THE AVAILABILITY OF THE ISOMERS AND SOME ANALOGUES OF TYROSINE IN REPLACING DIETARY PHENYLALANINE IN THE NUTRITION OF THE GROWING RAT

by

EDWARD CHARLES BUBL

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1948

APPROVED:

Redacted for Privacy

Professor of Chemistry

In Charge of Major

Redacted for Privacy

Head of Department of Chemistry

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Professor Joseph S. Butts for his helpful criticism and guidance in carrying out this study.

Special thanks are due also to Professor Vernon H. Cheldelin and Mr. S. C. Fang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

THE AVAILABILITY OF THE ISOMERS AND SOME ANALOGUES OF TYROSINE IN REPLACING DIETARY PHENYLALANINE IN THE NUTRITION OF THE GROWING RAT

SECTION I

INTRODUCTION

Early studies on the nutritional importance of the amino acids were hampered by the lack of good methods for separation and analysis. The magnitude of the problem can be realized when one considers that, upon hydrolysis, there are obtained ten to twenty individual components closely alike in chemical properties. Therefore, it is not surprising that by the end of the nineteenth century, ninety years after the first amino acid was isolated, only twelve naturally occurring amino acids were known (1). This lack served to stimulate widespread research and in the next fifteen years, all but two of the twenty-six amino acids now known had been isolated.

As the studies on amino acids progressed, it became apparent that the proteins and their constituent amino acids occupied a unique position in nutrition. Unlike the fats and carbohydrates, which are used mainly for fuel and energy, the amino acids are utilized in the synthesis of living tissue.

Soon another fact was noted: that higher nitrogen content did not indicate nutritional superiority. It was discovered that inferior proteins could be made to support growth by the addition of certain amino acids. In this manner, it was found by Osborn and Mendel (2) that tryptophane and lysine were essential for the growth of rats. These amino acids were added to zein, a protein deficient in tryptophane and lysine. Good growth occurred. However, the removal of either of the amino acids caused immediate cessation of growth. Thus, it was concluded that these were necessary for growth, regardless of the level of nitrogen intake.

The first attempts to compound an amino acid mixture which would support growth led to inconclusive results. Abderhalden (3) prepared a diet containing sixteen fairly pure amino acids which he fed orally to dogs. The animals refused the food, and even upon forced feeding, Abderhalden was unable to keep the animals in positive nitrogen balance. A few years later, Hopkins (4) prepared a similar diet from the amino acids known at the time (methionine and threonine missing) and found that rats lost weight slowly but survived for some time. Other efforts in this country (5) and in Japan (6) indicated the same

conclusion, that the amino acid mixtures thus prepared would not support growth.

In the early thirties, a series of experiments were initiated at the University of Illinois which changed the entire picture of amino acid nutrition. Professor W. C. Rose began to investigate the use of crystalline amino acid mixtures in the nutrition of the rat. The first major discovery of these workers was that growth promoting proteins must contain at least one amino acid in addition to those known to be essential. A search for this component led to the isolation and identification of the amino acid threonine (7). This amino acid proved to be essential for the growing rat and in all probability was the only one remaining to be discovered (8).

By using amino acid mixtures containing all of the known acids except the one being tested for its growth promoting action, Rose was able to prove that only ten amino acids are essential for the growth of the white rat. His mixtures were patterned after the amino acid composition of casein. His conclusions are listed in Table I (9, 10).

TABLE I

Naturally Occurring Amino Acids

Essential Amino Acids	Nonessential Amino Acids
*Valine	Glycine
*Isoleucine	Alanine
*Leucine	Serine
*Threonine	Norleucine
*Methionine	Aspartic acid
*Lysine	Glutamic acid
Arginine	Proline
Histidine	Hydroxyproline
*Phenylalanine	Tyrosine
*Tryptophane	Cystine
	Citrulline
	Thyroxine

These same amino acids were found by Rice and Rose (11) to be essential in canine nutrition. In a later series of papers, Rose and his co-workers (12, 13, and 14) were able to show that in adult humans, only eight of the above ten essential amino acids were needed to maintain positive nitrogen balance. Those starred in Table I are essential for maintenance in man.

With the expanding use of amino acids in experimental work, it became increasingly important to know the biological effects of the unnatural isomers. The natural occurring amino acids are of the L configuration; the D component is considered the unnatural isomer. Since many of the amino acids available were

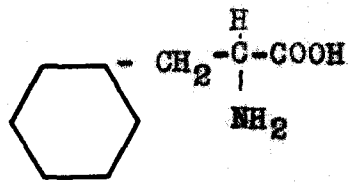
prepared synthetically and were DL mixtures the amount of the L component needed was supplied by doubling the requirement. Many workers considered this not only a waste of expensive amino acids, but feared that the feeding of the D component might cause detrimental results. Consequently, studies were undertaken to determine the nutritional availability of the D isomer.

Both enantiomorphs of the amino acid can be utilized for growth in the rat in the case of tryptophane (15), phenylalanine (16), methionine (17), and histidine (18), while the unnatural form fails to show growth responses for valine, leucine, isoleucine (10), threonine (19), and lysine (20).

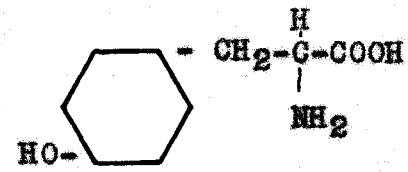
The work of Albanese and coworkers indicates that there may be a distinct species variation in the metabolism of D amino acids. These workers, using an excretion technic following the feeding of a large dose of an amino acid, found that the D forms of some amino acids are utilized by man, whereas others are almost completely excreted. DL-Arginine, non-essential for adult humans, seems to be catabolized totally upon ingestion (21). In the case of tryptophane (22, 23), these workers found that L- but not D-tryptophane is available to man, while the rat can utilize either stereoisomer. On the other hand, man can utilize both

forms of acetyl-tryptophane while only acetyl-L-tryptophane is available for the rat. Both isomers of methionine are used by man (24).

It may be noted from Table I that the amino acid tyrosine is listed as non-essential. This amino acid closely resembles the essential amino acid phenylalanine. It has been believed for many years



Phenylalanine



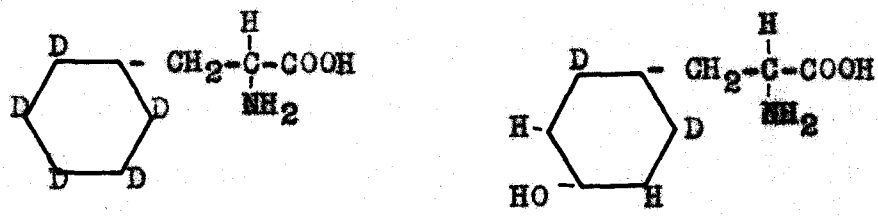
Tyrosine

that these two amino acids were interchangeable (25, 26, 27, 28). Womack and Rose (29) found that tyrosine could not replace phenylalanine in the diet of a rat, but that the inclusion of tyrosine had a sparing action upon the phenylalanine requirement of the animal (30). This evidence seemed to point to the conversion of phenylalanine to tyrosine but not the reverse. Additional evidence that the interconversion might be in one direction was the isolation of tyrosine from the animals whose diet had been devoid of tyrosine but contained phenylalanine.

Positive proof of the interconversion was shown by Moss and Schoenheimer in their isotopic studies (31). Using phenylalanine labeled with deuterium in the positions indicated in Figure 1, they were able to show that phenylalanine is rapidly converted into tyrosine, not only in the growing rat, but also in the full grown rat.

FIGURE 1

The Conversion of Isotopic Phenylalanine to Tyrosine



A most startling fact to come out of these studies was that the conversion of phenylalanine to tyrosine occurred even when the diet was high in tyrosine. Further studies (32, 33) indicated that tyrosine was available to the animal from two sources, dietary and metabolic.

Recently, it was shown by Heir (34) that upon feeding of a single amino acid by gavage, the blood level of that amino acid would rise sharply. Upon

the feeding of phenylalanine, it was found that the blood level of tyrosine as well as that of phenylalanine would rise within a few hours after ingestion. When tyrosine was fed, the phenylalanine level in the blood remained constant while the tyrosine level rose.

Thus good evidence has accumulated indicating that phenylalanine is oxidized to tyrosine but that the reverse of this reaction does not occur to any extent.

In the recent studies of Womack and Rose (35), the quantitative requirement of the rat for phenylalanine was determined. They found that 0.9 per cent phenylalanine would meet the requirements of the animal for this compound as long as the diet provided an abundance of amino acids other than tyrosine. Upon further study (36), they found that about one-half of the phenylalanine requirement of the animal could be met by L-tyrosine. Thus, an animal whose diet contained 0.4 per cent tyrosine and 0.5 per cent phenylalanine grew as well as the animals whose diet contained 0.9 per cent phenylalanine.

The papers existing in the literature seemed to indicate that D-tyrosine was not metabolized by animals. An early paper by Wohlgemuth (37) indicated

that when rabbits were fed racemic tyrosine, 76 per cent of the D-isomer was excreted into the urine.

Using human subjects, Albanese, Irby, and Lein (38), employing an excretion technic, report that sufficient tyrosine and aliphatic organic acids are found in the urine to account for most of the D-isomer. These workers fed 0.01 mol of DL-tyrosine and collected the urine for forty-eight hours. The urine was analyzed for tyrosine by the chemical method of Lugg (39) and for organic acids by the method of Van Slyke (40). The rise in organic acids and tyrosine following the ingestion of DL-tyrosine is appreciable. Upon the ingestion of L-tyrosine at the same level, there is little excretion of tyrosine or organic acids. The conclusion drawn from these data is that the D isomer is poorly or totally unavailable for normal physiological function in man. However, there is no evidence presented in the paper which would identify the urinary tyrosine to be of the D configuration. Albanese in another paper (23) indicates that "it is reasonable to assume that the unnatural component is more readily excreted because it is less readily utilized." If this is the case, apparently the renal and blood thresholds of the optical isomers must be different.

It should now be possible to answer the question of the nutritional availability of D-tyrosine since Rose and coworkers have shown that on amino acid diets, L-tyrosine can replace one-half of the phenylalanine requirement of the rat.

In the studies with these mixtures supplying the entire protein nitrogen intake, it is desirable to know if the growth response to an amino acid mixture is equal to the response to an intact protein containing the same amino acids. The literature contains a number of papers concerning this question and in some cases data are presented which seem to indicate that the intact protein may be superior to the amino acid mixture, even though the same acids are present in both diets and in the same amount.

Albanese (41) using a mixture of the ten essential amino acids plus cystine at a level of 14.7 per cent found that rats lost weight rapidly and death occurred after a few weeks. If the level of the nitrogen in the ration was increased to 29.4 per cent, the same results occurred. If the ration at the 14.7 per cent level was supplemented with a small percentage of casein, growth was obtained. The authors concluded that the lack of growth was due to the toxic effect of the D amino acids in the ration.

The results of Kinsey and Grant (42) are just the reverse. They found that the mixture of the ten essential amino acids would support growth when fed at 11.5 per cent level. However, much superior growth was noted when one-half of the nitrogen in the diet came from casein. The reason for these contrary results might be the difference in the vitamins supplied to the animals. In the experiments of Albanese, the vitamins were supplied as yeast and cod liver oil; in those of Grant, the vitamins were added as crystalline compounds and liver extract.

Silber (43) and Benditt (44) have shown also that mixtures of the ten essential amino acids give fair growth. Frazier (45, 46), by using protein depleted animals, was able to confirm these results but could not find any good indication that the inclusion of casein in the diet would insure better growth. Womack and Rose (47) in a short note, indicate that much better growth could be obtained when part of the diet was added as complete protein. Rose compares this growth factor to arginine; it is needed for the most rapid growth, but it is not completely essential.

Strepogenin is the name given to a growth factor, concentrated by Sprince and Woolley (48, 49), which is essential for the growth of certain hemolytic

streptococci of Lancefield Group A. This factor is a water-soluble, alcohol-insoluble, non-dialyzable compound found in liver and enzymatic digests of pure proteins, such as casein and crystalline insulin. Woolley (50) has correlated the strepogenin content of proteins with their growth promoting powers in mice on amino acid diets. This growth promoting effect could not be attributed to known amino acids. It appears that some proteins contain something of nutritional importance apart from the essential amino acids. This phenomenon has now been shown for rats (51), mice (52), and chickens (53, 54). From other experiments, Woolley has concluded that this factor may be a peptide which is not destroyed by enzymatic hydrolysis, but is completely destroyed by acid hydrolysis. This peptide may be a glutamic acid containing peptide (55).

Although the problem of growth on amino acid mixtures versus intact protein is not a major part of this study, information has been obtained concerning growth rates under these two conditions. These data are included in this thesis.

The following study was undertaken to determine the growth response of rats to an amino acid diet

containing a suboptimal level of phenylalanine plus the isomers or analogs of tyrosine.

SECTION II

EXPERIMENTAL

The animals used throughout this study were weanling rats, twenty-eight days of age. These animals had been born of the inbred females of the departmental stock colony. The colony was started originally from the Evans-Long strain. To our knowledge, the strain has been kept pure.

The experimental animals were placed in individual wire-bottomed cages and were allowed water and, except when noted, the experimental diets ad libitum. Animal weights were recorded daily. The animals were fed by placing a weighed amount of food in the containers each day. The food thus measured was always in excess of what might be eaten. The food intakes were calculated by difference after careful collection of spillage.

Stock Ration Experiments

The animals in the stock colony are fed routinely on a ration prepared by one of the local feed companies. This ration is composed as follows:

TABLE II

Composition of Stock Ration (Ration 1)

Material	Per cent
Whole yellow corn meal	38.0
Whole wheat flour	31.9
Ground alfalfa leaves	7.6
Skim milk powder	20.0
Irradiated yeast	1.0
Sodium chloride	0.5
Calcium carbonate	0.5
Cod liver oil	0.5

It seemed desirable at the beginning of the work to obtain the growth rate of weanling animals on this diet and to determine its adequacy for reproduction. The growth curve is shown in Figure 2. Table III summarizes the data obtained.

TABLE III

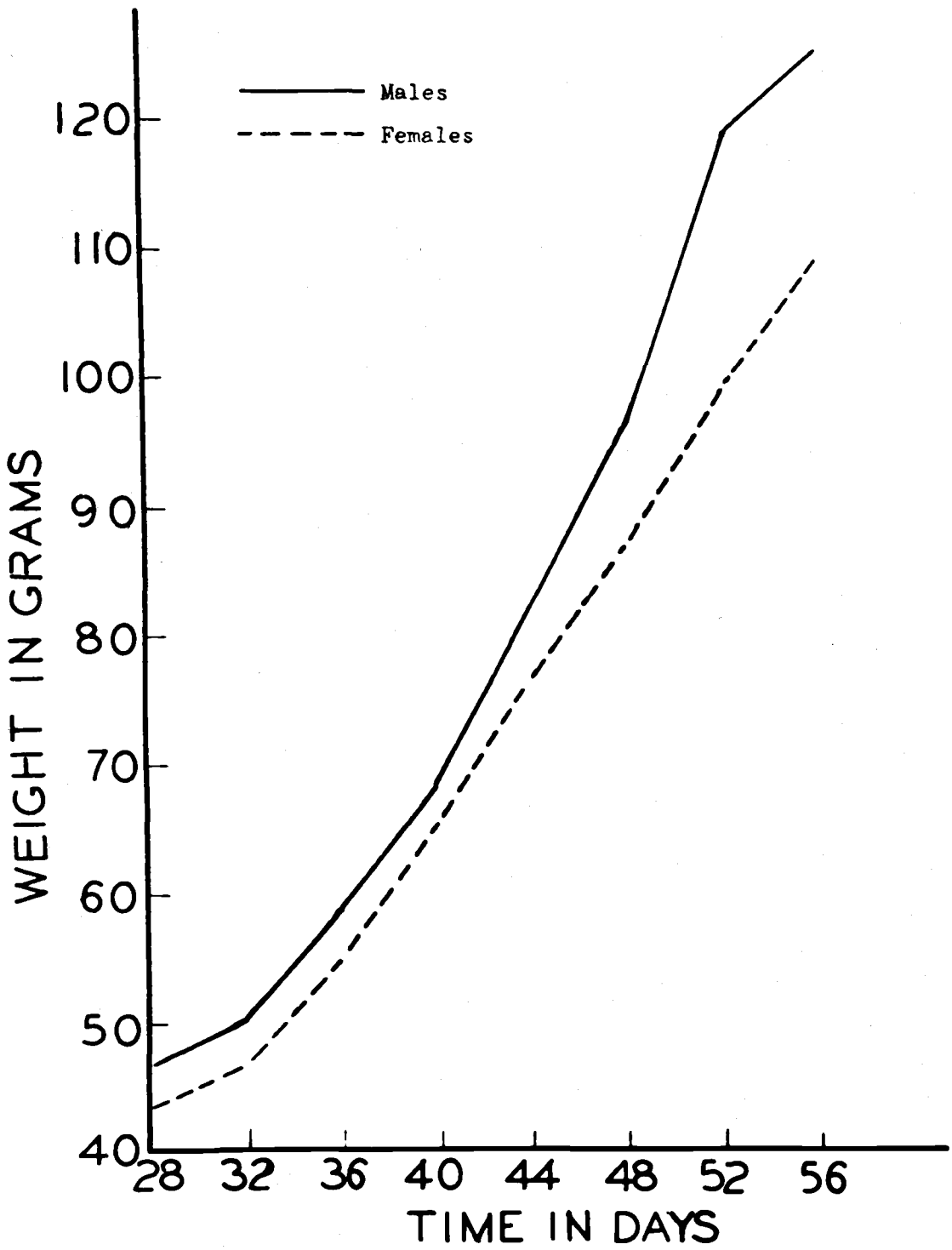
Growth Response of 28 Day Old Rats to Stock Diet (Ration 1), ad libitum Feeding

(Each experiment covered 28 days)

Sex	Number of rats used	Average total gain in weight in grams	Approximate food intake in grams	Grams food divided by weight gain
Male	5	65.6	336	5.12
Female	12	56.3	285	5.06

FIGURE 2

Growth Response of 28 Day Old Male and Female Rats Receiving Stock Ration Ad Libitum



This mixture proved to be adequate in respect to reproduction and lactation. There were seasonal variations noted, however, which might have been due to the freshness of the ground alfalfa leaves. Rats are known to need a source of vitamin E (56) and possibly unknown factors which are contained in green leafy material. This seasonal variation could be corrected if greens in the form of grocer's scraps were fed. If these were not available, the following mixture was found to give excellent results.

TABLE IV

Supplement to Stock Ration for Reproduction

Material	Per cent
Brewers yeast	50
Liver extract	49
Cystine	1

The Basal Diets

Since an amino acid diet is expensive, it was considered advisable to examine the growth rates of rats on a number of basal diets appearing in the literature in order to determine which would be most suitable for our purpose. In the main, there were two types of basal rations considered: the high and low fat diets.

The High Fat Diet

The high fat diet has been used by Rose and co-workers (10) for many years according to his published results on the essential amino acids. It is a thick paste-like mixture composed of the following ingredients.

TABLE V

High Fat Diet (Ration 2)

Material	Per cent
Cornstarch	31.3
Sucrose	15.0
Osborne-Mendel salts	4.0
Agar	2.0
Lard	26.0
Cod liver oil	5.0
Protein[1]	16.7

[1] Vitamin-free casein. General Biochemicals, Inc., Chagrin Falls, Ohio

The vitamins were given in the form of small pellets containing approximately the following level in each.

TABLE VI

Vitamin Supplements Used in High Fat Diet

Vitamin	Milligrams
Thiamine hydrochloride	0.01
Riboflavin	0.02
Niacin	0.01
Calcium pantothenate	0.05
Choline chloride	5.0
p-Aminobenzoic acid	1.5
Wheat germ oil[1]	25.0
Liver extract[2]	12.5

[1] Viobin Corporation, Montecello, Illinois
[2] Wilson's 1:20. Wilson and Company, Chicago, Illinois

Figure 3 shows the growth curve for this ration. Table VII summarizes the experiment.

TABLE VII

Growth Response of 28 Day Old Rats Receiving High Fat Diet ad libitum

(Each experiment covered 24 days)

Sex	Number of rats used	Average total gain in weight in grams	Average food intake in grams	Grams food divided by weight gain
Male	4	38.2	121.5	3.2
Female	6	37.9	132.5	3.7

The Low Fat Diet

In a recent paper from Rose's laboratory,

Borman et al (57) published data which indicated that rats made somewhat better gains when the ration contained a smaller proportion of fat. The studies indicated also that the Osborne-Mendel salt mixture used previously did not furnish enough phosphorus for maximum gain in the absence of casein or yeast. The Jones salt mixture is used now (58). Also, the vitamin intake of the diet has been increased. The composition of the diet is as follows:

TABLE VIII

Low Fat Diet (Ration 3)

Material	Per cent
Sucrose	71.74
'Cellu flour'[1]	2.00
Jones salts	4.00
Corn oil[2]	2.00
Vitamins A and D concentrate[3]	0.05
Choline chloride	0.20
Inositol	0.20
Liver extract[4]	0.40
Protein[5]	19.51

[1] Chicago Diabetic Supply Company, Chicago, Illinois
[2] 'Mazola Oil.' Corn Products Refining Company, Argo, Illinois
[3] Containing 400 I.U.D. and 300 I.U.A. per gram. Napco XX. National Oil Products Company
[4] Wilson's 1:20. Wilson and Company, Chicago, Illinois
[5] Vitamin-free casein. General Biochemicals, Inc. Chagrin Falls, Ohio

The vitamins in crystalline form were mixed directly into the ration, which eliminated the use of pills and made for a more uniform vitamin intake in relation to the food intake. The vitamin levels in a kilo of mixed ration are as follows:

TABLE IX

Vitamin Supplements Used in Low Fat Diet

Vitamin	Milligrams
Thiamine hydrochloride	5
Riboflavin	10
Pyridoxine hydrochloride	5
Nicotinic acid	5
Calcium d-pantothenate	25
p-Aminobenzoic acid	300
α-tocopherol	25
2-methyl-1,4-naphthoquinone	2

Biotin was omitted from the vitamin supplements because a source was not available when the experiment was begun. It was considered that the liver extract would supply this factor, or in any case, it would not be limiting in a twenty-eight day experiment.

Figure 3 shows the growth curve for this ration. Table X summarizes the experiment.

FIGURE 3

Growth Response of 28 Day Old Rats to High (Ration 2) and Low (Ration 3) Fat Diets. Ad Libitum Feeding.

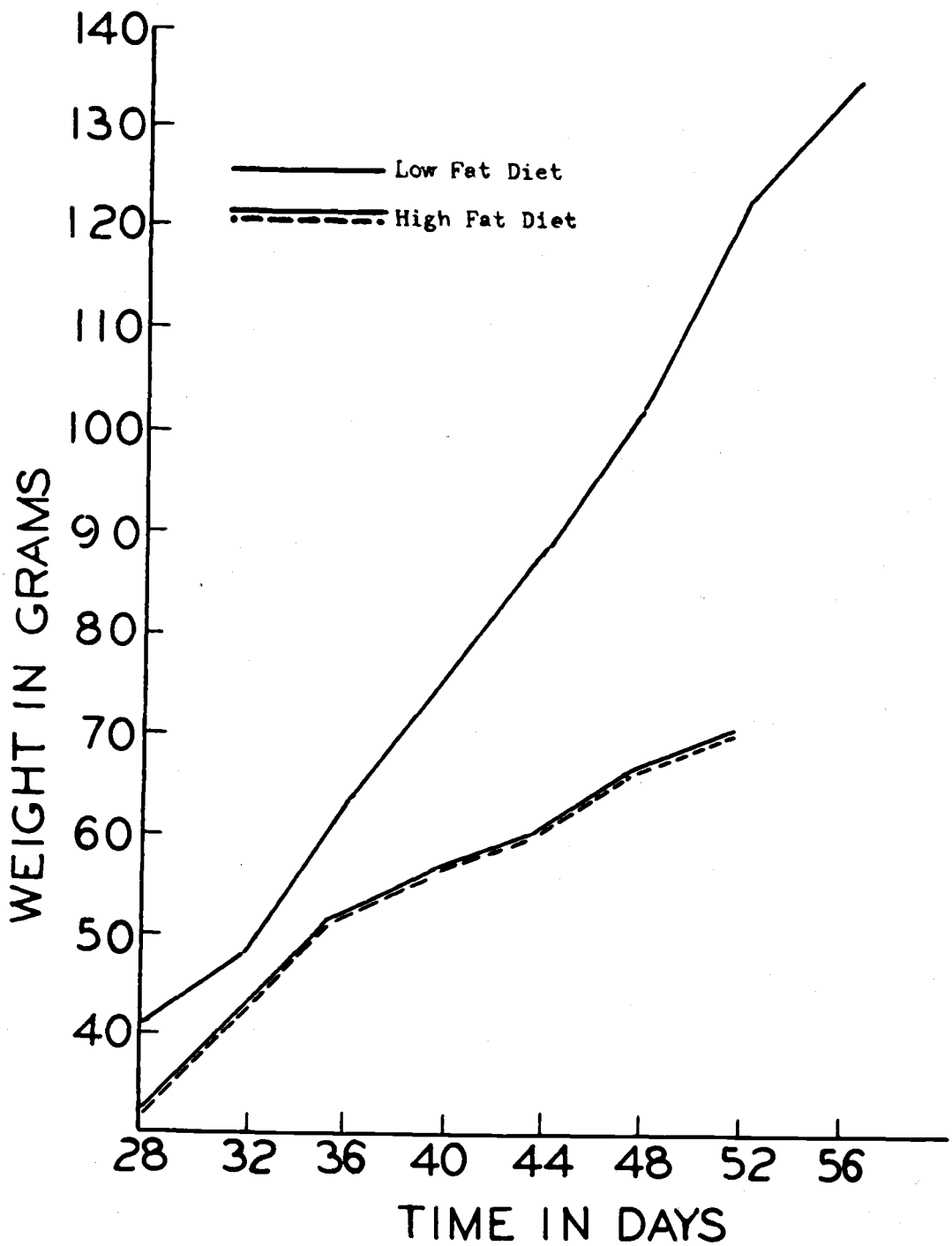


TABLE X

Growth Response of 28 Day Old Rats Receiving Low Fat Diet (Ration 3) ad libitum

(Each experiment covered 28 days)

Sex	Number of rats used	Average total gain in weight in grams	Average food intake in grams	Grams food divided by weight gain
Male	10	103.8	284.5	2.77
Female	3	85.0	280.0	3.3

Studies Using the Optical Isomers of Tyrosine

Four litters of rats were used in this experiment. They were distributed as evenly as possible with respect to weights, litters and sex.

The basal diet and the vitamin supplements were made up according to Borman et al (57); (Tables VIII and IX), in the same manner as in the previous experiment, since the low fat diet gave the best growth. The protein was supplied in the form of a mixture of crystalline amino acids (Ration 4).

The mixture, patterned after Borman's study (57), was prepared from amino acids, some of which were isolated or synthesized in this laboratory, while some were from commercial sources. In each case, these compounds gave excellent analytical values

(Table XI), indicating a high degree of purity. The composition of the amino acid mixture is listed in Table XII. This list differs from that of Borman (57) in that the amino acid arginine was added, while phenylalanine and tyrosine were omitted. It differed also in that hydroxyproline was omitted, because we were unable to obtain a supply of sufficient purity to warrant its use. This omission should have no effect upon the experiment (59).

TABLE XI

Nitrogen Analyses of Amino Acids Used in Mixture
Kjeldahl method used except when noted

Amino Acid	%, Theory	%, Found	%Deviation
Glycine	18.67	18.53	0.75
Alanine	15.83	15.77	0.44
Methionine	9.40	9.49	0.94
Glutamic Acid	9.52	9.78	2.66
Aspartic acid	10.53	10.50	0.02
Valine	11.96	11.90	0.05
Leucine	10.68	10.49	1.73
Isoleucine	10.68	10.50	1.73
Cystine	11.66	11.45	1.80
Threonine	11.80	11.87	0.06
Serine	13.33	13.35	0.02
Tryptophane	13.72	13.62*	0.58
Lysine monohydrochloride	17.66	17.30*	2.12
Histidine monohydrochloride	27.10	27.01	0.33
D-tyrosine	7.73	7.75	0.25
Phenylalanine	8.49	8.55	0.71
Arginine monohydrochloride	26.30	26.12	0.67

* Van Slyke method used.

TABLE XII

Composition of Amino Acid Mixture

Amino Acid	Grams
Glycine	0.1
Alanine	0.7*
Serine	0.2*
Valine	2.0*
Leucine	2.4*
Isoleucine	1.6*
Cystine	0.2
Methionine	0.8*
Threonine	1.4*
Tryptophane	0.4*
Aspartic acid	0.2
Glutamic acid	2.0
Lysine monohydrochloride	3.0*
Proline	0.2
Histidine monohydrochloride	0.95
Arginine monohydrochloride	0.60
Sodium bicarbonate	1.76
Tyrosine	0.50
Phenylalanine	0.50*
Total	19.51

* Denotes racemic acid

The DL-tyrosine used was prepared by the method of du Vigneaud and Meyer (60) from L-tyrosine.

Analysis:	$C_9H_{11}O_3N$	Found	Theory
	N (Kjeldahl)	7.70%	7.73%
	N (Van Slyke)	7.75%	7.73%

$(\alpha)_D^{26} = 0.00$ (4% tyrosine in 4% HCl)

The DL-tyrosine was resolved using l-brucine according to the directions of Sealock (61).

The D isomer gave the following analytical results:

Analysis: $C_9H_{11}O_3N$	Found	Theory
N (Kjeldahl)	7.75%	7.73%
N (Van Slyke)	7.77%	7.73%
C	59.62%	59.66%
H	6.16%	6.12%

$(\alpha)_D^{26}$ = + 9.5 (4% tyrosine in 1.0 N HCl)

The animals were divided into five groups as follows:

Group 1. Basal plus 0.5% phenylalanine plus 0.5% DL-tyrosine.

Group 2. Basal plus 0.5% phenylalanine plus 0.5% L-tyrosine.

Group 3. Basal plus 0.5% phenylalanine plus 0.5% D-tyrosine.

Group 4. Basal plus 0.5% phenylalanine plus 0.25% L-tyrosine plus 0.25% alanine.

Group 5. Basal alone.

The basal diet without any tyrosine and phenylalanine should not and did not give positive growth. This confirms the study of Womack and Rose (36). The second group is the control group for the experiment, since the demonstration (36) that L-tyrosine is capable of replacing one-half the phenylalanine.

Figure 4 shows the growth curve for rats on the diets containing the various optical isomers of tyrosine. Table XIII summarizes the data obtained.

FIGURE 4

Growth Response of 28 Day Old Rats to
Modified Low Fat Diet (Ration 4) Containing
0.5% DL-Phenylalanine plus D-, L-, or DL-Tyrosine

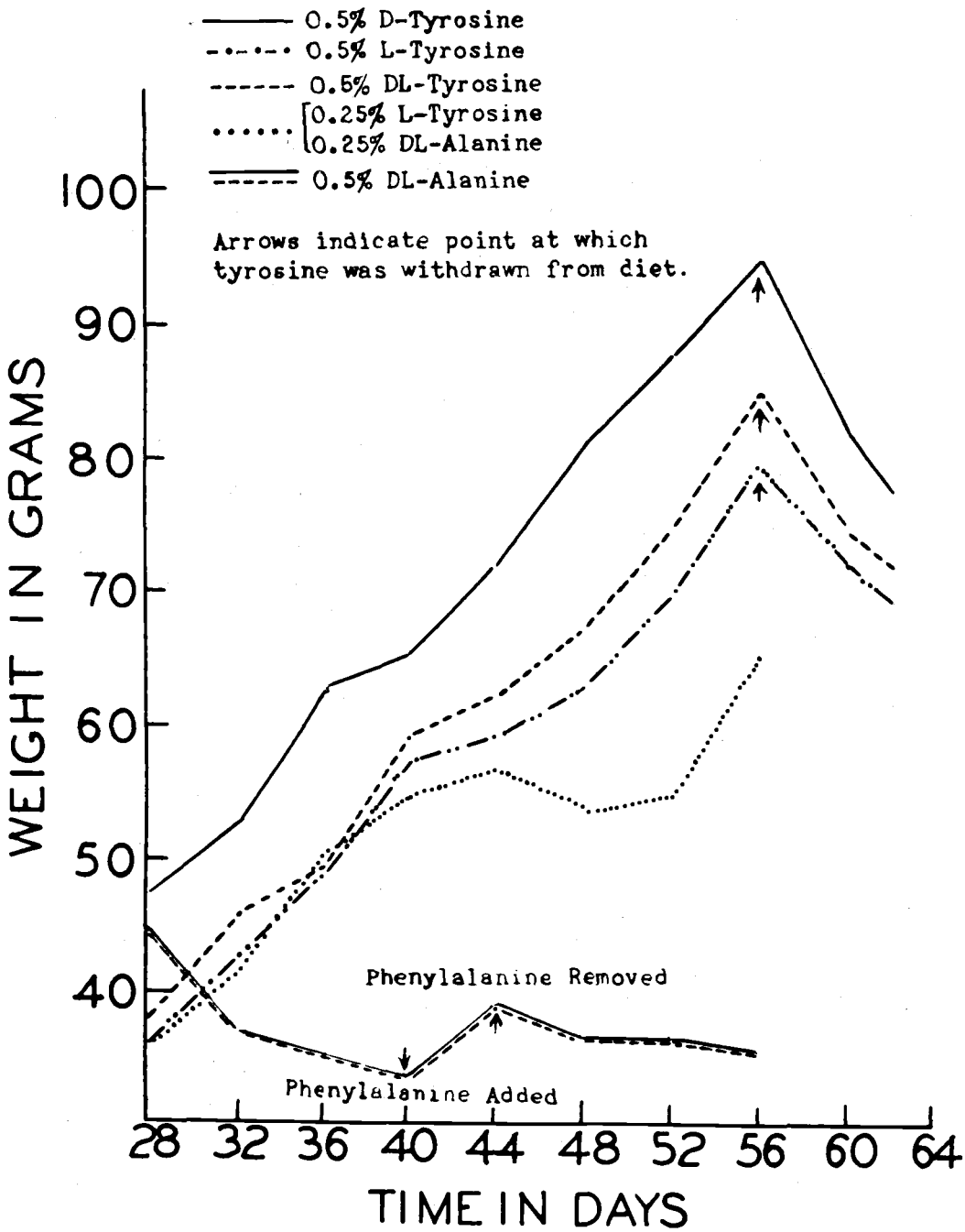


TABLE XIII

Growth Response of 28 Day Old Rats to Modified Low Fat Diet (Ration 4) Containing 0.5% DL-Phenylalanine Plus D-, L-, or DL-Tyrosine

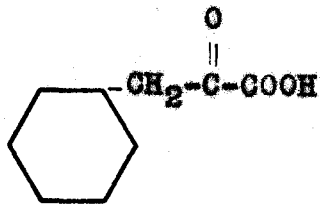
(Each experiment covered 28 days)

Litter Number	Rat Number and Sex	Total Gain in Weight in grams	Total Food Intake in grams	Amino Acid Supplements
6	34 ♂	45	182	
6	35 ♂	42	167	
6	36 ♂	26	170	0.5% DL-Phenylalanine
7	86 ♀	47	188	0.5% DL-Tyrosine
8	87 ♀	43	216	
8	88 ♀	53	223	
Average		42.6		
6	37 ♂	46	177	
6	38 ♀	47	199	0.5% DL-Phenylalanine
6	39 ♀	43	188	0.5% L-Tyrosine
7	85 ♂	32	196	
Average		42.0		
7	81 ♀	57	238	
7	82 ♀	25	202	0.5% DL-Phenylalanine
7	83 ♂	39	216	0.5% D-Tyrosine
7	84 ♂	59	232	
Average		45.0		
6	40 ♀	24	194	0.5% DL-Phenylalanine
6	41 ♀	36	173	0.25% L-Tyrosine
6	42 ♂	Died after 2 weeks		0.25% DL-Alanine
Average		30.0		
	101 ♀	-10	99	1.0% DL-Alanine
	102 ♀	-11	93	
Average		-10.5		

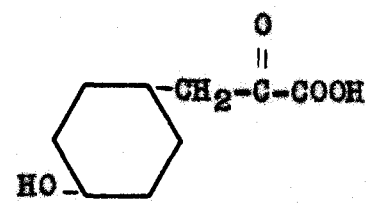
Growth Promoting Effects of the Keto Analogues of Tyrosine and Phenylalanine

The keto analogue can be substituted for the amino acid in the case of phenylalanine (9), methionine (62), tryptophane (63) and histidine (64). Apparently, the D-form of the four and only these four essential amino acids can be utilized by the rat. This striking correlation is considered to have biological significance.

The keto analogue of phenylalanine, phenylpyruvic acid, has been stated in a footnote by Rose (9) as being capable of promoting growth in the absence of



Phenylpyruvic acid



para-Hydroxyphenylpyruvic acid

phenylalanine. No experimental data are reported. Neither confirmation nor denial of this statement has appeared in the literature.

No reports of the substitution of the keto analogue of tyrosine, para-hydroxyphenylpyruvic acid, for tyrosine have appeared in the literature.

Phenylpyruvic acid was prepared according to directions given in Organic Syntheses (65). After recrystallization from chloroform, the compound had the following constants:

	Found	Literature Value
Melting point	152-154° C.	153-155° C.
Melting point of amide	190-192° C.	190-191° C.

The para-hydroxyphenylpyruvic acid was prepared in an analogous manner using para-hydroxybenzaldehyde as a starting material. The product, after recrystallization from chloroform had the following constants:

	Found	Literature Value
Melting point	210-212° C.	218-220° C.
Melting point of phenylhydrazone	165-166° C.	167-169° C.

The basal diet used in these experiments was the same as that used previously and is shown in Tables VIII and IX. The rations were prepared as follows:

Ration A. Basal plus 1.0% phenylpyruvic acid plus 0.5% alanine.

Ration B. Basal plus 0.5% para-hydroxyphenylpyruvic acid plus 0.5% phenylalanine plus 0.25% alanine.

Ration C. Basal plus 0.5% alanine.

The alanine is added to the ration to keep the nitrogen content of all of the rations equal.

The study is divided into two experiments. The growth curve for the first experiment is shown in Figure 5. Two animals were placed on each of the rations listed above. During the first nine days Period I) of feeding, the animals grew poorly and it was considered that more phenylalanine was required. To supply this need, 0.5% phenylalanine was added to each diet. The response to this addition was rapid and excellent growth was obtained (Period II). After 5 days, the supplementary 0.5% phenylalanine was removed from the diet. The animals continued to grow and the total growth was good (Period III), except on Ration C.

The growth data obtained in the second series of experiments are shown in Figure 6. Rations A and B were fed to the animals throughout the entire 28 days. No additional phenylalanine was ever added to the rations. Table XIV summarizes the data obtained on this experiment.

FIGURE 5

Growth Response of 28 Day Old Rats to a Basal Diet Containing the Keto Analogues of Tyrosine or Phenylalanine (Experiment 1).

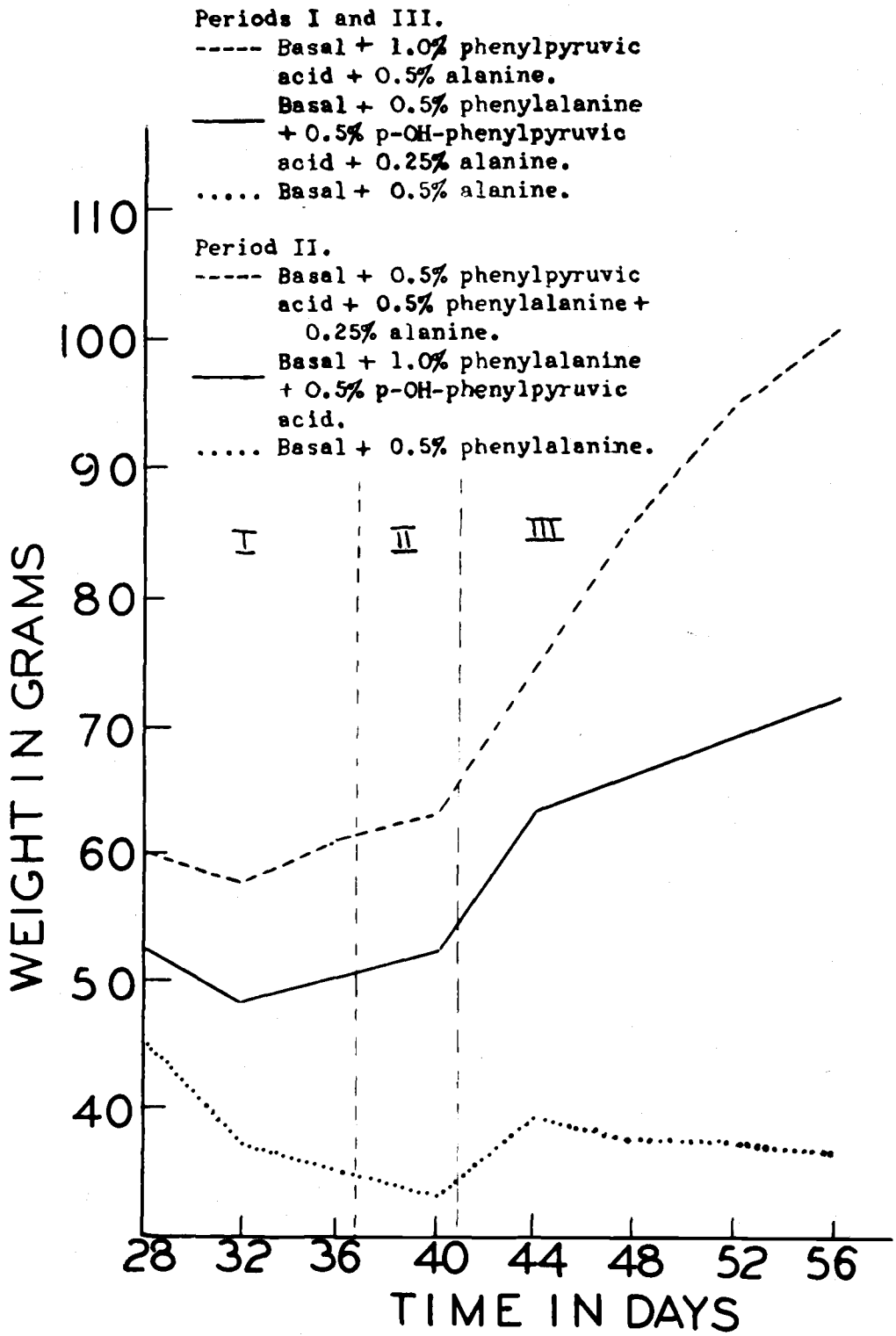


FIGURE 6

Growth Response of 28 Day Old Rats to a Basal Diet Containing the Keto Analogues of Tyrosine or Phenylalanine (Experiment 2).

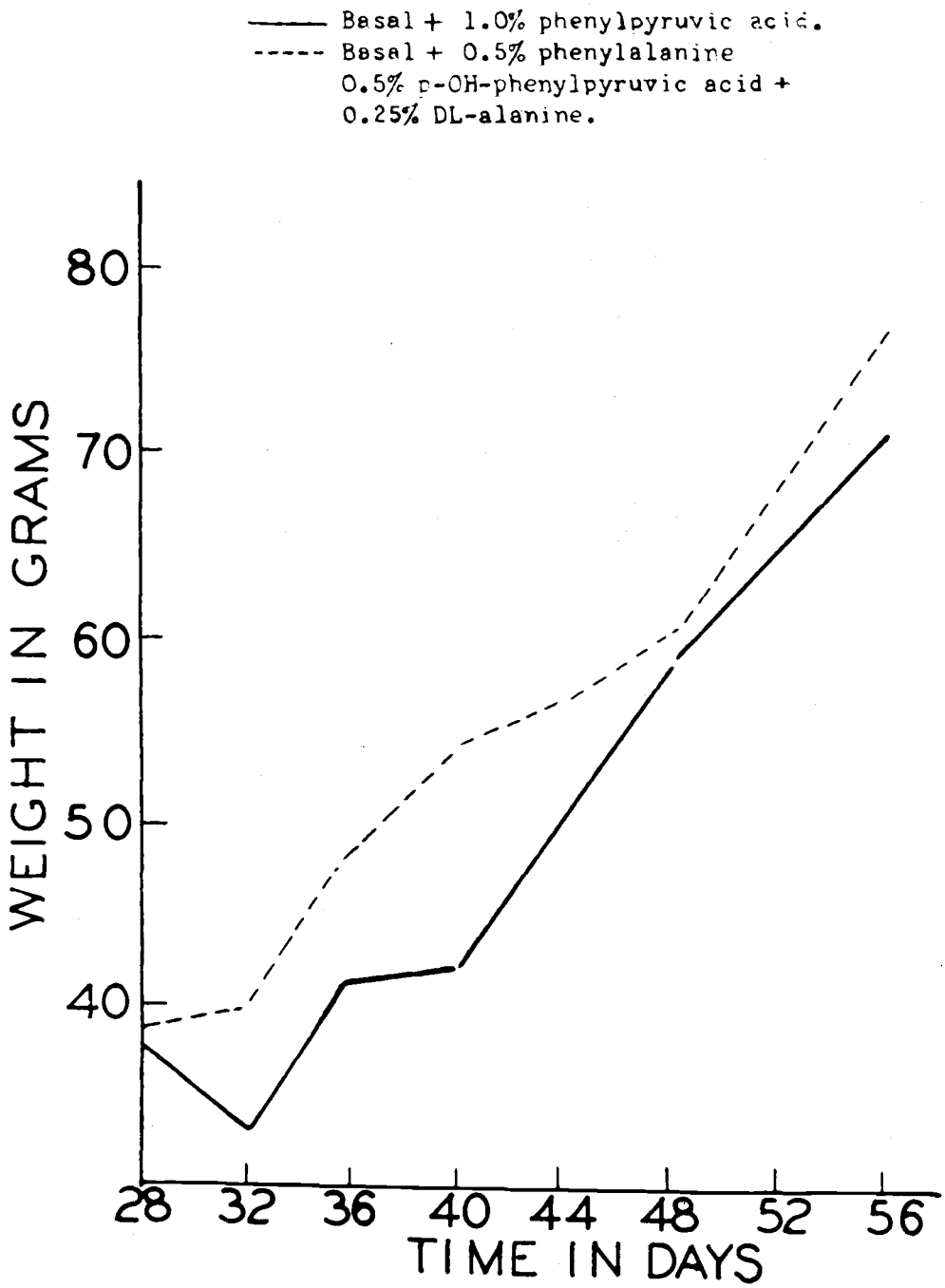


TABLE XIV

Growth Response of 28 Day Old Rats to Basal Diets Containing Phenylpyruvic Acid or para-Hydroxyphenylpyruvic Acid

(Each Experiment covered 28 days)

Diet	Number of animals	Average total gain in weight in grams	Average food intake in grams	Grams food divided by weight gain
Basal plus 1.0% phenyl-pyruvic acid	3	33.3	140	4.2
Basal plus 0.5% phenyl-alanine, plus 0.5% para-hydroxyphenyl-pyruvic acid, plus 0.25% alanine	3	35.6	149	4.1

Growth Comparisons of Casein and Casein Hydrolyzates with the Amino Acid Diets

The paired feeding technic was used in these experiments. In paired feeding, two groups of animals are selected, uniform as to age, weight and sex. The first group of animals is fed a control diet ad libitum. The second group is fed an experimental diet which is being compared to the control. The animals on the experimental diet are limited in their food intake to the same weight of food as the control

animals ate. In this manner, each group receives the same level of protein nitrogen, calories and vitamins. Since protein is the only variable, its biological value can be tested by measuring growth response.

The enzymatic hydrolysis of casein was prepared in the following manner. Five hundred g. of crude casein were placed in 2.5 l. of water; the mixture was adjusted to a pH of 8. To this was added 10 g. of a commercial proteolytic enzyme called "protease."[1] This mixture was allowed to digest for 72 hours at 40° C. Concentrated sodium hydroxide solution was added from time to time to keep the pH about 8. After digestion, the mixture was filtered and the filtrate was concentrated to a thick syrup under reduced pressure. The syrup was dried to a solid in a vacuum desiccator. The hydrolyzate had the following analysis:

N (Kjeldahl) = 12.2%
N (Van Slyke) = 11.6%
% Hydrolysis = 95.0%
Apparent

The acid hydrolysis of casein was carried out as follows. A suspension of 500 g. of casein in 1500 cc. of 9 N H_2SO_4 was autoclaved at 15 lbs. pressure for 8 hours. The hydrolyzed solution, after

[1] Takamine Laboratory, Inc. Clifton, New Jersey

filtration, was neutralized carefully with barium hydroxide. The clear solution was treated in the same manner as the enzymatic hydrolyzate to obtain a solid product.

The basal diet used in these experiments is shown in Tables VIII and IX. The protein source is the only component which is variable.

Comparisons between the following groups of diets were obtained:

Group I. Basal plus casein; ad libitum.

Basal plus amino acid mixture plus 0.5% tyrosine plus 0.5% phenylalanine; ad libitum.

Basal plus casein; paired feeding.

Group II. Basal plus casein enzymatic digest; ad libitum.

Basal plus amino acid mixture plus 0.5% tyrosine plus 0.5% phenylalanine; ad libitum.

Basal plus casein enzymatic digest; paired feeding.

Figure 7 shows the growth response for Group I; Figure 8 for Group II. Table XV summarizes the data obtained.

The acid-hydrolyzed casein was fortified by the addition of 0.4% tryptophane, 0.2% cystine, and 0.6% methionine, since these are the amino acids

which might be destroyed by acid hydrolysis. The animals did not tolerate this material very well, and the growth was so poor that the growth curves are not plotted.

FIGURE 7

Growth Response of 28 Day Old Rats to the Casein Ration Compared to the Amino Acid Ration

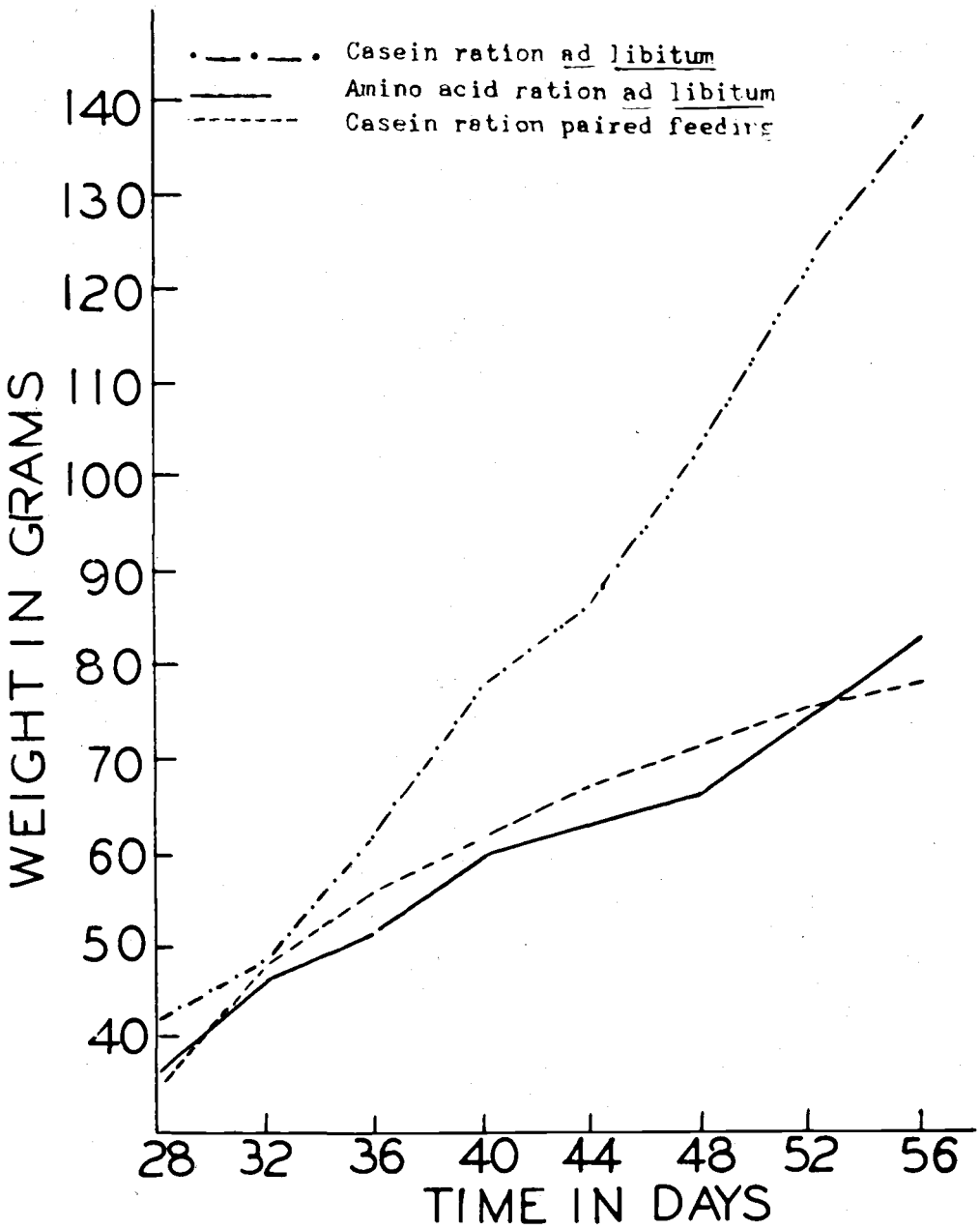


FIGURE 8

Growth Response of 28 Day Old Rats to the Casein Hydrolyzate Compared to the Amino Acid Ration

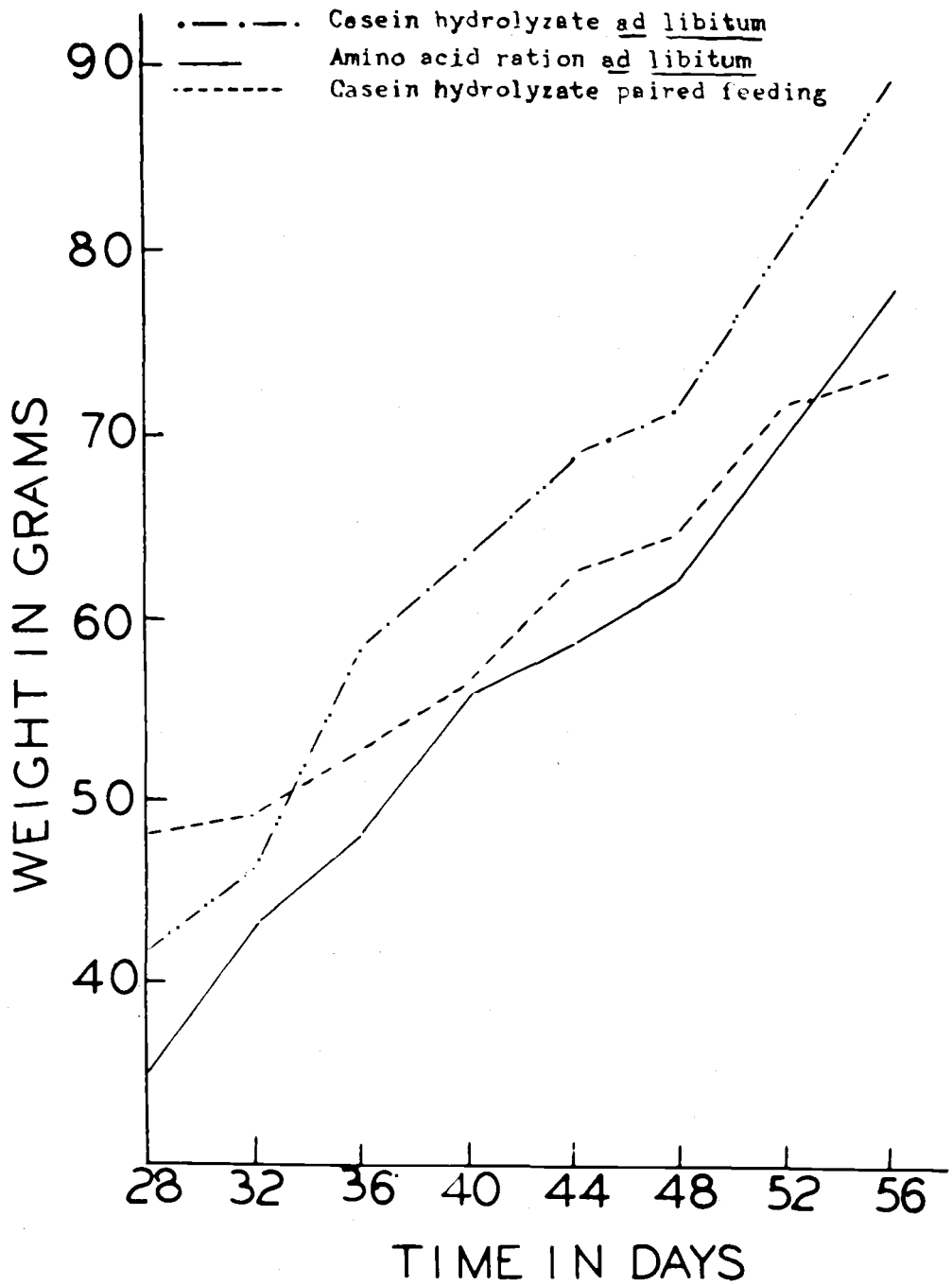


TABLE XV

Growth Response of 28 Day Old Rats to Amino Acid Diet (Ration 4), Casein Ration, and Casein Hydrolyzates

(Each experiment covered 28 days)

Protein source and feeding procedure	Number of rats used	Average total gain in weight in grams	Average food intake in grams	Grams food divided by weight gain
Amino acid mixture plus tyrosine and phenylalanine; ad libitum	4	42.0	174	4.1
Casein; ad libitum	13	94.0	282	3.0
Casein; paired feeding	8	38.8	174	4.4
Enzymatic casein digest; ad libitum	6	45.3	265.8	5.8
Enzymatic casein digest; paired feeding	6	26.7	174	6.7
Fortified[1] acid-hydrolyzed casein digest; ad libitum[2]	5	-2	-	-
Fortified[1] acid-hydrolyzed casein digest; paired feeding[2]	5	-3	-	-

[1] Fortified with 0.4% tryptophane, 0.2% cystine and 0.6% methionine.

[2] These animals were fed for 16 days and were discarded because of poor growth.

SECTION III

DISCUSSION

The demonstration of Womack and Rose that L-tyrosine stimulates the growth of rats on an adequate diet suboptimal with respect to phenylalanine was applied to the study of the nutritional availability of D-tyrosine.

The control experiments, shown in Table XIII, confirm the growth average of forty-five grams during a twenty-eight day experiment as reported by Womack and Rose (36). The growth obtained on the basal amino acid mixture supplemented with 0.5 per cent L-tyrosine and 0.5 per cent DL-phenylalanine appears to be optimum for these experiments in both our study and that of Womack and Rose.

When the L-tyrosine in the control experiment was substituted by an equal amount of DL-tyrosine, a growth rate equal to that of the control was obtained. When the L-tyrosine level in the diet was lowered to 0.25 per cent, the growth was markedly inferior. It should be pointed out that at this level, the per cent of L-tyrosine in this mixture would be the same as in the diet containing 0.5 per cent DL-tyrosine. If the D-isomer had no retarding effect and were not

available for growth, the responses in each of these experiments should be the same. This is not so, since growth was superior on the DL-tyrosine diet, thereby giving strong support to the thesis that the D-isomer is nutritionally available.

This concept was confirmed by the feeding of D-tyrosine. The unnatural isomer, when added in a 0.5 per cent concentration to a basal diet containing 0.5 per cent phenylalanine, gave the optimum growth of forty-five grams for the twenty-eight day experiment. The growth obtained by Rose (36) on a basal diet supplemented with only 0.5 per cent phenylalanine was fourteen and eight tenths grams for a like period. Since the inclusion of D-tyrosine gave such a marked stimulation, it indicates that the D-isomer can be used by the growing rat to meet its demands for tyrosine and phenylalanine.

After the completion of the twenty-eight day period, the tyrosine was removed from the diet of one animal in each group. This caused a rapid loss of appetite and weight. In a six day period, the loss averaged from ten to fifteen grams; in some cases, this was one-sixth of the body weight. It was noticed in most of these animals that one day after the removal of the essential component, the appetite and

food intake decreased. This quick break in food intake gives rise to much speculation. Is this lack of appetite due to flavor or to an expanding metabolic disturbance? The sensory stimulation of taste, if it is the reason, must be extremely acute for in many instances, the compound removed was in a concentration of less than five parts per thousand. A more logical conclusion is that in the animal there is a disruption of an important metabolic function upon the removal of the essential amino acid. In simpler terms, the animal becomes sick and to such an extent that he no longer has a desire for food.

These experiments show conclusively that the growing rat can use either isomer of tyrosine for growth purposes; in man, the unnatural isomer has been postulated by Albanese (38) to be completely unavailable for metabolic functions. This difference might be due to the following reasons: in the first place, a distinct species difference in the metabolism of the tyrosine molecule; secondly, dissimilar methods of testing. In the rat, the D-tyrosine was fed during its growing period; in man, it was fed in the maintenance stage. It is possible that the pathway of metabolism of tyrosine may change as the animal grows older and is no longer in the active growing stage.

If this is so, it is contrary to the theory of protein metabolism as presented by Schoenheimer (66). Another reason for this difference may be that the metabolism of D-amino acids is one of degree rather than an all or none process (67). In other words, the availability of these substances might be a function of two competing reactions: (a) the rate at which the organism can convert the unnatural into the natural form; (b) the speed at which the respective D and L-forms are excreted by the kidney.

In the metabolism of amino acids, the following reaction is of great importance:

This system, called transamination, has been shown to occur between a number of amino and keto acids. The specific enzymes needed for these reactions are found throughout body tissue. The amino acids methionine, histidine, tryptophane and phenylalanine are the only amino acids reported in the literature for which the D form is nutritionally available for growth in the rat. These same amino acids can be substituted by

the keto compound in the rat. Now, to this list may be added the amino acid tyrosine. The correlation that can be drawn between the availability of the D-isomers and the keto compounds is due perhaps to the presence of these enzyme systems in the animal. If the animal can carry out the above reaction, the keto compound is an intermediate and should be used with ease.

In the case of phenylpyruvic acid, there seems to be little doubt that the rat can use this compound with the same facility that it uses D- and L-phenylalanine. The results, shown in Table XIV, indicate this fact. The animals fed a diet devoid of phenylalanine, grew poorly and lost weight. The inclusion of 1 per cent phenylpyruvic acid with no phenylalanine present in the diet, gave excellent growth and the gains were almost optimum.

The situation with regard to para-hydroxyphenylpyruvic acid is not as clear. The weight gains obtained by Rose on a basal diet containing only 0.5 per cent phenylalanine were, on the average, fourteen grams. The addition of para-hydroxyphenylpyruvic acid to this diet gave an average gain of twenty-nine grams. This is an increased growth of fifteen grams. The gain is not optimum, but the stimulatory

effect is quite noticeable. This seems to indicate that the compound can be used by the rat but not as effectively as tyrosine.

In the feeding experiments using these compounds, there was noticed a marked induction period lasting from eight to ten days. The animals, in many instances, refused food for the first few days of the experiment. Soon, their appetites revived and their weight showed an increase. This type of phenomenon is infrequent in animal experimentation where one is dealing with individuals, but has been noted in many instances with microorganisms where populations are used.

The meaning of the induction period is not clear, but the animal seems unaccustomed to the keto compounds and does not seem to use them. After a period of readjustment, the animal resumes eating and the food intake and weight gains are normal.

Also, further consideration should be given to the microorganisms of the intestinal tract. The role of the intestinal flora in animal nutrition is little understood and, perhaps, vastly underestimated. The organisms may be inhibited by the keto compounds, thus destroying a source of an unknown growth factor. After a period of readjustment, the inhibited organisms

may resume growth, or a new one may flourish and good growth of the animal can continue. The data shown in Figure 7 indicate the nutritional superiority of the casein ration to the amino acid ration. However, it should be noted in Table XV that the food intake of the casein fed animals was larger by more than one hundred grams. If the casein ration is fed at the lower level of intake of the animals on the amino acid diet, the superiority disappears. A similar situation is shown in Figure 8 illustrating the growth response to the enzymatic casein hydrolyzates.

These facts may indicate that the animals receiving the casein diet at the lowered intake are limited in their growth by the lack of protein and calories. Cannon (68) has shown that if the caloric intake is too low, there is little nitrogen retention from ingested protein. Therefore, the protein could be adequate, but the caloric intake too low for efficient utilization of the protein.

Also, it might be pointed out that although we have no proof, there may be a source of the growth factor, strepogenin, in our rations. The 0.4 per cent liver extract (Wilson's 1:20) contained in the rations is a potent source of water-soluble vitamins known and unknown. Woolley (50) and Cary (51) have

shown liver to be a source of strepogenin-like materials. Thus, the presence of the liver extract may have masked any of the growth responses postulated by Woolley as being given by intact protein but not by amino acid mixtures.

The reason for the limited food intake on the amino acid diet is open to speculation. As has been discussed previously, it may be due to the flavor or to an expanding metabolic disturbance. The flavor of the amino acid ration is distasteful and this may be a most important factor. The possibility that casein may contain substances which are not present in the amino acid mixture is very real. Intact protein may contain materials which are not absolutely essential for rats but may be necessary for the most rapid growth and the total well-being of the animal. Arginine is an example of such a compound.

In the case of the acid-hydrolyzed casein, the lack of growth in the rat may be due to the destruction of some amino acids. Methionine, cystine and tryptophane were added to the ration, but possibly other amino acids may have been destroyed. The ration may contain in addition a slight excess of acid which might cause intestinal disorders in the animal.

These questions are not answered in this thesis but sufficient data are presented to make it evident that this problem is worthy of further investigation.

SECTION IV

SUMMARY

1. When suboptimal amounts of phenylalanine are incorporated into an amino acid mixture otherwise adequate for the growth of the white rat, the additional phenylalanine requirement is met equally well with D-, L-, or DL-tyrosine.

2. Phenylalanine can be substituted by its keto analogue, phenylpyruvic acid, in the amino acid requirement of the growing rat.

3. The keto analogue of tyrosine, para-hydroxyphenylpyruvic acid, can replace tyrosine required to meet the demands of the rat on an amino acid mixture suboptimal in phenylalanine. However, this analogue does not appear to be utilized quite as well as the keto analogue of phenylalanine.

4. By use of the paired feeding technic, no growth stimulation was shown by casein and casein hydrolysates when compared with an amino acid mixture under our experimental conditions.

SECTION V

BIBLIOGRAPHY

1. Vickery, H. B., and Schmidt, C. L. A., Chem. Rev., 9, 169 (1931)

2. Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 17, 325 (1914).

3. Abderhalden, E., Z. physiol. Chem., 77, 22 (1912).

4. Hopkins, F. G., J. Chem. Soc., 109, 629 (1916).

5. Mitchell, H. H., J. Biol. Chem., 26, 231 (1916).

6. Suzuki, U., Inst. Phys. and Chem. Res., 4, 1 (1926).

7. McCoy, R. H., Meyer, C. E., and Rose, W. C., J. Biol. Chem., 112, 283 (1935).

8. Meyer, C. E., and Rose, W. C., J. Biol. Chem., 115, 721 (1936)

9. Rose, W. C., Science, 86, 298 (1937).

10. Rose, W. C., Physiol. Rev., 18, 109 (1938).

11. Rose, W. C., and Rice, E. E., Science, 90, 186 (1939).

12. Rose, W. C., Haines, W. J., and Johnson, J. E., J. Biol. Chem., 146, 683 (1942).

13. Rose, W. C., Haines, W. J., Johnson, J. E., and Warner, D. T., J. Biol. Chem., 148, 457 (1943).

14. Rose, W. C., Proc. Am. Phil. Soc., 91, 112 (1947).

15. Berg, C. P., J. Biol. Chem., 104, 373 (1934).

16. Rose, W. C., and Womack, M., J. Biol. Chem., 166, 104 (1946).

17. Jackson, R. W., and Block, R. J., Proc. Soc. Exp. Biol. and Med., 30, 587 (1932).

18. Cox, G. J., and Berg, C. P., J. Biol. Chem., 107, 497 (1934).

19. West, H. D., and Carter, H. E., J. Biol. Chem., 122, 611 (1938).

20. Berg, C. P., J. Nutr., 12, 671 (1936).

21. Albanese, A. A., Irby, V., and Frankston, J. E., J. Biol. Chem., 160, 25 (1945).

22. Albanese, A. A., and Irby V., J. Biol. Chem., 155, 101 (1944).

23. Albanese, A. A., Bull. Johns Hopkins Hosp., 75, 175 (1944).

24. Albanese, A. A., and Frankston, J. E., J. Biol. Chem., 158, 101 (1945).

25. Abderhalden, E., Z. physiol. Chem., 96, 1 (1915).

26. Dakin, H. D., J. Biol. Chem., 44, 499 (1920).

27. Shambaugh, N. F., Lewis, H. B., and Tourtelotte, D., J. Biol. Chem., 92, 499 (1931).

28. Embden, G., and Baldes, K., Biochem. Z., 55, 301 (1913).

29. Womack, M., and Rose, W. C., J. Biol. Chem., 107, 449 (1934).

30. Womack, M., and Rose, W. C., J. Biol. Chem., 112, 275 (1935).

31. Moss, A. R., and Schoenheimer, R., J. Biol. Chem., 135, 415 (1940).

32. Schoenheimer, R., Ratner, S., and Rittenberg, D., J. Biol. Chem., 127, 333 (1939).

33. Moss, A. R., J. Biol. Chem., 137, 739 (1941).

34. Heir, S. W., J. Biol. Chem., 171, 813 (1947).

35. Rose, W. C., and Womack, M., J. Biol. Chem., 166, 103 (1946).

36. Womack, M., and Rose, W. C., J. Biol. Chem., 166, 429 (1946).

37. Wohlgemuth, J., Ber. chem. Ges., 38, 2064 (1905).

38. Albanese, A. A., Irby, V., and Lein, M., J. Biol. Chem., 166, 513 (1946).

39. Lugg, J. W. H., Biochem. J., 31, 1423 (1937).

40. Van Slyke, D. D., and Palmer, W. W., J. Biol. Chem., 41, 567 (1920).

41. Albanese, A. A., and Irby, V., Science, 98, 286 (1943).

42. Kinsey, V. E., and Grant, W. M., Science 99, 303 (1944).

43. Silber, R. H., Clark, I., Howe, E. E., and Porter, C. C., Federation Proc., 6, 290 (1940).

44. Benditt, E. P., Humphreys, E. M., Staube, R. L., Wissler, R. W., and Steffee, C. H., J. Nutr., 33, 65 (1947).

45. Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R., J. Nutr., 33, 65 (1947).

46. Cannon, P. R., Steffee, C. H., Frazier, L. E., Rowley, D. A., and Stepto, R. C., Federation Proc., 6, 390 (1947).

47. Womack, M., and Rose, W. C., J. Biol. Chem., 162, 735 (1946).

48. Woolley, D. W., J. Biol. Chem., 159, 753 (1945).

49. Sprince, H., and Woolley, D. W., J. Am. Chem. Soc., 67, 1734 (1945).

50. Woolley, D. W., J. Biol. Chem., 162, 383 (1946).

51. Cary, C. A., Garbman, L. P., Dryden, A. M., and Likely, G. D., Federation Proc., 5, 128 (1946).

52. McClendon, J. F., Proc. Soc. Exp. Biol. and Med., 28, 915 (1930-1931).

53. Scott, M. L., Norris, L. C., and Heuser, G. F., J. Biol. Chem., 167, 261 (1947).

54. Luckey, T. D., Moore, P. R., Elvehjem, C. A., and Hart, E. B., Proc. Soc. Exptl. Biol. and Med., 64, 348 (1947).

55. Woolley, D. W., J. Biol. Chem., 172, 71 (1947).

56. Evans, H. M., and Bishop, K. S., Science, 56, 650 (1922).

57. Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M., and Rose, W. C., J. Biol. Chem., 166, 585 (1946).

58. Jones, J. H., and Foster, C., J. Nutr., 24, 245 (1942).

59. Womack, M., and Rose, W. C., J. Biol. Chem., 171, 37 (1947).

60. du Vigneaud, V., and Meyer, C. E., J. Biol. Chem., 98, 295 (1932).

61. Sealock, R. R., J. Biol. Chem., 166, 1 (1946).

62. Cahill, W. M., and Rudolph, G. G., J. Biol. Chem., 145, 201 (1942).

63. Bauguess, L. C., and Berg, C. P., J. Biol Chem., 104, 675 (1934).

64. Cox, G. J., and Rose, W. C., J. Biol. Chem., 68, 781 (1926).

65. Organic Syntheses, New York, Coll. 2, 519 (1943).

66. Schoenheimer, R., The Dynamic State of Body Constituents, Cambridge, 1942.

67. Albanese, A. A., in Advances in Protein Chemistry, III, New York, 1946.

68. Cannon, P. R., J. Am. Med. Assoc., 135, 1043 (1947).