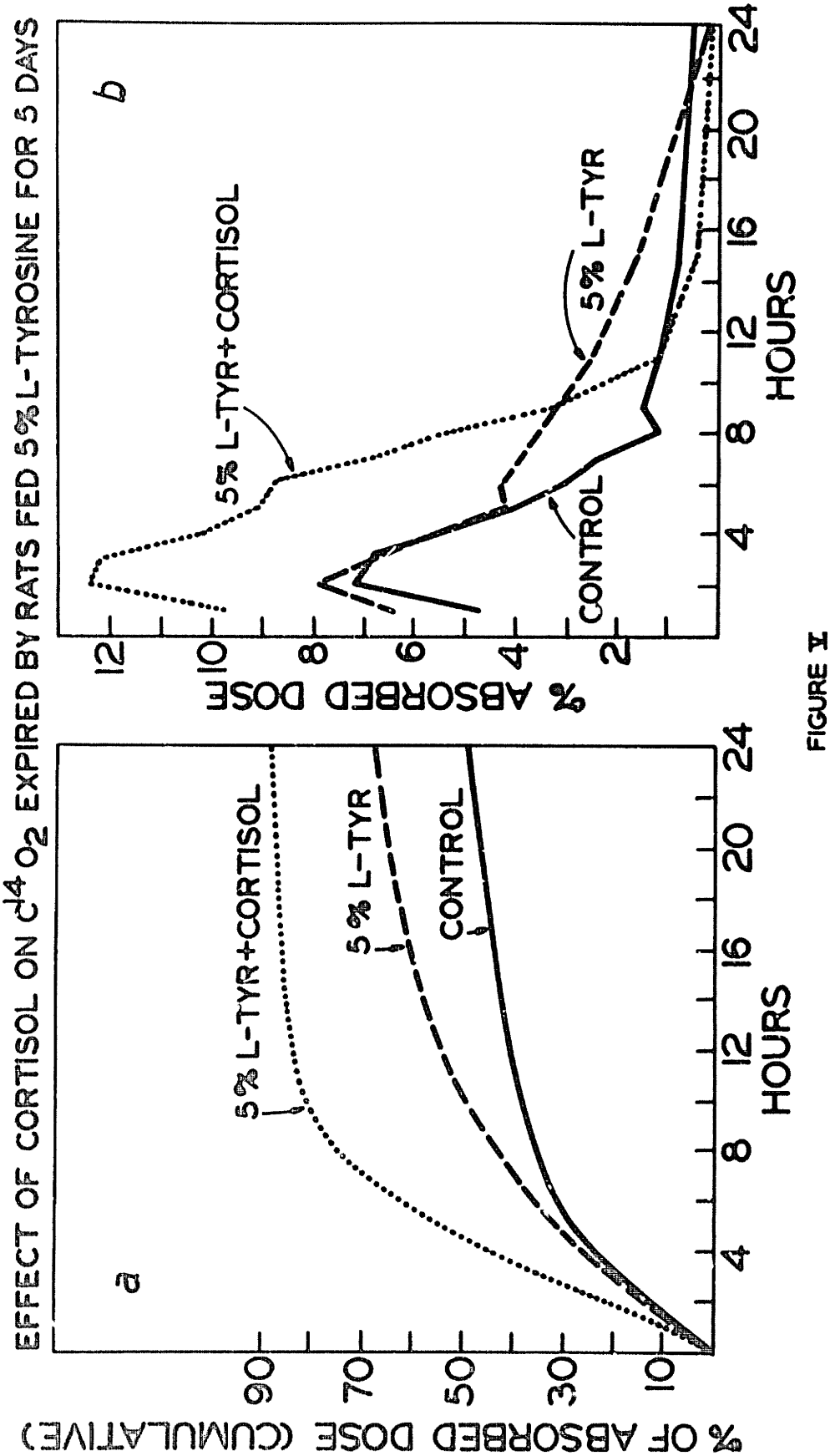

EFFECT OF CORTISOL ON $C^{14}O_2$ EXPIRED BY RATS FED 5% L-TYROSINE FOR 5 DAYS
a
% OF ABSORBED DOSE (CUMULATIVE)
90
70
50
30
10
4
8
12
16
20
24
HOURS
5% L-TYR+CORTISOL
5% L-TYR
CONTROL
b
% ABSORBED DOSE
12
10
8
6
4
2
4
8
12
16
20
24
HOURS
5% L-TYR+CORTISOL
5% L-TYR
CONTROL

FIGURE I

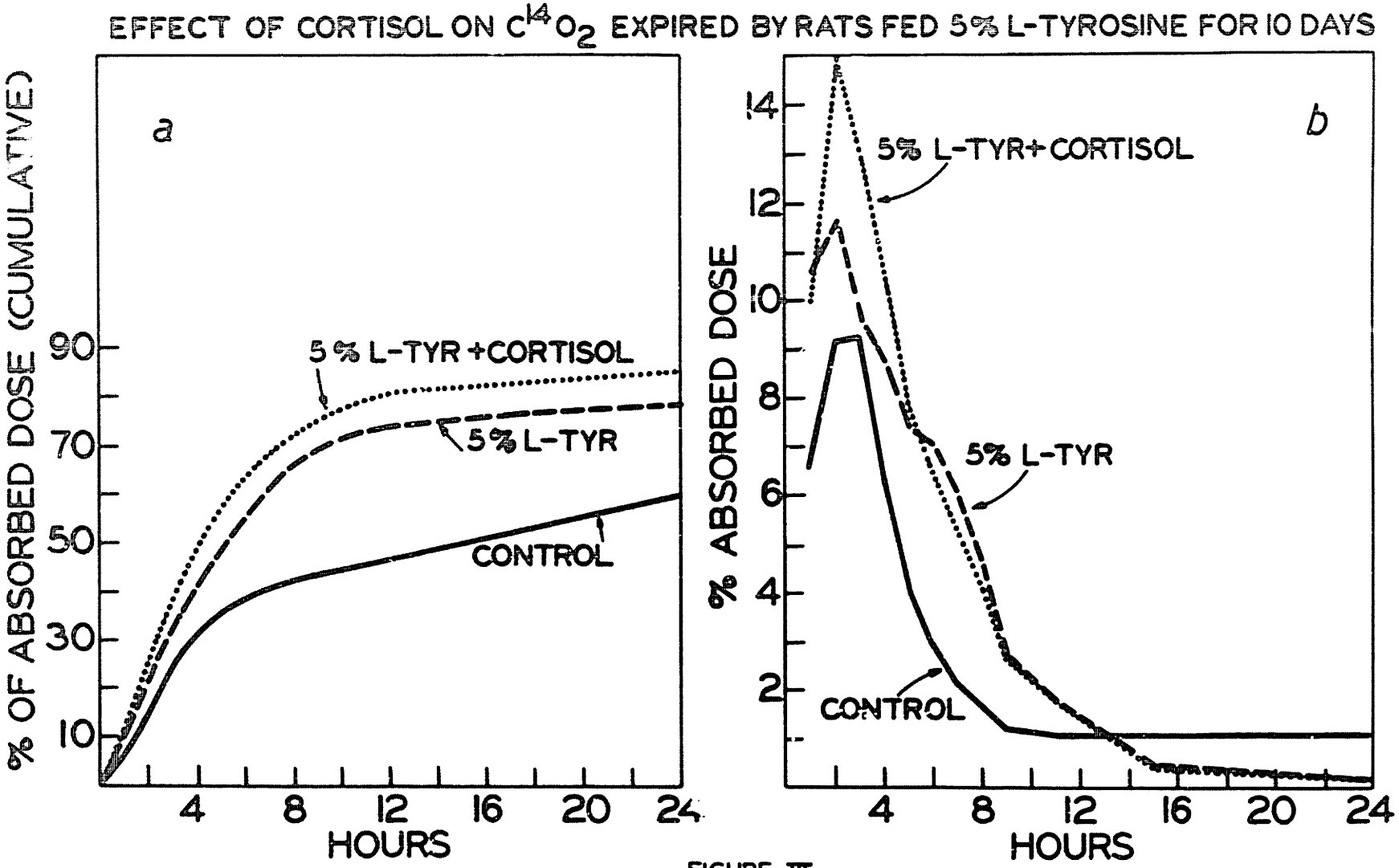

EFFECT OF CORTISOL ON $C^{14}O_2$ EXPIRED BY RATS FED 5% L-TYROSINE FOR 10 DAYS
a
% OF ABSORBED DOSE (CUMULATIVE)
90
70
50
30
10
5% L-TYR + CORTISOL
5% L-TYR
CONTROL
4
8
12
16
20
24
HOURS
b
% ABSORBED DOSE
14
12
10
8
6
4
2
5% L-TYR+CORTISOL
5% L-TYR
CONTROL
4
8
12
16
20
24
HOURS

FIGURE VI

TABLE 6

Effect of cortisol on the distribution of radioactivity in feces, urine and carbon dioxide excreted by rats fed high Tyr. diet [1,2]

Diet	Treatment	Days on Diet	Average Weight of rats	Feces	Urine	CO_2
Basal	Saline		66.4 ± 1.6	3.8 ± 1.26	10.5 ± 4.6	51.0 ± 8.1
Basal + 5% L-Tyr	Saline	5 d	50 ± 1.2	3.4 ± 0.58	15.6 ± 2.8	67.8 ± 5.9
" + "	Cortisol		54.8 ± 0.6	1.94± 0.38	2.5 ± 0.7	87.8 ± 1.9
Basal	Saline		74.8 ± 0.6	4.8 ± 1.2	10.9 ± 2.1	59.9 ± 3.6
Basal + 5% L-Tyr	Saline	10 d	46.9 ± 1.0	5.0 ± 1.5	7.8 ± 2.3	77.1 ± 5.3
" + "	Cortisol		54.9 ± 0.6	3.1 ± 0.7	7.9 ± 0.88	85.2 ± 1.8

[1]Values calculated as the average of % of absorbed dose of 4 rats ± standard error of the mean.

[2]24-hour experimental period.

TABLE 7

Effect of cortisol on the distribution of radioactivity in feces, urine and carbon dioxide excreted by rats fed 5% L-tyrosine[1,2]

Diet	Treatment	Days on diet	Feces	Urine	CO_2	% Retained
Basal	saline		3.8 ± 1.3	9.6 ± 4.6	49.1 ± 8.0	38.6
Basal + 5% L-Tyr	"	5 d	3.4 ± 0.6	15.0 ± 4.0	64.9 ± 6.0	16.7
" + "	cortisol		1.9 ± 0.4	2.5 ± 0.6	87.4 ± 2.2	8.2
Basal	saline		4.8 ± 1.2	10.3 ± 2.0	57.3 ± 3.8	27.6
Basal + 5% L-Tyr	"	10 d	5.0 ± 1.5	7.5 ± 2.2	75.8 ± 5.8	11.7
" + "	cortisol		3.1 ± 0.7	7.6 ± 0.8	82.4 ± 1.6	6.9

[1]Values calculated as the average of % of ingested dose of 4 rats ± standard error of the mean

[2]24-hour experimental period.

Amounts of labelled material excreted in the urine are of interest. Rats fed the control diet excreted relatively high amounts of radioactivity in the urine at 5 and 10 days. Also rats fed the high tyrosine diet excreted a substantial amount of radioactivity in the urine at 5 days. On the contrary, rats fed the high tyrosine diet and injected with cortisol acetate excreted very little at 5 days. At 10 days, there was a marked decrease in the amount of radioactivity excreted by the animals fed the high tyrosine diet and there was some increase in the urinary excretion of radioactivity by rats fed the high tyrosine diet and injected with cortisol.

Table 7 also shows that rats fed the high tyrosine diet and injected with cortisol retained the least amount of the load of tyrosine in their tissues. However, rats fed the control diet for 5 days retained close to 40% of the radioactivity in their tissues. This decreased to about 30% at 10 days.

Table 8 shows the plasma and liver tyrosine concentrations of rats fed the different experimental diets. At zero time liver tyrosine concentration of rats fed the high tyrosine diet was 6-fold and plasma tyrosine concentration more than 20-fold that of rats previously fed the control diet. Rats previously fed the high tyrosine diet and injected with cortisol had more or less the same liver and plasma tyrosine concentrations as animals fed the control diet.

Three hours after administering the tyrosine load, there was a substantial increase in tyrosine concentration of plasma and liver of rats that were fed the control diet for 5 days.

TABLE 8

Effect of cortisol[1] on plasma and liver tyrosine concentrations of rats fed 5% L-Tyr for 5 days and fed the tyrosine load

Diet	Treatment	Zero time			After 3 hours		
		Tyr. concentration		Liver / Plasma	Tyr. concentration		Liver / Plasma
		Liver[2]	Plasma[3]		Liver[2]	Plasma[3]	
(6% Casein + 0.2 L-Met)	Saline	18	5.1	3.5	395	412	0.96
" + 5% L-Tyr	Saline	119	118.6	1.0	849	946	0.90
" + "	2 mg cortisol	17	7.5	2.27	293	428	0.68

[1]Experimental conditions as mentioned under "Oxidation Study."

[2]μ moles tyrosine per 100 g of pooled liver samples.

[3]μ moles tyrosine per 100 ml pooled plasma samples.

Also rats previously fed the high tyrosine diet showed more increase in tyrosine concentration than the corresponding controls. Cortisol injected rats fed the high tyrosine diet showed substantially lower liver tyrosine concentration than the saline injected rats fed the high tyrosine diet and much lower than the animals fed the control diet. Cortisol injection also caused a substantial decrease in plasma tyrosine concentration of rats fed the high tyrosine diet as compared to the saline injected rats fed the high tyrosine diet. However, the value was more or less the same as that for the controls.

Discussion

It is clear that cortisol treatment alleviates the external lesions of rats fed a diet containing as much as 5% of L-tyrosine. However, the response depends on the type of cortisol ester injected. The semisuccinate ester did not prevent the development of the external lesions. Even the well known cortisol-induction of liver tyrosine transaminase was not observed in rats fed the basal diet and injected with 4 mg cortisol semisuccinate daily for 9 days. However, the rate of growth of rats fed the basal diet was depressed. Frenkel and Havenhill (1963) compared the effect of solubility as well as the route of administration of cortisol esters on body weight of hamsters. Animals were given 2.5 mg of the drug daily for 10 days. They found that cortisol was most effective in producing weight loss by the subcutaneous route. The acetate was most potent after intraperitoneal injection and the water soluble sodium semisuccinate derivative was least effective by any route. (Solubility of these drugs are: 28 mg%, 1 mg% and 5000 mg% respectively.) Melby and Cyr (1961) found that when the semisuccinate was injected intramuscularly or subcutaneously into humans, the plasma level peaked after 60 minutes. Lin and Knox (1958) studied the effect of injecting the semisuccinate derivative on rat tyrosine transaminase activity. They found that the enzyme activity reached a maximum at 5 hours after injection and then decreased until it reached the basal level by 12 hours. In our experiments, there was about 17 hours between the time of injecting

the drug and the time of obtaining the liver sample. Another interesting observation was made by Frenkel and Havenhill (1963). They found that after injection of hamsters with the least soluble cortisol acetate was stopped weight loss continued for almost two weeks and it took 7 weeks for the animals to regain their starting weight. After stopping the administration of the alcohol the hamsters gained 14 g during the first week. Increasing the solubility of the drug seems to increase the rate of its absorption, however, the rate of excretion or metabolism is increased and this results in a short-acting effect.

Daily injection of 3 mg cortisol acetate for 5 days depressed the growth of rats fed the basal diet to a great extent. This led us to cut down the dose injected to 2 mg daily from the 6th-15th day of the experiment. There is a definite beneficial effect of cortisol acetate injection of rats fed the high tyrosine diet. However, if during the first 5 days of the experiment a 2 mg dose instead of a 3 mg dose had been injected daily, the rats might have gained more weight.

The liver weights of rats fed the basal as well as the high tyrosine diets were generally increased by cortisol acetate injection. This is probably due to increased glycogen deposition in the liver (Greengard et al., 1963).

Rats fed the high tyrosine diet with or without cortisol acetate injection for 5 days showed similar tyrosine transaminase activities to that of the controls that were injected with cortisol. The effect of cortisol acetate on tyrosine transaminase

activity of rats fed the basal diet decreased gradually until by the 15th day of the treatment there was little evidence of an effect. This decrease in response with time may be due to increased capacity of the liver to catabolize the drug. This may explain also the gradual decrease in tyrosine transaminase activity of rats fed the high tyrosine diet and injected with cortisol acetate. Tyrosine feeding may elicit both substrate-type induction (Knox, 1964) and the hormonal-type induction of tyrosine transaminase through stimulating the adrenocortical hormone output. The latter is a fast process while the former one is a slow process.

Cortisol acetate injection for 5 days of rats fed the control diet caused a significant increase in p-hydroxyphenylpyruvate hydroxylase activity as compared to the saline-injected controls. It seems that cortisol injected rats fed the high tyrosine diet have a higher enzyme activity than the saline injected group fed this diet. This enzyme activity in rats fed the control diet and the high tyrosine diet tended to decrease with time. This would also fit with the assumption that the rate of catabolism of the drug increases with time.

The greatest change in plasma amino acid concentration was in tyrosine concentration. At 5 and 15 days cortisol greatly lowered plasma tyrosine of rats fed the high tyrosine diet.

The capacity of the rat to oxidize tyrosine was greatly increased by cortisol administration. The capacity to oxidize carbon number one of the tyrosine molecule depends upon both tyrosine transaminase and p-hydroxyphenylpyruvate hydroxylase activities.

It is clear that cortisol injection tends to keep the ratio of the first two enzymes close to 1 (Table 4). So the oxidation rate should be a function of the limiting enzyme activity. These two enzymes were quite high in rats fed the high tyrosine diet and injected with cortisol acetate. Similarly the rate of oxidation of the labelled tyrosine over the 24-hour period was also high at 5 and 10 days. This resulted in less tyrosine being retained (Table 8). On the other hand, high tyrosine feeding alone resulted in tyrosine transaminase to p-hydroxyphenylpyruvate ratio higher than 1, which means that the hydroxylase activity is more limiting than the transaminase and in fact its absolute value was less than that of rats fed the high tyrosine diet and injected with cortisol.

It is difficult to compare the capacity of the animals to oxidize tyrosine in these experiments for three main reasons: 1) in the first experiment, the enzymes were measured under ad libitum feeding while in the oxidation study the animals were fasted for 9 hours before starting the study; 2) rats were injected with cortisol acetate or saline at 17 hours before liver samples were obtained for enzyme assay while in the oxidation study the animals were injected at zero time too; and 3) the enzymes were assayed at 30°C while the oxidation study was carried out with rats having body temperature of about 38°C. However, the trend in both experiments illustrates very clearly that cortisol administration can increase the rate of oxidation of tyrosine.

Feeding a high tyrosine load resulted in a temporary tyrosyluria-type condition. This is illustrated by increased urinary excretion of the load at 5 and 10 days by rats previously fed the control diet or the high tyrosine diet.

The study of the plasma and liver tyrosine concentrations is quite interesting. The so-called "fasting level" of tyrosine in plasma and liver from rats fed the high tyrosine diet and injected with cortisol was almost the same as that of the control animals. It seems that the beneficial effect of cortisol can be related to its ability to maintain a more or less normal level of tyrosine in the animal tissues.

General Discussion

An excessive intake of tyrosine results in gross external symptoms as well as retardation of growth or death of the rat depending on the protein level (Schweizer, 1947; Graham, 1950; Sauberlich, 1961) and the level of tyrosine in the diet (Sullivan Hess and Sebrell, 1932 and Schweizer, 1947). Our data show that rats fed a 6% casein diet (+ 0.2% L-methionine) supplemented with 3% of L-tyrosine gained less weight than controls and that external lesions appeared in 7-10 days. On the other hand, raising the tyrosine supplement to 5% resulted in severe body weight loss, earlier onset of the external lesions and higher mortality.

Cortisol injection as well as supplementation with some amino acids especially threonine and glycine alleviated these symptoms partially or completely while thyroxine injection aggravated the condition.

In his studies, Alam (1965) tried to elucidate the reason for the beneficial effect observed when rats fed the high tyrosine diet were injected with cortisol or fed such a diet supplemented with 1.25% L-threonine by studying the oxidation of labelled tyrosine under these conditions. He did not observe any difference in the rate of oxidation of the labelled tyrosine. This was probably due to the experimental design. He carried out his measurements during the first day of feeding the experimental diet and hence was probably measuring the capacity of the normal rat to oxidize excess tyrosine. Such a result is to

be expected if the process of adaptation requires some time. It was suggested then that the beneficial effect of threonine supplementation might be mediated through increased urinary excretion of tyrosine and its metabolites.

The data presented here show that each amino acid supplement exerts its beneficial effect to a different extent and possibly through different mechanisms. Threonine supplementation alleviated both the external lesions and the growth depression due to a 3% L-tyrosine diet. However, it did not increase urinary total phenol excretion,while methionine supplementation increased it to some extent. It seems that the effect of glycine on urinary total phenol excretion is similar to that of threonine. The increased homogentisic acid excretion by rats fed threonine and glycine supplements points to a possible increase in tyrosine catabolism under these conditions and suggests that homogentisic acid oxidase may become limiting.

Feeding a high tyrosine diet alone or together with some amino acids elevated tyrosine transaminase quite substantially. However, the elevation was not always accompanied by a corresponding decrease in plasma tyrosine concentration. Cortisol acetate injection and threonine supplementation were the only treatments to bring about substantial decreases in plasma tyrosine concentration, yet such treatments did not elevate tyrosine transaminase activity above the level attained by rats fed the high tyrosine diet alone. It appears then that the increase in tyrosine transaminase activity may be ineffective unless it is accompanied by a corresponding increase in the enzymes which

further catabolize tyrosine. The relative ratios of such enzymes under these conditions appear to be quite important (Knox et al., 1964). High tyrosine feeding resulted in ratios (tyrosine transaminase to p-hydroxyphenylpyruvate hydroxylase activities) higher than 1, while cortisol administration tended to keep such ratio close to 1. In contrast, thyroxine administration pushed the ratio much higher than 1. However, one cannot overlook the importance of higher absolute enzyme values in this connection as low activities may still give a ratio of one. Thyroxine injection of rats fed the basal diet showed a ratio of 0.71 as compared to the untreated control of a value of 0.40. This increase was primarily due to a decrease in the hydroxylase activity.

The possibility should be considered that thyroxine production and adrenocortical hormone production may be elevated in rats fed a high tyrosine diet. It has been shown by Simon et al. (1962) that the addition of tyrosine to a protein-deficient diet significantly increased the iodine uptake of the thyroid. We have observed marked increase in adrenal weight of rats fed the high tyrosine diet. The findings obtained by Munro et al. (1963, 1965) that the administration of certain amino acids increases adrenocortical function lend support to the hypothesis that high tyrosine may elicit a stress-induced adrenocortical hormone production. If this is the case, then adrenal-thyroid interactions may become critical under these experimental conditions. Massive administration of cortisone to hyperthyroid patients resulted in lowering PBI values (Frederickson, 1951). In rats,

there was a definite decrease in absolute thyroid weight after cortisone treatment (Money, 1954). Adrenal atrophy occurs following surgical throidectomy or thiouracil treatment, but this effect can be corrected by small simultaneous doses of thyroxine (Zarrow and Money, 1949; Freedman and Gordon, 1950). Also it was shown that the administration of cortisone to euthyroid rats decreased thyroid protein as well as RNA/DNA and they suggested that cortisone affected protein metabolism in the thyroid gland.

We have found that cortisol acetate injection alleviated the pathological lesions and improved the weight of rats fed the high tyrosine diet. On the other hand administration of thyroxine aggravated the condition of such rats while thiouracil improved it to some extent. The limited beneficial effect of thioracil might be explained if at the same time as thyroxine formation was inhibited, adrenocorticoid production was depressed too.

Another problem is the adaptation to daily injections of cortisol acetate. Of great interest was the finding that liver tyrosine transaminase activity as well as p-hydroxyphenylpyruvate activities of rats fed the basal diet and injected with cortisol progressively decreased until they reached the basal level by the end of the 15-day experimental period. This may reflect an adaptive increase in the capacity of animals to catabolize cortisol. It has been shown that thyroxine administration increased the rate of catabolism of corticosteroids (McGuire and Tomkins, 1959; Rivlin, 1963).

It is of interest that animals fed the high tyrosine diet showed progressive increases in tyrosine transaminase activity as compared to rats fed the basal or the high tyrosine diet and injected with cortisol acetate. Tyrosine may exert its effect on tyrosine transaminase by 2 separate mechanisms simultaneously: a fast mechanism, i.e., hormonal-type one which will lose its effect with time due to adaptation to increased circulating corticosterone; and a substrate-type mechanism which is a slow one (Knox, 1964). Hormonal induction of tyrosine transaminase has been shown to result in increased synthesis of the apoenzyme (Kenney, 1962), while substrate type induction may involve decreased rate of degradation of the enzyme as in the case of arginase (Shimke et al., 1963), where the rate of degradation was decreased during starvation. If the hormonal induction of tyrosine transaminase takes place at an early stage of adaptation followed by the substrate-type induction later on, this may explain the progressive increase in this enzyme activity of rats fed the high tyrosine diet alone.

Increasing the tyrosine intake by force feeding the high tyrosine diet is good evidence that the appearance of the toxicity symptoms were not primarily due to depressed food intake.

All the data so far obtained suggest more strongly than ever before that the accumulation of tyrosine in tissues is the main reason for the syndrome. The results obtained by feeding p-hydroxyphenylpyruvic acid instead of tyrosine provides evidence that neither this metabolite nor any of the next metabolites

in this scheme of tyrosine catabolism can be responsible for at least the appearance of the external lesions. If such animals were kept for longer periods on such a diet, the pathological lesions might have appeared because of the limited capacity of these rats to degrade p-hydroxyphenylpyruvic acid with the result that it was transaminated to tyrosine as reflected by the increase in plasma tyrosine concentration, and probably in other tissues.

Therefore, one may conclude that products of the transamination pathway of tyrosine metabolism are not responsible for the development of the toxicity syndrome. It seems important to study in some detail the other pathways, namely those leading to formation of tyramine, epinephrine, and thyroxine. It may be that excessive feeding of tyrosine results in some sort of hormonal imbalance.

Summary

Feeding a 6% casein diet supplemented with 0.2% L-methionine (basal) and containing 3% or 5% L-tyrosine to weaning rats resulted in growth depression or body weight loss and increased plasma tyrosine concentration. The degree and time of onset of the external lesions depended on the level of tyrosine in the diet.

Addition of threonine, glycine, tryptophan, a mixture of leucine, isoleucine and valine, and methionine alleviated the pathological lesions partly or completely with only threonine stimulating growth. Adrenal weights were increased by all of these treatments except threonine supplementation. The degree of beneficial effect appeared to depend upon the ability of the treatment to depress plasma tyrosine concentration. High tyrosine feeding alone or together with these amino acid supplements increased liver tyrosine-α-ketoglutarate transaminase activity (tyrosine transaminase) to different degrees at 2, 10 and 22 days of the experimental period. The beneficial effects of the threonine, methionine and glycine supplements were not accompanied by increased excretion of tyrosine and its metabolites. The increased urinary homogentisic acid excretion by rats fed threonine and glycine supplements suggests that the rate of catabolism of tyrosine increased under these conditions.

There appear to be some diurnal changes in tyrosine transaminase activity. The peak of activity was at 9 a.m. for rats

fed the high tyrosine diet and 9 p.m. for rats fed the basal diet. The diurnal changes in the latter followed the diurnal rhythm of plasma coticosterone as reported in the literature. The rhythm exhibited by rats fed the high tyrosine diet followed a rhythm in plasma tyrosine concentration which accompanied a diurnal change in food intake.

Feeding the low protein diet with 5% of p-hydroxyphenylpyruvic acid substituted for 5% of L-tyrosine did not result in development of external lesions despite increased food intake. However, plasma tyrosine concentration was elevated yet it was far less than that of rats fed the 5% L-tyrosine diet. Liver tyrosine transaminase activity was significantly elevated by such treatment. However, none of the different dietary regimens affected the activity of p-hydroxyphenylpyruvate hydroxylase. These results indicate that the metabolites of this pathway of tyrosine metabolism are not responsible for the development of the external lesions.

When rats were force-fed the 5% L-tyrosine diet, there was no body weight loss but the animals gained less weight than rats fed an equal amount of the basal diet. The pathological lesions appeared in mild form yet the animals died very shortly thereafter.

Daily injection of as little as 5 γ L-thyroxine daily aggravated the condition of rats fed a high tyrosine diet. Thiouracil administration alleviated the external lesions partly and improved to some extent the growth of the animals. Administration of thyroxine to rats fed the high tyrosine diet for 15 days resulted in a highly elevated plasma tyrosine concentration which

was accompanied by a corresponding increase in liver tyrosine transaminase activity. P-hydroxyphenylpyruvate hydroxylase activity was somewhat depressed by thyroxine administration for 9 days.

Injection of cortisol acetate depressed the rate of growth of rats fed the basal diet more than injection of cortisol semisuccinate. On the other hand, injection of rats fed the 5% L-tyrosine diet with cortisol acetate alleviated the pathological lesions while the semisuccinate form was without effect. The inducing effect of cortisol acetate on tyrosine transaminase activity of liver of rats fed the basal diet decreased with time until it reached the basal level after 15 days of daily injection. Also tyrosine transaminase activity of rats fed the high tyrosine diet and injected daily with cortisol acetate showed a decreased response as the experiment went on. There was some increase in liver p-hydroxyphenylpyruvate hydroxylase activity after cortisol injection of rats fed the basal diet which fell to the basal level at the 15th day. Cortisol acetate injection tended to keep the ratio of tyrosine transaminase activity to that of the hydroxylase close to 1 while high tyrosine feeding alone increased it above 1.

Daily injection of rats fed the high tyrosine diet with cortisol resulted in a substantial decrease in plasma tyrosine concentration especially at 5 days of the experimental period, as compared to the animals fed the high tyrosine and injected with saline.

Rats were fed the basal or the high tyrosine diet with or without cortisol acetate injection for 5 and 10 days and their capacity to oxidize a load of tyrosine labelled with L-tyrosine-1-C^{14} over a 24 hour period was measured. At 5 days, the animals fed the high tyrosine diet and injected with cortisol showed a significant increase in their capacity to oxidize the tyrosine load as compared to the other two groups. At 10 days, there was no significant effect of cortisol injection on rats fed the high tyrosine diet as compared to those fed such a diet and injected with saline. The latter group showed a marked increase in capacity to oxidize the tyrosine load at that time. When animals fed the basal diet were given the tyrosine load they retained a substantial amount of the dose and excreted a significant amount of it in the urine at 5 and 10 days. Cortisol injection tended to keep the liver tyrosine level close to normal.

The results thus far obtained indicate that the development of the tyrosine toxicity syndrome is associated with elevated tyrosine concentration in plasma and other tissues and its alleviation is associated with reduction in tyrosine concentration.

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PART II

FACTORS AFFECTING AVAILABILITY OF LYSINE IN FOODS

Introduction

The primary function of dietary protein is to furnish amino acids for the synthesis of tissue proteins. A measure of the efficiency with which the amino acids of highly digestible proteins can be utilized is readily obtained by calculating the chemical score (Block and Mitchell) from an amino acid analysis of the protein. However, particularly in heat-processed proteins, some amino acids may be unavailable owing to interactions among themselves or with other components of the food or feed or to alteration of the amino acid molecule itself (Donoso et al., 1962). Lysine and methionine are the amino acids most subject to such changes.

Lysine is the indispensable amino acid that is most limiting in many foods of plant origin, especially in cereal grains which represent the major source of protein for most of the developing countries (F.A.O., 1955). Consequently, particular attention has been paid to determination of availability of lysine. Carpenter (1960) developed a method based on the reaction of the ε-amino group of lysine with dinitrofluorobenzene (FDNB) for measuring the availability of lysine. Many biological methods have been used also. Enzymatic methods have been developed by Mauron et al. (1955) and Sheffner et al. (1956). Schweigert and Guthneck (1953) and Guthneck et al. (1953) determined lysine available for the growth of the protein depleted adult rat. Kuiken and Lyman (1948) and Kuiken (1952) measured the amount of ingested lysine

that was excreted in the feces of rats fed different food stuffs. Gupta et al. (1958) used the rate of growth of the young rat over a two-week period as a measure of lysine available in the diet. Ousterhaut et al. (1959) estimated the availability of various essential amino acids in proteins by using a basal diet containing crystalline forms of all of the essential amino acids except the one being assayed and by adding the protein under test as the only source of this amino acid.

Microbiological assay methods have been devised and used for the estimation of available lysine in foods. Ford (1964) compared different test microorganisms as a means of assessing the availability of methionine tryptophane, lysine. Stott and Smith (1966) used the microorganisms Tetrahymena pyriformis and found that microbiological assays of meat meals generally gave lower values than the FDNB-method. They thought other factors might have influenced the assay besides the unavailability of amino acids.

Several of these methods have been used to study the effect of heat treatment on the nutritive value of proteins. Moderate heat treatment has been shown to improve the food value of certain plant proteins, mainly those of legumes (Kahade and Evans, 1966), whereas severe heat treatment usually lowers the nutritive value. Observations in vitro have shown that the rates of enzymatic release of lysine from proteins decreased when the proteins were heated dry (Pader, et al., 1948 and Mauron et al., 1955). Autoclaving at 250°F produced a marked decrease in the

utilization of the lysine by animals for growth (Heller et al., 1961).

Carpenter et al. (1963) measured the amount of lysine present in different fish proteins using standard chromatographic methods and the FDNB-method. Values obtained with the FDNB-method were lower than those obtained by the chromatographic method.

The present studies were undertaken in an attempt to assess the reliability of the growth rate of rats as a means of estimating the amount of available lysine in foods and feeds and to study some of the factors affecting estimates of available lysine in heat-treated proteins.

Experimental

Chemical estimation of lysine and other amino acids:

The FDNB-method as developed by Carpenter (1960) was used, with slight modifications, to measure the available lysine.

A sample of about 0.2 g protein was placed in a round-bottomed flask. 8 ml of 8% sodium bicarbonate solution (W/V) were then added and stirred for a few minutes using a magnetic stirrer. 0.3 ml of 1-fluro-2:4 dinitrobenzene (FDNB) was dissolved in 12 ml ethanol (FDNB was obtained from Eastman Organic Chemicals, Rochester, New York), added to the flask and the mixture was stirred for about two hours in the dark. The alcohol was then removed by heating on a steam bath. 24 ml of 8.1 N HCl solution were added cautiously and the flask was heated under reflux for 18 hours. Excess acid was then removed by concentration to dryness at 40°C using a rotatory flask evaporator. The residue was then dissolved in 1 N HCl, filtered and made to 500 ml with 1 N HCl.

An aliquot was then extracted 4 times with ether. Residual ether was removed by warming the solution. Portions (10 ml) were then pipetted into a tube (A); another in a separating funnel (B) and a third one in an Erlenmeyer flask. The latter portion was neutralized with 2 N sodium hydroxide. The same volume was added to portion B carefully to avoid effervescence followed by 10 ml of buffer solution of pH 8.5. 0.25 ml methoxycarbonyl chloride was then added and the mixture was vigorously shaken and left to stand for about 5 minutes. 3.75 ml

concentrated hydrochloric acid was then added cautiously and then the solution was again extracted three times with ether. The residual ether was evaporated by warming the solution. The density of the color of portions A and B was then read at 435 mu using a Beckman spectrophotometer. Sample B serves as a blank and "A - B" value is the optical density of the colored -DNP-lysine. A standard curve was constructed by plotting the optical density against the concentration of pure -DNP-lysine.

In some other experiments lysine as well as other amino acids, in protein samples or fecal materials, were measured by the usual acid hydrolysis. About 0.1 - 0.2 g sample was hydrolysed with 10 ml 6 N hydrochloric acid in sealed tube and under nitrogen atmosphere at 105 C in an oven for 18 hours. Excess acid was removed by concentrating the hydrolysate to dryness at 40 C using a flash evaporator. The hydrolysate was then dissolved in water, filtered and made to a known volume. Samples were kept in the freezer till analysed. Aliquot was then introduced in the top of the column of the Technicon amino acid analyzer.

Heat treatment of proteins:

Samples of egg albumin were first autoclaved for different time intervals at 121 C. Other samples were prepared to contain graded levels of glucose and were then autoclaved for 12 hours During later studies the egg albumin to which 2% glucose was added and autoclaved was used throughout.

In another type of experiment, fresh lean beefsteak obtained commercially was autoclaved for different time intervals in the same way as described above. The samples were freeze-dried and then extracted with diethyl-ether for 24 hours. Samples were aerated and ground in a ball-mill.

Growth experiments:

Male weanling rats (Holtzman and Sprague Dawley) weighing 50-55 g were used throughout these investigations. The rats were kept for 2 days in individual suspended screen-bottom cages and fed a basal wheat gluten diet. Wheat gluten was found to contain 11.3% butanal-extractable material, so this was taken into account in preparing the basal diet which contained 25% wheat gluten, 5% fat, 0.5% vitamin mixture, 5% salt mixture (Harper, 1959) and was made to 100% with a starch-dextrin mixture of 2:1 ratio.

To develop a standard curve for available lysine, graded levels of lysine hydrochloride were added to the basal diet. In other instances different amounts of the protein under test were added at the expense of the dietary carbohydrate. Groups of 6 animals each were fed ad libitum for a period of 2 weeks at 25°C. Rats were weighed every 2 days and food intake was recorded either for the group or for individual rats.

Carcass analysis:

At the end of the feeding period, some animals were bled, eviscerated and freeze-dried; moisture content was calculated. Carcass nitrogen was determined by the Kjeldahl method (using

mercury as a catalyst).

Digestibility measurement:

Groups of 3 animals each were fed the test protein at a level of 5% in the 25% wheat gluten diet for 6 days. The rats were then transferred to stainless steel metabolic cages where feces were collected for 4 days during which food consumption was recorded daily. A control group receiving 4% whole egg protein was used. Nitrogen in feed and feces was then determined. Correction for endogenous nitrogen as related to total body weight was done for animals fed the test diet.

Amino acid analysis:

Feces were dried and ground into a powder. Samples were hydrolyzed with 6 N hydrochloric acid under nitrogen in a sealed tube and kept in an oven at 105° for 18 hours. Excess acid was removed by repeated vacuum distillation at 40°C. Protein samples were also treated similarly. Amino acids were measured using a Technicon amino acid analyzer.

Results

Chemical analysis:

The amounts of available lysine in the different proteins used as determined by the FDNB-method are shown in Table 1. Although it is very low in lysine, 25% of wheat gluten can supply the requirements of the rat for all of the indispensible amino acids except lysine. For this reason wheat gluten was used as the protein source in the basal diet.

TABLE 1

Amounts of lysine available[1] in the proteins analyzed

Protein	gm %
Egg albumin	6.24
Casein	8.70
Wheat gluten	1.54
Meat	9.10

[1]Determined by the FDNB-method.

No change was observed in the amount of available lysine in egg white autoclaved alone for 2 hours, but when samples containing different amounts of glucose were autoclaved for 12 hours, the loss of available lysine increased as the amount of glucose added was increased (Table 2). The sample which contained 2% of glucose was analyzed for amino acid composition

TABLE 2

Effect of autoclaving[1] on the availability of lysine[2] in egg albumin-glucose mixture

g% glucose added	% loss of available lysine
1.0	26.1
1.5	40.0
2.0	43.9

[1]12 hour autoclaving.

[2]Determined by FDNB-method.

and not only was the value for lysine low, but also those for methionine, histidine and arginine (Table 3). Autoclaving

TABLE 3

Amino acid analysis of egg albumin[1]

Amino acid	Unautoclaved g %	Autoclaved g %	g % recovered
Val	5.99	4.99	83.3
Met	3.04	0.601	19.8
Ileu	4.58	3.67	80.1
Leu	7.6	6.07	79.9
Phe	5.19	4.72	90.9
Lys	6.59	4.53	69.7
His	2.20	1.49	67.7
Arg	5.74	4.02	70.0

[1]Determined by the amino acid analyzer.

beef muscle for different periods of times also resulted in a loss of available lysine, as determined by FDNB-method of 3.5 to 16.5% (Table 4).

TABLE 4

Effect of autoclaving period on "availability" of lysine in beef meat steak

Time of autoclaving	g% lysine[1]	% loss in availability
Non-autoclaved meat	9.10	0
30 minutes	8.87	3.5
3 hours	8.76	3.7
12 hours	7.60	16.5

[1]Determined by FDNB-method.

Reliability of standard curve for growth method of determining lysine availability:

The reliability of the growth method of assessing the amount of available lysine was estimated from the values for a standard curve relating weight gain to amount of lysine consumed. For each experiment there were 6 groups receiving 6 different levels of lysine which resulted in 6 different amounts of lysine consumed as shown in Table 5. The slope and the intercept of the simple regression line were calculated for each set of experiments (Snedecor, 1957). The regression lines obtained for the 3 experiments are shown in Figure 1. The standard deviation of the regression coefficient and significance

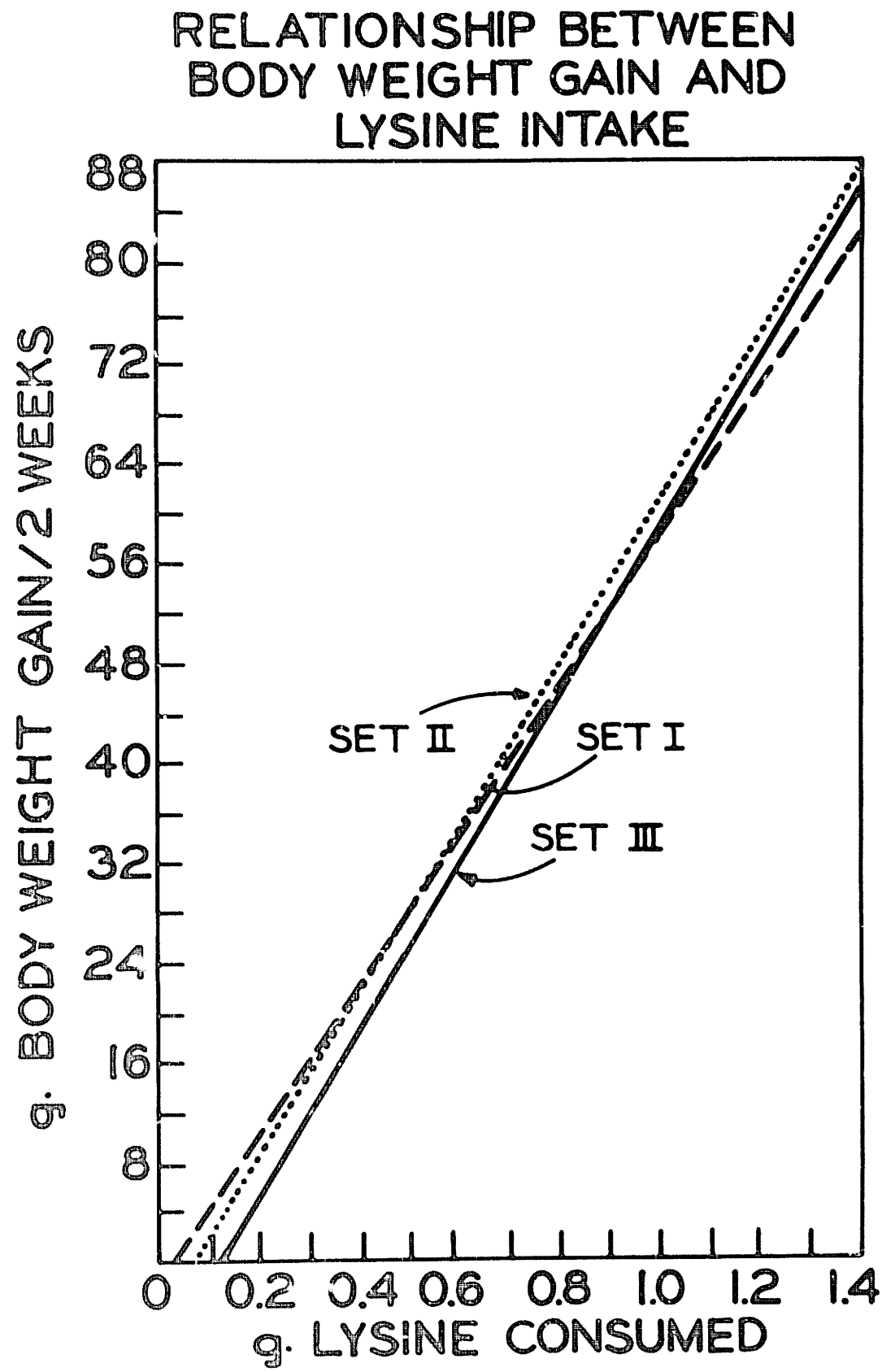
RELATIONSHIP BETWEEN
BODY WEIGHT GAIN AND
LYSINE INTAKE
g. BODY WEIGHT GAIN/2 WEEKS
88
80
72
64
56
48
40
32
24
16
8
SET II
SET I
SET III
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
g. LYSINE CONSUMED

FIGURE I

TABLE 5

Results of feeding different levels of lysine to rats for 2 weeks

Set No.	LYS level	Food consumed	LYS consumed	Weight gain[1]	P.E.R.
	g	g	g	g	
I	0.385	111.3	0.429	21.6 ± 2.19	0.88
	0.485	145.7	0.707	43.3 ± 1.78	1.35
	0.585	152.4	0.892	50.1 ± 2.25	1.48
	0.685	151.2	1.036	65.4 ± 3.04	1.96
	0.785	141.2	1.108	63.3 ± 2.15	1.77
	0.885	137.8	1.219	68.2 ± 2.98	2.19
II	0.385	127.6	0.491	25.7 ± 1.7	0.92
	0.435	144.1	0.627	35.7 ± 1.02	1.12
	0.535	163.2	0.873	55.6 ± 3.22	1.54
	0.635	180.3	1.145	71.1 ± 3.39	1.62
	0.735	176.7	1.299	86.7 ± 1.88	1.99
	0.835	174.4	1.456	85.3 ± 1.41	1.98
III	0.385	107.9	0.415	20.8 ± 1.39	0.88
	0.435	120.7	0.525	25.6 ± 1.57	0.96
	0.535	142.8	0.764	42.7 ± 2.94	1.35
	0.635	170.2	1.081	61.0 ± 1.61	1.61
	0.735	169.8	1.248	77.0 ± 3.48	2.02
	0.835	168.2	1.404	85.3 ± 3.71	2.25

[1]Average of 6 rats ± standard error of the mean.

of the regression and the correlation coefficient were calculated. A summary of the values obtained are tabulated in Table 6. Using the 18 individual groups to calculate the average standard curve for the three sets of experiments combined, the confidence belt was calculated for y on the x axis. This is illustrated in Figure 2.

TABLE 6

Statistical analyses of regression lines relating weight gain to lysine consumed

	Regression coefficient b	t = b/Sb*	r
Set I	59.98	11.14	0.984
Set II	65.74	12.92	0.988
Set III	66.96	32.36	0.998
Pooled sets	66.0	27.92	0.990

*t = test of significance of b. All have $p < 0.001$.

The same procedure was applied to test the relation between P.E.R. and lysine consumed. The correlation coefficients for the 3 sets of experiments are 0.985, 0.978 and 0.991 respectively.

Percentage nitrogen retention was calculated using the following equation:

$$= \frac{\text{carcass } N_2 \text{ of test group} - \text{carcass } N_2 \text{ of control}}{N_2 \text{ intake}}$$

The results are shown in Table 7. Similar statistical analyses showed a correlation coefficient between percentage nitrogen

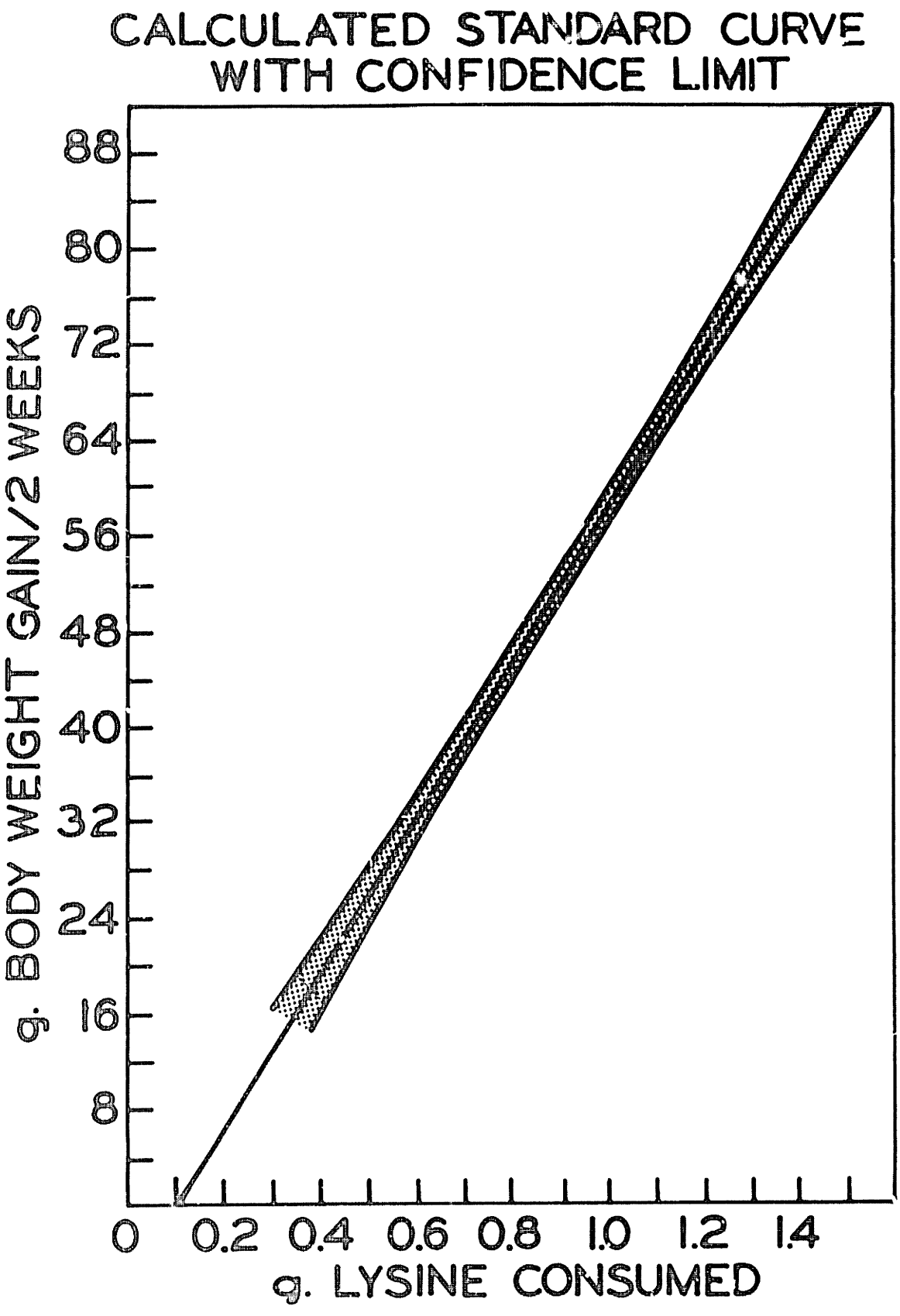
CALCULATED STANDARD CURVE
WITH CONFIDENCE LIMIT
g. BODY WEIGHT GAIN/2 WEEKS
88
80
72
64
56
48
40
32
24
16
8
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
g. LYSINE CONSUMED

FIGURE II

TABLE 7

Carcass analysis[1] of groups of rats fed graded levels of lysine

Lys.cons. g	% carcass analyzed	% H_2O in carcass	% N in carcass	Carcass N_2 corrected for original B. weight	g N gain in carcass	% N retention
0.43	81.4	61.8	2 89	2.08	0.64	16.5
0.71	87,0	61.0	2.68	2.52	1.08	21.0
0.89	83.0	61.1	2.85	2.88	1.44	26.6
1.04	82.9	62.9	2.78	3.24	1.80	33.2
1.11	83.3	64.8	2.96	3.38	1.94	38.2
1.22	82.4	62.0	2.97	3.55	2.11	42.4
0.49	78.0	64.1	2.45	1.98	0.54	14.3
0.63	79.6	62.9	2.52	2.29	0.85	18.7
0.87	78.6	63.0	2.46	2.72	1.28	24.7
1.14	81.3	63.8	2.49	3.16	1.71	28.2
1.30	80.2	64.7	2.61	3.70	2.26	37.6
1.46	84.3	65.5	2.80	3.94	2.50	41.3

[1]Pooled 6 carcass samples were analyzed.

retained and lysine consumed to be 0.973 and 0.984 for the two sets of experiments studied.

Measurements of biologically available lysine:

It was quite important to determine the availability of lysine in wheat gluten biologically and compare the result with the values obtained by the FDNB-method. Gluten was incorporated into the diet to furnish 2 different lysine levels. Food intake, weight gain and the computed weight gain from the standard curve and the amount of lysine estimated by the FDNB-method are shown in Table 8.

TABLE 8

Results of feeding wheat gluten alone[1]

Lysine level	Food consumed	Lysine consumed	Weight gain	
			found	expected[2]
g%	g	g	g	
0.485	133.0	0.645	36.3	36.0
0.635	134.9	0.857	45.6	49.2

[1]Average per rat.

[2]Computed from the standard curve of g weight gain vs. g lysine consumed.

The different proteins examined were similarly supplied in the diet at levels ranging from 2 to 7% replacing carbohydrate in the basal diet. Lysine availability has been calculated using the following two equations:

$$(1)\quad \%\ \text{lysine available} = \frac{\text{Total lysine as detd. by rat assay}}{\text{Total lysine as detd. chemically}} \times 100$$

This gives the availability of the total dietary lysine

$$(2)\quad \%\ \text{lysine available} = \frac{\text{Total lysine as detd. by rat assay} - \text{gluten lysine consumed}}{\text{Total lysine as detd. chemically} - \text{gluten lysine consumed}} \times 100$$

This gives the value of available lysine in the test sample only.

It is clear that the values obtained for autoclaved egg albumin by the second equation were extremely low (Table 9). Those for autoclaved meat were below the values obtained by the FDNB-method. The low values for the autoclaved products were thought to be due to the presence of a toxic material formed during autoclaving of the protein which might have affected the body weight gain of the animals.

Groups of 6 animals each were fed a basal diet containing 18% casein supplemented with 0.2% L-methionine and the same diet with 2, 4 or 9% autoclaved egg albumin added. No depression in body weight gain was observed over the 2-week feeding period (72.5 g to 74.8 g) and the feed efficiency ranged from 45.7 to 51.7 (g weight gain/100 g food consumed). Therefore, it seemed unlikely that any toxic substance was formed du ing autoclaving.

Using values for the amino acid composition of egg albumin, a 25% gluten diet was prepared to contain the amino acids that

TABLE 9

Biologically available lysine in proteins studied as calculated by different methods*

Protein level	Weight Gain	Lysine consumed	% available lysine	
			Method I	Method II
g%	g	g/rat/ 2 weeks		
Unautoclaved egg albumin				
1.6	52.8	0.98	100.1	100.2
4.8	53.3	0.934	97.6	94.0
5.0	57.1	0.99	98.0	94.6
Autoclaved egg albumin				
5	25.4	0.785	62.7	16.8
5	29.5	0.884	66.5	25.6
5	29.3	0.902	61.5	13.4
7	31.9	1.004	59.0	20.0
7	33.6	1.124	55.0	15.2
7	32.2	1.102	54.6	13.5
Nonautoclaved meat				
2	46.0	0.784	102.7	106.3
4	63.5	1.161	93.6	91.7
Autoclaved meat				
2	32.5	0.687	87.3	61.4
4	55.5	1.159	81.5	65.5
Nonautoclaved casein				
1.8	35.8	.648	96.0	85.6
4.3	61.0	1.028	94.1	87.6

*c.f. text for equations

would be supplied by 5% of unautoclaved egg albumin but with the amount of lysine that would be supplied by 5% of autoclaved egg albumin as determined by the FDNB-method. Body weight gain of the group that received the amino acid mixture coincided with the computed value (g weight gain/2 weeks - g lysine consumed). This shows that values obtained for FDNB-method coincide with the biological values for untreated samples.

In another attempt to study this problem, methionine was added to diets containing the autoclaved and unautoclaved egg albumin and beef. The availability of lysine was calculated by the previously mentioned methods and the results showed that the addition of methionine was without effect as shown in Table 10.

TABLE 10

Effect of addition of methionine on availability of lysine of autoclaved proteins

	Weight gain	Lysine consumed	Method I	Method II
Autoclaved egg albumin	g.	g.		
5%	26	0.759	66.3	32.6
5% + 0.2% L-meth.	25	0.723	67.5	27.2
Autoclaved meat				
2%	34.3	0.699	90.1	62.5
2% + 0.2% L-meth.	34.0	0.710	88.9	56.1

Digestibility of egg albumin:

The digestibility values were calculated for the autoclaved and nonautoclaved samples:

$$\text{True digestibility} = \frac{\text{N intake} - (\text{fecal N of test group} - \text{fecal } N_2 \text{ of control})}{\text{N intake}}$$

It is clear that this equation measures the digestibility of the total dietary protein. Therefore, in order to calculate the digestibility of the protein supplement, the following equation was used:

Gluten N consumed X $\frac{100}{100}$ + test protein N consumed X $\bar{\bar{Y}}$ =

Total dietary protein N X observed digestibility, where $\bar{\bar{Y}}$ is the digestibility value we are interested in (and assuming 100% digestibility of gluten). Results obtained (Table 11) show that the digestibility of the autoclaved egg albumin

TABLE 11

Digestibility of autoclaved and unautoclaved egg albumin[1]

Protein	Observed true digestibility of the total dietary protein	Calculated[2] digestibility of the test protein
Unautoclaved	99.2	94.4
Autoclaved	87.6	18.1

[1]Average of three experiments.

[2]Using the equation mentioned:

N-supplied by gluten X $\frac{100}{100}$ + N_2-supplied by test protein = total N_2Xobserved true digestibility.

was greatly decreased. The amino acid analysis of the feces showed that not only was lysine excretion greatly increased but also excretion of other essential amino acids as shown in Table 12.

TABLE 12

Excretion of fecal amino acids by groups* of rats fed 5% autoclaved I and 5% non-autoclaved II egg albumin samples

Amino acid	Mg/100 g food consumed I	Mg/100g food consumed II	I/II
Val	343	84	4.1
I Leu	261	69	3.8
Leu	419	99	4.2
Phe	288	58	5.0
Lys	309	94	3.3
His	102	26	3.9
Arg	231	64	3.6

*Amino acids recovered in pooled feces from 4 rats excreted in 4 days.

Discussion

Gupta et al. (1958) used a basal diet containing 20% of wheat gluten supplemented with some essential amino acids for measurement of availability of lysine by the growth method. Munaver and Harper (1959) suggested that a 30% wheat gluten diet would provide the accepted amino acid requirements for the rat except lysine. We found that a 25% wheat gluten supplemented with lysine to give a lysine content of 0.835%, supported a rate of weight gain that compared well with that obtained with an 18% casein diet supplemented with 0.2% L-methionine.

From Table 4 it is clear that addition of lysine to the basal diet stimulated food intake. Also body weight gain increased with increasing lysine consumption. However, total food intake increased with increasing dietary lysine level only up to about 0.6% and after that it remained more or less constant, but lysine intake and growth rate continued to increase. Therefore, a higher correlation should be expected between lysine consumed and body weight gain (DeMuelenaere, Chen and Harper, 1967), and a highly significant regression coefficient and a very high correlation coefficient (almost 1.00 in one set of experiments) were obtained. This shows that the standard curve for the growth method provides a reliable measure of free lysine consumed. Also a high correlation was obtained between P.E.R. and lysine consumed as well as between percentage nitrogen retained and lysine consumed. DeMuelenaere et al.

(1967) suggested that the increase in the protein content of the diet may tend to decrease the value for availability determined by growth. However, values obtained for the untreated protein samples show that this does not occur when a 25% gluten diet is used and the protein supplement is included at a 5% level. Also the possibility that an amino acid imbalance depressed growth under such conditions seems unlikely as the amino acid mixture simulating egg albumin in its composition, except for lysine, did not depress growth.

The amino acid composition of a protein can be altered by severe heat treatment and even more if it is heated in the presence of free aldehyde groups of glucose. This is shown clearly in Tables 2, 3 and 4. However, values for availability of lysine vary with the method used (DeMuelenaere et al., 1967). Available lysine in autoclaved egg albumin as determined by Carpenter's method was about 56% while that determined by the growth method was about 20%. Similar but smaller differences were observed for autoclaved beef muscle. The reason for such differences is presumably that the presence of bound sugars interfered with enzymatic hydrolysis in the intestine, in such a way that adjacent amino acids were not hydrolyzed (Donoso et al., 1962). This is indicated by our observation that about 80% of the autoclaved egg albumin-lysine was excreted in feces (calculated on the assumption that lysine of wheat gluten was completely available). In a comparison of a chick assay method with the FDNB-method for determining the availability of lysine in foods, Carpenter and associates (1963) used a non-purified

diet to which were added different protein supplements. The FDNB-method gave values that were lower than the values obtained by the chick assay. On the other hand, when total lysine in the samples was determined chromatographically, the values came out higher than the values obtained by the FDNB-methods, which is to be expected. In fact, we found that the FDNB-value of heat treated egg albumin was about 14% less than the value recovered by acid hydrolysis (Tables 2 and 3). The discrepancy in Carpenter's results is probably due to the fact that the dietary lysine content was based on calculation. Also the availability values given by the chick assay is a measure of the total lysine available in the diet and not of the protein under test. In fact, when they doubled the amount of herring meal added in the chick assay there was a drop in available lysine when measured by that biological technique.

Stott and Smith (1966) compared values for lysine available in some foods as determined microbiologically with those obtained by the FDNB-method. It was found that values obtained microbiologically were much lower than those obtained by the FDNB-method and in some instances the microbiological values were about 60% of those given by that chemical method. Ford in 1964 mentioned that the availability of lysine in a leaf protein concentrate was low using a microbiological assay procedure, while the FDNB-method gave higher values. Interestingly, using more rigorous enzymatic digestion with pepsin or higher concentrations of papain, higher values were obtained. This indicated that the protein as a whole was digested slowly. However,

microbiological results were consistent with biological assay using the rat (Henry and Ford, 1965). Our results show similar differences between the rat assay and the Carpenter procedure in determination of available lysine in heat treated proteins. (FDNB-method gave a value of 56% available lysine in autoclaved egg albumin while the rat-assay gave about 20% available.) Also, when availability was calculated using the equation:

$$\text{Total availability of lysine in diet} = \frac{\text{lysine intake} - (\text{lysine excreted by test} - \text{lysine excreted by control})}{\text{lysine intake}}$$

(values from fecal excretion experiment). The value came out as 65%, which is almost the same as the value obtained by using equation 1 for the total lysine available in the diet using the growth experiment.

Our results also show a high correlation between the values obtained by the FDNB-method and the growth assay procedure for unautoclaved egg albumin, beef protein and casein. The discrepancy occurred only with the autoclaved protein preparations. The FDNB-method as recommended by Carpenter would take no account of rate of digestion nor the possibility that, after digestion of a severely processed "food," many of the lysine moieties reacting as "available" would remain bound in indigestible peptide residues and thus inaccessible to the rat.

Summary

Chemical (FDNB) as well as biological methods (rat-growth assay, carcass analysis and fecal analysis) for determining the availability of lysine in foods were studied.

A 25% gluten diet was used as a basal diet to which graded levels of lysine were added. There was good correlation between lysine consumed and weight gain, carcass nitrogen and protein efficiency ratio of rats fed these diets for 14 days.

Autoclaving egg albumin to which 1-2% glucose was added for 12 hours resulted in decrease in the availability value (as determined by the FDNB-method) depending on the amount of glucose added. However, the rat-growth assay for the sample that had 2% glucose gave a far lower value than the FDNB procedure. This lowering in growth response was not due to the formation of toxic compounds or due to loss of methionine during autoclaving.

Measurement of digestibility of the autoclaved egg albumin gave a value similar to that obtained by the growth assay for lysine availability.

Fecal analysis showed that not only was lysine excretion by rats fed autoclaved egg albumin increased, but also other amino acids were excreted in greater amounts.

The value for available lysine in autoclaved beef muscle as determined by the growth-assay was lower than that obtained by the FDNB-method.

The use of the FDNB-method cannot be recommended for the estimation of available lysine in heat-treated foods.

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Suggestions for Future Work

The bases for the beneficial effects due to addition of different amino acid supplements to a high tyrosine diet seem quite complex. All of the effective amino acid supplements acted to depress plasma tyrosine concentration and to stimulate tyrosine-α-ketoglutarate transaminase activity to different degrees. For example, threonine was the only amino acid that alleviated completely the toxicity syndrome while glycine alleviated only the external lesions yet it resulted in higher tyrosine transaminase activity. Threonine supplementation did not increase adrenal weight while glycine did. However, the excretion patterns (total phenol and homogentisic acid in urine) by rats fed these two supplements were similar. Threonine as mentioned before being the limiting amino acid may increase tyrosine utilization for tissue synthesis while glycine supplementation may act to increase its catabolism. Such conditions can be studied in some detail using an approach similar to the one used to elucidate the role of cortisol in tyrosine toxicity. It is advisable to study the uptake of labelled tyrosine by different tissues especially after threonine supplementation.

Adrenal weights of rats were found to be increased by high tyrosine feeding. Experiments are needed to show whether this truly represents increased synthesis and secretion of glucocorticoids. This can be done by measuring the amount of adrenal cortical hormone in the adrenal as well as its metabolites in urine at different time intervals.

The decrease in tyrosine transaminase activity with time in rats injected daily with cortisol acetate is of great interest. The capacity of the animal to catabolize this compound needs to be investigated thoroughly. This can be done by injecting labelled compound at different time intervals, e.g., at 5 and 10 days of the experimental period and following its metabolites in the urine. The role of liver in catabolizing the compound can be studied in vitro or in vivo using hydrocortisone-1,2-H^3 and following its metabolites chromatographically.

Because of the exclusion of the transaminase pathway of tyrosine metabolism as a possible source of toxic agent in tyrosine toxicity, other pathways need to be investigated. In particular, thyroxine synthesis under such experimental conditions is of great importance. We gathered, indirectly, evidence for a role of thyroxine, however, the direct approach then should be whether tyrosine in excess influences thyroxine synthesis and secretion by the thyroid gland. Measurement of protein-bound iodine or preferably the butanol-extractable iodine in blood from rats fed the experimental diets, is very important, especially the latter as representing the active form of thyroxine. ^{131} uptake by the thyroid gland might also be measured, however, this will not furnish direct proof of active synthesis and secretion of thyroxine by the gland.

Epinephrine effect should also be studied under such conditions as it represents an important intermediate of other pathways of tyrosine metabolism. Measuring mandelic acid excretion in urine is appropriate for such investigation.

With respect to measurement of the availability of amino acids in foods, especially lysine, the FDNB-method is attractive. It proved to be reliable when used to estimate available lysine in unheated foods especially of low carbohydrate content. However, it does not appear reliable for heat processed food or foods that are rich in carbohydrates.

In order to overcome the destructive effect of carbohydrates on DNP-lysine during acid hydrolysis, Mauron and Bujard (1963) suggested guanidation as an alternative approach for determining lysine in foods. Guanidation with o-methylisourea transforms lysine molecule with a free ϵ-amino group into homoarginine which is stable during acid hydrolysis. This technique needs further investigation to see if it is more useful than the FDNB-method.

In fact, the main problem in availability measurements of amino acids in foods is the need for appropriate and reliable methods. To avoid inherent problems when using bioassay techniques, chemical methods are greatly needed. The sulfur/nitrogen ratio in proteins deserves thorough investigation. Tryptophan, which is limiting in many foods especially of plant origin, has not received much attention. This may be due to lack of methods suitable to estimate this amino acid in foods. Also one should not overlook the importance of the bioassay methods, as they are the ultimate goal. They need to be refined. Formulae must be developed to calculate the availability of amino acids in the test-protein rather than the value for the total available amino acid in the diet.

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Biography

The author was born in Assiut, Upper Egypt, in March, 1935. He completed his high school education in 1951. Then he went to the Faculty of Science, Cairo University, where he graduated with honors in May, 1955. After that he spent one semester studying psychology of education. In 1956 he got a scholarship for "training in research" at the National Research Center, Cairo and in 1958 he was appointed as an Assistant Researcher in the Nutrition Research Unit, National Research Center, Cairo. He got his master's degree at the Faculty of Science, Cairo University, Cairo, in 1961.

In 1962 he got an Egyptian scholarship to study at the Department of Nutrition and Food Science, M.I.T., Cambridge, Mass. After January, 1966, the author was permitted to complete his thesis in absentia at the Department of Biochemistry, University of Wisconsin, Madison, Wis., when his thesis supervisor, Prof. A. E. Harper, moved to the latter University.

It is sad that the author's father died in January, 1965, without seeing the author. His mother is still living, together with a brother and sister.