THE METABOLISM
OF
PHENYLALANINE

by

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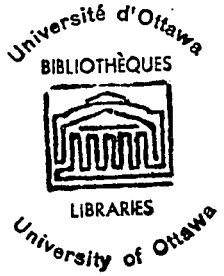

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TABLE OF CONTENTS PAGE NO.

TABLE OF CONTENTS CONT'D. PAGE NO.

TABLE OF CONTENTS CONT'D. PAGE NO.

TABLE OF CONTENTS CONT'D. PAGE NO.

ABSTRACT

The metabolism of phenylalanine-C^{14} was studied in three different groups of rats: normal animals, phenylalanine-deficient animals and rats which had been maintained on a high (5 and 7%) phenylalanine diet from 21 days of age and considered to be "experimentally phenylketonuric".

In normal rats the injection of DL-phenylalanine-1-C^{14} resulted in the excretion of more radioactivity in the CO_2 and urine than did the injection of DL-phenylalanine-3-C^{14}. However, the radioactivity in the proteins was higher after the injection of DL-phenylalanine-3-C^{14}. Para-fluorophenylalanine-3-C^{14}, an analogue of phenylalanine, produced higher levels of radioactivity in the CO_2 and urine than did DL-phenylalanine-3-C^{14}. This analogue was incorporated into the proteins in significant quantities and was also rapidly transformed into tyrosine-C^{14}. The tyrosine-C^{14} was then incorporated into the proteins. A second phenylalanine analogue, beta-2-thienylalanine-3-C^{14} produced only trace quantities of radioactive $C^{14}O_2$ but highly radioactive urine. In all of the organs, except the pancreas, this analogue was only incorporated into the proteins in trace amounts.

After the addition of either non-radioactive para-fluorophenylalanine or beta-2-thienylalanine to the

injected phenylalanine-C^{14}, there was, in general, an increase in the ratio of tyrosine-C^{14}/phenylalanine-C^{14} incorporated into protein. More radioactive CO_2 and urine were produced, and the radioactivity of the protein was decreased. Both of these effects were most pronounced when a third analogue, 2-amino-3-phenylbutanoic acid, was used. This compound was also incorporated into the proteins.

After treatment with a low-phenylalanine diet the radioactive CO_2 and urine produced when phenylalanine-3-C^{14} was injected indicated that there was a general reduction in the rate of phenylalanine metabolism. The level of incorporation of radioactivity in the proteins of the phenylalanine-deficient rats varied little from normal values. More phenylalanine than tyrosine was incorporated into protein.

When attempts were made to induce an experimental phenylketonuria it was found that if the rats were placed on the high phenylalanine diet too early, the phenylalanine was toxic. Injection of phenylalanine-C^{14} into phenylketonuric rats resulted in an increase in the release of the radioactivity in the CO_2 and urine and a decrease in the radioactivity of the proteins compared to the results obtained in normal rats. The ratio of tyrosine-C^{14}/phenylalanine-C^{14} incorporated into proteins in these phenylketonuric rats was also lower.

DL-tryptophan-3-C^{14} was also injected into phenylketonuric rats. The only difference from the values obtained for normal rats was a slight elevation of the radioactive content of the urine in the phenylketonuric rats.

RESUME

Le métabolisme de la phénylalanine a été étudié chez le rat normal, chez le rat carencé en phénylalanine et chez le rat recevant un régime contenant 5 à 7% de L-phénylalanine. Ce dernier traitement produit chez le rat une phénylcétonurie expérimentale.

Le rat normal excrète plus de carbone radioactif dans le CO_2 expiré et dans l'urine après injection de DL-phénylalanine-1-C^{14} qu'après injection de DL-phénylalanine-3-C^{14}, cependant plus de phénylalanine radioactive est incorporée dans les protéines des différents organes dans le deuxième cas. Une certaine quantité de tyrosine radioactive dérivée de la phénylalanine injectée est aussi incorporée dans les protéines.

Le rat normal métabolise la DL-para-fluorophénylalanine-3-C^{14} un analogue de la phénylalanine. Dans ce cas plus de carbone radioactif est excrété dans le CO_2 et l'urine contrairement à ce qui se produit lorsque la phénylalanine est utilisée. L'analogue est aussi incorporé dans les protéines et transformé rapidement en tyrosine qui est aussi incorporée dans les protéines.

La DL-béta-2-thienylalanine-3-C^{14} n'est pas transformée en $C^{14}O_2$ par le rat. Beaucoup plus de carbone radioactif est exrété dans l'urine. Cet analogue radioactif n'est pas incorporé dans les protéines du rat de façon appréciable sauf dans les protéines du pancréas.

Lorsque les analogues non-radioactifs de la phénylalanine sont injectés en même temps que la phénylalanine radioactive et en plus grandes quantités, il y a une augmentation de l'excrétion de carbone radioactif dans le CO_2 et dans l'urine. L'acide amino-2 phényl-3 butanoique produit l'augmentation la plus importante. Cet analogue est aussi incorporé dans les protéines du rat. Plus de tyrosine radioactive est aussi incorporée dans les protéines lorsque les analogues non-radioactifs sont ajoutés à la phénylalanine radioactive injectée.

Le rat carencé en phénylalanine catabolise la phénylalanine radioactive plus lentement que le rat normal. Moins de carbone radioactif est excrété dans le CO_2 et dans l'urine. Il y a cependant autant de radioactivité incorporée dans les protéines mais la radioactivité est due presqu'uniquement à l'incorporation de la phénylalanine radioactive.

Un régime contenant 5 à 7% de L-phénylalanine s'est avéré toxique chez les très jeunes rats. Le rat phénylcétonurique métabolise la phénylalanine radioactive plus rapidement que le rat normal. Moins de phénylalanine radioactive et beaucoup moins de tyrosine radioactive sont incorporées dans les protéines du rat phénylcétonurique. Ces rats métabolisent le DL-tryptophane-3-C^{14} de la même façon que le rat normal sauf qu'il y a un peu plus de produits radioactifs dans l'urine.

PART ONE

INTRODUCTION

Phenylalanine is an essential aromatic amino acid from which the body proteins, the thyroid hormones, the sympathoadrenal neurohumors and melanin are synthesized. The main metabolic route of phenylalanine involves a conversion to the non-essential amino acid, tyrosine, which in adults is capable of sparing approximately one half the daily requirement of phenylalanine for growth and three quarters of the requirement of phenylalanine for nitrogen equilibrium (1).

Rose (2) has found that the minimum and maximum daily requirement of L-phenylalanine for nitrogen balance in normal, adult human males is 0.8 and 1.1 g respectively, while Block and Mitchel (3) suggest a requirement of 40.3 mg per day of phenylalanine and tyrosine for a male albino rat with a basal metabolic rate of 27.6 Calories per day. However, as Armstrong (4) has pointed out, the actual minimum daily requirement of L-phenylalanine is less than that observed when phenylalanine is relied upon to meet the requirement for tyrosine as well. Mertz (5) has stated that phenylalanine need not compose more than 0.5% of a 20% protein diet.

Although it is recognized that the amino acid

content of the blood depends upon various factors, especially the components of the diet, Harper (6) has derived values for the amino acid level in plasma from normal individuals who were under comparable experimental conditions. These figures show a range of 1.1-4.0 g for phenylalanine and 0.9-2.4 g for tyrosine.

Urinary excretion of phenylalanine in the normal subject is 10-30 mg per day, while that of tyrosine is 15-50 mg per day (7).

1. THE METABOLISM OF PHENYLALANINE

The metabolism of phenylalanine is primarily concerned with an irreversible conversion to tyrosine. Some minor routes unrelated to tyrosine metabolism are also known; these become quantitatively more important in the metabolic error, phenylketonuria. The metabolic pathways of phenylalanine are shown in Figure 1. The metabolic maps described in Figures 1, 3, 4, 5 and 8 were derived from Figures given in "Comparative Biochemistry" (8). Figure 2 was described in "The Enzymes" (9) while Figure 7 was derived from Chapter 4 in "Phenylketonuria" (10).

The conversion of phenylalanine to tyrosine occurs predominently in the liver and is outlined in detail in Figure 2. Some evidence has been presented indicating that this reaction also occurs in muscle (11). Moss and Schoenheimer (12) have shown that, in the rat, deuterium-labelled phenylalanine is converted to tyrosine by a process which is not affected by the amount of tyrosine available. A specific enzyme system which catalyzes this reaction was described in rats and in humans (13, 14). Actually, two enzymes are involved in this reaction (15). The first, a labile enzyme found only in liver, requires molecular oxygen and a co-factor, which Kaufman (16) has suggested may be a

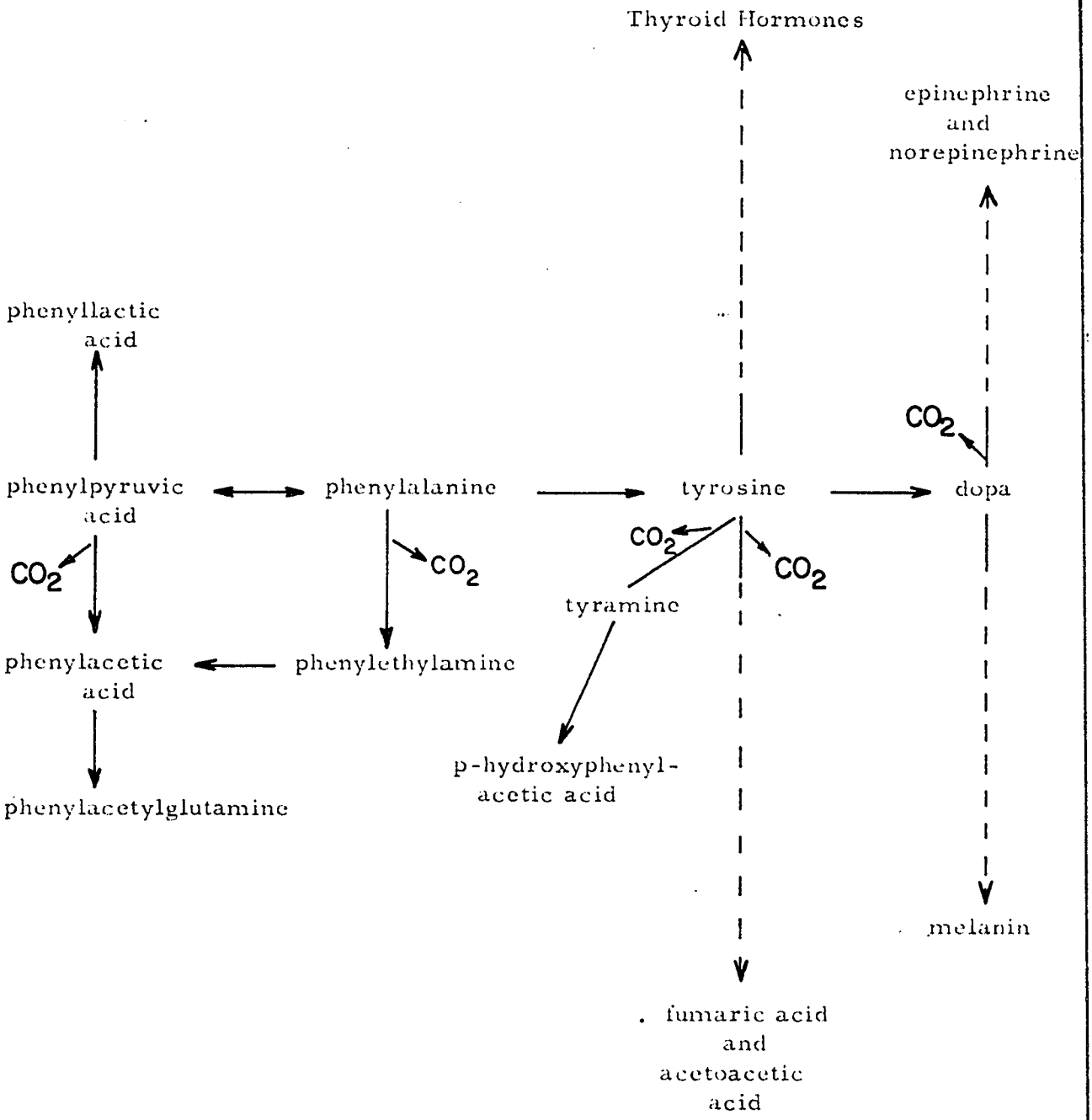


Figure 1

The Metabolism of Phenylalanine

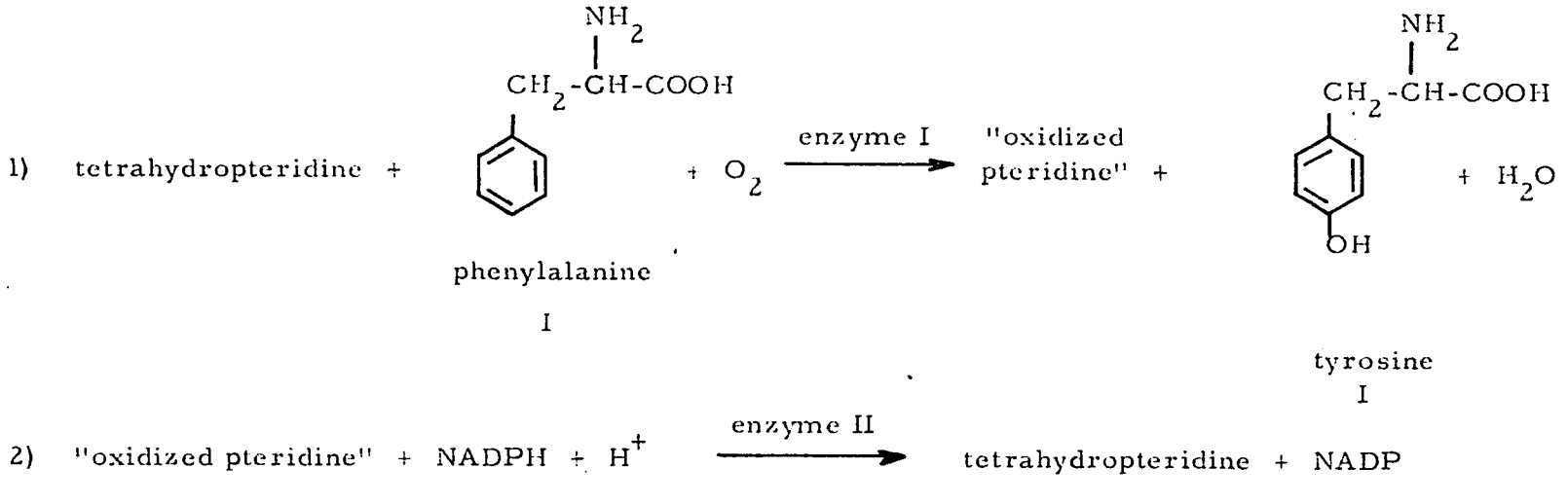


Figure 2

The Conversion of Phenylalanine to Tyrosine

tetrahydropteridine. The second enzyme is more stable and has a wider distribution, being found in liver and several other tissues: it regenerates the co-factor for the first enzyme (17, 18). Kaufman (19) has suggested that the oxidized co-factor in reaction 1 is a 5,6-dihydropterdine. The phenylalanine hydroxylase system is absent in fetal liver, but rapidly appears after birth (20, 21).

Normally, the route from tyrosine to acetoacetate and fumarate is quantitatively the most important accounting for most of the several grams of phenylalanine and tyrosine catabolized per day. It also accounts for the classification of these amino acids as both ketogenic and glucogenic. This route, which involves the formation of homogentisic acid, is illustrated in Figure 3. Tyrosine (II) is reversibly converted into p-hydroxyphenylpyruvic acid (III) by transamination with alpha-ketoglutaric acid. The enzyme involved is tyrosine-alpha-ketoglutarate transaminase. It is an adaptive enzyme found in liver and kidney and is pyridoxal phosphate dependent (22). To a minor extent, p-hydroxyphenylpyruvic acid may be reduced to p-hydroxyphenyllactic acid or decarboxylated to p-hydroxyphenylacetic acid; neither is believed to be further metabolized (23). Williams and Babuscio (24) have found that only 0.68% of administered DL-C^{14}-tyrosine is transformed into

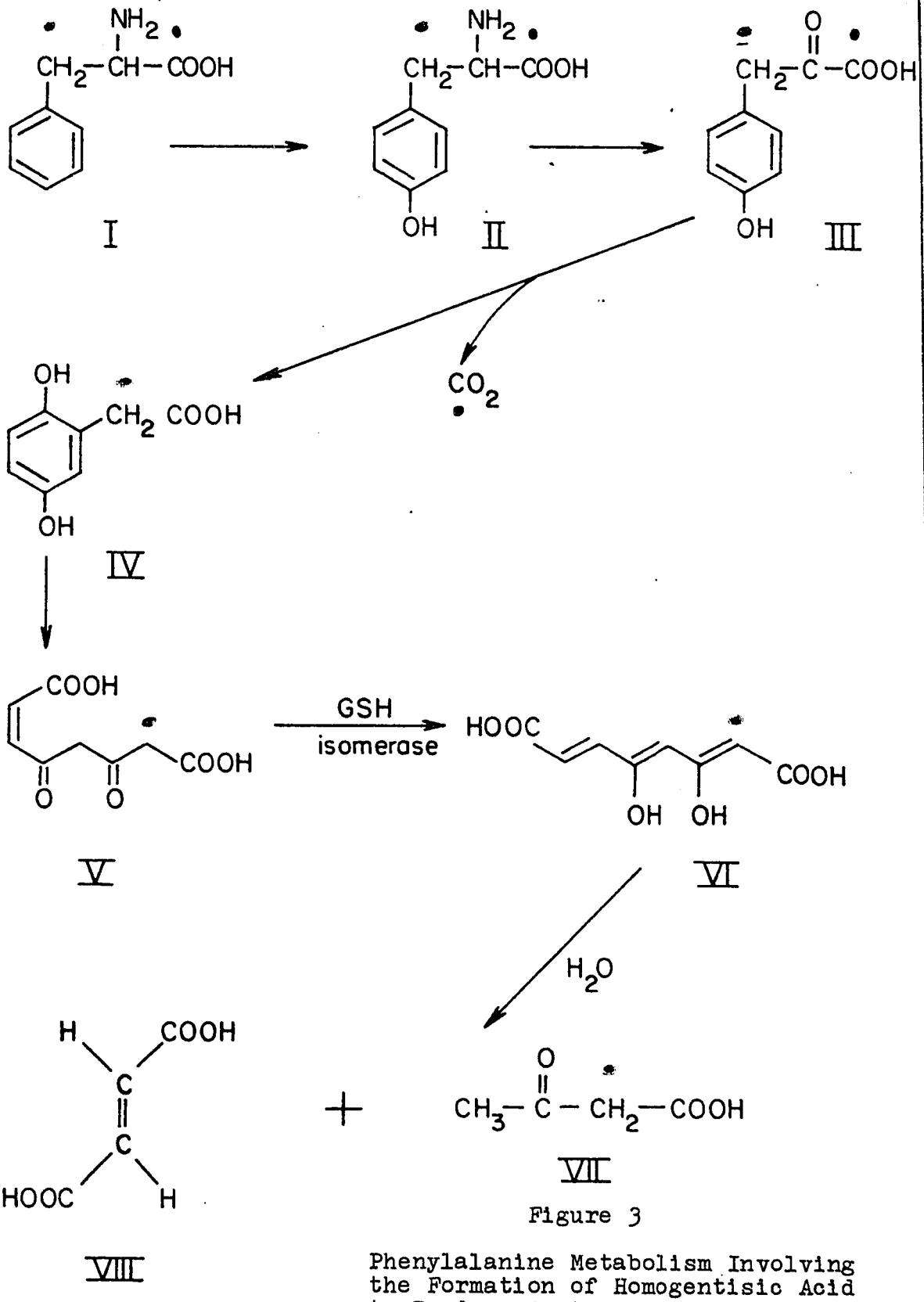


Figure 3

Phenylalanine Metabolism Involving the Formation of Homogentisic Acid to Produce Acetoacetic Acid and Fumaric Acid.

p-hydroxyphenylacetic acid, via that route. They have also found that only 0.32% of the tyrosine is decarboxylated to tyramine showing that direct decarboxylation of tyrosine to tyramine is a very minor pathway of tyrosine catabolism.

The conversion of p-hydroxyphenylpyruvic acid to homogentisate (IV) involves three reactions: ring oxidation, oxidative decarboxylation and migration of the side chain, all of which appear to be catalyzed by a single enzyme (25). It appears that the migration of the side chain must occur before its conversion from a pyruvate to an acetate moiety (26). Then homogentisic acid is oxidized and its ring opened to form maleylacetoacetic acid (V) through the action of homogentisic acid oxidase (26). This enzyme requires Fe++ and molecular oxygen and is found in both the liver and the kidney. An isomerase, requiring reduced glutathione as co-factor, converts this cis compound to fumarylacetoacetate (VI) (27). Fumarylacetoacetate, but not its cis isomer (23), is hydrolyzed to fumarate (VIII) and acetoacetate (VII) by a soluble liver enzyme (27) which hydrolyses many diketo acids, but whose natural substrate is apparently fumarylacetoacetic acid (28). Fumaric acid can then enter the Krebs cycle where it is converted to malic acid. Acetoacetate will produce two acetyl-CoA which will be broken down to CO_2 and H_2O.

Tyrosine can also be a precursor of the thyroid hormones, the adrenal hormones, or the pigment, melanin. These reactions are described in Figure 4 and are well explained in Chapter 6 of "Comparative Biochemistry" (29) from which they have been summarized as follows:

If tyrosine is oxidized by tyrosinase to 3,4-dihydroxyphenylalanine (dopa) (IX) an intermediate common to the synthesis of both melanin and epinephrine is formed. The mechanism of the tyrosinase reaction is quite complex involving either a single enzyme or enzyme complex. In both plants and animals, the enzyme contains copper which is believed to undergo changes in valence during the reaction. Traces of dopa are necessary for initiation of the oxidation. In animals, tyrosinase is found in the skin and liver and in melanomas. The product of this reaction, dihydroxyphenylalanine, may then enter into either of two alternate pathways.

A decarboxylating enzyme for dopa is found in several tissues but is particularly potent in the kidney. Pyridoxal phosphate is the co-enzyme and hydroxytyramine, or dopamine (XIII), is the product of the reaction. The same decarboxylase is believed to act upon dopa, tyrosine, and 5-hydroxytryptophan. After decarboxylation the proximal carbon atom in the side chain of dopamine is oxized to produce norepinephrine (XIV). This

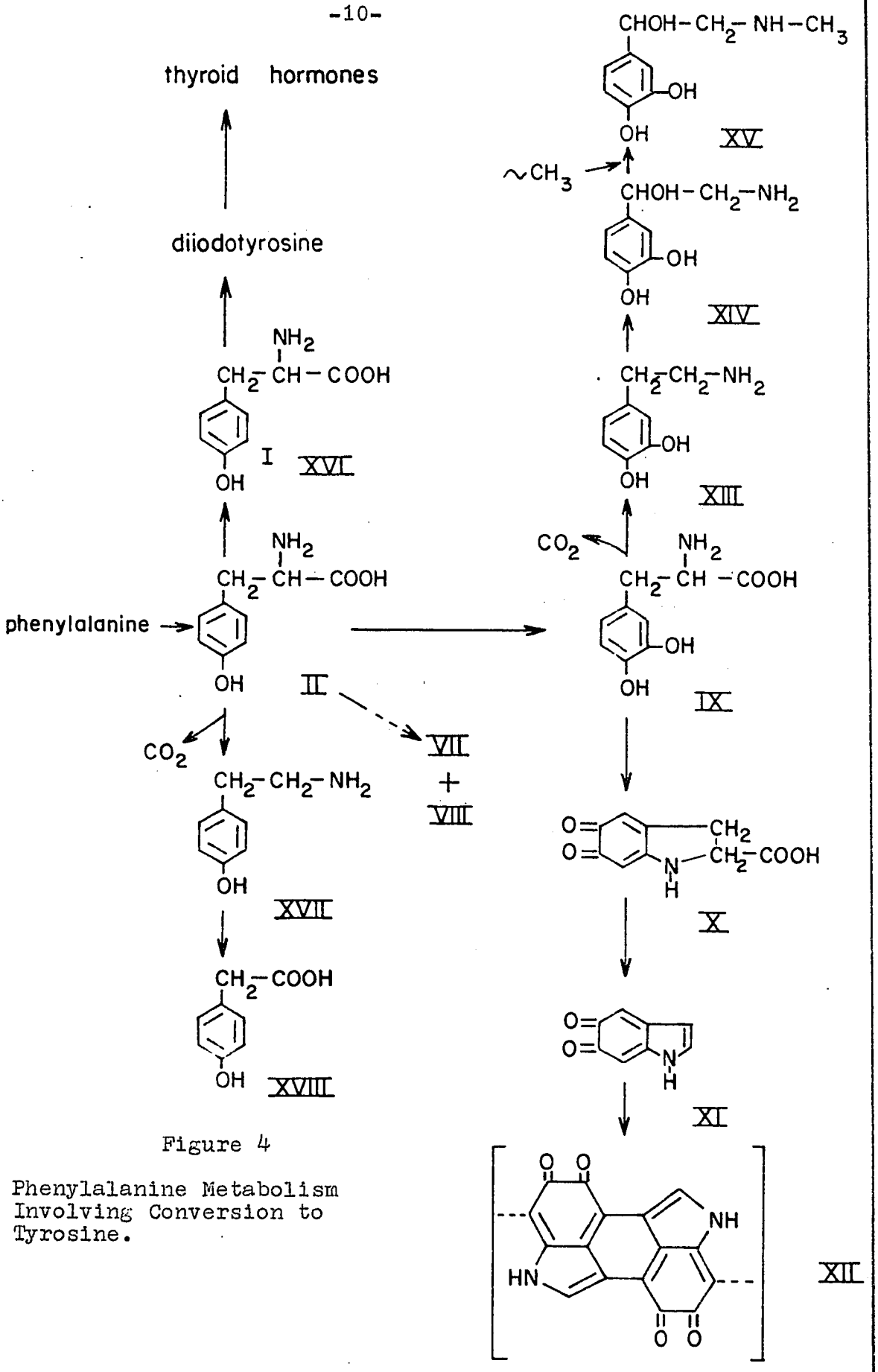


Figure 4

Phenylalanine Metabolism Involving Conversion to Tyrosine.

reaction is catalyzed by dopamine-beta-oxidase and requires molecular oxygen and ascorbic acid. Epinephrine (XV) is synthesized from norepinephrine by transmethylation, S-adenosylmethionine being the source of the methyl group.

Melanin (XII) may be defined as a brown to black pigment resulting from the polymerization of oxidation products of dopa. Its formation is apparently the result of the action of a single enzyme, tyrosinase, which functions in a specialized cell, the melanocyte. Briefly, dopa is oxidized, cyclized, and then further oxidized to the red pigment dopachrome (X). This compound is spontaneously decarboxylated to 5,6-dihydroxyindole which is oxidized to its corresponding quinone. Polymerization of indole-5,6-quinone (XI) produces melanin.

Tyrosine may also be converted to thyroxine. Iodination of tyrosine to mono and diiodotyrosine occurs in the thyroid gland. Two molecules of diiodotyrosine may then be coupled, with the loss of one alanine side chain, to form thyroxine. Thyroxine itself constitutes only a minor fraction of the total thyroxine-related metabolites excreted in feces and urine, the feces being the major avenue of excretion.

The reactions of phenylalanine which are not related to the metabolism of tyrosine are described in

Figure 5. Phenylalanine may undergo decarboxylation by the enzyme, aromatic-L-amino acid decarboxylase (30) to form phenylethylamine (XXVII), a reaction which occurs in micro-organisms and in mammalian tissues (31, 32). Phenylethylamine is excreted in the urine of normal patients (33). There is also evidence that this compound may be further converted to phenylacetic acid (XXI) which is found in the urine as phenylacetylglutamine (34).

Phenylalanine may also be converted to phenylpyruvic acid (XIX) by the action of an L-amino acid oxidase or a transaminase. Although some formation via the oxidase seems possible, the transaminase pathway was shown by Meister et al (35) to be quantitatively of greater importance. Phenylpyruvic acid is a normal excretory product in urine (33). There is also some evidence that the kidney has a limited ability to transform phenylpyruvic acid to some other substance (36) and that only when this capacity is exceeded is phenylpyruvic acid excreted. Possible metabolites of phenylpyruvic acid which would be formed in the kidney are: phenylalanine, phenyllactic acid (XX) and phenylacetic acid. A suggestion has been made that phenylpyruvic acid might be a precursor for o-hydroxyphenylacetic acid (XXVI) (37). This latter compound is also formed directly from phenylalanine via an ortho-hydroxylation to form o-tyrosine (XXIV).

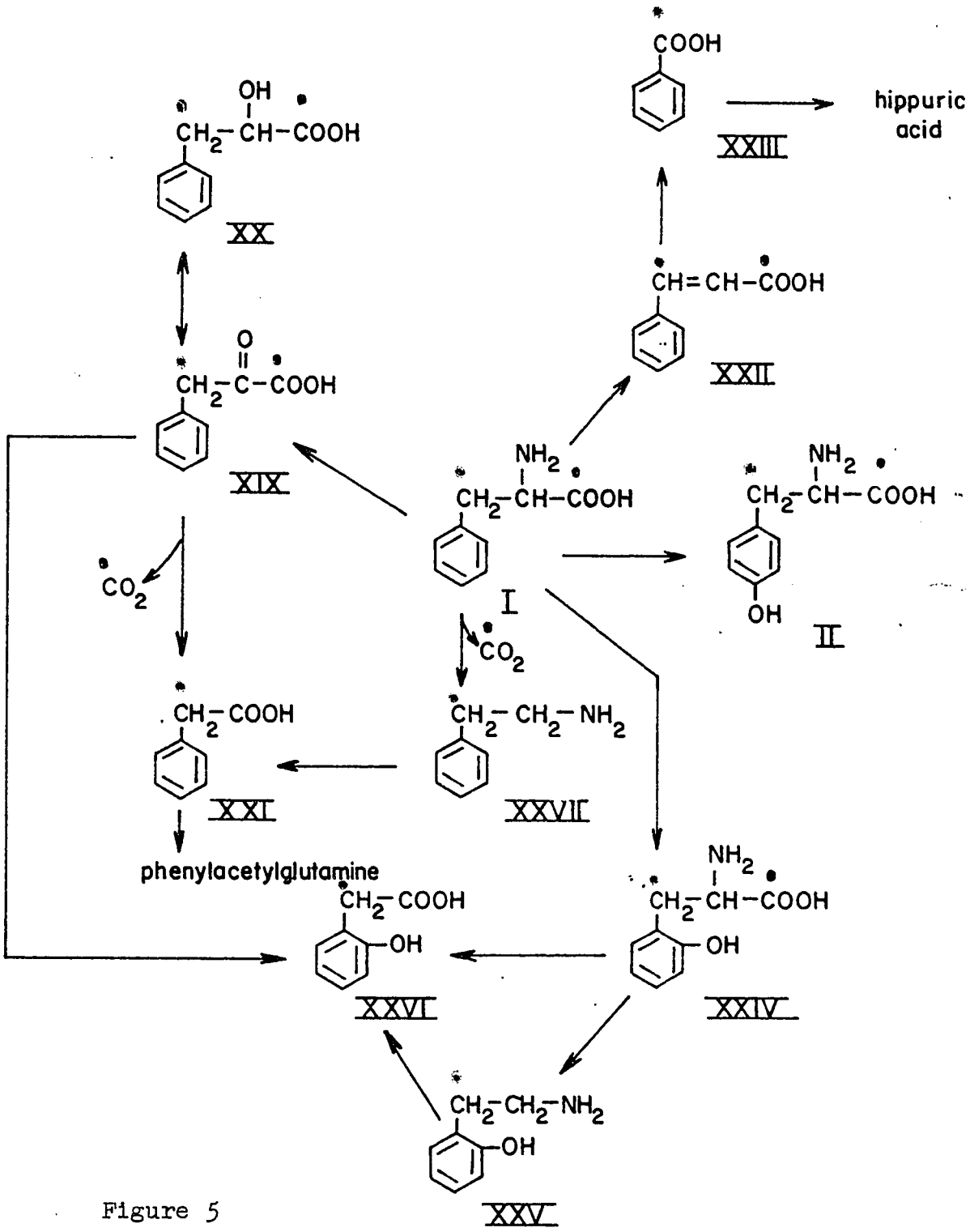


Figure 5

Phenylalanine Metabolism
Not Involving a Conversion
to Tyrosine.

Phenyllactic acid and phenylacetic acid are the metabolites most likely formed from phenylpyruvic acid. Phenylacetic acid could arise from phenylpyruvic acid by the action of a decarboxylase followed by an aldehyde dehydrogenase. Phenylacetic acid is conjugated with glutamine and then excreted in the urine (34).

Closs and Folling (38) first commented on the presence of phenyllactic acid in the urine and Zeller (39) has shown that it has the L-configuration. This indicates that it is probably formed by a lactic dehydrogenase, since in animals this enzyme acts only upon L-alpha-hydroxyacids.

Some phenylalanine may also be converted to benzoic acid (XXIII) via cinnamic acid (XXII) in mammals (40, 34) since hippuric acid, the glycine conjugate of benzoate, is found in the urine of fasted humans (34). In all, Armstrong et al (42) and Dalgleish (42) have detected more than 40 phenolic compounds in human urine.

2. AMINO ACID ANALOGUES

An amino acid analogue is a compound which is analogous in structure to the natural amino acid. Because of this similarity, it has been possible, in certain cases, to replace the natural amino acid in the diet by a structural analogue. In several instances, this has resulted in the incorporation of the analogues into proteins. In other cases, the amino acid analogue interfered with the metabolism or function of the natural amino acid producing metabolic antagonism or inhibition. The structural features which permit this metabolic antagonism include: 1) the presence of certain functional groups which are necessary if the analogue is to be bound to the enzyme in the same way as the metabolite was and 2) a distance between such functional groups similar to that in the metabolite.

Competitive enzyme inhibition by structural analogues was demonstrated with succinic dehydrogenase in 1928 (43). However, it was not until Woods (44, 45) reported that sulfonamide drugs exerted their effect by competing with p-aminobenzoic acid for an essential enzyme that the concept of a competitive enzyme relationship by growth inhibitory analogues and their corresponding amino acids became prevalent.

The variety of possible structural modifications

which could be made while still retaining a structural similarity to phenylalanine has resulted in the preparation of numerous analogues many of which are competitive inhibitors. Examples of possible structural modifications of phenylalanine are given by para-fluorophenylalanine (p-F-phe), beta-2-thienylalanine (BTA) and 2-amino-3-phenylbutanoic acid (APBA), as follows:

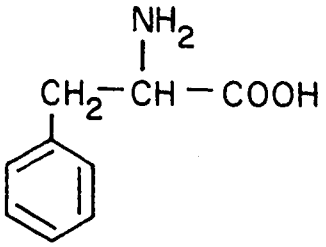
BTA..... modified by replacement of the phenyl ring of phenylalanine by an isoteric ring.

p-F-phe..... modified by substitution on the phenyl ring.

APBA..... modified by placement of a substituent group on the side chain.

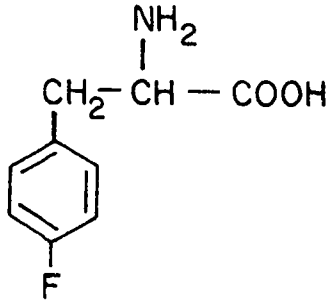
Their structure is illustrated in Figure 6.

In 1948, Bennet (46, 47) indicated that p-fluorophenylalanine could serve as a substrate for the phenylalanine hydroxylating enzyme. Then, in the same year, Beerstecher and Shiver (48) found that p-fluorophenylalanine was incorporated in the protein of Lactobacillis arabinosus when phenylalanine was present in suboptimal amounts. However Armstrong and Lewis (49, 50) found p-fluorophenylalanine to be toxic when added to the diet of normal rats. This toxicity was attributed to the formation of fluorides. Other researchers have



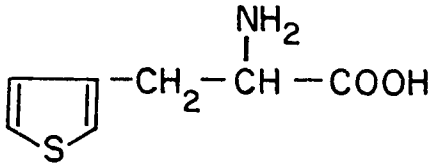
Phenylalanine

(phe)



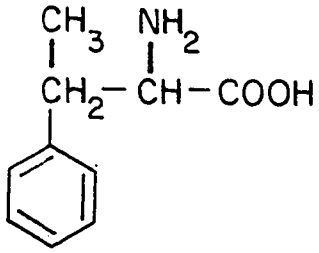
p-Fluorophenylalanine

(p-F-phe)



beta-2-Thienylalanine

(BTA)



2-amino-3-phenylbutanoic

(APBA)

Figure 6

The Structure of Phenylalanine and Some of its Analogues.

since indicated that p-fluorophenylalanine could be incorporated into diverse bacterial and animal proteins. In 1959, Kruh and Rosa (51) obtained an *in vitro* incorporation of p-fluorophenylalanine-C^{14} in rabbit haemoglobin while the evidence of Cohen and Munier (52) indicated that in *Escherichia coli* p-fluorophenylalanine may replace as much as 72.5% of the phenylalanine. P-fluorophenylalanine is incorporated into hen ovalbumin and lysozyme (53), into cat pancreatic amylase (54, 55, 56), into alkaline phospatase in *E. coli* (57) and into rabbit aldolase and 3-phosphoglyceraldehyde dehydrogenase (58). In 1964, Arnstein and Richmond (59) demonstrated that p-fluorophenylalanine was utilized for protein synthesis by the phenylalanine incorporating system from rabbit reticulocytes. Previc (60) further substantiated the fact that p-fluorophenylanine can be incorporated into *E. coli* as a partial phenylalanine substitute. Conway (61) found that the inhibition of phenylalanine incorporation into virus and bacteria, caused by p-fluorophenylalanine (62, 63) only occurred when the concentration of p-fluorophenylalanine was sufficient to prevent formation of 85-90% of the intracellular pool. Selection of phenylalanine over p-fluorophenylalanine no longer occurred after the two compounds were incorporated into the internal (conversion) pool

of yeast (64). To date, the fluorophenylalanines have been found to be the most effective antagonists of phenylalanine (59).

Not only does evidence show that p-fluorophenylalanine is incorporated into protein, both in rats and in micro-organisms, but Kaufman (65) has recently found that p-fluorophenylalanine is hydroxylated to tyrosine by the enzyme phenylalanine hydroxylase. This is a direct route which does not involve an intermediary conversion to phenylalanine. The phenylalanine hydroxylase is able to catalyze this reaction to produce equal amounts of L-tyrosine and F^-. However, tyrosine is formed from p-fluorophenylalanine at only 1/6 the rate of its production from phenylalanine (65). The reaction requires oxygen, two enzymes, NADPH and tetrahydropteridine (66).

In 1945, Ferger and du Vigneaud (67) found that BTA could not replace phenylalanine in the diet of rats; then a few years later, Beerstecher (48) reported the inhibition, by BTA, of growth in E. coli. At this time, Ferger and du Vigneaud (68) showed that in their earlier experiment, the action of BTA in rat growth inhibition was essentially an anti-phenylalanine effect. BTA alone caused a loss of weight, an effect which was reversed by the addition of phenylalanine to the diet, thus illustrating an antagonism between these two

compounds. However, Cohen and Munier (52) found that radioactive BTA was incorporated into the proteins of E. coli. In 1960, Wolfe and Hahn (69) showed that BTA underwent activation but was generally not involved in protein or nucleic acid biosynthesis in E. coli. Further, Kaufman (70) observed a loss of weight accompanied by pathological change in tissues of rats after four weeks on a 1-2% BTA diet. High concentrations of phenylalanine may prevent the toxicity of BTA in E. coli presumably by preventing an irreversible, essential step in the assimilation of BTA in proteins.

APBA has been shown to be an effective phenylalanine antagonist for Leuconostoc dextranicum but not for E. coli. However by competitively inhibiting the incorporation of BTA into E. coli proteins, APBA was able to prevent the normally toxic effect of BTA (71). Phenylalanine can also perform this inhibition.

3. METABOLISM OF PHENYLALANINE UNDER ABNORMAL CONDITIONS

a. Phenylalanine Excess

In 1952 Lewis (72) reported that a large dietary excess of cystine was toxic. Since then there have been many reports that excess dietary intakes of most dispensible and several of the indispensible amino acids depress growth under some conditions. However the investigations have been done under such a variety of conditions that meaningful comparisons are difficult (73, 74, 75).

The toxicity of a high dietary intake of tyrosine is well documented (76, 77, 78, 79, 80). Diets which contain 9.5 to 20% tyrosine together with 4 to 18% protein have been tested. Schweizer (80) found that feeding young rats 1% or more tyrosine in a diet containing 12% protein produced lesions of the eyes characterized by vascularization, erosion, and cloudiness of the corneal epithelium, conjunctivitis and keratitis. The last can be quite severe, resulting in complete closing of one or both eyes. The toes are also affected, particularly those on the front paws. They become swollen, red and encrusted, with histologic examination revealing hyperkeratosis and edema. In severe cases the toes atrophy. Mature rats are much

more resistant to tyrosine toxicity than are young rats.

The basis for the toxicity is not known. It has been suggested that excessive tyramine formation may be the cause and Bernhard and Zilliken (81) found a small increase in tyramine excretion after feeding excess tyrosine. However, after feeding tyramine in quantities as high as 1% of the diet, no signs resembling tyrosine toxicity were produced. The inclusion of an amine oxidase inhibitor in a diet containing excess tyrosine did not increase the severity of the signs. Harper (82) states that phenylalanine in excess does not cause comparable signs. An excess of phenylalanine has been reported to produce a depression in growth in a low protein diet, and an amino acid imbalance was suggested as the cause of this growth depression (75). In fact, an intraperitoneal injection of phenylalanine was used by Carver et al (83) to produce an amino acid imbalance in rats.

In 1934, Asbjorn Fölling discovered an inborn error of phenylalanine metabolism which he called phenylketonuria (PKU) (84, 85). After autopsy of phenylketonuric patients, Jervis (86) was able to report that in this disease the phenylalanine oxidizing system was lacking. Subsequent studies by others (87, 88) have suggested that specifically it is the labile

enzyme I of Figure 2 which is deficient in phenylketonuria. Thus, the conversion of phenylalanine to tyrosine does not occur, causing a consequent rise in blood phenylalanine level from 1-3 mg/100 ml serum to 20-30X the normal value. Where renal functions are normal, high levels of phenylalanine (15-20 mg/100 ml or more) results in the urinary excretion of phenylpyruvic acid (89, 90). This loss of the ability to effect the oxidation of phenylalanine to tyrosine is not complete in phenylketonuria since experiments with C^{14} phenylalanine showed that as much as 10% of the amino acid may still be converted to tyrosine (91). Still, the actual conversion level in the average phenylketonuric is so small that, practically, it may be considered that there is almost a complete loss of the ability to oxidize phenylalanine. Consequently, the plasma level of phenylalanine is increased. Phenylpyruvate excretion increases and there is a concomitant modification in the appearance of the other normal metabolites. In Figure 7 are outlined the transformations of phenylalanine which must be considered in relation to phenylketonuria.

Because of the scanty information available, it is only possible to make rough approximations of the proportion of ingested phenylalanine disposed of in the form of the five major metabolites of phenylalanine in

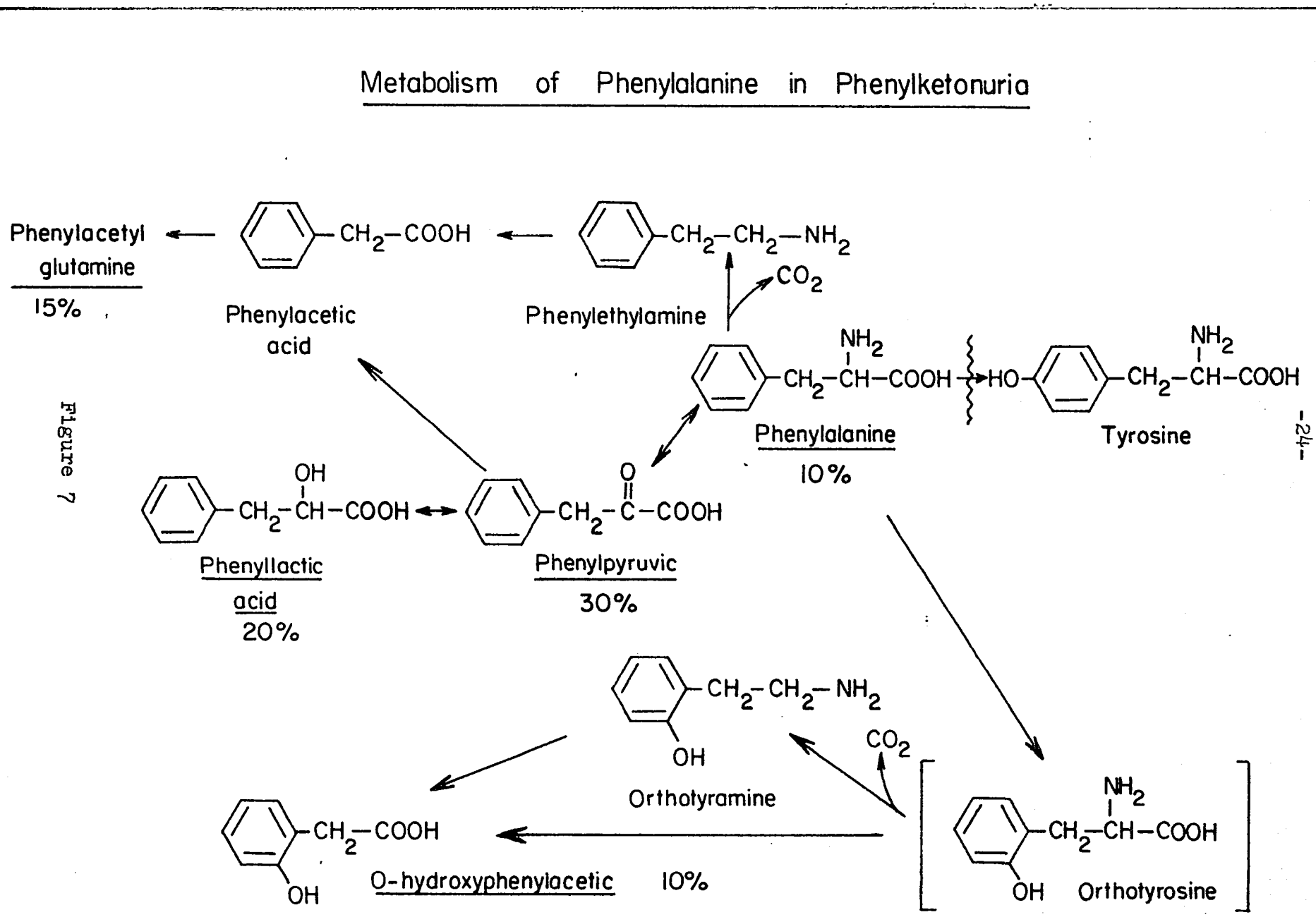

Metabolism of Phenylalanine in Phenylketonuria
Phenylacetyl glutamine
15%
Phenylacetic acid
Phenylethylamine
CO2
Phenylalanine
10%
Tyrosine
Phenyllactic acid
20%
Phenylpyruvic
30%
Orthotyramine
CO2
O-hydroxyphenylacetic
10%
Orthotyrosine

Figure 7

phenylketonuria. The best estimates possible at present are that 10% is oxidized to tyrosine which is further metabolized (92). A small amount of phenylalanine and phenylpyruvic acid is excreted in sweat (93) and the remainder of the dietary phenylalanine appears in the urine in the form of four major products as follows: phenylalanine itself, 10%; phenylpyruvic acid, 30%; phenyllactic acid, 20% (93) and phenylacetylglutamine, 15% (94); a total of about 75% phenyl compounds. The remaining product, o-hydroxyphenylacetic acid, accounts for approximately 10% of the ingested phenylalanine (95). Thus, these substances, on the basis of crude estimates from the available information, account for most of the ingested phenylalanine. A normal excretory product, m-hydroxyphenylacetic acid, is either greatly diminished in quantity or is absent. It was the identification of one of these excretory products which led to the discovery of phenylketonuria. When Følling added a 10% $FeCl_3$ solution to a urine sample from one patient he noticed the development of a dark green colour. He eventually found that phenylpyruvic acid was responsible for the formation of this green colour. Since then the ferric chloride test has become the best known and most widely used of any of the diagnostic urine tests for phenylketonuria (84). Acidified solutions of 10% $FeCl_3$ react with a phenylketoacid to produce a blue green

colour. A definitely positive, immediate colour reaction can disappear in less than a minute, therefore observations and evaluations must be made immediately following addition of the $FeCl_3$. Clinically, tests are obtained by drying urine samples on filter paper then applying several drops of the ferric chloride solution to the spot. Extensive testing has demonstrated that this is a sensitive and accurate test, yielding only 5% false-negative tests on urine samples standing four hours at room temperature (84).

Another colour test has been developed for the testing of possible phenylketonuric patients and is often used in conjunction with the ferric chloride test. This is the dinitrophenylhydrazine (DNPH) test which has given less than 1% false-negative results (96). A positive reaction depends on the presence of a keto group and is indicated by the rapid formation of a yellow precipitate in the test tube.

Excess phenylalanine can be decarboxylated to form phenylethylamine, by a normal pathway of metabolism. However, since the Michaelis constant for phenylalanine decarboxylation in mammalian tissue is greater than 10^{-2} molar, enhanced formation of phenylethylamine is expected in the presence of high phenylalanine levels (97). Phenylethylamine is excreted in the urine of phenylketonurics (98), but curiously enough, this level

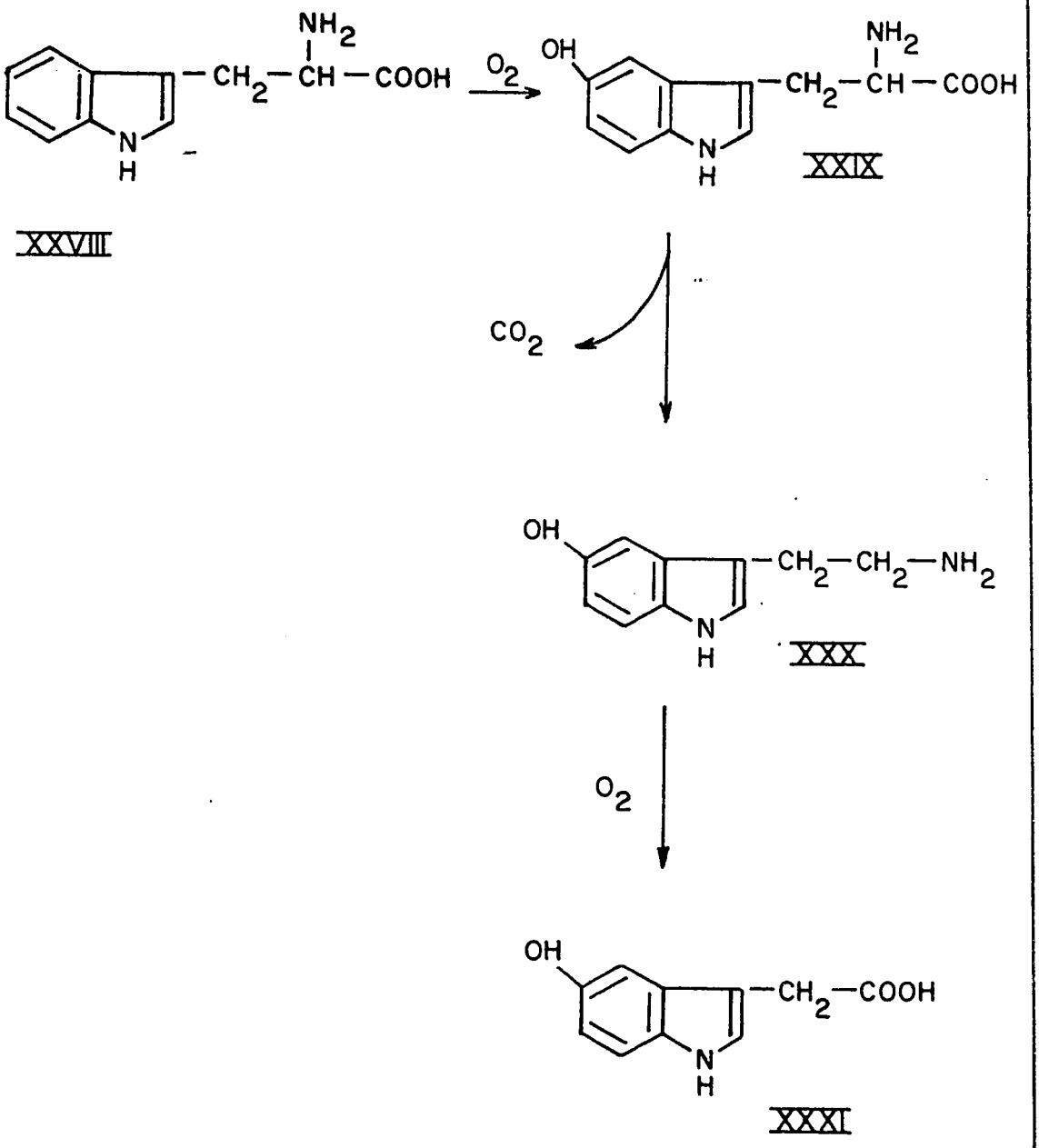
does not seem to be increased above normal (99). The phenylethylamine excess in phenylketonuria is probably well controlled by the monoamine oxidase system. Administration of an inhibitor of the monoamine oxidase elevated the phenylethylamine levels in the urine (97). Phenylethylamine can produce phenylacetic acid which is excreted in the urine as the glutamine conjugate. However, phenylacetylglutamine is mainly formed from excess phenylpyruvic acid since the plasma levels of this compound are greatly increased in phenylketonuria. As in the normal metabolic pattern, phenylpyruvic acid can produce phenyllactic acid, phenylacetic acid or o-hydroxyphenylacetic acid with consequent alteration in the levels of each of these products. Recently two new metabolites derived from phenylalanine and pyridoxine have been isolated from the urine of animals on a high phenylalanine diet (100). These have been tentatively named compounds A and B. Compound A has only been found in the urine of phenylketonuric rats while compound B has been detected in the urine of both normal and phenylketonuric rats. It is not yet known what, if any, is the significance of compound A in the pathogenesis of the mental disease (100).

While it is known that the specific defect in phenylketonuria is the lack of the labile enzyme 1 in

the phenylalanine hydroxylase system (87, 88), the actual cause of the mental defect is not yet known. Jervis (101) has suggested four hypotheses:

1) the direct toxic effect caused by overflow of phenylalanine or its derivatives
2) the effects of phenylalanine and its derivatives on enzyme systems
3) the structural alteration of brain constituents
4) an amino acid imbalance

One of these hypotheses, the effects of phenylalanine and its derivatives on enzyme systems, involves a consideration of the resultant alteration in tryptophan metabolism. Normally the metabolism of tryptophan proceeds via several routes, one of which results in the formation of nicotinamide. Another pathway involves the formation of serotonin. This compound is a neurohormone found in the gastro-intestinal tract and the central nervous system. Its role is presently under intense investigation. Serotonin is more concentrated in the basal grey masses of the brain, while little is found in the cerebral cortex (103). Generally, the distribution of serotonin in the central nervous system closely resembles that of adrenalin (103). The formation of serotonin is described in Figure 8. Tryptophan (XXVIII) is hydroxylated by the enzyme tryptophan hydroxylase to 5-hydroxytryptophan (XXIX) (104);



XXVIII XXIX XXX XXXI

Figure 8

The "Serotonin Pathway" of Tryptophan Metabolism.

subsequent decarboxylation of this compound by the enzyme, 5-hydroxytryptophan decarboxylase, results in formation of 5-hydroxytryptamine or serotonin (XXX). Further oxidation results in the formation of 5-hydroxy-indolacetic acid (XXXI) which is normally excreted in small amounts in the urine (105).

In phenylketonuria tryptophan metabolism appears to be altered. Armstrong and Robinson (106) have noted the excretion of abnormal indole derivatives in phenyl-ketonuric patients. These compounds were identified as indolelactic acid and indoleacetic acid, neither of which is found in the urine of a normal individual. They do however appear in the normal urine following ingestion of tryptophan. Pare et al (107) noted a defect in the 5-hydroxyindoleacetic acid pathway of tryptophan metabolism in phenylketonurics and suggested that the failure in serotonin production might play some part in the pathogenesis of the mental deficiency. This serotonin deficiency in phenylketonuria was first attributed to an inhibition of tryptophan hydroxylase (107, 108) and later to an inhibition of aromatic L-amino acid decarboxylase (105, 98, 109) by high tissue concentrations of phenylalanine. Perry et al (110) have shown that defective 5-hydroxylation of tryptophan does occur in phenylketonurics and have concluded that it may be primarily responsible for the decreased serotonin

production in these patients. Further evidence is supplied by the fact that in mice it is possible to prevent the mental defect of phenylketonuria by the administration of serotonin congeners such as hydroxytryptophan or melatonin (111).

Udenfriend et al (112) have shown that the tryptophan and phenylalanine hydroxylase activities in liver extracts represent one and the same enzyme. However, it is worthwhile to note that Freedland et al (113, 114) have found that although the phenylalanine hydroxylase enzyme system of mammalian liver can convert tryptophan to 5-hydroxytryptophan, the rate of hydroxylation of phenylalanine by this system is 30 times as great as that of tryptophan. Freedland (115) indicates that this difference in the rate of reaction of the phenylalanine hydroxylase may be explained by the fact that there are two different binding sites on the enzyme surface for phenylalanine and tryptophan. There is also some evidence of an enzymatic conversion of tryptophan to 5-hydroxytryptophan in brain (116, 117). However, Renson et al (114) indicate that in brain there is no hydroxylation of phenylalanine itself. Some investigators feel that the ability of the liver phenylalanine hydroxylase to hydroxylate tryptophan has little physiological significance (104).

Another possibility is disclosed by the fact that

high concentrations of phenylalanine and other amino acids interfere with the active transport of 5-hydroxytryptophan and tyrosine into rat brain both *in vitro* and in the living animal (118, 119, 120). Still to be determined is the relative importance of the defective 5-hydroxylation of tryptophan and of impaired transport of 5-hydroxytryptophan into brain in producing a serotonin deficiency in the human phenylketonuric brain. Also, the question remains: how does a deficiency of serotonin in brain during the period of rapid growth, if such occurs, contribute to the mental defect characteristic of phenylketonuria?

b. Phenylalanine Deficiency

Elimination of single amino acids from an otherwise complete diet indicates that certain amino acids are essential to the diet, that is, the amino acid can't be synthesized by the organism from substances ordinarily available to the cells at a rate commensurate with optimal physiological requirements. Such an amino acid is phenylalanine. Tyrosine can, in part, act as a substitute for phenylalanine; nevertheless there is an essential role for phenylalanine in protein formation since the phenylalanine to tyrosine pathway is irreversible.

The ommission of any one essential amino acid from

the diet will result in a failure of the body to use the other amino acids except as a source of calories; within one day in young and growing animals this results in loss of appetite and growth failure and, in mature animals, in severe negative nitrogen balance indicating loss of body nitrogen (121, 122, 123). In fact, the effects of omitting one indispensable amino acid from the diet for only a few hours can be detected (124, 125, 126).

An amino acid deficient diet causes a depression in the actual food intake (121, 122). Also, it appears that such a diet is consumed in smaller quantities than is a protein-free diet (122, 127). Several investigators claim that this depression of food intake is a protective mechanism (128, 129). This is indicated by the fact that force-feeding a diet deficient in a single amino acid results in severe tissue changes and early death of the rat whereas animals allowed to eat the deficient diet ad libitum survive much longer and show no obvious abnormalities.

One actual use for a deficient diet is in the treatment of certain metabolic disorders. A phenylalanine-restricted diet has so far proved to be the only therapeutic measure of value in the management of phenylketonuria. It is difficult to compose such a diet, as most of the natural proteins contain about four to six per-

cent phenylalanine, and there is no protein with a low phenylalanine concentration and a normal concentration of the other essential amino acids. However, several preparations of low phenylalanine content are now commercially available.

After the institution of the low phenylalanine diet, various biochemical changes take place, leading to a practically complete normalization of the metabolites in plasma and urine. However, the primary enzyme block between phenylalanine and tyrosine remains unchanged. The phenylalanine concentration of the plasma decreases from 15-60 mg/100 ml to the normal level of 0.8-1.5 mg/100 ml within a period of three to five days. At the same time, the excretion of phenylalanine in the urine is reduced from 70-500 mg/day and more to normal levels of 10-20 mg/day. The excretion of phenylpyruvic acid, starting from levels similar to those of phenylalanine, becomes too small for detection by the ferric chloride reaction at phenylalanine plasma levels below 15 mg/100 ml. Phenyllactic acid, phenylacetic acid and its glutamate, being derived from phenylpyruvic acid, disappear from the urine at about the same time as phenylpyruvic acid (130).

If the phenylalanine intake is too severely restricted there will be an initial fall of the plasma phenylalanine level to subnormal values. This will be

followed by an increase leading to phenylalaninuria, with an increase of various other amino acids leading to generalized aminoaciduria. Finally, phenylpyruvic acid will appear in the urine again. Clinical symptoms may be relatively mild and chronic, consisting of unsatisfactory gain of weight, anorexia and inactivity over a period of weeks or months. Severe phenylalanine deficiency will lead within a few days to acute metabolic crises, with rapid loss of weight, vomiting, dehydration, acidosis and lethargy (131). Thus, use of the phenylalanine restricted diet in phenylketonuria, though definitely effective, should be carefully supervised.

One of these phenylalanine-deficient diets, Lofenalac, brings phenylalanine levels down to four to six micromoles per liter. Cockburn et al (132) have attempted to determine what, if any, are the effects of Lofenalac on the bone marrow and on the amino acid metabolism. They have found that within 24-48 hours there is a change in the bone marrow characterized by cytoplasmic vaculization. Within 48 hours this effect may be reversed by adding 100 mg/kg of phenylalanine to the diet. Addition of valine, alanine, or methionine to normal and phenylalanine-deficient diets does not produce these marrow changes or other toxic effects.

4. THE STUDY OF A METABOLIC PATHWAY

The study of a metabolic pathway *in vivo* requires that a method of following the chemical changes in the metabolite, the distribution in the tissues, and the end product excretion be found. Subsequent identification of these products would aid in the elucidation of the intermediary metabolism of the compound. The use of isotopically labelled compounds and the development of advanced radioisotopic measurement techniques have provided an efficient means for tracing the pathway of an administered substance.

Jeffay and Alvarez (133) have developed a method for trapping the CO_2 released by the experimental subject in order to determine the amount of radioactivity it contains. Techniques have also been found to permit the counting of radioactivity in the urine (134) and blood (135, 136) as well as in the body tissues (136, 137, 138, 139). By use of protein hydrolysis, autoradiography and amino acid analysis it is possible to determine the specific intermediary metabolites which contain the radioactivity. By placing the C^{14} atom in different known positions in the compound to be tested, it is possible to determine the manner in which the compound undergoes degradation. At the present time, a very wide variety of methods is available for the

investigation of a metabolic route.

In 1948, Schepartz et al (140) used DL-phenylalanine containing C^{14} at different positions on the benzene ring and the side chain to investigate the route by which homogentisic acid is formed from tyrosine. Using this technique, they were able to study the mechanisms by which the ring is cleaved to form acetoacetic acid. Other investigators employed radioactive tracer techniques to show the formation of radioactive ketone bodies from tyrosine in the rat (141, 142). More recently Sourkes et al (143) and Porter et al (144) investigated the formation of respiratory $C^{14}O_2$ from L-tyrosine-1-C^{14} and from DL-tyrosine-3-C^{14} when an inhibitor of dopa decarboxylase was used. Gey (145) investigated the distribution and metabolism of DL-3, 4-dihydroxyphenylalanine (dopa)-2-C^{14} in the rat. In 1962, Porter and Titus (134) studied the distribution of radioactivity of C^{14} labelled L-3,4-dihydroxy-alpha-methylphenylalanine in the tissues. They found that the kidneys contained the highest concentration of radioactivity and that this concentration did not change with the administration of methyldopa labelled with carbon 14 on either the first or the second carbons. However the livers showed more radioactivity when the compound labelled on the second carbon was given. This study included trapping the expired CO_2 and urine as well

as investigating the tissue concentrations. Further evidence of selectively increased radioactivity in particular body organs was given by Michel et al (146) who found that the injection of L-thyroxine into rats increased the in vivo incorporation of radioactive amino acids into the protein of liver, kidney and heart, but not into the protein of spleen, testis or brain.

Weiss and Rossi (147) have studied the metabolism of dopa-C^{14} both in normal and in alpha-methyldopa treated mice. This compound, alpha-methyldopa, was shown by Sourkes (148) to be an extremely potent inhibitor of dopa decarboxylase. In mice, the duration of the decarboxylase inhibitory action of alpha-methyldopa is from eight to sixteen hours after an interperitoneal injection (147). It is now known that alpha-methyldopa is, in general, an active decarboxylase inhibitor (149). Smith (150) reported that, in mice, the levels of serotonin in the brains decreased after the parenteral administration of alpha-methyldopa. He has shown that the DL-alpha-methyldopa inhibits 5-hydroxytryptophan decarboxylase (151). That alpha-methyldopa will inhibit the hydroxylation of tryptophan and phenylalanine has also been shown (152). There appears to be a decrease in amino acid production due to development of a transient sedation and lowered blood pressure (153). Recently Oates and his co-workers (154) studied the production of

phenylethylamine by using both alpha-methyldopa and a monoamine oxidase inhibitor. Studies on phenylketonuric patients have also been done using a monoamine oxidase inhibitor, pargyline hydrochloride (155). This monoamine oxidase inhibitor slows the breakdown of the amine, therefore the excretion of phenylethylamine in the urine is greatly increased.

Using the DL-form of an amino acid instead of the L-form complicates the study of the metabolism of the amino acid since the body proteins contain only L-amino acids and most of the enzymes involved in the metabolic processes are specific for the L-form of the amino acid. Therefore the utilization of the D-amino acid by the animal becomes a problem. Since the D-isomer cannot be used as such, it must first be converted into the L-amino acid. The inversion of the D-isomer of an essential amino acid was first shown by Conrad and Berg (156) in 1937. D-phenylalanine is deaminated by the action of a D-amino acid oxidase, an enzyme found mainly in the kidney of the rat (157). This results in the formation of phenylpyruvic acid. Subsequent transamination of the phenylpyruvic acid to form L-phenylalanine, a reaction which also takes place in the kidney (158), completes the change from the D to the L-isomer.

The ability of D-amino acids to maintain the animal

and to promote growth is subject to great species and individual variation (159) as well as a variation which depends on the nature of the particular amino acid involved (159). In man, when the L-phenylalanine of the diet is replaced by D-phenylalanine, there is some conversion of the D to the L-isomer, but the rate of this conversion is not sufficient to maintain nitrogen balance (160). However Rose and Womack (161) have found that, in rats, the inclusion of D-phenylalanine instead of L-phenylalanine in the diet promoted growth almost as well as did L-phenylalanine. They also found that it was possible to produce maximum growth in the rats by adding DL-phenylalanine to the diet (161).

Information concerning the conversion of D-phenylalanine to the L-isomer in rats was supplied by Armstrong (162). He found that on a 5% D-phenylalanine diet there was no loss of the D-isomer in the inversion process, that is, there was no increase in the quantity of phenylpyruvic acid which was excreted. Further, Meister et al (163) and Babe et al (164) found that phenylpyruvic acid can completely replace L-phenylalanine in the diet. Recently it has been shown that undernourished rats can use the D-isomer of phenylalanine better than normal rats (165).

From these results it appears that the D-phenylalanine which is converted to phenylpyruvic acid is

not lost via the urine and that the phenylpyruvic acid is capable of being converted into L-phenylalanine in sufficient quantities to permit optimal growth in the normal rat.

In phenylketonuria the study of the metabolic pathway of phenylalanine has been complicated by the fact that diagnosis of this disease is now followed by a treatment which significantly modifies the biochemical correlates indicative of phenylketonuria.

To permit the continued study of the conditions of phenylketonuria Waisman and his collegues developed a technique for inducing an experimental phenylketonuria in rats (166, 167, 168) and in monkeys (169, 170, 171). In 1958, Auerbach and Waisman (166) found that feeding 5% DL-phenylalanine to young rats resulted in a 200% increase in the plasma phenylalanine. However they felt that a better increase would be obtained if only the L-isomer were used since, they argued, D-phenylalanine might contribute to the excretion of phenylpyruvic acid and interfere with the absorption of L-phenylalanine. It has since been established that if 21 day old rats of the Holtsman-Sprague Dawley strain are placed on a 5% L-phenylalanine diet for one week, then put on a 7% L-phenylalanine diet, it is possible to establish an experimental phenylketonuria which shows maximum values at 52 days of age (169, 172). That is, the blood phenyl-

alanine levels are high and the urine gives positive $FeCl_3$ (84) and DNPH tests (96), indicative of high phenylpyruvic acid excretion.

Wooley et al (173) have induced an experimental phenylketonuria in mice by the continuous oral administration of DL-phenylalanine and L-tyrosine from birth to maturity. Scores on maze learning and discriminatory tests of animals made experimentally phenylketonuria indicated that there was mental impairment (174, 175). Recently, Waisman (175) has indicated that the optimum phenylalanine diet for inducing experimental phenylketonuria in the rat is either 7% DL or 4% DL + 4% L-phenylalanine commencing at 21 days of age and continuing for 21 days.

5. STATEMENT OF THE PROBLEM

Some of the amino acid analogues are not able to replace the natural amino acid in the diet while other analogues effectively substitute for the amino acid to such an extent that they are incorporated into the proteins. *In vivo* studies indicate that certain amino acid analogues can act as competitive inhibitors in enzymatic reactions involving the amino acids. Some of the phenylalanine analogues are known to be competitive inhibitors of phenylalanine.

Therefore, it was decided to study the metabolism of some radioactive phenylalanine analogues in rats and to compare the metabolic pattern obtained with that of radioactive phenylalanine. In order to study the metabolism of these compounds the level of radioactivity in the CO_2, urine, and tissue proteins was determined.

In addition, the effect of an excess of the non-radioactive analogues on the oxidative metabolism of phenylalanine was studied to determine whether the analogues could inhibit the normal catabolism of the amino acid.

In some animals inborn metabolic errors alter normal phenylalanine metabolism. One such metabolic error, phenylketonuria, involves an increase in the plasma phenylalanine level and, in rats, can be experi-

mentally induced by feeding the animals a high phenylalanine diet. In human phenylketonurics the disease is treated by maintaining the patient on a low phenylalanine diet. The metabolism of radioactive phenylalanine in rats fed either a high phenylalanine diet or a low phenylalanine diet was compared with the metabolism of radioactive phenylalanine in normal rats.

PART TWO

EXPERIMENTAL

A. METHODS

1. MATERIALS

a. Chemicals

1) DL-phenylalanine-3-C^{14}

2) DL-phenylalanine-1-C^{14}

3) L-phenylalanine-1-C^{14}

4) DL-para-fluorophenylalanine-3-C^{14}

5) DL-beta-2-thienylalanine-3-C^{14}

6) DL-tryptophan-3-C^{14}

All of these compounds were chromatographically pure and had a specific activity of one microcurie per mg. They were obtained from Calbiochem.

7) DL-phenylalanine, L-phenylalanine, D-phenylalanine, DL-para-fluorophenylalanine, DL-beta-2-thienylalanine, 2-amino-3-phenylbutanoic acid and DL-alpha-methyldopa, all of which were obtained from Nutritional Biochemical Company.

8) A monoamine oxidase inhibitor, 2-phenylcyclopropylamine, or parnate was obtained from Dr. Belleau, Department of Chemistry, Ottawa University.

b. Animals

Male albino rats of the Wistar strain obtained from Romain Robidoux Company, St. Constant, P.Q. were used in the experiment.

c. Diets

Three types of diets were used:

1) Purina Lab Chow

2) Lofenalac, available commercially from Mead Johnson and Company, and containing less than 0.1% phenylalanine.

3) A high phenylalanine diet prepared by addition of either 50 g of L-phenylalanine to 950 g of a "Normal Powered Protein Test Diet" obtained from General Biochemicals Company, to make a 5% L-phenylalanine diet, or 70 g of L-phenylalanine plus 930 g of the test diet for a 7% diet.

2. METHODS

a. Preparation and Maintenance of the Animals

Animals were used in 3 groups, each one prepared in a different way.

1) Normal:

Male albino rats received from Romain Robidoux Company were maintained on Purina Lab Chow ad libitum

until they reached a body weight of 150 g.

2) Phenylalanine-deficient:

Normal male albino rats were maintained on Purina Lab Chow until they had attained a body weight of 200 g. They were then placed on Lofenalac and fed ad libitum until they had lost 25% of their body weight.

3) Phenylketonuric:

These animals were prepared in 2 ways although only rats subjected to the second method were tested with chemicals.

A. Following the methods of Woolley and his co-workers (173) infant rats were fed several drops of a solution containing 7% L-phenylalanine commencing 2 days after birth. At 21 days of age they were put on a solid 7% phenylalanine diet.

B. Following Waisman's (167, 172) method, 21 day old male albino rats were placed on the solid diet containing 5% L-phenylalanine for one week then they were put on the 7% phenylalanine diet until positive $FeCl_3$ (84) and DNPH (96) tests were obtained.

After injection, the normal rats and the rats which had been maintained on a Lofenalac diet received only H_2O during the period of experimental interest. However, the rats fed the 7% phenylalanine diet continued to receive their phenylalanine-supplemented diet as well as water for the duration of the experiment.

b. Injections

1) Chemical Preparation and Administration:

A solution containing 8 mg of the radioisotope in 4 ml of saline was prepared for injection of a 0.5 cc sample into each rat. Each experimental group was composed of 8 rats. When a non-radioactive substance was to be studied, these compounds were added to the radioactive solution in either a 1:10 or 1:20 ratio as follows:

A. DL-phenylalanine-3-C^{14} plus p-fluorophenylalanine in both 1:10 and 1:20 ratios

B. DL-phenylalanine-3-C^{14} plus beta-3-thienylalanine in both 1:10 and 1:20 ratios

C. DL-phenylalanine-3-C^{14} plus 2-amino-3-phenylbutanoic acid in both 1:10 and 1:20 ratios

D. DL-phenylalanine-3-C^{14} plus D-phenylalanine in a 1:20 ratio only

E. DL-phenylalanine-3-C^{14} plus L-phenylalanine in a 1:20 ratio only

The test solution (0.5 cc) was administered by direct injection into the left jugular vein, an operation requiring minor surgical techniques.

2) Inhibitor Preparation:

Alpha-methyldopa was injected intraperitoneally at a dosage of 20 mg in 0.5 cc of saline one hour before administration of the radioactive test substance. The

monoamine oxidase inhibitor, 2-phenylcyclopropylamine, was injected intraperitoneally ½ hour before operation at a dosage of 0.2 mg in 0.5 cc of saline.

c. CO_2 and Urine Collection

Immediately following injection of the radioisotope solution the rats were placed in glass metabolic cages (176) for a period of 24 hours to permit the collection of expired $C^{14}O_2$ and urine.

At the top of each cage, 2 glass tubes projecting from a rubber stopper provided a means for the intake of fresh air and the collection of air from the cage.

Air from the cage was drawn out by a suction apparatus and passed through 200 ml of a solution containing a 1:2 ratio of ethanolamine and ethylene glycol monomethylether as described by Jeffay and Alvarez (133). This "CO_2 trapper" removed the CO_2 from the air and effectively retained it in the solution.

At periods of 2, 6, 12 and 24 hours, two samples of 1 ml each were taken from each flask containing the CO_2 trapper. These samples were placed in counting vials then 15 ml of the scintillation counting mixture was added. This mixture was composed of a 1:2 ratio of ethanol to a scintillation mixture. The scintillation mixture contained 0.5% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)

dissolved in a 1:2 ratio of ethylene glycol monomethyl ether and toluene as outlined by Cuppy et al (177). A 2:1 combination of scintillation mixture and ethanol, as first described by Hayes and Gould (178), produced the final counting mixture.

At the bottom of each cage a connecting apparatus equipped with a stopcock led to a removable flask in which the urine was collected under 5 ml of toluene. Closure of the stopcock permitted maintainence of a closed system when the urine flask was changed 6, 12 and 24 hours after injection.

After removal from the cage, the urine was filtered to remove possible fecal contamination, the total volume was read and recorded, then two samples of 0.2 ml each were taken and placed in 15 ml of the standard counting mixture.

d. Urea

In some cases the urea was extracted from the urine following a procedure outlined in "Practical Physiological Chemistry" (179) except that the urine was first freeze-dried then the residue was extracted with boiling acetone and the urea crystallized.

e. Organs

At 2, 6 or 24 hours, according to the time of ex-

perimental interest, the animals were sacrificed by decapitation and the blood collected over a beaker containing potassium oxalate.

The blood was then centrifuged and the plasma removed. Two plasma samples of 0.05 ml each were placed in the counting vials with 2 ml of hyamine. The counting vials were incubated for 24 hours at 55°C (180). The remaining plasma was frozen and stored for eventual extraction of the protein. The liver, kidneys, pancreas and brain were removed and the total weight of each organ recorded. From each organ, two samples of approximately 50 mg were obtained. Care was taken to ensure that kidney samples were cut from the cortex at the anterior end of the kidney while in liver the extremeties of the left lateral lobe were chosen. Most of the brain samples were taken from the anterior portions of the cerebral hemispheres. Because of the very diffuse nature of the rat pancreas, samples of this organ were taken at random. The weight of each tissue slice was recorded, then the samples were placed in a counting vial containing 2 ml of hyamine. These samples were incubated at 55°C until complete solution of the organ was attained (about 24 hours). The remainder of each organ was frozen to permit subsequent extraction of the protein.

When the incubation period was completed, the

samples were bleached with 10 drops of 30% hydrogen peroxide according to the method of Herberg (180) and the bottles were allowed to stand for 20 minutes. Then the counting mixture was added followed by acidification of each sample to forestall quenching caused by chemiluminescene (181). Since bleaching of the samples did not effectively remove all of the colour, expecially in the liver and kidney samples, it was necessary to apply a correction factor for the resulting colour quenching. These factors were obtained from curves prepared according to Toporek (182). An additional correction factor for quenching caused by the hyamine was obtained by determining the quenching of known quantitites of radioisotopes by different amounts of hyamine.

f. Protein Extraction

To extract the trichloroacetic acid (TCA) insoluble protein from the organ samples, each organ was first homogenized in H_2O in a glass homogenizer and the homogenate spun on an International Refrigerated Centrifuge at 18,000 X g. The liver was spun for 75 minutes - the other organs for 20 minutes.

The debris was discarded and the supernatant transferred to a test tube to which was added a sufficient quantity of a 25% TCA solution to obtain a

final TCA concentration of 10%. The blood plasma was thawed, measured directly into the test tube and the TCA added in the same way. Then the tubes were centrifuged for 20 minutes until the protein precipitate was spun down. The supernatant was discarded and the protein precipitate was washed with 10 ml each of 10% TCA, acetone (twice) and ether (twice). This procedure was efficient in removing all of the absorbed amino acid. A control in which phenylalanine-3-C^{14} was added to a plasma sample and the plasma proteins extracted in the above manner showed no radioactive contamination of the proteins. The proteins were dried over concentrated H_2SO_4, then 2 samples of 20 mg each were weighed into counting vials and the weights recorded. These samples were incubated with 1 ml of hyamine at 55°C until solution was affected. They were then bleached, the counting mixture was added and the bottles were acidified as was described for the organ samples.

g. Supernatant

For some samples, the TCA supernatant (from the protein extraction process) was retained. To this was added the various washings of 10% TCA, acetone and ether. The solution was allowed to evaporate, was diluted to 10 ml with H_2O, then two samples of 0.5 cc each were taken and placed in the counting mixture.

h. Amino Acid Analysis

Plasma and pancreas protein samples of approximately 20 mg were weighed into glass tubes sealed at one end. Five ml of 6N HCl were added, and the tubes were sealed under vacuum. Heating for 18 hours at 100°C completed hydrolysis. The samples were then filtered and evaporated to dryness in a flash evaporator. The sample was dissolved in 1 ml of a 0.10% HCl solution and 0.5 ml of this solution was placed on the column of a Technicon Automatic Amino Acid Analyser. Drops of column effluent, corresponding to the regions in which the radioactive peaks were expected, were collected from the bypass tube. Twenty drops were collected in each vial then counting mixture was added. The quantity of radioactivity per bottle was plotted to obtain a picture of the radioactivity in the amino acids in the protein. Establishment of the ratios of the radioactivity of tyrosine/phenylalanine or tyrosine/analogue peaks permitted an evaluation of the level of incorporation.

i. Statistical Analysis

The first statistical procedure which was applied to all of the values obtained for CO_2, urine, organs and proteins was the Dixon Test for extreme values (183, 184). This test was done at the 10% level (to permit

removal of possible outliers). The mean and standard deviation were obtained for each group and an analysis of variance (185) was done on the experimental groups.

However, it was suspected that the sample deviations were not constant. Bartlett's Test (186) for homogeneity of variance was applied to the protein results and confirmed this suspicion.

Dr Peter Robinson (Head of the Statistical Consultant Division, Dominion Experimental Farm) was then consulted. He advised that the analysis of variance would give a poor estimate of significance especially for the experimental error- a value used in the Duncan's Multiple Range Test (187). This, he claimed, was due to the heterogeneity of the variances. Thus it would be necessary to use a square root transformation if the analysis of variance were to be done on these values. Further, the analysis of variance compares all of the treatments with one another but this was not required in this experiment. Only specific comparisons were of interest.

Since only certain comparisons were to be made and since some of these groups were obviously different (there was no overlap between the values at all) it seemed that even if these transformations were made, the calculations involved would be tedious and rather unnecessary.

Dr Robinson therefore suggested that a simple t test would be the only procedure that would compensate for the unequal variances with unequal sub-class numbers (in the CO_2, urine and proteins). Because repetitive use of this t test would increase the possibility of error, the test was only applied to those values which seemed to overlap. Any treatments whose ranges didn't overlap could be considered statistically different as the number of animals adequately represented a population.

One other possible test, Dunnet's Procedure (188) was considered. However, this does not take into account the individual variances and is subject to the same faults in estimating the single difference as is the analysis of variances. Therefore, the t test (189) was applied at both the 1% and 5% levels in order to determine significance between the pertinent experimental groups.

j. Determination of Radioactivity

The samples were counted in a Nuclear Chicago Liquid Scintillation Counter model number 8401, system 703 for which the counting efficiency was 45%. Determinations were made to a probable standard error of 2%.

B. DISCUSSION AND RESULTS

1. THE PREPARATION OF THE ANIMALS

a. Phenylketonuric Rats

In an initial attempt to produce experimental phenylketonuria in rats, the method of Woolley et al (173) was followed since it was hoped that early induction of phenylketonuria would effect the best results with regard to brain damage.

Unfortunately, severe toxicity was observed and most of the young rats died soon after being placed on the 7% solid diet. External signs of toxicity were: eye lesions, swelling of the front paws accompanied by atrophy of the toes and a thick urinary excretion. Some of these signs are shown in Figure 9. These signs seem to be similar to those listed by Schweizer (80) as indications of tyrosine toxicity. In addition, at autopsy, the bladder was found to be swollen and obstructed by white crystals. These crystals were also found in the ureters (Figure 10). The crystals and the urine were both analyzed on the Technicon Auto Analyser (Table 1).

In both plasma and urine, the levels of tyrosine and phenylalanine were very high. There was ten times as much tyrosine as phenylalanine in the plasma and six

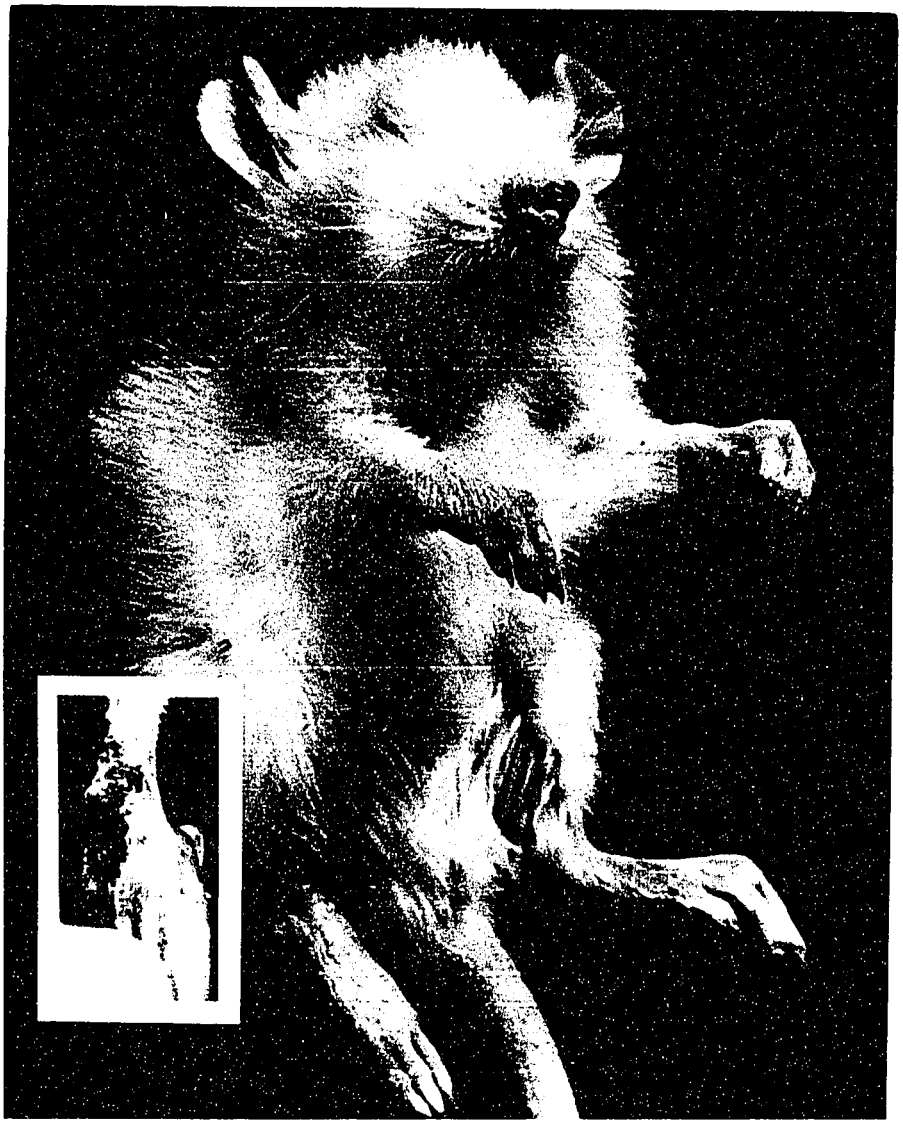


Figure 9

Some External Signs of the Toxic effects of a High Phenylalanine Diet.

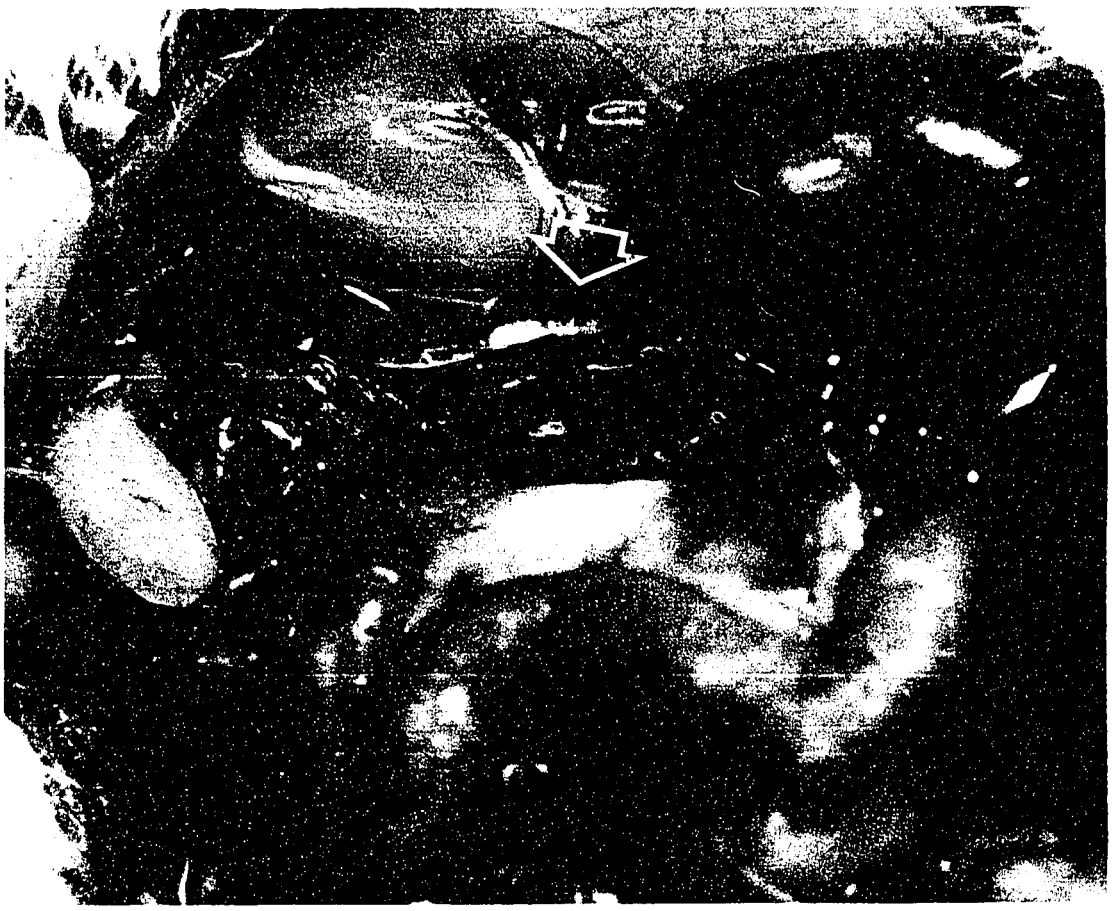


Figure 10

Amino Acid Crystals in the Ureter

TABLE 1

The Free Amino Acid Content of the Urine, Crystals and Plasma in a Case of Phenylalanine Toxicity

AMINO ACID	URINE	CRYSTALS	PLASMA
PHENYLALANINE	5.39[1]	14.20	4.53
TYROSINE	31.70	41.90	42.40
GLYCINE	0.86	0.89	0.74
ALANINE	1.83	1.11	2.00
ISOLEUCINE	0.47	0.66	0.55
LEUCINE	1.00	1.00	1.00

[1]results are expressed as ratios of the number of moles of each amino acid to that of leucine.

times as much in the urine. These levels are much higher than those found in normal rats or even in phenylketonuric rats as will be discussed later in this section. The high plasma tyrosine levels agreed with Carver's (83) finding that 30 minutes after the injection of 100 mg of phenylalanine into a rat, the free tyrosine level of the plasma was raised about five fold. Also, in normal rats, the liver phenylalanine hydroxylase is a very active enzyme (190). However, the high plasma tyrosine level differed from the condition found in human phenylketonuria since in that case, the enzyme for the conversion of phenylalanine to tyrosine is absent (86).

The crystals which were isolated from the bladder and ureters contained about three times as much tyrosine as phenylalanine. As the level of these two amino acids in the urine increased, they crystallized out. Obstruction of the urethra by the crystals appeared to be the immediate cause of death. However, the toxic effect was probably the result of the transformation of phenylalanine into tyrosine- an amino acid of known toxicity when in dietary excess (80). In these rats the ratio of tyrosine to leucine in the plasma was approximately 42, while that of phenylalanine to leucine was only 4.5.

To the best of my knowledge, this is the first experimental proof of phenylalanine toxicity. In

"Mammalian Protein Metabolism", Harper (82) has claimed that excess dietary phenylalanine does not elicit any signs of toxicity. A depression in growth, caused by a possible amino acid imbalance, was reported by Sauberlich (75) after feeding DL-phenylalanine at the 5% level in a low protein diet. This effect could be prevented by dietary supplements of protein. In the experiment discussed in this thesis, the diet contained an adequate amount of protein, however, considering the very large excess of dietary phenylalanine, it is possible that an amino acid imbalance may have been involved. Nonetheless, the conversion of phenylalanine into tyrosine was probably the main cause of the toxicity.

When Waisman's method (167, 172) was used to produce experimental phenylketonuria, phenylalanine toxicity was rarely observed and then only when the rats were very small (weighing less than 40 gm at 21 days). Consequently, the injections were done in rats which had been subjected to Waisman's method (167, 172) for induction of experimental phenylketonuria. All of these animals gave a positive $FeCl_3$ test by 35 days of age, that is, when the rats had been on the high-phenylalanine diet for two weeks.

This was found to be the optimum age for use of the experimentally phenylketonuric rats because when dietary treatment was continued after this time the

rats no longer gave the positive $FeCl_3$ test. Instead of the dark green colour characteristic of phenylketonuria (84) a dark brown colour was formed. Testing of phenylpyruvic acid, homogentisic acid or phenylethylamine with $FeCl_3$ did not produce this brown colour. Further, the DNPH test was no longer positive in these older rats. Either the yellow precipitate was not formed at all, or it appeared only after the test tube had stood for many hours at room temperature. Thus, the rats could no longer be considered to be biochemically phenylketonuric and therefore no longer fulfilled the necessary experimental requirements although the phenylalanine and tyrosine blood levels were very high. For example, one rat which was tested after 40 days on the high phenylalanine diet no longer gave positive $FeCl_3$ nor DNPH tests. However, amino acid analysis of the plasma showed that the tyrosine/leucine ratio was still 8.1 while the phenylalanine/leucine level was 1.8. The fact that Waisman's rats reached optimal experimental conditions at 58 days of age may be explained by the fact that different strains of rats were used.

This method of expressing the results of the amino acid analysis of the protein hydrolyzates as ratios of the amino acid/leucine was adopted to provide an easier comparison between samples. This was especially necessary when consecutive blood samples were obtained from

the rat's tail. The original sample volume was usually quite small and was further reduced by removal of the blood cells and proteins before analysis on the amino acid analyser. When the blood volume was used as a basis for comparison it was difficult to obtain reproducible results.

After only one day on the 5% phenylalanine diet some rats showed higher tyrosine than phenylalanine levels in the plasma. After 3 days on the diet, this was true for most of the rats. The variations in the ratios of tyrosine to phenylalanine found in the first days of treatment were probably caused by individual differences in food consumption. By 6 days, the ratios of these amino acids to leucine were 7 and 1.6 respectively. Four days later, when the rats were on a 7% diet and gave both positive $FeCl_3$ and DNPH tests, the ratios were around 9 and 3 respectively. This ratio of tyrosine/leucine was much lower than that obtained when Woolley's method (173) was used (when the ratio was approximately 42). However, the phenylalanine to leucine ratios in these two groups of rats were not very different: the value obtained with Woolley's method (173) was 4.5. In the control rats maintained on Purina Lab Chow, these ratios were found to be 0.41 and 0.52. It was therefore possible that the rats on a phenylalanine diet went into a state of tyrosyluria very rapidly. This

state has been obtained in young rats by feeding 5% tyrosine and is characterized by an excretion of p-hydroxyphenylpyruvate (191). It has been shown that such a state involves a change in the activity of two of the enzymes involved in tyrosine metabolism (191). First, there is an increase in the tyrosine transaminase activity and secondly there is a decrease in the hydroxyphenylpyruvate hydroxylase of the liver. The net result is an accumulation of p-hydroxyphenylpyruvate which is excreted in the urine. In order to determine exactly what state these rats were in, a complete time study of enzyme activity and p-hydroxyphenylpyruvate excretion would have had to be done using rats which had been maintained on the 7% phenylalanine diet for at least 40 days. As the time did not permit this, the rats were used when they gave a positive $FeCl_3$ test for phenylketonuria.

A brief study of the level of several of the free amino acids of the plasma and in the plasma proteins was made. In the phenylketonuric rats used in the experiment, the levels of phenylalanine and tyrosine in the plasma proteins were found to be approximately 2.4 micromoles and 1.8 micromoles respectively per 10 mg of protein. These values are quite close to those obtained in normal rats (Table 2). The close similarity of the amino acid composition of the plasma proteins in normal

TABLE 2

The Effect of the Diet on the Amino Acid Level of the Protein and Non-protein Fraction of Blood Plasma

	Fraction		Glycine	Alanine	Isoleucine	Leucine	Tyrosine	Phenylalanine
Control	Non-protein micromoles/ml	±SD	0.36 0.04	0.41 0.09	0.08 0.00	0.14 0.02	0.10 0.03	0.19 0.02
	plasma proteins micromoles/10 mg protein	±SD	1.73 0.87	2.55 0.95	1.33 0.55	4.26 0.44	1.78 0.21	2.22 0.87
Phenylalanine-Deficient								
	Non-protein micromoles/ml	±SD	0.31 0.02	0.31 0.03	0.05 0.00	0.08 0.01	0.04 0.01	0.06 0.02
	plasma proteins micromoles/10 mg protein	±SD	2.84 0.55	3.43 0.93	1.23 0.41	3.89 0.51	1.58 0.51	2.14 0.33
Phenylketonuric								
	Non-protein micromoles/ml	±SD	0.15 0.00	0.36 0.04	0.07 0.00	0.11 0.01	1.66 0.02	0.30 0.01
	plasma proteins micromoles/10 mg protein		—	—	—	—	1.80	2.40

n=4

and phenylketonuric rats agreed with the results obtained in haemoglobin (192) and various tissues (193) of human phenylketonuric patients.

The levels of the free amino acids in the plasma of phenylketonuric rats are different from those of normal and phenylalanine-deficient rats (Table 2). The free amino acid level in the plasma is very susceptible to changes in the concentration of a given amino acid. Two hours after the injection of 100 mg of phenylalanine Carver (83) observed an increase in the phenylalanine and tyrosine levels in the plasma while, with only a few exceptions, the levels of the other amino acids remained constant or were slightly depressed. Carver et al (194) have also reported a distorted amino acid pattern in the plasma of phenylketonuric subjects and suggested that the amino acid imbalance produced by excess phenylalanine could contribute to the pathology of phenylketonuria.

In my work, the dietary treatment of the phenylketonuric rats was continued throughout the 24 hour experimental period. This was necessary in order to maintain high plasma levels of phenylalanine. When phenylketonuric rats were starved for periods of 24 or even 6 hours, changes in the phenylalanine and tyrosine levels of the non-protein fraction of the plasma could be detected. Table 3 illustrates the different ratios

TABLE 3

The Effects of Fasting on the Free Amino Acid Level of the Blood Plasma of a Rat Maintained on a High (7%) Phenylalanine Diet

number of days since dietary treatment first initiated	treatment	tyrosine/leucine	phenylalanine/leucine
27 days	fed 7% phenylalanine diet	1.50	0.90
30 days	fasted for 24 hours	0.07	0.03
37 days	subsequently fed 7% phenylalanine diet	0.94	0.35
39 days	fasted for 6 hours	0.49	0.09
42 days	subsequently fed 7% phenylalanine diet	0.39	0.86

of tyrosine/leucine and phenylalanine/leucine obtained from a phenylketonuric rat which had been maintained on the 7% phenylalanine diet then was subjected to certain intervals of starvation. Initially, the ratios were very high but were drastically depleted after 24 hours of fasting. During a subsequent ten day feeding period in which the 7% phenylalanine diet was again fed ad libitum the ratios increased considerably although they did not reach their former high level. Even a six hour period of food deprivation resulted in a substantial decrease in the tyrosine/leucine and phenylalanine/leucine ratios.

b. Phenylalanine-deficient Rats

Phenylalanine deficiency was produced by feeding the rats Lofenalac, a diet with a low phenylalanine content (less than 0.10%). This produced a decline in the food intake, so the animals were force-fed once or twice daily. However, force-feeding produced vomiting, diarrhoea, and weight loss, followed, in many cases, by death. It is probable that the amino acid imbalance produced by force-feeding these animals was too severe.

When the rats were fed ad libitum, the 25% weight loss, desired for experimentation, was attained after approximately 21 days. Then, a fairly constant weight was maintained for several weeks. No external signs

of deficiency were visible except that the coats became scruffy and harsh and there was some loss of fur.

Analysis of the amino acid content of the non-protein fraction of the plasma and the plasma proteins of these phenylalanine-deficient animals was made after 25% of the initial weight had been lost (Table 2). The tyrosine and phenylalanine levels of the non-protein fraction of the plasma were much lower in the deficient animals than in the normal animals. Some of the other amino acids studied also showed a decrease over normal values although this decrease was not so large as that of phenylalanine and tyrosine. These decreases could be expected since the rats were suffering from starvation and an amino acid imbalance. Little difference was noted in the amino acid content of the plasma proteins in the normal and deficient animals even with regard to the phenylalanine and tyrosine levels. This was expected since it is well known that the amino acid content of proteins is very stable; further, it should be remembered that an excess of phenylalanine produced no change in the phenylalanine level in the protein (195, 196).

2. THE INJECTION OF THE ANIMALS

Initially, the amino acids were injected intraperitoneally in a saline solution. However, the rate of absorption of the 0.5 cc volume seemed to vary greatly from one animal to another.

Consequently, the rate of appearance of the C^{14} in the blood and its subsequent metabolism were subject to great variation with time. Approximately one in four animals displayed only background radioactivity at 2 hours although by 24 hours all of the samples had a comparable radioactive content.

Since the experiment was to be conducted within certain time limitations, the amino acid-saline solution was introduced directly into the jugular vein thus eliminating possible errors due to variable absorption rates in the body cavity.

3. THE RADIOACTIVITY OF THE CO_2 AND URINE

a. Discussion of Technique

The metabolic cages were considered to be closed systems since it was probable that the constantly applied suction eliminated any loss of CO_2 via the air intake. The suction was maintained by the use of alternate water and electric pumps either of which was applied to all of the CO_2 trapping flasks drawing air from the cages directly through the trapping solution. Thus, adjustment of the cage suction permitted a fairly even flow of air through all of the cages used in one run (usually 4 or 6).

That the CO_2 trapper, an ethanolamine solution in ethylene glycol monomethyl ether (1:2 v/v), was effective in removing all of the CO_2 from the air was shown by a trial experiment in which the air was first drawn through the trapper solution and then through a 200 ml solution of 2N NaOH. Testing of this NaOH solution did not reveal the presence of any radioactivity.

Some difficulty was encountered with the standard trapping solution which yellowed upon standing. This same effect was achieved by bubbling a stream of air through the mixture of trapper and CO_2 at room temperature. However, this reaction did not occur when air

was bubbled through either ethylene glycol monomethyl ether or ethanolamine alone, or in the absence of CO_2. Since any colouration would adversely affect the counting of the radioactivity, care was taken to prevent its occurrence. Solutions were mixed immediately before use. The cage suction was adjusted so that the air flow, while sufficient to ventilate the cage, would not be so great as to affect the solution.

Urine samples were taken at 6, 12 and 24 hours but were not taken at 2 hours since at that time little or no urine had been excreted.

b. Discussion of Results

1) NORMAL RATS

A. Injected with Phenylalanine-C^{14}

The phenylalanine-C^{14} compounds used in the experiment were labelled on the first or third carbons of the alanine side chain. This provided a means for studying the $C^{14}O_2$ production from different parts of the molecule. Using phenylalanine-1-C^{14} permitted the estimation of the rate of oxidative or non-oxidative decarboxylation of

the amino acid since any CO_2 thus produced would contain the radioisotope. The carbon number 1 is marked ( ) on Figures 3 and 5 in the introduction to indicate the possible fate of the C^{14}. The production of $C^{14}O_2$ from phenylalanine-3-C^{14} would involve reactions at the level of the Krebs cycle. The position of the third carbon is also marked ( ) on Figures 3 and 5.

The first series of experiments involved the injection of DL-phenylalanine-3-C^{14} into normal animals. The curves obtained from plotting the $C^{14}O_2$ expired after the injection of DL-phenylalanine-3-C^{14} or several other compounds are shown in Figure 11. The values obtained for the levels of radioactivity in the CO_2 and urine are shown in Table 4.

For both CO_2 and urine the level of radioactivity became increasingly higher as time progressed until the rate of increase slowed and became constant. At 24 hours, the total $C^{14}O_2$ excretion was approximately three times the value obtained at two hours. Since the DL-form of the amino acid was used, it is probable that the D-phenylalanine was rapidly transformed to L-phenylalanine via deamination by the D-amino acid oxidase of the kidney to phenylpyruvic acid, then transamination to the L-form of the amino acid (157, 158). It is therefore possible that a portion of the radioactivity of the CO_2 and urine was provided by the

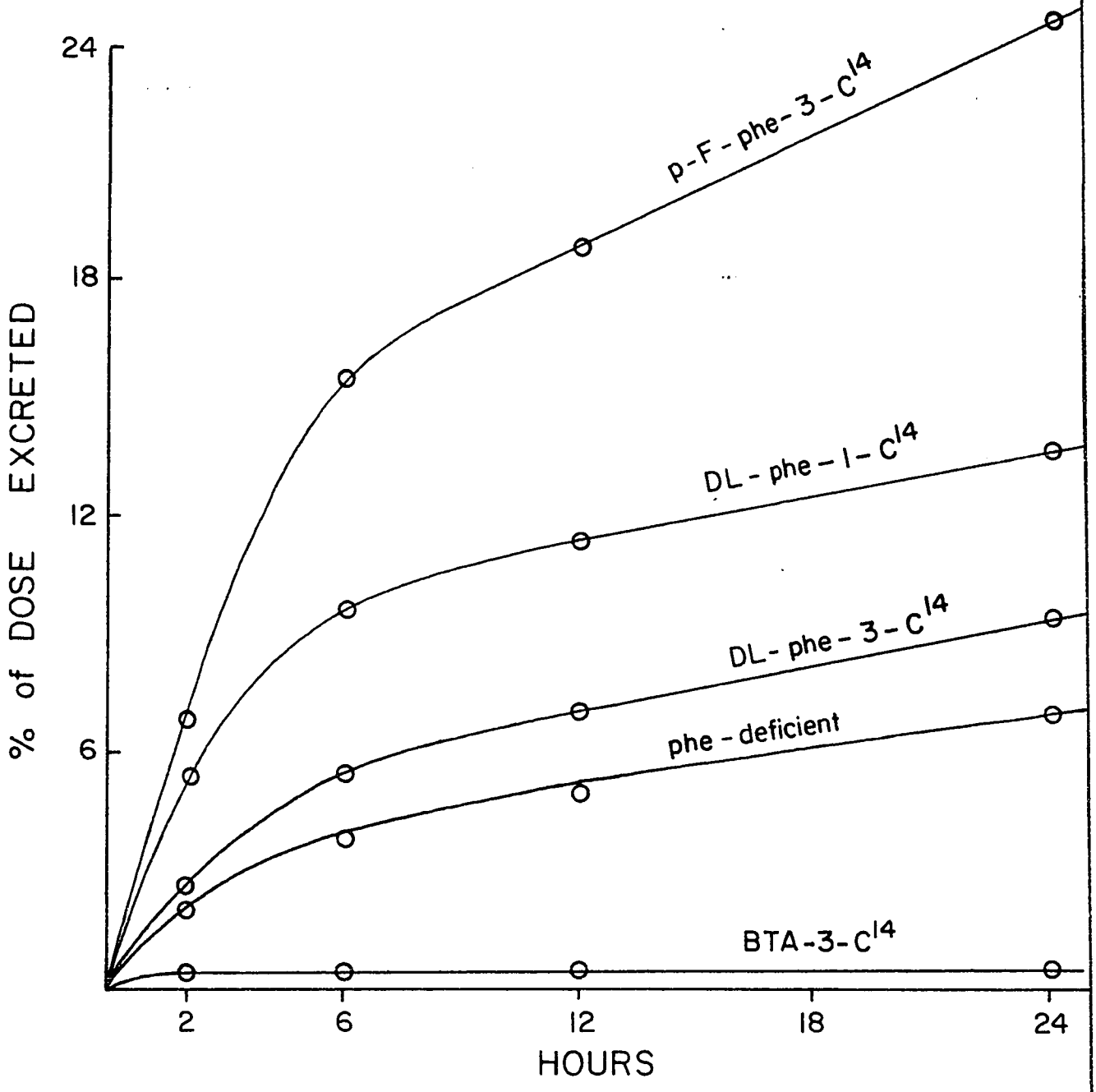


Figure 11

$C^{14}O_2$ Excretion in Normal and Phenylalanine-deficient Rats after the Injection of the Radioactive Compound.

TABLE 4

Excretion of C^{14} in the CO_2 and Urine of Normal Rats after the Injection of Phenylalanine-C^{14}

Amino Acid Injected		CO_2				Urine		
		2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
DL-phe-3-C^{14}		2.85[1]	5.64	7.17	9.55	2.15	2.79	3.51
	±SD	0.90	1.47	1.87	2.07	0.81	1.09	1.02
DL-phe-1-C^{14}		5.66	9.75	11.87	13.78	3.01	3.38	3.63
	±SD	1.03	1.15	1.22	2.02	1.07	0.90	1.46
L-phe-1-C^{14}		9.18	12.18	13.87	15.41	1.17	1.36	1.46
	±SD	1.76	2.09	2.22	2.27	0.33	0.32	.33
DL-phe-3-C^{14} + L-phe 1:20		10.61	18.99	21.11	22.94	3.93	4.49	5.39
	±SD	1.01	1.34	0.99	2.03	1.32	1.20	1.26
DL-phe-3-C^{14} + D-phe 1:20		5.54	11.09	13.05	14.97	6.47*	7.10*	7.87
	±SD	1.07	0.89	0.78	1.11	1.11	0.75	1.41

[1] expression of cumulative % of administered dose excreted giving mean ±SD for each group

n=8 unless marked * where n=7

D-phenylalanine which had been converted to L-phenylalanine, or in the urine, by the excretion of D-phenylalanine either as such, as phenylpyruvic acid or one of its metabolites.

When DL-phenylalanine-3-C^{14} was used, one could expect production of the $C^{14}O_2$ only at the level of the Krebs cycle that is, slightly later than if the amino acid had been labelled on the first carbon. It is also possible that the total $C^{14}O_2$ excretion, at least within a period of hours after injection, would be smaller when phenylalanine-3-C^{14} was used. This would be explained by the possible formation of other C^{14}-containing metabolites at the level of acetyl-CoA rather than release of the radioactive carbon as CO_2.

A second series of experiments was done on normal rats by injecting DL-phenylalanine-1-C^{14}. Since the C^{14} was on the first carbon, the radioactive CO_2 could result from the production of homogentisic acid, phenylethylamine, tyramine or dopamine, all of which are formed quite early along the metabolic pathway. Consequently, at two and 24 hours the level of radioactivity in the CO_2 was much higher after the injection of DL-phenylalanine-1-C^{14} than after the injection of DL-phenylalanine-3-C^{14} (Table 4 and Figure 11).

The investigation of $C^{14}O_2$ production from phenylalanine-1-C^{14} involves the consideration of the possible

direct decarboxylation of either phenylalanine or tyrosine. Little is known concerning the decarboxylation of phenylalanine to produce phenylethylamine, but the literature review presented in the introduction indicates that in normal rats, very little phenylethylamine is produced. Williams and Babiscio (24) found that only 0.32% of the injected tyrosine resulted in the formation of tyramine. Therefore, when using phenylalanine-1-C^{14}, most of the $C^{14}O_2$ was probably produced by the oxidative decarboxylation of p-hydroxyphenylpyruvic acid - not by the decarboxylation of phenylalanine or tyrosine to the corresponding amines.

The urine values obtained from phenylalanine-1-C^{14} and phenylalanine-3-C^{14} were quite similar at 6 hours; by 24 hours they did not differ at all (Table 4). 24 hours after the injection of either DL-phenylalanine-1-C^{14} or DL-phenylalanine-3-C^{14} the total quantity of radioactivity was quite low - 3.63% and 3.51% of the dose respectively. Therefore the quantity of the D-isomer which was excreted as such or as a metabolite was very low since the D-isomer represented 50% of the radioactivity of the racemic mixture which was injected. In the group of rats injected with phenylalanine-1-C^{14} there was a slight increase in the radioactivity of the urine at 6 hours. This increase may have been the result of the larger quantity of $C^{14}O_2$ available for

possible incorporation into urea. Evidence concerning this possibility was obtained in the case of phenylketonuric rats and will be discussed later in section 2) C.

A third group of normal rats was injected with L-phenylalanine-1-C^{14} (Table 4) in order to determine the quantity of radioactivity in the CO_2 and urine derived from the D and the L-isomers when DL-phenylalanine-1-C^{14} was injected. The percent contribution of each isomer to the radioactivity (Table 5) was calculated as follows: since DL-phenylalanine contains equal amounts of each isomer and since equal amounts of DL-phenylalanine and L-phenylalanine-1-C^{14} were injected it was reasoned that the contribution of the L-phenylalanine to the radioactivity formed after injection of the DL-phenylalanine-1-C^{14} could be determined by calculating 50% of the radioactivity obtained from injection of L-phenylalanine-1-C^{14}. Subtraction of this value from the total radioactivity excreted when DL-phenylalanine-1-C^{14} was used (Table 4) would represent the contribution of the D-isomer to the radioactivity of the CO_2.

Comparison of the values of $C^{14}O_2$ for the DL and L-forms indicated that at 2 hours, 81% of the radioactivity was derived from the L-phenylalanine and 19% came from the D-phenylalanine. At 6 hours the relative ratios

TABLE 5

Calculated % Contributions of the D and L-isomers of Phenylalanine-1-C^{14} to the Radioactivity of the CO_2 and Urine of Normal Rats

Amino Acid	CO_2				Urine		
	2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
L-phenylalanine-1-C^{14}	81.00[1]	62.46	60.90	55.95	19.60	20.12	20.11
D-phenylalanine-1-C^{14}	19.00	37.54	39.10	44.05	80.40	79.88	79.89

[1] calculated from C^{14} O_2 and urine C^{14} results obtained after injection of DL-phenylalanine-1-C^{14} or L-phenylalanine-1-C^{14} as given in Table 4.

were closer (62% and 37% of L and D respectively); 24 hour values were 56% and 44%.

The results obtained with phenylalanine-1-C^{14} are different from those obtained by Sourkes and Moran (197). In their comparative study of $C^{14}O_2$ production from DL-tyrosine-1-C^{14} and L-tyrosine-1-C^{14} equal percentages of $C^{14}O_2$ were obtained for the L and D-isomers for the first 3 hours. However, in tyrosine catabolism, there is one less step than in phenylalanine catabolism, namely, the hydroxylation of phenylalanine to tyrosine. Also, before D-phenylalanine can form L-tyrosine, it must be transformed to L-phenylalanine (157, 158). This could explain the initially lower percent of the C^{14} which came from the D-phenylalanine.

The relative contributions of each isomer to the radioactivity of the urine did not approximate each other even after 24 hours (Table 5). The high values for the D-isomer would be expected because of the high concentration of phenylpyruvic acid formed during the conversion of the D-isomer to the L-form of the amino acid (157, 158). Also, some D-phenylalanine may have been excreted in the urine.

The percent contributions of the D and L-isomers to the radioactivity of the CO_2 and urine after injection of DL-phenylalanine-3-C^{14} were studied in another group of normal rats. As L-phenylalanine-3-C^{14} was not avail-

able, the DL-phenylalanine-3-C^{14} was injected simultaneously with either non-radioactive D or L-phenylalanine in a 1:20 ratio. It was hoped that by increasing the amount of the L or D-isomer it would be possible to estimate the contribution of the other isomer to the C^{14} excretion. The expectation was that after the addition of an excess of non-radioactive L-phenylalanine, the resultant C^{14} excretion would be lower since the radioactive L-phenylalanine would be greatly diluted by the non-radioactive L-phenylalanine. However, the opposite effect was found: there was a 270% increase in the $C^{14}O_2$ production after 2 hours (Table 4). This increase in the $C^{14}O_2$ was coupled with an increase in the radioactivity of the urine and, as will be discussed later in section 5, with a decrease in the radioactivity of the proteins. The high level of L-phenylalanine could cause an activation of the transformation of phenylalanine to tyrosine and a stimulation of phenylalanine and tyrosine catabolism. Such an activation is quite possible considering the high level of phenylalanine hydroxylase in the rat liver (190). In addition, the catabolism of tyrosine could be activated by the increase in tyrosine levels. Recently, Sourkes and Moran (197) have observed a 50% increase in the $C^{14}O_2$ production following injection of DL-tyrosine and L-tyrosine-1-C^{14}. They attributed the

increase to an activation of the tyrosine-alpha-ketoglutarate transaminase by the excess tyrosine.

The first samples taken after the injection of DL-phenylalanine-3-C^{14} plus D-phenylalanine showed a 94% increase in the $C^{14}O_2$ and a 200% increase in the level of radioactivity in the urine (Table 4) over the values obtained after the injection of DL-phenylalanine-3-C^{14} alone. The differences may be explained by the fact that D-phenylalanine will not be incorporated into protein nor converted to tyrosine as such but must be first converted into L-phenylalanine (157, 158). During this conversion process the high levels of the D-isomer would result in higher than normal levels of phenylpyruvic acid. Therefore, more of this latter compound would be excreted in the urine than would normally be expected. This could explain the relatively high level of radioactivity in the urine. The eventual conversion of D to L-phenylalanine would account for the elevation in $C^{14}O_2$ in the same way as did injected L-phenylalanine.

B. Injected with Radioactive Analogues of Phenylalanine

Normal rats were also used to study the metabolism of DL-para-fluorophenylalanine-3-C^{14} and DL-BTA-3-C^{14}, known phenylalanine analogues (46-70) (Table 6). For

TABLE 6

The Excretion of C^{14} in the CO_2 and Urine of Normal Rats after the Injection of Phenylalanine-3-C^{14} or its Radioactive Analogues

Amino Acid Injected		CO_2				Urine		
		2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
DL-phe-3-C^{14}		2.85(1)	5.64	7.17	9.55	2.15	2.79	3.51
	±SD	0.9	1.47	1.87	2.07	0.81	1.09	1.02
DL-p-F-phe-3-C^{14}		6.87	15.55	18.89	24.77	5.24	6.39	6.85
	±SD	2.45	3.10	4.04	2.43	1.46	0.74	1.06
DL-BTA-3-C^{14}		0.45	0.44	.41	0.58	29.54	36.56	38.50
	±SD	1.29	1.24	1.28	0.39	8.66	7.58	8.04

(1) expression of cumulative % of administered dose excreted giving mean ±SD for each group

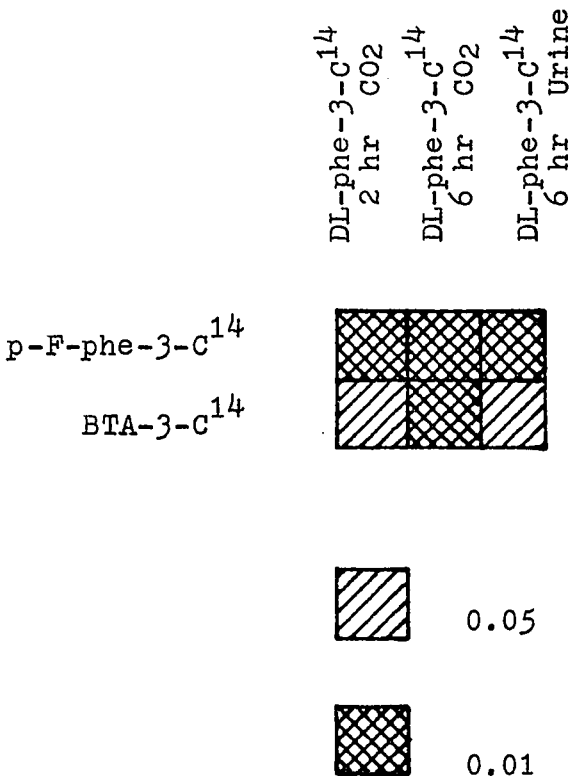


Figure 12

Summary of the Results of the T Test for Significance between Values for the Radioactivity in the CO_2 and Urine Obtained from Normal Rats Injected with DL-phenylalanine-3-C^{14}, or a Radioactive Analogue.

this and subsequent experiments figures have been prepared in order that one may derive a quick determination of the significance between two experimental values. The charts are used by first locating the heading for one compound in the horizontal position and the heading for the other in the vertical position. By obtaining the point of intersection of these two one can determine the significance for any differences between the two compounds.

Compared with that of DL-phenylalanine-3-C^{14}, the radioactivity of DL-p-fluorophenylalanine-3-C^{14} was eliminated faster and in greater quantity in both the CO_2 and urine (Table 6 and Figures 11 and 12). This increase was probably the result of a lower incorporation into protein (section 5. 1) B). Rapid transformation of the analogue into tyrosine, a reaction which is known to occur <u>in vitro</u> (65), would make greater amounts of the latter amino acid available for metabolism. Since the C^{14} label was again on the third carbon, production of the $C^{14}O_2$ would occur at the level of the Krebs cycle, therefore only after transformation to tyrosine.

The radioactivity of the urine reached a maximum of 6.85% of the dose at 24 hours. Since the D-isomer represented 50% of the radioactivity injected, this low level of radioactivity in the urine indicated that much

of the D-fluorophenylalanine was metabolized by a route which did not result in excretion of the metabolites in the urine.

Another group of normal animals was injected with BTA-3-C^{14}. However, this compound resulted in the production of negligible quantities of $C^{14}O_2$ (Figure 11). These values are significantly different from those of DL-phenylalanine-3-C^{14} at all levels tested (Figure 12). The urine values, even after six hours, were very high. At 24 hours there was a mean value of 38.5% (Table 6) compared with 3.5% in the case of injected DL-phenylalanine-3-C^{14}. Chromatograms were run on the urine samples using butanol, acetic acid and water (4:1:5). The radioautographs done with the chromatograms showed the presence of a very large number of radioactive compounds in addition to intact BTA. The nature of these other compounds is not known, nor is it known whether they were metabolic breakdown products of BTA or the products of bacterial degradation in the urine.

C. Injected with Phenylalanine-C^{14} and its Non-radioactive Analogues

From the first series of experiments using radioactive phenylalanine or its radioactive analogues, it seemed possible that p-fluorophenylalanine was metabo-

lized by the same route as phenylalanine only at a faster rate, while BTA followed a completely different route. From the variety of work discussed in the introduction, it was also known that these two analogues could replace phenylalanine in animal and bacterial proteins but could not completely replace phenylalanine in the diet of animals (46-70). In order to have some indication of the site at which the analogues could compete with phenylalanine in vivo, an excess of any one of the non-radioactive analogues, p-fluorophenylalanine, BTA or APBA was injected simultaneously with the phenylalanine-C^{14}. Effective concentrations of the analogues were tested by using phenylalanine-3-C^{14} to analogue ratios of 1:10 and 1:20.

a) Phenylalanine-3-C^{14} and its Non-radioactive Analogues

The simultaneous injection of a non-radioactive analogue and phenylalanine-3-C^{14} resulted in an increase in the radioactivity excreted at 24 hours compared to the values obtained when only phenylalanine was injected. These values are given in Table 7 while the significance of the values is shown in Figures 13, 14 and 15.

The addition of APBA to the injection material resulted in the largest increases in the radioactivity of both the CO_2 and urine. BTA was the next most effective analogue in increasing the amount of radioacti-

TABLE 7

The Excretion of C^{14} in the CO_2 and Urine of Normal Rats After the Injection of Phenylalanine-3-C^{14} and its Non-radioactive Analogues

Amino Acid			CO_2				Urine		
			2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
DL-phe-3-C^{14}			2.85(1)	5.64	7.17	9.55	2.15	2.79	3.51
		±SD	0.90	1.47	1.87	2.07	0.81	1.09	1.02
DL-phe-3-C^{14} + p-F-phe	1:10		2.53	6.08	7.44	9.96	3.66	4.11	4.80
		±SD	0.60	0.49	0.91	0.76	0.75	0.30	0.93
	1:20		3.51	7.91	9.47	11.36	5.32	5.76	6.28
		±SD	1.40	1.75	1.66	1.96	1.73	1.67	1.65
DL-phe-3-C^{14} + BTA	1:10		3.07	6.21	7.28*	10.37	4.11	4.72	5.38
		±SD	1.05	1.63	1.67	2.77	1.65	1.49	1.43
	1:20		4.94	8.24	10.06	11.67	6.04	6.87	8.40
		±SD	1.11	1.43	1.59	1.60	2.16	1.99	0.94
DL-phe-3-C^{14} + APBA	1:10		8.47	16.14	18.69	21.68	4.88	5.56	6.27
		±SD	2.60	4.13	4.38	4.78	1.19	1.24	1.24
	1:20		11.45	20.49	24.05	27.53	4.27	5.12	5.96
		±SD	2.37	21.99	2.21	2.60	1.44	1.25	1.36

(1) expression of cumulative % of administered dose excreted giving mean ±SD for each group

n=8 unless marked * where n=7

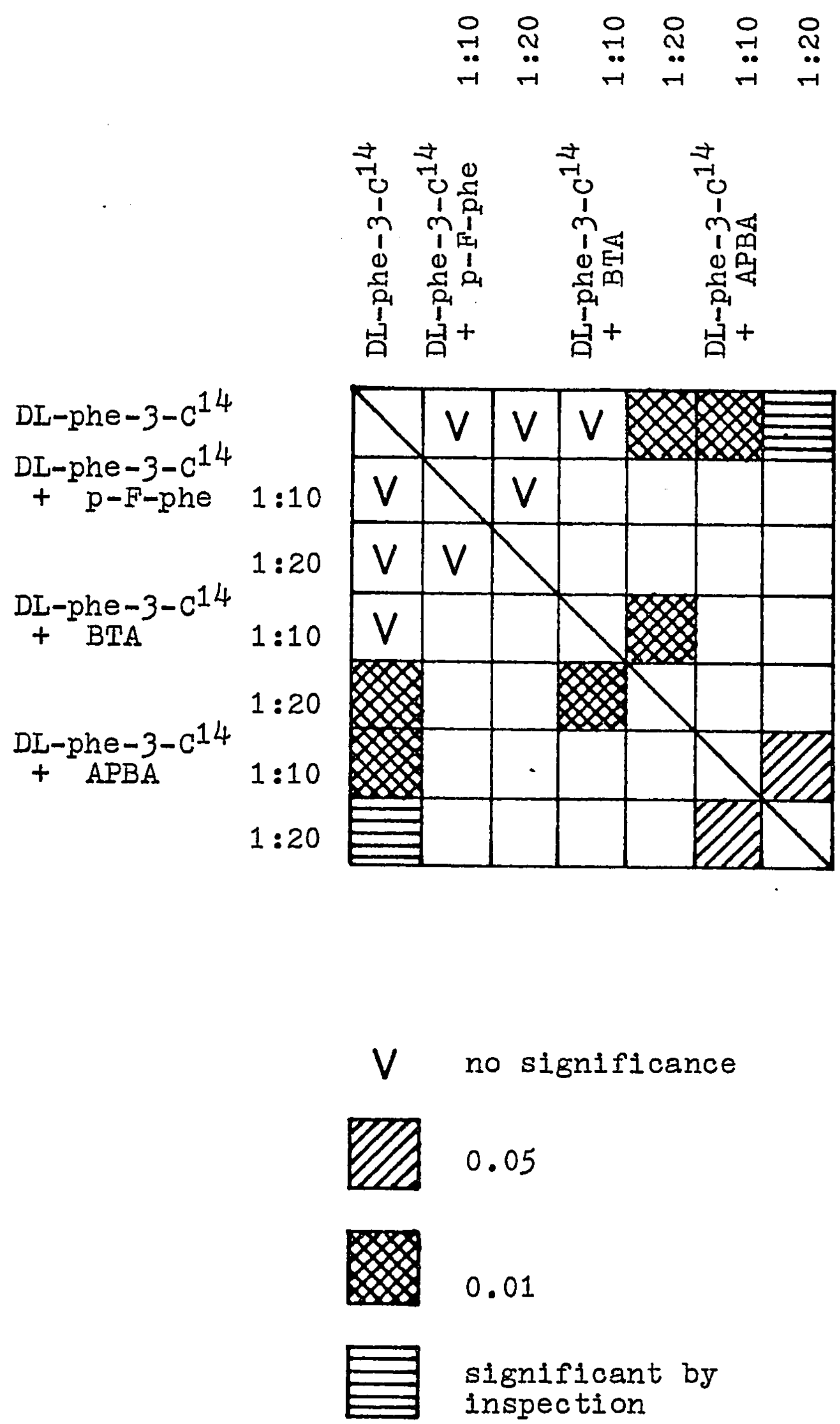


Figure 13

A Summary of the Results of the T Test for Significance between Values of $C^{14}O_2$ at 2 Hours Obtained from Normal Rats Injected with Phenylalanine-3-C^{14} + a Non-radioactive Analogue or Phenylalanine-3-C^{14}.

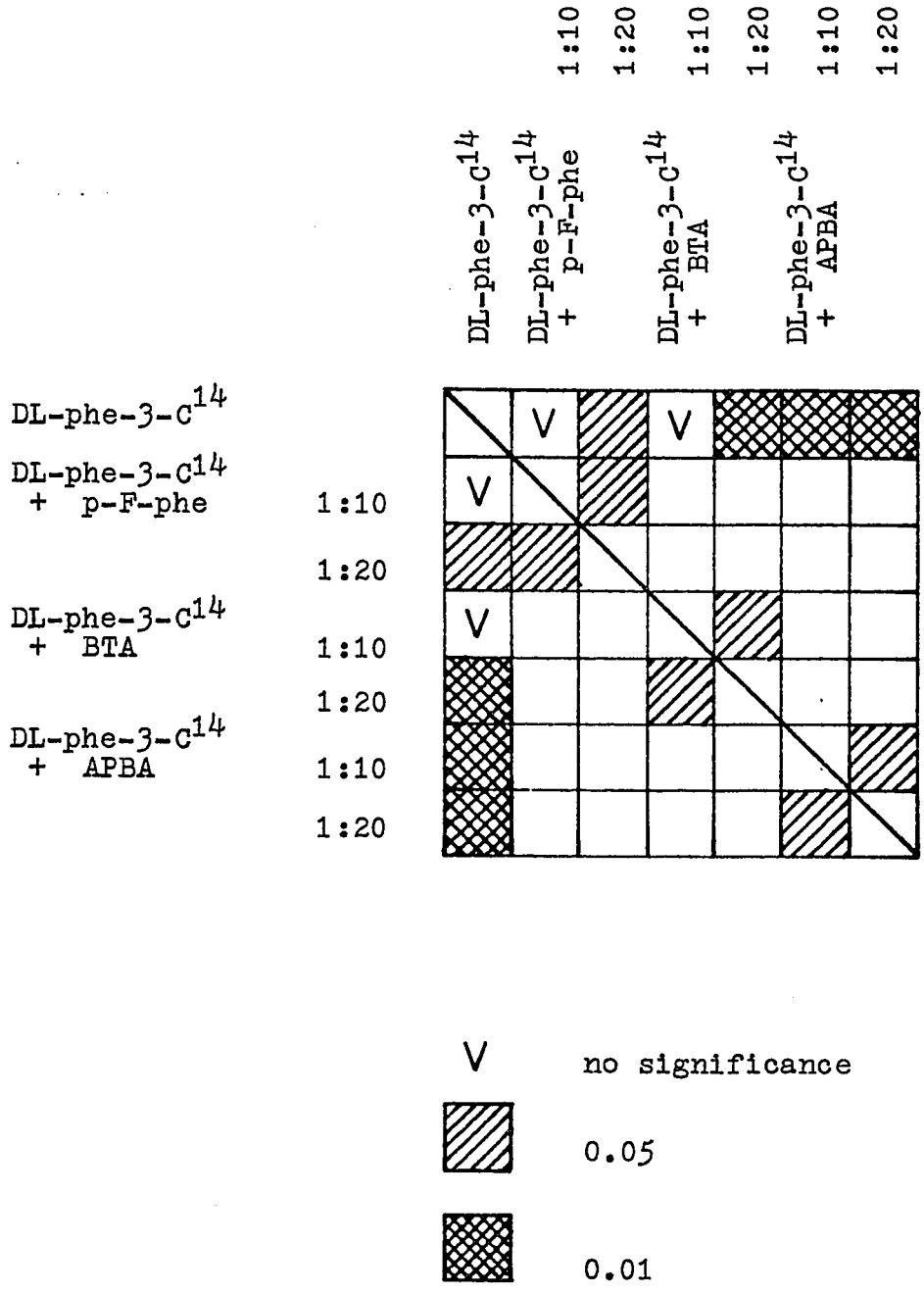


Figure 14

A Summary of the Results of the T Test for Significance between Values of $C^{14}O_2$ at 6 Hours Obtained from Normal Rats Injected with Phenylalanine-3-C^{14} + a Non-radioactive Analogue or Phenylalanine-3-C^{14}.

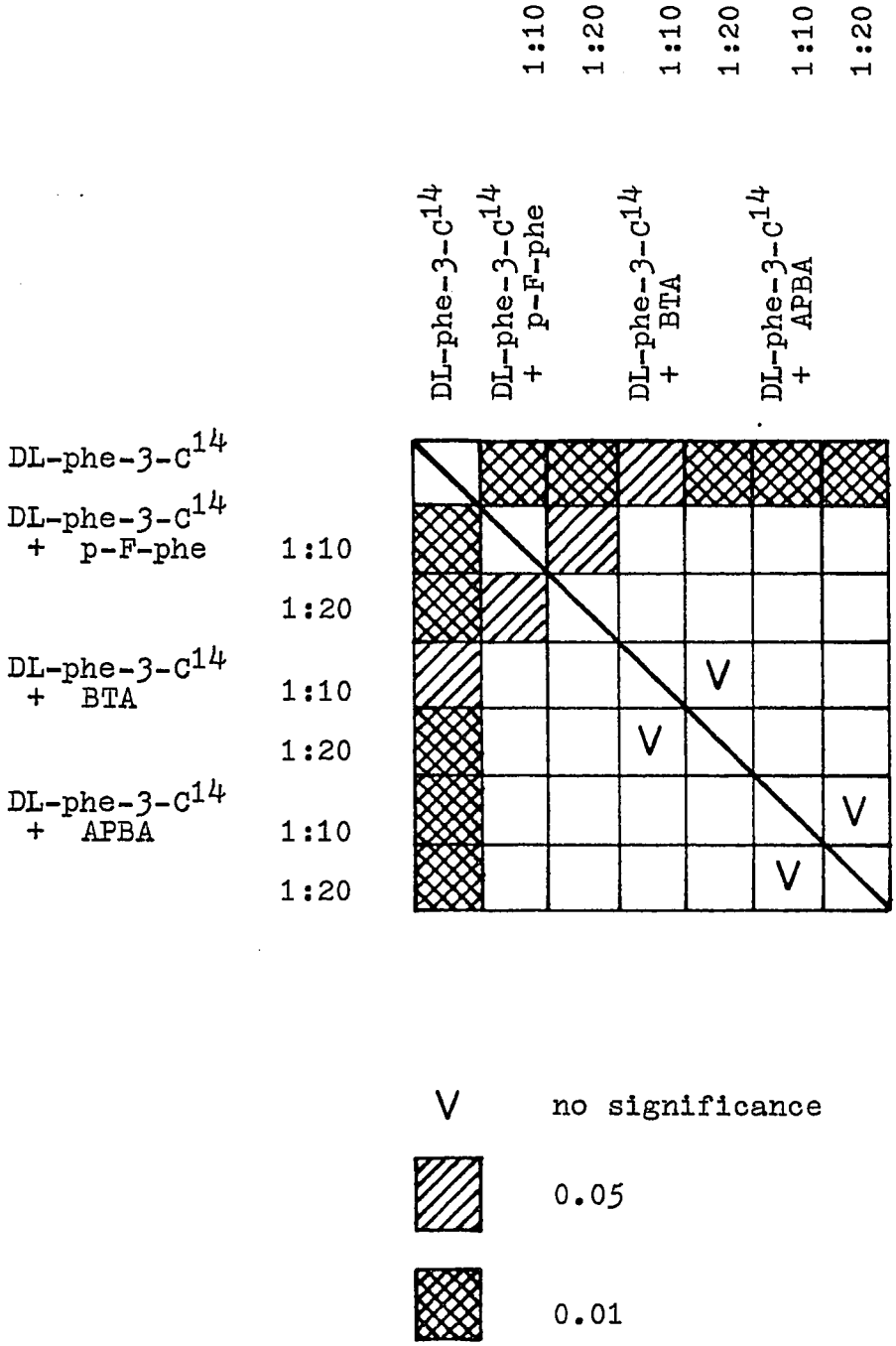


Figure 15

A Summary of the Results of the T Test for Significance between Values of Radioactivity in the Urine at 6 Hours Obtained from Normal Rats Injected with Phenylalanine-3-C^{14} + a Non-radioactive Analogue or Phenylalanine-3-C^{14}.

vity excreted. P-fluorophenylalanine produced the smallest increases.

Different values for both radioactive CO_2 and urine were obtained when the analogue was injected in concentrations of 10 and 20 times that of radioactive phenylalanine. The difference between the $C^{14}O_2$ produced by the 1:10 and 1:20 ratios of phenylalanine to APBA was significant even at 2 hours and continued to differ at 6 and 24 hours. The C^{14} content of the urine samples from the two different ratios seemed quite similar (Table 7). When BTA was added to the injection, the value obtained from the 1:10 ratio at 2 hours was 3.07% while that from the 1:20 ratio was 4.94% (Table 7). These values are significantly different (Figure 13). As was the case when APBA was added, the 6 hour urine samples showed no significant difference between the two ratios. Only p-fluorophenylalanine produced no significant difference in the $C^{14}O_2$ values between the two ratios at 2 hours although by 6 hours there was some significant difference (Figure 14). However, the two different ratios of p-fluorophenylalanine produced significantly different values for the radioactive content of the urine (Figure 15).

This increase in the amount of C^{14} excreted in both the CO_2 and urine, produced by the addition of a non-radioactive analogue to the radioactive phenylalanine,

indicated that there was an increased conversion of phenylalanine into tyrosine and that subsequently more tyrosine was available for catabolism. The high C^{14} levels in the urine, which by 24 hours were considerably increased over the values obtained when phenylalanine-3-C^{14} was used alone may have been the result of a greater excretion of phenylalanine by-products.

When APBA was added to the injection material, there was a very great increase in the amount of radioactivity in the CO_2 - almost 300% after only 2 hours (Table 7). Again, more pronounced effects were attained with a higher concentration of the analogue. After 2 hours the addition of APBA produced a much greater increase in radioactivity than did either of the other two analogues even after 24 hours. APBA must have exercised a strong competition at the level of incorporation into protein. This would permit a much greater transformation of phenylalanine-C^{14} into tyrosine and would also leave more phenylalanine-C^{14} to be metabolized and eventually eliminated via the urine. It is also possible that the excess APBA stimulated the metabolism of phenylalanine and tyrosine in the same way as an excess of L or D-phenylalanine stimulated $C^{14}O_2$ production from DL-phenylalanine-3-C^{14} (Table 4) or as an excess of DL-tyrosine stimulated the catabolism of L-tyrosine-C^{14} (196). This would imply

that an excess of APBA could increase the activity of some of the enzymes involved in the catabolic pathways and that probably APBA would be metabolized via the same route as phenylalanine. The metabolism of APBA could not be studied since it was not possible to obtain any radioactive APBA commercially.

If it were true that the presence of an analogue stimulated the metabolism of phenylalanine by increasing the activity of the enzymes, one would expect a larger increase in the amount of $C^{14}O_2$ produced after the addition of p-fluorophenylalanine to the injection. At the 1:20 ratio, the addition of p-fluorophenylalanine to the injection only resulted in a 0.66% increase in the radioactivity after 2 hours. By 6 hours, only the 1:20 ratio produced any significant increases in the level of $C^{14}O_2$ although by 24 hours, the 1:20 ratio produced a 20% increase in the value for $C^{14}O_2$. After the addition of p-fluorophenylalanine, the level of radioactivity in the urine was only slightly elevated at the 1:10 level. However, the 1:20 ratio produced twice as much radioactivity as did the injection of phenylalanine-3-C^{14} alone.

It is interesting that BTA, an analogue which did not appear to be metabolized by the tyrosine pathway, as was indicated from the very low $C^{14}O_2$ production after injection of BTA-3-C^{14} (Table 6), favoured a

slightly higher $C^{14}O_2$ production than did p-fluorophenylalanine when either analogue was added to the injection of phenylalanine-C^{14}. The increase produced by BTA was significant after only 2 hours when the 1:20 ratio was used (Figure 13). Higher values for the radioactivity of the urine were produced when BTA rather than p-fluorophenylalanine was injected with the radioactive phenylalanine.

This increase in the level of radioactivity in the CO_2 and urine caused by the addition of a non-radioactive analogue to the injection material differs from the results of Moran, Sourkes and Chavez (199). These investigators found that the simultaneous injection of DL-tyrosine-3-C^{14} and two of its non-radioactive analogues produced a decrease of almost 40% in the $C^{14}O_2$ excretion within the first two hours. In the present experiment the competition between phenylalanine and its analogues was probably not at the level of the oxidation process but was at the level of protein synthesis since the level of radioactivity in the CO_2 and urine was higher, and as will be discussed later in section 5, the C^{14} content of the proteins was lower.

b) Phenylalanine-1-C^{14} and its Non-radioactive Analogues

To determine whether these phenylalanine analogues could compete with phenylalanine or tyrosine for decarb-

oxylation, the experiments were repeated using phenylalanine-1-C^{14}. Only the 1:10 ratio of phenylalanine to analogue was used. The results for these experiments are given in Table 8 while significance of the values is shown in Figure 16.

Compared to the values obtained when only phenylalanine-1-C^{14} was used, the simultaneous injection of any one of the three non-radioactive analogues resulted in a significant increase in the $C^{14}O_2$ excretion after 2 hours in two cases (Table 8 and Figure 16). Administration of DL-phenylalanine-1-C^{14} + p-fluorophenylalanine produced no significant increase in the $C^{14}O_2$ levels at 2 or 6 hours. When APBA was added to the injection, all of the $C^{14}O_2$ values were significantly increased (Table 8). Again, this increase in the $C^{14}O_2$ produced by the addition of APBA or BTA to the phenylalanine-C^{14} injection differed from the results of Sourkes et al (199). They found that the addition of some tyrosine analogues, such as the hydrazino analogues of 2-methyldopa, to the tyrosine-1-C^{14} injection produced an 80% inhibition of the tyrosine decarboxylase. Some other analogues, such as alpha-methyl-dimethoxyphenylalanine derivatives stimulated the $C^{14}O_2$ production. In the present experiment, BTA and APBA did not inhibit the $C^{14}O_2$ production; they appeared instead to stimulate it - especially APBA. P-fluorophenylalanine was probably

TABLE 8

The Excretion of C^{14} in the CO_2 and Urine of Normal Rats after the Injection of Phenylalanine-1-C^{14} and its Non-radioactive Analogues

Amino Acid			CO_2				Urine		
			2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
DL-phe-1-C^{14}			5.66[1]	9.75	11.38	13.78	3.01	3.38	3.63
		±SD	1.03	1.15	1.22	2.02	1.07	0.90	0.86
DL-phe-1-C^{14} + p-F-phe	1:10		5.76	9.41	11.23	13.26	2.92	3.25	3.84
		±SD	0.51	1.30	1.41	1.69	0.98	1.13	1.20
DL-phe-1-C^{14} + BTA	1:10		8.59	10.62	11.87	14.71	3.98	4.19	4.48
		±SD	2.93	1.18	1.29	1.20	1.52	1.51	1.50
DL-phe-1-C^{14} + APBA	1:10		13.29	17.96	19.17	21.38	3.88	4.37	4.61
		±SD	1.53	1.22	1.04	1.63	0.70	0.95	0.79

[1] expression of cumulative % of administered dose excreted giving mean ±SD for each group

n=8

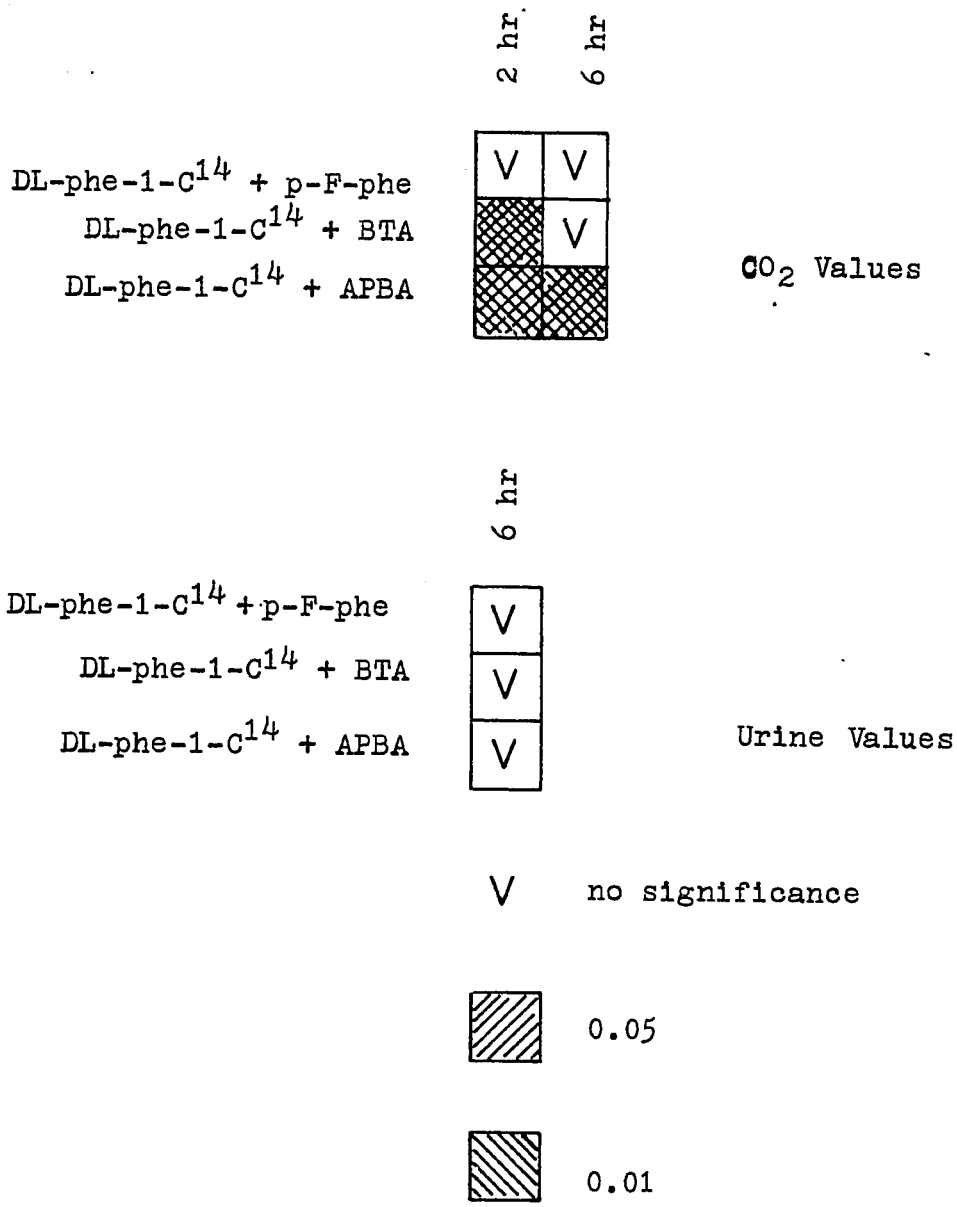


Figure 16

A Summary of the Results of the T Test for Significance between Values of Radioactivity in the CO_2 and Urine Obtained from Normal Rats Injected with Phenylalanine-1-C^{14} + a Non-radioactive Analogue or Phenylalanine-1-C^{14}.

rapidly transformed into tyrosine, as will be discussed in section 6, therefore it is possible that it competed for decarboxylation. This competition may not have been great enough to produce a decrease in the CO_2 production, as was the case for the tyrosine analogue (199), but may have been just sufficient to prevent any significant increase in the $C^{14}O_2$ (Table 8).

The C^{14} content of the urine differed from that obtained with phenylalanine-3-C^{14} since even after 24 hours the amino acid labelled in the carbon 1 position did not produce a big increase in the radioactivity. The loss of the C^{14} through formation of $C^{14}O_2$ must have been responsible for the lower radioactivity of the urine when phenylalanine-1-C^{14} was used. It is probable that the over-all increase in the radioactivity of the urine, obtained after the injection of phenylalanine-3-C^{14} plus an analogue, was not caused by the excretion of phenylalanine non-decarboxylated by-products since, if this were so, the same increases would have been obtained with phenylalanine-1-C^{14}. The increase in the first case was probably the result of the excretion of decarboxylated by-products such as phenylacetic acid or phenylethylamine or of the corresponding tyrosine derivatives.

2) PHENYLKETONURIC RATS

As was previously discussed in section 1. a. the high phenylalanine diet produced an alteration in the level of the amino acids in the non-protein fraction of the plasma. The levels of phenylalanine and, particularly, of tyrosine were greatly increased. Therefore, when phenylalanine-C^{14} was injected into these phenylketonuric rats some change in the pattern of C^{14} release was expected. The curves obtained by plotting the $C^{14}O_2$ produced after the injection of DL-phenylalanine-3-C^{14} and DL-phenylalanine-1-C^{14} into phenylketonuric rats are shown in Figure 17.

A. $C^{14}O_2$ after the Injection of DL-phenylalanine-3-C^{14}

When the C^{14} was on the third carbon of phenylalanine, the excretion of radioactivity in the CO_2 after 6, 12 and 24 hours was greatly increased in the phenylketonuric rat (Table 9) compared to values obtained in the normal rat (Table 5). The significance of the results is discussed in Figures 18, 19, 20. The excretion of radioactivity in the urine was nearly double the normal values (Table 9).

In the first two hours, there was a lag period in the $C^{14}O_2$ production in which no significant difference

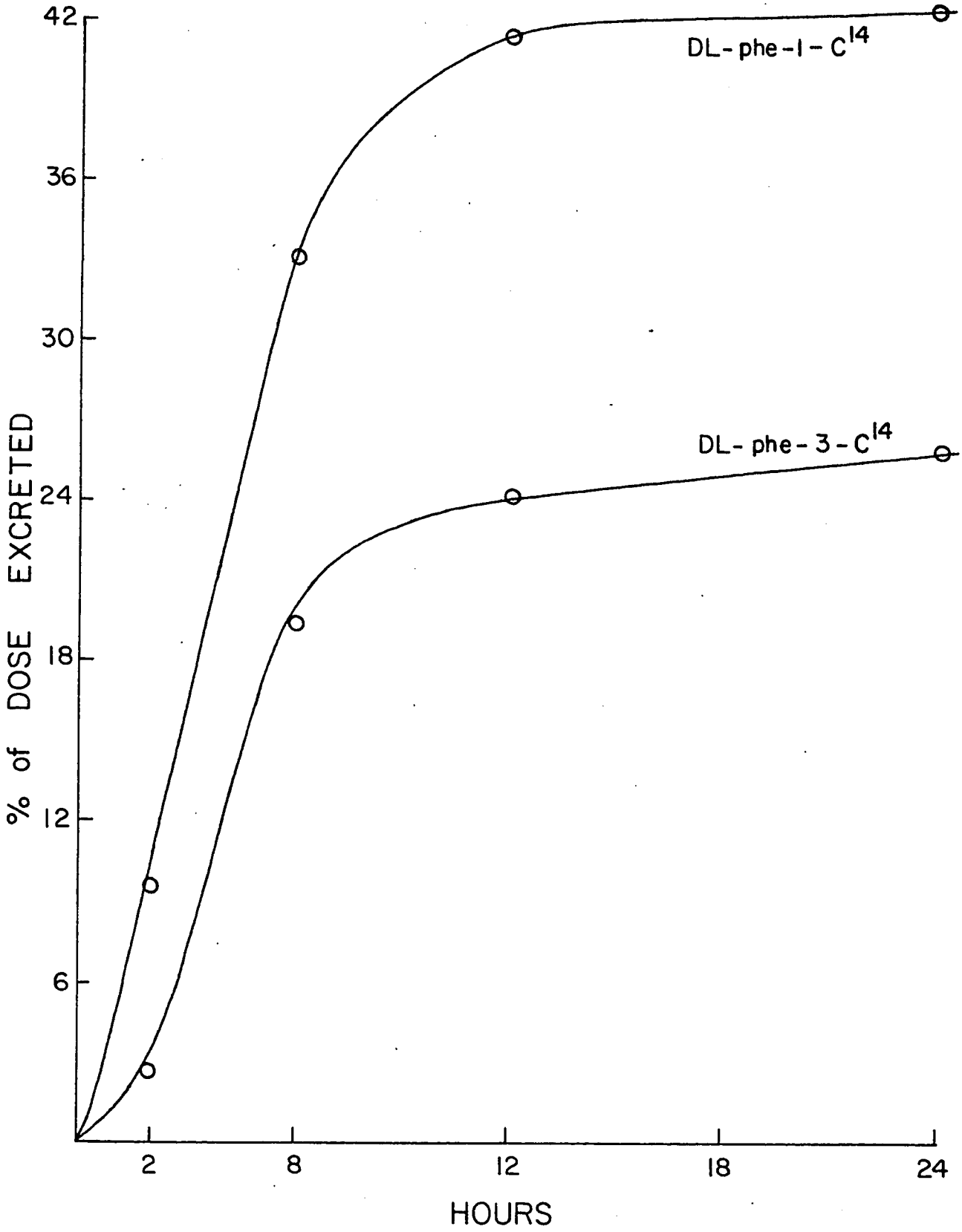


Figure 17

$C^{14}O_2$ Excretion in Phenylketonuric Rats after the Injection of DL-phenylalanine-C^{14}.

TABLE 9

The Excretion of C^{14} in the CO_2 and Urine of Phenylketonuric Rats and Normal Rats after the Injection of Phenylalanine-C^{14}

Amino Acid		CO_2				Urine		
		2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
DL-phe-3-C^{14}	N	2.85[1]	5.64	7.17	9.55	2.15	2.79	3.51
	±SD	0.90	1.47	1.87	2.07	0.81	1.09	1.02
	PKU	2.63	17.33	24.22	25.77	2.38	4.86	7.37
	±SD	0.87	3.63	4.12	3.62	1.49	1.90	2.36
DL-phe-1-C^{14}	N	5.66	9.75	11.38	13.78	3.01	3.38	3.63
	±SD	1.03	1.15	1.22	2.02	1.07	0.90	0.86
	PKU	9.58	33.09	41.20	42.20	8.50	10.42	11.86
	±SD	2.58	3.94	4.90	4.53	1.90	3.24	3.82
L-phe-1-C^{14}	N	9.18	12.18	13.87	15.41	1.17	1.36	1.46
	±SD	1.76	2.09	2.22	2.27	0.33	0.32	0.33
	PKU	10.37	42.95	51.39	55.65	4.49	4.79	6.44
	±SD	2.58	6.99	5.64	6.84	2.81	2.86	3.22
DL-phe-1-C^{14} + alpha-methyldopa	PKU	6.87	25.89	33.00	39.07	5.85	7.10	7.63
	±SD	2.10	10.68	9.90	8.66	1.30	1.69	1.89
DL-phe-1-C^{14} + parnate	PKU	6.65	22.65	30.06	32.54	4.57	5.65	7.07
	±SD	1.72	3.79	5.92	4.68	1.58	1.59	1.87
DL-tryptophan-1-C^{14}	N	3.71	8.51	10.96	13.84	4.08	4.78	5.96
	±SD	1.75	3.13	2.90	3.41	1.64	1.52	1.51
	PKU	4.20	8.48	10.02	11.76	8.03	13.36	14.55
	±SD	0.97	1.78	2.83	2.83	2.83	5.00	4.99

[1] expression of cumulative % of administered dose excreted giving mean ±SD for each group N=normal rats PKU=phenylketonuric rats n=8

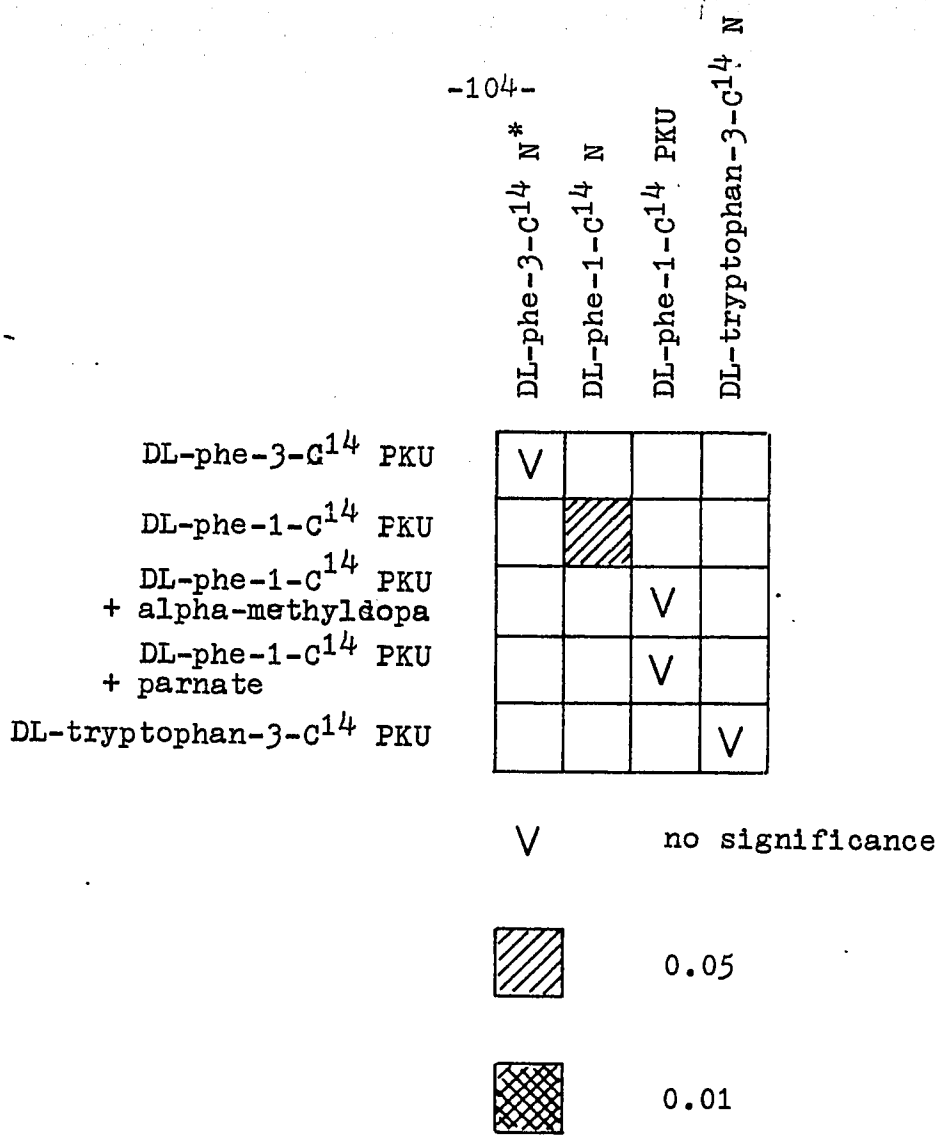


Figure 18

A Summary of the Results of the T Test for Significance between Values of $C^{14}O_2$ at 2 Hours in Phenylketonuric and Normal Rats.

*normal rats

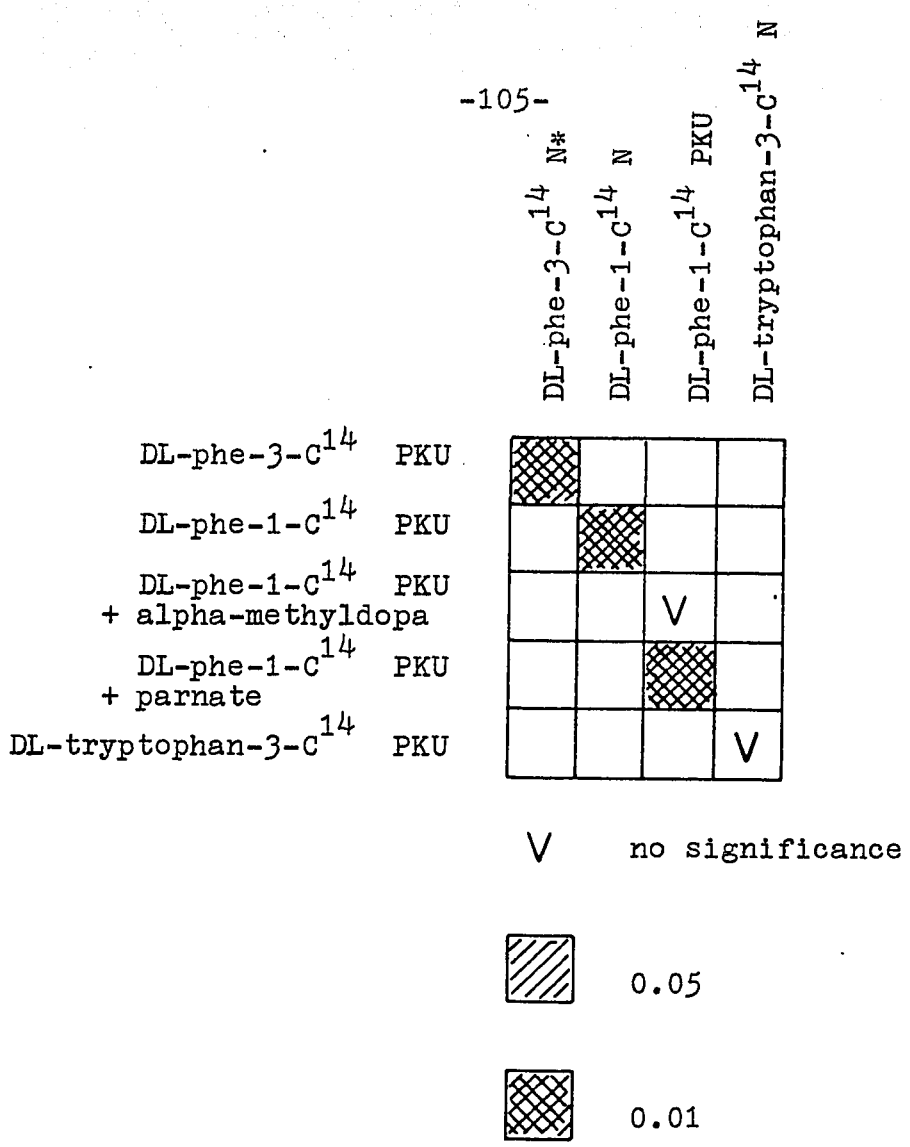


Figure 19

A Summary of the Results of the T Test for Significance between Values of $C^{14}O_2$ at 6 hours in Phenylketonuric and Normal Rats.

*normal rats

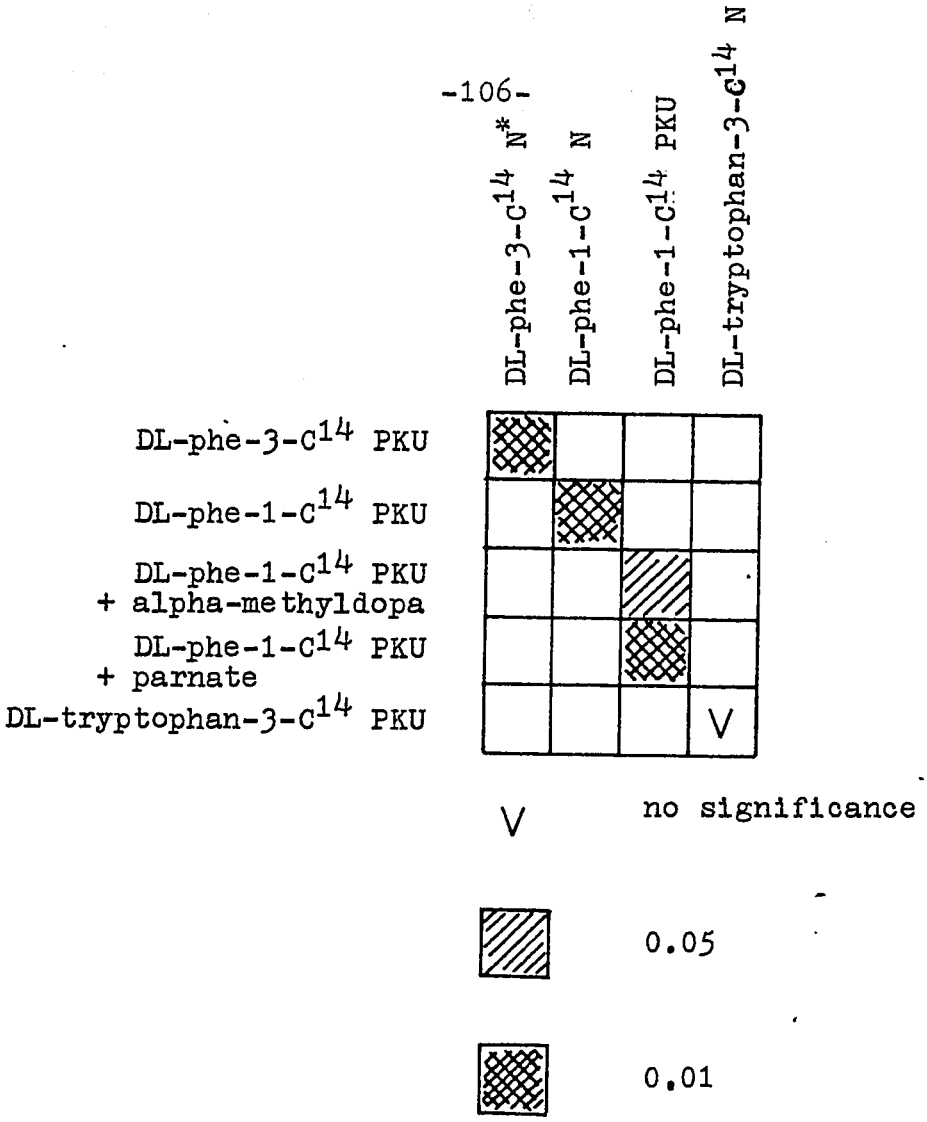


Figure 20

A Summary of the Results of the T Test for Significance between Values of Radioactivity in the Urine at 6 Hours in Phenylketonuric and Normal Rats.

*normal rats

from normal values was noted. This was followed by a rapid increase for the next four to five hours until a plateau was reached. This initial lag period may have been caused by the dilution of the phenylalanine-C^{14} by the high levels of phenylalanine and tyrosine already present in the free amino acid pool of the plasma as a result of the diet.

The high $C^{14}O_2$ levels in phenylketonuric rats (Table 9), coupled with the observation that the crystals from the bladder and ureters of rats made phenylketonuric by the method of Woolley et al (173) consisted mainly of tyrosine (Table 1) indicated that there was an increased conversion of phenylalanine to tyrosine. As previously mentioned, this differs from the situation found in true phenylketonuria, a disease in which there is no phenylalanine hydroxylase.

The high rate of conversion of phenylalanine to tyrosine was contradictory to the discovery of Freedland et al (198). These investigators found that when phenylalanine was added to the diet in moderate amounts, there was ordinarily an increase in the activity of phenylalanine hydroxylase but when an excess of phenylalanine (7%) was included in the diet it caused a marked decrease in enzyme activity in rats fed ad libitum. One possible reason for the apparent difference in phenylalanine hydroxylase activity is the use of different strains of

rats.

The significant increase in the radioactivity of the urine compared to that of normal rats (Figure 20) was expected since, in phenylketonuric rats, there is an increased excretion of phenylpyruvic acid, phenyllactic acid, phenylacetic acid and, possibly, of phenylethylamine (93, 94). When phenylalanine-3-C^{14} was used, all of these compounds would be radioactive in the urine.

B. $C^{14}O_2$ after the Injection of DL-phenylalanine-1-C^{14}

When phenylalanine-1-C^{14} was injected, there was a very large increase in the radioactivity of the CO_2 in the phenylketonuric rat after only 2 hours (Table 9 and Figure 18). There was no noticeable lag period in CO_2 production although the plasma levels of phenylalanine and tyrosine were very high. From results mentioned earlier (secion 1. a.) it is known that there was more tyrosine than phenylalanine in the plasma of the phenylketonuric rat. This may mean that the dilution effect, mentioned with regard to injection of phenylalanine-3-C^{14} into these animals, occurred mainly at the level of tyrosine or its derivatives. The CO_2 produced by the decarboxylation of phenylalanine-1-C^{14} or radioactive phenylpyruvic acid would contain the C^{14}. This, of

course, would not be true for phenylalanine-3-C^{14}. Any possible dilution effect, ascribed to the tyrosine pool, would not effect the production of $C^{14}O_2$ from decarboxylation of either DL-phenylalanine-1-C^{14} or phenylpyruvic acid. This could explain the different results obtained when phenylalanine-3-C^{14} and phenylalanine-1-C^{14} were used.

Since the $C^{14}O_2$ expired after injection of phenylalanine-3-C^{14} would be mainly produced during the process of tyrosine catabolism (at the level of the Krebs cycle), the lag period in the $C^{14}O_2$ release (found with this compound) would be an indication of dilution of the radioactive phenylalanine derivatives by the high levels of tyrosine in the plasma.

The increase in $C^{14}O_2$ when phenylalanine-1-C^{14} was used indicated that there was an activation of the decarboxylation of phenylalanine, tyrosine and dopa. Production of phenylethylamine, tyramine and dopamine would then be increased. The $C^{14}O_2$ could also come from the decarboxylation of phenylpyruvic acid or hydroxyphenylpyruvic acid or from the oxidative decarboxylation of hydroxyphenylpyruvic acid since both of these compounds are produced in larger quantities in phenylketonuric rats (93). Pogrund et al (200) and Williams and Babuscio (24) have shown that, under normal conditions, the decarboxylation of tyrosine is a very minor pathway of tyrosine

metabolism. It is possible that when the blood concentration of this amino acid was increased to many times the normal value, decarboxylation also increased.

This same possibility was considered by Sourkes et al (201, 197). However evidence from several sources, including their work with three inhibitors of L-dopa decarboxylase, led them to conclude that tyrosine was mainly metabolized via the tyrosine-alpha-ketoglutarate transaminase and that dopa was mainly degraded via decarboxylation. These investigators concluded that it was unlikely that oxidative decarboxylation of 3,4-dihydroxyphenylpyruvic acid (formed from administered dopa) played more than a minor role in metabolism.

In a third group of phenylketonuric rats the results obtained from the injection of DL-phenylalanine-1-C^{14} were further investigated using two specific enzyme inhibitors. These were alpha-methyldopa, an inhibitor of the aromatic amino acid decarboxylase and parnate, a monoamine oxidase inhibitor. The results of the $C^{14}O_2$ and radioactive urine production are given in Table 9 while their significance is shown in Figures 18, 19 and 20.

When alpha-methyldopa was used, there was a decrease of only 37% in the radioactivity of the CO_2 after 2 hours (Table 9). This relatively low level of inhibition indicated that while decarboxylation of phenylalanine,

phenylpyruvic acid, and possibly tyrosine in phenylketonuric rats was increased over the level found in normal rats (24), most of the $C^{14}O_2$ came from the oxidative decarboxylation of p-hydroxyphenylpyruvic acid to give homogentisic acid. If the level of $C^{14}O_2$ had been decreased by a greater amount, it would have indicated that the decarboxylation process was of greater significance since alpha-methyldopa inhibits decarboxylation of phenylalanine, phenylpyruvic acid, tyrosine and dopa. Initially, the high $C^{14}O_2$ values obtained for phenylketonuric rats were interpreted to mean that high levels of phenylethylamine were being formed. This was expected because, in human phenylketonurics, there is a very great decrease in the amount of phenylalanine converted to tyrosine (91). However, it appeared that, in rats made experimentally phenylketonuric the conversion of phenylalanine to tyrosine still occurred - probably at an increased rate. The injection of parnate, a monoamine oxidase inhibitor, produced a 50% decrease in the radioactivity of the urine and a 32% decrease in the expired $C^{14}O_2$ (Table 9). The decrease in the $C^{14}O_2$ was unexpected since decarboxylation should not be affected by a monoamine oxidase inhibitor. The decrease in the radioactivity of the urine was also unexpected since parnate should only cause an accumulation of the amines.

Because of the position of the radioactive carbon these amines would not be radioactive. From these limited results it seemed that this inhibitor had the ability to slow down the entire process of phenylalanine metabolism in the phenylketonuric rat.

C. The Radioactivity of the Urine

Injection of either phenylalanine-3-C^{14} or phenylalanine-1-C^{14} into phenylketonuric rats resulted in higher values for urine radioactivity than were found in normal rats (Table 9). This was not expected in the case of phenylalanine-1-C^{14} since decarboxylation of this compound to produce phenylethylamine or phenylacetic acid would lead to the excretion of non-radioactive derivatives in the urine. A possible explanation for this increased excretion of radioactivity would be that more radioactive urea was excreted. This radioactive urea could be formed from the larger quantity of $C^{14}O_2$ available as a result of the increased decarboxylation (indicated by the very high $C^{14}O_2$ values shown in Table 9).

To test this possibility, urea was isolated from the 6 hour urine samples and crystallized (Table 10). The melting point for these crystals was found to be 134-135°C. A standard urea sample melted between 131 and 136°C. The melting point given in the handbook is

TABLE 10

The Radioactivity of Urea Crystals Isolated from the Urine of Phenylketonuric Rats

Injection	cpm per mg of Urea
DL-phenylalanine-3-C^{14}	28
DL-phenylalanine-1-C^{14}	748
DL-phenylalanine-1-C^{14} + alpha-methyldopa	648

133°C.

When phenylalanine-1-C^{14} was used, the urea was about 25 times more radioactive than when phenylalanine-3-C^{14} was used. That is, the CO_2 produced by decarboxylation of phenylalanine and tyrosine derivatives in these first stages of their catabolism was available for formation of urea earlier than the CO_2 produced later at the level of the Krebs cycle. When alpha-methyldopa was used with phenylalanine-1-C^{14}, a 14% decrease in the radioactivity of the urea was obtained. This small decrease would confirm earlier observations on respiratory $C^{14}O_2$ which indicated that oxidative decarboxylation of phenylalanine and tyrosine derivatives was more important in phenylketonuric rats than was straight decarboxylation of phenylalanine and tyrosine. This conclusion was supported by the fact that the total radioactivity of the urine of phenylketonuric rats injected with DL-phenylalanine-1-C^{14} and alpha-methyldopa was also decreased by about 30% after 6 hours.

D. The Contribution of the L and D-isomers of Phenylalanine to $C^{14}O_2$ Production

In order to study the contribution of the D and L-isomers of DL-phenylalanine-1-C^{14} to the $C^{14}O_2$ production in phenylketonuric animals the same approach

was used as for normal rats. Following injection of DL or L-phenylalanine-1-C^{14}, comparisons of the C^{14} excretion were made. The main difference between normal and phenylketonuric rats was that in the phenylketonuric rats, the quantity of C^{14} which came from the D-isomer nearly equalled that which came from the L-isomer (Table 11). These results also pointed to an increased rate of phenylalanine metabolism in phenylketonuric rats. The greater quantity of $C^{14}O_2$ arising from the D-isomer, compared with that found in normal rats (Table 10), could have been the result of decarboxylation of phenylpyruvic acid, a compound formed during the conversion of the D to the L-isomer (157, 158). It is also possible that the conversion of D to L-phenylalanine proceeded at a very rapid rate in the phenylketonuric rats and that the higher $C^{14}O_2$ results were partially accounted for during tyrosine catabolism - since this is still a possible metabolic pathway.

The formation of phenylpyruvic acid would also explain the very high percent of radioactivity in the urine which came from D-phenylalanine. A smaller quantity of L-phenylalanine would have been transaminated to phenylpyruvic acid and excreted in the urine.

TABLE 11

Calculated % Contributions of the D and L-isomers of Phenylalanine-1-C^{14} to the Radioactivity of the CO_2 and Urine of Phenylketonuric Rats

Amino Acid	CO_2				Urine		
	2 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
L-phenylalanine-1-C^{14}	54.17[1]	64.91	62.37	65.94	26.47	23.03	27.15
D-phenylalanine-1-C^{14}	45.83	35.09	37.63	34.06	73.53	76.97	72.85

[1] calculated from $C^{14}O_2$ and urine C^{14} results obtained after injection of DL-phenylalanine-1-C^{14} or L-phenylalanine-1-C^{14} as given in Table 8

E. The Radioactivity of the CO_2 and Urine after the Injection of DL-tryptophan-3-C^{14}

Because of the evidence implicating the metabolism of tryptophan with the condition of phenylketonuria (119, 120), both phenylketonuric and normal rats were injected with tryptophan-3-C^{14} (Table 9 and Figures 18, 19 and 20).

Over the 24 hour period, the $C^{14}O_2$ values for normal rats were comparable to those of phenylketonuric rats. No significant differences were obtained (Figures 18 and 19). This would mean that the pathways leading from the third carbon of tryptophan to CO_2, after cleavage of the alanine side chain from the indole moiety, were not affected by excess phenylalanine.

There was a large increase in the C^{14} excretion in the urine of phenylketonuric rats compared to that of normal rats (Table 9). This was possibly the result of an increased excretion of normal tryptophan metabolites which still contained the third carbon atom such as kynurenine, hydroxykynurenine, kynurenic acid and 8-hydroxykynurenic acid. The excess phenylalanine or its metabolites could have interfered with some of the enzymes involved in the catabolism of kynurenine or hydroxy-kynurenine such as the kynurenine-3-hydroxylase or kynureninase. The increased radioactivity could also

have been caused by the excretion of radioactive indoleacetic acid or indolelactic acid - metabolites which are formed from tryptophan in phenylketonuria as described by Armstrong et al (106). Since the urinary excretion of 5-hydroxyindoleacetic acid, the oxidation product of serotonin, is greatly decreased in phenylketonuria (107), it would appear that the increased level of radioactivity, found in the urine of phenylketonuric rats after the injection of tryptophan-3-C^{14}, would be the result of the catabolism of tryptophan via the nicotinamide (normal) or indoleacetic acid (abnormal) routes.

3) PHENYLALANINE-DEFICIENT RATS

A. Injected with Phenylalanine-3-C^{14}

When phenylalanine-3-C^{14} was injected into phenylalanine-deficient rats, the excretion of radioactivity in the CO_2 was slightly lower than that of normal rats (Table 12). This difference became significant only after 6 hours (Figure 21). The low $C^{14}O_2$ production indicated that the catabolism of phenylalanine was quite slow in these phenylalanine-deficient rats. This could be expected since, after the period of dietary treatment, the level of phenylalanine in the non-protein

TABLE 12

The Excretion of C^{14} in the CO_2 and Urine of Phenylalanine-deficient Rats after the Injection of Phenylalanine-C^{14}

Amino Acid		CO_2				Urine		
		2 hr	6 hr	12 hr	24 hr	2 hr	6 hr	12 hr
Normal Rats								
DL-phe-3-C^{14}		2.85[1]	5.64	7.17	9.55	2.15	2.79	3.51
	±SD	0.90	1.47	1.87	2.07	0.81	1.09	1.02
Phenylalanine-deficient Rats								
DL-phe-3-C^{14}		1.99	3.72	5.03	6.91	1.15	1.62*	2.08
	±SD	0.78	1.49	1.54	1.78	0.45	0.49	0.69

[1] expression of cumulative % of administered dose excreted giving mean ±SD for each group

n=8 unless marked * where n=7

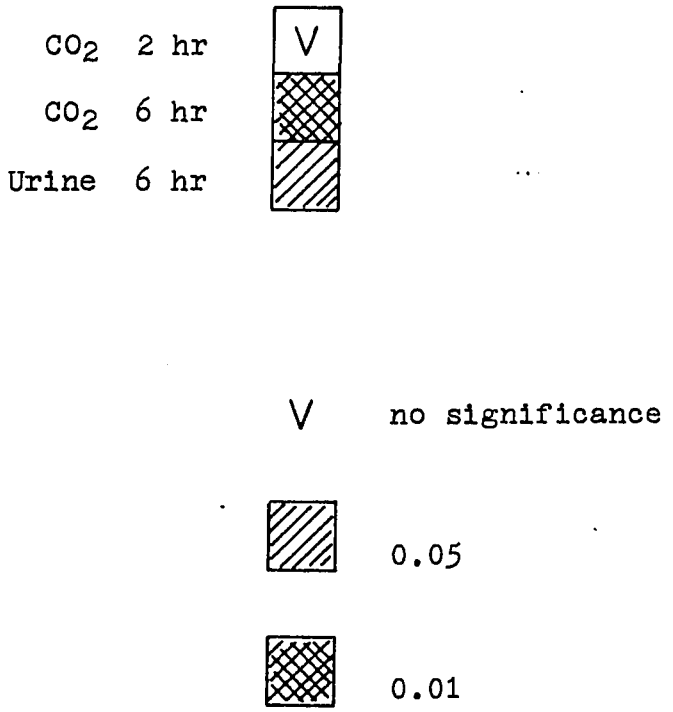


Figure 21

A Summary of the Results of the T Test for Significance between Values of Radioactivity in the CO_2 and Urine after Injection of Phenylalanine-3-C^{14} in Normal and Phenylalanine-deficient Rats.

fraction of the plasma was very low (Table 3). It is probable that there was a great demand for this essential amino acid for protein synthesis.

The level of excretion of radioactivity in the urine was also lower than for normal rats (Table 12). This decrease in the quantity of radioactivity excreted in the urine could have been the result of a more efficient use of D-phenylalanine in these deficient rats since de Gasquet et al (165) have shown that phenylalanine-deficient rats use D-phenylalanine better than normal rats. This decrease was also a reflection of the greater need for phenylalanine for protein synthesis. From these limited results on phenylalanine-deficient rats, one would expect a greater incorporation of phenylalanine into tissue proteins since, compared to normal animals, very little radioactivity was excreted by the phenylalanine-deficient animals.

4. THE RADIOACTIVITY OF THE ORGANS AND THE PLASMA

a. Discussion of Technique

By injecting a radioactive compound into the body, it is possible to trace the route by which the compound is metabolized. In this experiment, the radioactive material was introduced directly into the jugular vein therefore the plasma should show a high level of radioactivity for the first few hours. Since the C^{14} compound was an essential amino acid or one of its analogues, the radioactivity would enter the amino acid pool of the tissues. The C^{14} could be incorporated into the protein or distributed into one of many possible metabolites. The loss of the C^{14} in the CO_2 or urine has already been discussed in section 4.

By sampling each organ, it was possible to obtain some idea of the total retention of the radioactivity in the organs or plasma however, it was difficult to draw any conclusions as to the significance of these results since the radioactivity represented the total C^{14} present in the sample whatever its form. There was no indication as to whether the C^{14} was in the protein or non-protein fraction or was merely en route to another site.

Phenylalanine-1-C^{14} was used only to determine the

pattern of distribution of radioactivity in the CO_2 and urine compared with that obtained with phenylalanine-3-C^{14} therefore no 2 or 6 hour organ or plasma samples were taken after the injection of phenylalanine-1-C^{14}.

b. Discussion of Results

1) NORMAL RATS

A. Injected with Phenylalanine-C^{14}

The results for the total retention of radioactivity in the organs after the injection of DL-phenylalanine-3-C^{14} into normal rats are shown in Table 13. Comparison of the 2, 6 and 24 hour samples shows that the radioactivity of the plasma and the organs was relatively high at 2 hours, fell at 6 hours, then regained the former high levels by 24 hours. The pancreas was the only exception to this pattern. At 2 hours, the level of radioactivity in the pancreas was much higher than that of the other organs; this value slowly declined during the next 22 hours. The initially high level of radioactivity in the pancreas may be explained by the large capacity of this organ for concentrating amino acids and analogues (203).

Table 13 also shows the results of the injection of

TABLE 13

The Retention of C^{14} in the Organs and Plasma of Normal Rats after the Injection of Phenylalanine-C^{14}

Amino Acid		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14}	2 hr	1.23[1]	2.56*	4.35	0.24	0.60*
	±SD	0.23	0.35	0.79	0.08	0.07
	6 hr	0.76*	1.85	1.85	0.14*	0.29
	±SD	0.08	0.61	0.59	0.03	0.06
	24 hr	1.64*	2.92	1.01	0.18	0.61*
	±SD	0.15	0.63	0.13	0.06	0.11
DL-phe-1-C^{14}	24 hr	1.54	3.37	0.81	0.31	0.67
	±SD	0.42	0.64	0.18	0.07	0.10
L-phe-1-C^{14}	24 hr	1.29*	1.16	0.76	0.27	0.45
	±SD	0.11	0.17	0.06	0.05	0.09

[1] expressed as % of administered dose

n=8 unless marked * where n=7

DL-phenylalanine-1-C^{14} into a second group of normal animals. These values, which are only available for the 24 hour series, compare quite closely to those obtained when DL-phenylalanine-3-C^{14} was used.

The third experimental group of animals was injected with L-phenylalanine-1-C^{14}. Compared to the values found after the injection of DL-phenylalanine-1-C^{14}, there was a decrease in the level of the radioactivity in the plasma at 24 hours (Table 13). The reason for the decrease in radioactivity at 6 hours and the increase at 24 hours was not clear. After 24 hours there had been a considerable recycling of the radioactivity and a redistribution of the C^{14} into different compounds found in the cells. The high level of radioactivity found in the organs at 24 hours probably represented radioactive compounds other than phenylalanine and tyrosine. The pancreas, an organ mainly concerned with the synthesis and secretion of proteins and other substances, was the only organ which did not show an increase in the radioactivity at 24 hours.

Another group of normal rats was used to investigate the effect of the simultaneous injection of either non-radioactive D or L-phenylalanine and phenylalanine-3-C^{14}. The addition of the non-radioactive compound reduced the amount of radioactivity present in all of the organs (Tables 13 and 14). The plasma values were also lower except at 6 hours when both the D and L-isomers produced

TABLE 14

The Retention of C^{14} in the Organs and Plasma of Normal Rats after the Injection of Phenylalanine-C^{14} + either Non-radioactive D or L-phenylalanine

Amino Acid		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14} + L-phe 1:20	2 hr	0.77[1]	1.29	1.75	1.48	0.32
	±SD	0.17	0.26	0.59	0.05	0.05
	6 hr	0.68*	1.33	1.29*	0.12**	0.34
	±SD	0.11	0.16	0.19	0.01	0.06
	24 hr	1.23	2.00	0.71	0.24	0.52
	±SD	0.08	0.24	0.11	0.04	0.06
DL-phe-3-C^{14} + D-phe 1:20	2 hr	0.66	0.67	2.36	0.08*	0.31
	±SD	0.14	0.24	0.78	0.02	0.09
	6 hr	1.07	1.14	3.20*	0.22	0.62*
	±SD	0.15	0.11	0.61	0.03	0.07
	24 hr	0.97	0.86*	0.58	0.15	0.43
	±SD	0.23	0.10	0.15	0.05	0.10

[1] expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD

n=8 unless marked * where n=7

**n=6

a higher level of radioactivity than the value obtained when only phenylalanine-3-C^{14} was used. This was especially true with phenylalanine-3-C^{14} + D-phenylalanine. The main difference between the two treatments was that when an excess of non-radioactive L-phenylalanine was used, the same general picture was obtained as when only phenylalanine-3-C^{14} was used. That is, the radioactivity of all of the organs except that of the pancreas decreased at 6 hours then increased again at 24 hours. When an excess of D-phenylalanine was used, the radioactivity of the plasma and all of the organs increased at 6 hours then decreased by 24 hours. It is possible that the difference was caused by the conversion of the D-isomer to the L-form. This process normally requires some time but could have been increased in the presence of a large excess of D-phenylalanine (157, 158).

B. Injected with Radioactive Analogues of Phenylalanine

In general, the radioactivity of the plasma and organs of the animals which received radioactive phenylalanine-3-C^{14} (Table 13) was higher than the radioactivity of the organs of the animals which received p-fluorophenylalanine-3-C^{14} or BTA-3-C^{14} (Table 15). This seems logical since these analogues were oxidized or excreted more rapidly than were the natural amino acids -

TABLE 15

The Retention of C^{14} in the Organs and Plasma of Normal Rats after the Injection of the Radioactive Analogues of Phenylalanine

Amino Acid		Liver	Kidney	Pancreas	Brain	Plasma
p-F-phe-3-C^{14}	2 hr	1.11[1]	0.91	3.59	0.21	0.57
	±SD	0.32	0.25	0.92	0.04	0.09
	6 hr	0.98*	0.69	1.66	0.10	0.46
	±SD	0.16	0.16	0.73	0.06	0.19
	24 hr	1.41	1.12	0.83	0.22	0.48
	±SD	0.38	0.36	0.15	0.06	0.11
BTA-3-C^{14}	2 hr	0.46	0.97	1.47	0.08	0.14
	±SD	0.15	0.58	0.58	0.04	0.04
	6 hr	0.17	0.55	0.25	0.04*	0.05
	±SD	0.09	0.20	0.09	0.01	0.03
	24 hr	0.03	0.16	0.03	0.00	0.00
	±SD	0.02	0.06	0.01	0.00	0.00

[1] expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD

n=8 unless marked * where n=7

as shown by the higher levels of radioactivity in the CO_2 and urine (Table 6). When p-fluorophenylalanine-3-C^{14} was injected, the distribution of radioactivity was the same as that obtained with phenylalanine-3-C^{14}. The radioactivity of the plasma and of all the organs, except the pancreas, decreased at 6 hours then increased again at 24 hours. In all cases, the quantity of radioactivity in the samples taken after the injection of BTA-3-C^{14} decreased from 2 hours to 24 hours. By 24 hours almost no radioactivity was found in the organs or plasma.

C. Injected with Phenylalanine-C^{14} and its Non-radioactive Analogues

When the non-radioactive analogue was added to the injection material there were some definite changes in the quantity of radioactivity retained in the tissues and plasma (Tables 16 and 17). Usually the values were lower than those obtained with phenylalanine-C^{14} alone (Table 13).

a) Phenylalanine-3-C^{14} and its Non-radioactive Analogues

Although the radioactive content of the organs was lower when the analogue was added to the injection, in all of the samples the pancreas still had the highest level of C^{14} (Table 16). The plasma values for 2 hour samples

TABLE 16

The Retention of C^{14} in the Organs and Plasma of Normal Rats after the Injection of the DL-phenylalanine-3-C^{14} + a Non-radioactive Analogue

Amino Acid			Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14} + p-F-phe	1:20	2 hr	0.73(1)	0.94	1.60*	0.06	0.19
		±SD	0.18	0.18	0.37	0.04	0.10
		6 hr	0.69	1.00	1.51	0.17*	0.46
		±SD	0.20	0.15	0.53	0.05	0.15
		24 hr	1.20	1.24	0.77	0.18	0.41
		±SD	0.16	0.14	0.36	0.06	0.10
DL-phe-3-C^{14} + p-F-phe	1:10	24 hr	1.31*	1.72	0.85	0.15	0.56
		±SD	0.09	0.27	0.17	0.03	0.14
DL-phe-3-C^{14} + BTA	1:20	2 hr	0.95**	1.61	2.06	0.09	0.33*
		±SD	0.11	0.24	0.39	0.04	0.02
		6 hr	2.14*	3.88*	3.69**	0.36*	1.01*
		±SD	0.32	0.51	0.18	0.08	0.13
		24 hr	1.44*	2.06	0.76	0.20	0.49
		±SD	0.20	0.31	0.20	0.00	0.21
	1:10	24 hr	1.47	2.49	0.76**	0.25*	0.58
		±SD	0.37	0.57	0.09	0.04	0.12
DL-phe-3-C^{14} + APBA	1:20	2 hr	0.79	2.15	2.26	0.13	0.19
		±SD	0.33	0.85	1.04	0.07	0.03
		6 hr	0.55*	1.73	0.95	0.10	0.17**
		±SD	0.08	0.36	0.16	0.03	0.02
		24 hr	0.91	1.30	0.81	0.15*	0.40
		±SD	0.22	0.80	0.27	0.03	0.09
	1:10	24 hr	1.01*	1.39	0.84	0.16	0.37
		±SD	0.13	0.79	0.22	0.04	0.11

(1) expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD
n=8 unless marked * where n=7 **n=6

of phenylalanine-3-C^{14} + p-fluorophenylalanine and phenylalanine-3-C^{14} + APBA were identical while those of phenylalanine-3-C^{14} + BTA were somewhat higher.

When phenylalanine-3-C^{14} + p-fluorophenylalanine was used in the 1:20 ratio, a comparison of the 2, 6 and 24 hour samples showed that there was a gradual increase in the level of C^{14} in the liver, kidney and the brain. The C^{14} in the plasma was higher at 6 hours while the radioactivity of the pancreas decreased with time. All of the BTA samples, including the pancreas, showed an increase in the radioactivity at 6 hours then a decrease with time. When APBA was added to the injection the situation was more complex. The radioactivity of the kidneys and of the pancreas decreased with time while that of the liver, brain and blood decreased at 6 hours then increased at 24 hours.

With all three analogues, there was an extremely close agreement between values obtained when the 1:10 and 1:20 ratios were used. The only values which varied to any extent were those of the plasma.

b) Phenylalanine-1-C^{14} and its Non-radioactive Analogues

Generally, the addition of the non-radioactive analogue produced a decrease in the C^{14} retention compared to the values obtained when phenylalanine-1-C^{14} was injected alone (Table 17). There were only three exceptions to this decrease

TABLE 17

The Retention of C^{14} in the Organs and Plasma of Normal Rats after the Injection of Phenylalanine-1-C^{14} and a Non-radioactive Analogue

Amino Acid		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-1-C^{14}	24 hr	1.54(1)	2.37	0.81	0.31	0.66
	±SD	0.42	0.64	0.18	0.07	0.10
DL-phe-1-C^{14}	24 hr	2.11	2.32*	1.05*	0.37*	0.63
+ p-F-phe 1:10	±SD	0.41	0.35	0.10	0.05	0.15
DL-phe-1-C^{14}	24 hr	1.39	2.21*	0.75	0.20*	0.38
+ BTA 1:10	±SD	0.28	0.18	0.13	0.05	0.11
DL-phe-1-C^{14}	24 hr	1.16*	3.12	0.66	0.22	0.38*
+ APBA 1:10	±SD	0.06	0.53	0.10	0.07	0.10

(1) expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD

n=8 unless marked * where n=7

**n=6

in the radioactivity after the addition of the analogue - notably in the liver and pancreas when p-fluorophenylalanine was added and the kidney when APBA was added. The injection of BTA resulted in a reduction in the quantity of radioactivity retained.

The 24 hour values are difficult to interpret since the radioactive carbon of phenylalanine-1-C^{14} was rapidly removed by decarboxylation (Table 8) therefore the $C^{14}O_2$ was available for the synthesis of other cellular components.

2) PHENYLKETONURIC RATS

The values for retention of radioactivity in the organs and plasma of phenylketonuric rats are given in Table 18. These may be compared to the values obtained for normal rats as shown in Table 13.

A) Injected with Phenylalanine-3-C^{14}

The organs of the phenylketonuric animals contained less radioactivity than those of normal animals. The liver values differed since they were very similar to those obtained from a normal rat. The plasma of the phenylketonuric animal showed a high level of radioactivity at 2 hours; this level decreased at 24 hours to below-normal values.

TABLE 18

The Retention of C^{14} in the Organs and Plasma of Phenylketonuric Rats after the Injection of Phenylalanine-C^{14} or DL-tryptophan-3-C^{14}

Amino Acids		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14} in PKU	2 hr	1.30*(1)	1.82	2.95	0.50	0.86
	±SD	0.45	0.71	1.08	0.24	0.46
	24 hr	0.70	1.24	0.59	0.25	0.36
	±SD	0.23	0.60	0.27	0.06	0.13
DL-phe-1-C^{14} in PKU	2 hr	1.43	1.69	3.67	0.36	0.81
	±SD	0.38	0.51	1.77	0.09	0.32
	24 hr	0.66	0.65	0.40	0.18	0.28
	±SD	0.22	0.24	0.15	0.04	0.07
L-phe-1-C^{14} in PKU	24 hr	7.08	6.05	0.49	0.18	0.25
	±SD	0.19	0.23	0.27	0.04	0.06
Tryptophan-3-C^{14} normal	24 hr	1.32	1.61	1.04	0.36	0.53
	±SD	0.35	0.54	0.31	0.01	0.06
Tryptophan-3-C^{14} in PKU	24 hr	0.96	1.12	0.85	0.21	0.35
		0.14	0.32	0.27	0.05	0.14

(1) expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD

n=8 unless marked * where n=7

In the phenylketonuric rats all of the values decreased with time. This was opposite to the situation found in the normal rat. The radioactivity in the CO_2 and urine (Table 9) implied that the phenylketonuric rat excreted the C^{14} carbon faster than normal rats therefore less of it was recycled into other compounds.

B) Injected with Phenylalanine-1-C^{14}

Every value for the total of C^{14} in the organs of phenylketonuric rats was below that of normal rats (Tables 13 and 18). This was especially noticeable in the plasma. Comparison of the 2 and 24 hour values for phenylketonuric animals revealed a very large reduction in the C^{14} content between these two time intervals. Again there was a faster excretion of C^{14} in the CO_2 and urine of the phenylketonuric rat (Table 9) compared to that of normal rats (Table 4)

In another group of phenylketonuric rats the injection of L-phenylalanine-1-C^{14} produced only a 11.7% decrease in the radioactivity of the plasma (Table 18) compared to that of normal rats (Table 13).

C. Injected with DL-tryptophan-1-C^{14}

There was little difference in the radioactivity of

the tissues of phenylketonuric and normal rats but the plasma values of the phenylketonuric animals were considerably lower than those of normal animals (Tables 13 and 18).

3) PHENYLALANINE-DEFICIENT RATS

A. Injected with DL-phenylalanine-3-C^{14}

Compared to the values obtained in normal rats the injection of phenylalanine-3-C^{14} into the phenylalanine-deficient rats, produced a large increase in the radioactivity of all of the organs except the pancreas and the plasma (Table 19). At 6 hours the plasma values were almost four times higher than normal. This value became closer to normal by 24 hours although the values were still considerably different. In the deficient animals there was a gradual decrease in the radioactivity of the organs with time. This was different from the decrease at 6 hours and the increase at 24 hours found in the normal rat. The pancreas behaved differently from all of the other organs. The radioactivity of this organ was very low at 2 hours while in the normal rat it was very high at this time. By six hours the pancreas of the phenylalanine-deficient rat showed a very large increase in radioactivity.

TABLE 19

The Retention of C^{14} in the Organs and Plasma of Phenylalanine-deficient Rats after Injection of DL-phenylalanine-3-C^{14}

Amino Acid		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14}	2 hr	3.14(1)	4.55	0.74	0.46	0.76
	±SD	0.36	0.71	0.08	0.10	0.10
	6 hr	2.89*	3.99*	3.77*	0.51*	1.06
	±SD	0.41	0.84	1.16	0.11	0.09
	24 hr	2.59	3.15	2.08	0.59	0.90
	±SD	0.16	0.56	0.42	0.14	0.10

(1) expressed as % of administered dose per gm of tissue or ml of plasma giving mean ±SD

n=8 unless marked * where n=7

5. THE RADIOACTIVITY OF THE PROTEINS

a. Discussion of Technique

The sample of the organ or plasma indicated the total C^{14} concentration but did not give any information as to whether the radioactivity had become incorporated into the protein of the tissue or was free within it. A partial answer was provided by counting the C^{14} in the TCA insoluble fraction. The amount of radioactivity in the soluble fraction of this homogenate was determined by counting the TCA supernatant. This latter value would represent the total radioactivity contained in the free amino acid pool of the tissues.

However, without running chromatograms, it was not possible to determine whether the radioactivity in the proteins was in the form of the amino acid analogue, phenylalanine, tyrosine, or another amino acid. When phenylalanine-1-C^{14} was used, there was the further complication of the possible utilization of the excess $C^{14}O_2$ in the synthesis of some other radioactive compound. The C^{14} compound may also have been lost to the proteins via incorporation of the C^{14} into fatty acids or carbohydrates. This could have occurred when DL-phenylalanine-3-C^{14} was used since the radioactive carbon is lost at the level of the Krebs cycle.

These many possibilities made the evaluation of the significance of the radioactivity in the organs and plasma very difficult. In this chapter the results for the radioactivity found in the water-soluble proteins will be given.

The results have been expressed per g of protein therefore they may appear to be rather high. However, for the pancreas and brain, the extraction process usually produced a yield of only 10 to 50 mg of proteins. When considering the actual quantity of protein obtained from the organs the values assume their proper perspective.

Since the results for the radioactivity of the CO_2, urine and organs were expressed as a percent of the administered dose, the same method was used for the proteins. This permitted a faster comparison of values for the different experimental groups.

In some cases, the TCA supernatant and the results of the ethanol and acetone washings were pooled and retained in order to estimate the amount of radioactivity present in the water-soluble, non-protein portion of the sample. The results obtained from the injection of DL-phenylalanine-3-C^{14} into normal rats are shown in Table 20. The radioactivity decreased with time; at 2 hours the values were approximately 1/10 those for the total C^{14} retention (Table 13). By 24 hours the quantity of radioactivity in the supernatant represented only 3 to 5% of the total C^{14} retained in the organs and plasma (Table 13). This indicated that, by 24

TABLE 20

The Radioactivity in the TCA Supernatant after the Injection of DL-phenylalanine-3-C^{14} into Normal Rats

Time	Liver	Kidney	Pancreas	Brain	Plasma
2 hr	0.15[1]	0.31	0.35	0.01	0.08
6 hr	0.08	0.20	0.09	0.03	0.04
24 hr	0.06	0.09	0.05	----	0.01

[1] expressed as % of administered dose in the total supernatant

hours, only trace quantities of free radioactive amino acids were found in the tissues.

b. Discussion of Results

1) NORMAL RATS

A. Injected with Phenylalanine-C^{14}

The results of the injection of DL-phenylalanine-3-C^{14} are given in Table 21. Expressed as counts per minute per mg of water-soluble protein the 2 hour values would be: liver, 2.0; kidney, 50.5; pancreas, 210.0; brain, 10.7 and plasma 52.2. This is for an injected dose of 653,840.0 counts per minute.

The total retention of radioactivity in the plasma and in all of the organs, except the pancreas, showed an initial high, a decrease by 6 hours, then a return to the previous high (Table 13). Since the $C^{14}O_2$ production (Figure 11) was highest within the first 6 hours and the level of radioactivity in most of the proteins was at its highest by 2 hours (Table 21) it is possible that a considerable proportion of the injected amino acid was rapidly incorporated into proteins then was catabolized as soon as it was released from the proteins.

Two hours after the injection of DL-phenylalanine-3-C^{14} the level of radioactivity in the plasma proteins was very high (Table 21). By 6 hours this value had slightly decreased; this decrease continued to 24 hours. This same

TABLE 21

The Incorporation of C^{14} into the Proteins of Normal Rats after the Injection of Phenylalanine-C^{14}

		Liver	Kidney	Pancreas	Brain	Plasma
		(1)				
DL-phe-3-C^{14}						
	2 hr	3.20*	7.23	34.31	1.74*	8.51*
	±SD	0.34	1.16	4.78	0.28	1.44
	6 hr	2.99	8.04	16.72	1.61	7.01*
	±SD	0.57	1.55	5.39	0.33	0.69
	24 hr	3.38*	9.96*	3.82*	1.45*	6.51
	±SD	0.50	1.58	0.47	0.12	1.13
DL-phe-1-C^{14}						
	24 hr	2.78	6.70	2.73	1.05	5.61
	±SD	0.74	1.69	0.72	0.48	1.81
L-phe-1-C^{14}						
	24 hr	3.18*	3.35	3.50*	1.88	3.95
	±SD	0.33	0.57	0.43	0.19	0.58

(1) expressed as % of administered dose per gm of protein giving mean ±SD

n=8 unless marked * where n=7

situation occurred in the pancreas and the brain although, in the latter case, the initial level of radioactivity was quite low and the decrease was small - indicating a very low protein turnover. It has been shown that, in vivo, the brain takes up amino acids more slowly than the other tissues (204). In the pancreas, the initial level of incorporation was the highest of any organ. There was also a considerable decrease in the C^{14} content over the 24 hour period.

The radioactivity in the proteins of the liver varied slightly over the 24 hour period while that of the kidneys showed a slight but constant increase with time. The liver, kidney and plasma had a higher total C^{14} retention at 24 hours than at 2 hours (Table 13). This was probably the result of the accumulation of radioactive metabolites derived from the injected radioactive amino acid. The liver and kidneys seemed able to use the injected amino acid or the radioactive compounds derived from the injected compound for protein synthesis over a longer period of time. In the kidney, D-phenylalanine can be converted to L-phenylalanine (157, 158). Therefore more L-phenylalanine-C^{14} would be available to the kidney for a longer time.

It is also possible that the increase in the radioactivity of the proteins of the liver and the kidney at 24 hours was caused by the incorporation of radioactive

tyrosine derived from injected phenylalanine-C^{14}. Recently, Ito, Guroff and Udenfriend (205) reported the results of their experiments in dogs. They found a difference between the behavior of injected tyrosine-C^{14} (tyrosine-T) and tyrosine which was derived from injected phenylalanine-C^{14} (tyrosine-P). In plasma albumin and globulin the specific activity of the tyrosine-P either increased or remained constant with time for several days while the specific activities of phenylalanine and tyrosine-T decreased with time. This indicated that there was a contribution of protein-bound phenylalanine to the protein-bound tyrosine and it was suggested that a pool of amino acids, derived from protein catabolism and not in equilibrium with the blood amino acids, could exist in some organs.

The results in Table 21 suggested the existence of such a pool in the liver and in the kidneys. Considering the results of Ito et al (205), the increase in the radioactivity of the protein after 24 hours could have been caused by the incorporation of tyrosine-P which was not diluted by the blood tyrosine. Such a pool would not operate in the pancreas because of the rapid protein synthesis there. In the pancreas, most of the synthesized protein is distributed outside of the organ. Therefore the amino acids necessary for this protein synthesis must be constantly supplied by the blood.

A second group of normal rats was injected with DL-phenyl-

alanine-1-C^{14} (Table 21). Since no 2 or 6 hour organ and plasma samples were taken, no conclusions could be made concerning the variation of protein radioactivity with time. After 24 hours, the level of radioactivity in most of the proteins of the rats injected with DL-phenylalanine-1-C^{14} was lower than that of the animals injected with DL-phenylalanine-3-C^{14}. This is reasonable since DL-phenylalanine-1-C^{14} lost its C^{14} in the CO_2 much more rapidly than did DL-phenylalanine-3-C^{14} (Table 4)

When L-phenylalanine-1-C^{14} was injected into the third series of normal rats, only the kidney proteins had a significantly lower specific activity than that obtained when the racemic mixture was used (Table 21). As was stated above, this may be an indication that, when the racemic mixture was used, the L-phenylalanine-C^{14} was available to the kidney for a longer period of time since the transformation of the D to the L-isomer occurs in the kidney (157).

Another group of normal rats was injected with DL-phenylalanine-3-C^{14} + either the D or L isomer in a 1:20 ratio. Compared to the values obtained when only DL-phenylalanine-3-C^{14} was used (Table 21) the addition of the D or L-isomer generally reduced the quantity of radioactivity in all the proteins sampled (Table 22).

Addition of the L-isomer to the injection material resulted in a decrease in the radioactivity of the proteins

TABLE 22

The Incorporation of C^{14} into the Proteins of Normal Rats after the Injection of DL-phenylalanine-3-C^{14} + either Non-radioactive D or L-phenylalanine

		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14} + L-phe 1:20	2 hr	2.44*(1)	5.32*	14.58*	1.07	4.85
	±SD	0.17	0.17	2.43	0.13	1.26
	6 hr	2.35	5.75	10.09	1.41	5.48
	±SD	0.24	0.54	1.63	0.26	1.00
	24 hr	2.48	5.05*	2.59	1.36	4.32
	±SD	0.56	1.01	0.58	0.29	1.26
DL-phe-3-C^{14} + D-phe 1:20	2 hr	2.25	2.50*	16.56**	0.88	4.51
	±SD	0.28	0.31	1.54	0.29	1.05
	6 hr	2.12	3.27	13.10	1.20	6.18
	±SD	0.53	0.35	1.41	0.37	1.06
	24 hr	2.80*	3.36	4.48	1.30*	5.28
	±SD	0.17	0.48	1.35	0.19	0.26

(1) expressed as % of administered dose per gm of protein giving mean ±SD

n=8 unless marked * where n=7

**n=6

(Table 22) and an increase in the radioactivity of the CO_2 (Table 4). In the discussion of the $C^{14}O_2$ results, it was postulated that the excess of L-phenylalanine increased the catabolism of phenylalanine. However the excess L-phenylalanine diluted the radioactive phenylalanine therefore reducing the incorporation of radioactive phenylalanine into the proteins. The pancreas, which was actively synthesizing proteins, was affected the most since there was a very great reduction in the level of the radioactivity in this organ. By 2 hours, the level of incorporation in the pancreas was higher than that in the other organs; it was greatly reduced by 24 hours. Over the 24 hour period, the incorporation of radioactivity into the proteins of the liver and kidney was not greatly affected by the large excess of phenylalanine. This relatively small decrease in the radioactivity in the proteins of the liver and kidney may be evidence in favour of the existence, in these organs, of a pool of free amino acids not in equilibrium with the blood amino acid pool as suggested by Ito et al (205).

When an excess of non-radioactive D-phenylalanine was used, the radioactivity in the proteins of the liver, pancreas, brain and plasma was similar to that produced when an excess of L-phenylalanine was used (Table 22). As the $C^{14}O_2$ production was lower when an excess of the D-isomer, rather

than the L-isomer, was used (Table 4) the excess D-phenylalanine must have activated the metabolism of phenylalanine to a lesser extent but decreased the incorporation of the phenylalanine-C^{14} into proteins to the same extent as did L-phenylalanine. This could be explained by a rapid transformation of the D to the L-isomer in these organs or to a competition between the D and L-phenylalanine at some stage of protein synthesis or of amino acid absorption. This second possibility was more likely because, as indicated by the values for the CO_2 and urine (Table 4), phenylalanine metabolism was not greatly stimulated. In the pancreas, the radioactivity of the protein in the 2 and 6 hour samples was high; these values were slightly higher than those obtained when the L-isomer was added. In the kidney the level of radioactivity at 2 hours was low indicating the importance of the transformation of D to L-phenylalanine. By 24 hours, the radioactivity of the kidney proteins was slightly increased. This reiterates the case for the existence of a separate amino acid pool in this organ.

B. Injected with Radioactive Analogues of Phenylalanine

When p-fluorophenylalanine-3-C^{14} was injected into normal rats, there was some incorporation of a radioactive compound into the protein (Table 23). At 2 hours, the

TABLE 23

The Incorporation of C^{14} into the Proteins of Normal Rats after the Injection of Radioactive Analogues of Phenylalanine

		Liver	Kidney	Pancreas	Brain	Plasma
p-F-phe-3-C^{14}						
	2 hr	2.79[1]	2.42	19.52	0.96	5.11*
	±SD	0.33	0.58	7.61	0.29	0.79
	6 hr	3.12*	3.37*	16.23	1.30**	9.02*
	±SD	0.61	0.46	6.62	0.18	0.72
	24 hr	2.36	2.66	2.80	0.99	5.16
	±SD	0.53	0.53	0.80	0.37	1.25
BTA-3-C^{14}						
	2 hr	0.28	0.87	3.64*	0.16*	1.01*
	±SD	0.11	0.20	1.96	0.12	0.18
	6 hr	0.30	0.89	1.29*	0.20	0.53
	±SD	0.09	0.15	0.51	0.13	0.10
	24 hr	0.13*	0.57	0.31	0.11	0.14
	±SD	0.09	0.29	0.16	0.05	0.13

[1] expressed as % of administered dose per gm of protein giving mean ±SD

n=8 unless marked * where n=7

**n=6

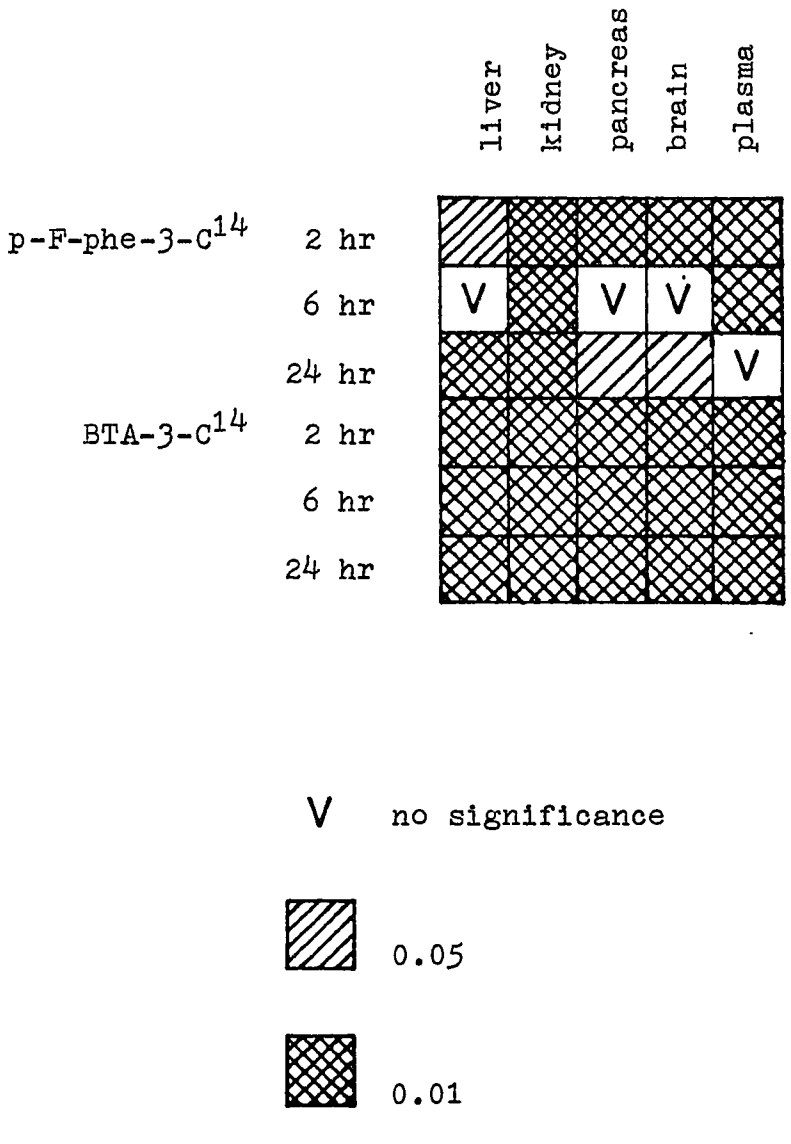


Figure 22

Summary of the Results of the T Test for Significance between Values for C^{14} Content of the Proteins of Normal Rats after the Injection of DL-phenylalanine-3-C^{14} or a Radioactive Analogue.

values for liver, brain and plasma were only slightly below those obtained after the injection of DL-phenylalanine-3-C^{14} (Table 21). The significance of the results is shown in Figure 22. The 2 hour values for kidney and pancreas proteins are considerably lower than those given in Table 21. The pancreas had the highest level of radioactivity of all of the samples. This agreed with the results obtained by Hausson et al (56) during their investigation of the mouse pancreas.

There was less radioactivity in the proteins when p-fluorophenylalanine-3-C^{14} rather than DL-phenylalanine-3-C^{14} was injected. This was expected since the radioactive content of the CO_2 and urine was higher in the former case (Tables 4 and 6). Further, in vivo, Westhead and Boyer (58) obtained only 16-25% replacement of phenylalanine by p-fluorophenylalanine for two rabbit enzymes. Previc (60) reported that p-fluorophenylalanine can be incorporated into E. coli proteins only as a partial substitute for phenylalanine.

The values given in Table 23 present a rather interesting picture of the quantity of radioactivity incorporated over the 24 hour period. For all except the pancreas samples there was an increase in the radioactivity from 2 to 6 hours followed by a decrease by 24 hours.

The radioactivity in the proteins at 2 hours could be the result of the incorporation of p-fluorophenylalanine-C^{14}.

This possibility will be discussed in chapter 6. The increase in the radioactivity at 6 hours may have been caused by the incorporation of more tyrosine-3-C^{14} which was derived from the injected p-fluorophenylalanine-3-C^{14}. The slower *in vitro* rate of conversion of p-fluorophenylalanine into tyrosine, compared with that of phenylalanine into tyrosine (65), could have partially accounted for the fact that when phenylalanine-3-C^{14} was injected the level of radioactivity was generally at its highest after 2 hours (Table 21).

The decrease in the radioactivity in the pancreas proteins from 2 to 6 hours was not so great as that obtained when DL-phenylalanine-3-C^{14} was used. This was possibly caused by the incorporation into protein of tyrosine-C^{14} derived from p-fluorophenylalanine-C^{14}. Also, the radioactivity of the kidney proteins did not show the gradual increase with time found when DL-phenylalanine-3-C^{14} was injected. This may indicate that the D-p-fluorophenylalanine could not be efficiently used by the organism. It is possible that the higher level of radioactivity in the urine (Table 23) was caused by the excretion of D-p-fluorophenylalanine.

After the injection of a second phenylalanine analogue, BTA-3-C^{14}, into normal rats the amount of radioactivity incorporated into the proteins was very low (Table 23). The significance of these results compared to those obtained

after the injection of DL-phenylalanine-3-C^{14} is shown in Figure 22. The total retention of radioactivity in the organs of animals injected with BTA-3-C^{14} was also extremely low (Table 15).

Only trace amounts of radioactivity were found in the proteins of the liver, kidney and brain by 2 hours. These values decreased with time. The pancreas was the only organ able to use BTA for protein synthesis to any appreciable extent. This was probably because the amino acid pool concerned with protein synthesis was in equilibrium with the plasma amino acid pool of the pancreas but not with that of the liver and kidneys. Therefore, in the kidneys and liver the BTA would not have been able to enter the amino acid pool for protein synthesis and would not be incorporated into the proteins. If this did occur, an excess of non-radioactive BTA should not influence the incorporation of phenylalanine in the liver and kidney but should influence the incorporation in the pancreas.

The level of radioactivity in the pancreas was relatively high at 2 hours then decreased to 1/10 the original value by 24 hours. This same ratio was true for the plasma proteins. There was no increase in the radioactivity of the proteins of the pancreas or the plasma at 6 hours.

These results, indicating that BTA was incorporated into the proteins in very small quantities, are substantiated by the findings of Wolfe and Hahn (69). These

investigators found that although BTA underwent activation it did not support protein or nucleic acid synthesis. Also Ezekiel (206) reported that BTA drastically inhibited protein synthesis in E. coli within 5 minutes. However, Cohen and Munier (52) found that BTA was incorporated into E. coli proteins while Janeck and Richenberg (207) reported that BTA replaced as much as 50% of the phenylalanine in beta-galactosidase in E. coli.

Although the metabolic pathway of BTA in rats is not known, it is clear that incorporation of BTA itself or of its immediate metabolites into protein was not of much significance in these rats (Table 23).

C. Injected with Phenylalanine-C^{14} and its Non-radioactive Analogues

If the phenylalanine analogue were effective in replacing the phenylalanine in the protein one would expect a decrease in the quantity of radioactivity in the protein and an increase in that of the CO_2 and urine compared with values obtained when only phenylalanine-C^{14} was injected. If the analogue itself were incorporated, the decrease in the radioactivity of the protein would occur earlier than if one of the analogue metabolites were incorporated. The same result would be achieved if the analogue were to inhibit one or more of the enzymes involved in protein

synthesis. The results of this experiment (Tables 24 and 25) may be compared with the results of the injection of DL-phenylalanine-C^{14} alone (Table 21).

a) Phenylalanine-3-C^{14} and its Non-radioactive Analogues

The results from this experiment are shown in Table 24 and discussed statistically in Figure 23. Figure 24 shows the significance of the differences between the 1:10 and 1:20 ratios of amino acid:analogue.

When DL-phenylalanine-3-C^{14} and non-radioactive p-fluorophenylalanine were injected into the rat, there was a decrease in the C^{14} content of the proteins at 2, 6 and 24 hours compared to the values obtained when only DL-phenylalanine-3-C^{14} was used. Coupled with the results obtained with p-fluorophenylalanine-3-C^{14} (Table 23) and from amino acid analysis (section 6) this decrease in the radioactivity indicated that some of the p-fluorophenylalanine or tyrosine derived from p-fluorophenylalanine was incorporated into the protein of the rat. This subject will be discussed in more detail in the chapter on amino acid analysis.

The addition of p-fluorophenylalanine to the DL-phenylalanine-3-C^{14} produced a significant difference in the radioactivity of all of the proteins at 2 and 6 hours (Figure 23). By 24 hours both ratios showed significantly decreased values for the kidney proteins. Only the 1:20

TABLE 24

The Incorporation of C^{14} into the Proteins of Normal Rats after the Injection of DL-phenylalanine-3-C^{14} + a Non-radioactive Analogue

	Ratio		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-3-C^{14} + p-F-phe	1:20	2 hr	2.17(1)	3.43	11.50	0.91	3.61
		±SD	0.36	0.40	4.77	0.14	1.18
	1:20	6 hr	1.87	3.28	9.09	1.08	4.00
		±SD	0.39	0.36	1.80	0.24	0.76
	1:20	24 hr	3.06	4.38	2.84	1.67	5.27
		±SD	0.19	0.46	0.55	0.30	1.18
	1:10	24 hr	3.08	5.89	3.70	0.94	6.37
		±SD	0.51	0.62	1.02	0.76	0.72
DL-phe-3-C^{14} + BTA	1:20	2 hr	3.64	9.31	21.56	0.98	7.34
		±SD	0.47	0.85	2.27	0.19	1.52
	1:20	6 hr	4.32	11.02	15.98	1.24	8.55
		±SD	0.48	2.00	2.66	0.44	1.71
	1:20	24 hr	3.30	6.79	2.71	1.17	5.07
		±SD	0.60	0.99	0.23	0.21	1.49
	1:10	24 hr	3.99	10.36	4.28	3.59	6.81
		±SD	0.46	1.34	1.74	1.84	1.24
DL-phe-3-C^{14} + APBA	1:20	2 hr	2.19	9.79	16.56	1.12	5.65
		±SD	0.31	2.38	3.87	0.33	0.59
	1:20	6 hr	2.04	9.25	8.96	1.01	4.51
		±SD	0.59	1.88	2.53	0.30	0.81
	1:20	24 hr	2.05	4.30	4.72	1.10	3.62
		±SD	0.49	1.79	1.64	0.17	0.25
	1:10	24 hr	2.04	4.45	5.27	1.07	3.87
		±SD	0.18	3.92	2.02	0.32	0.58

(1) expressed as % of administered dose per gm of protein giving mean ±SD
n=8

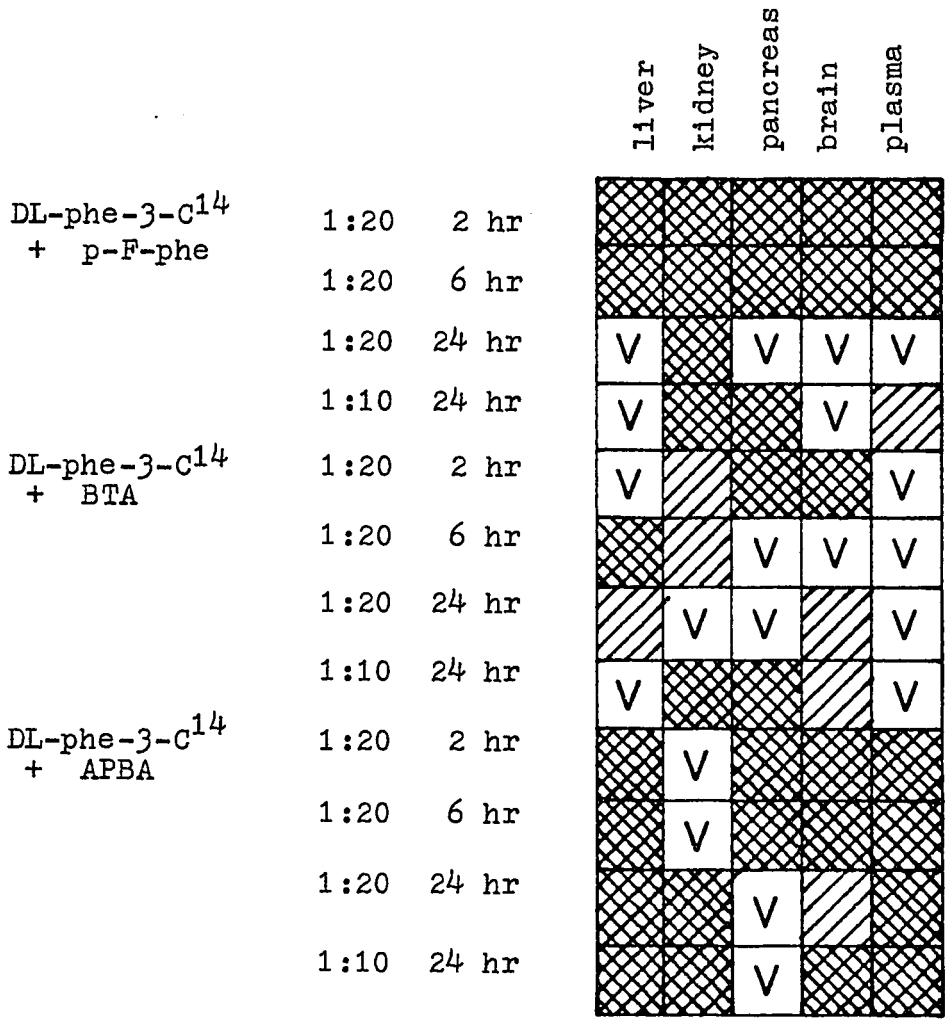


Figure 23

Summary of the Results of the T Test for Significance between Values for C^{14} Content of the Proteins of Normal Rats after the Injection of DL-phenylalanine-3-C^{14} + a Non-radioactive Analogue or DL-phenylalanine-3-C^{14}.

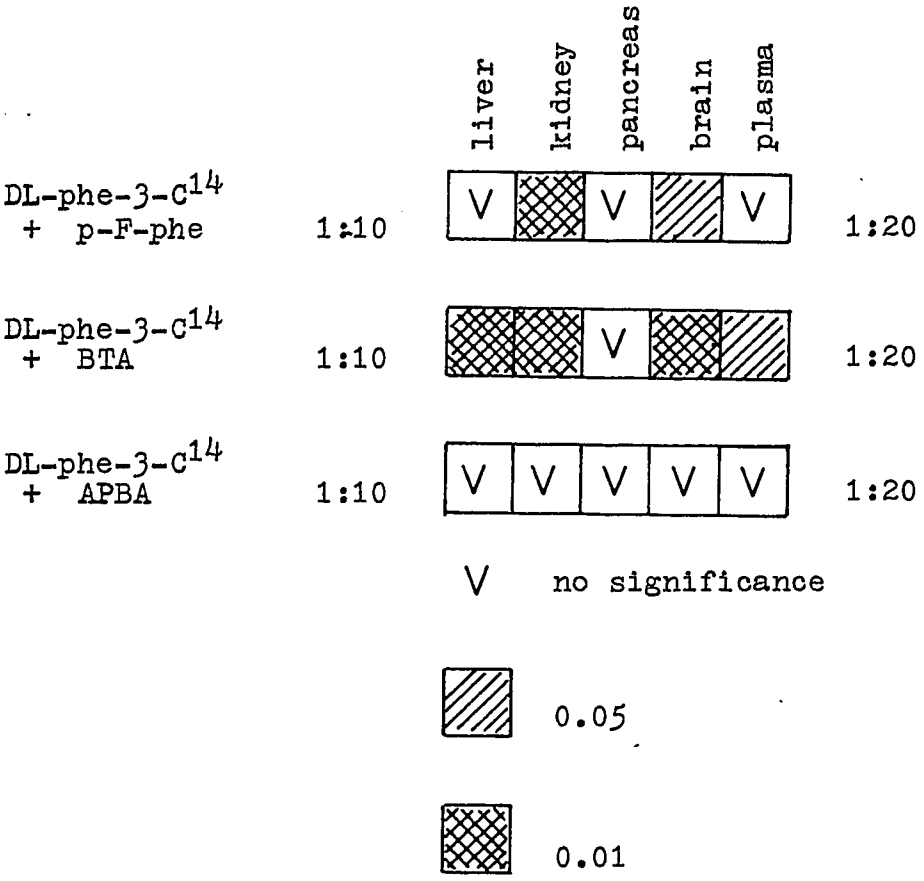


Figure 24

Summary of the Results of the T Test for Significance between Values for C^{14} Content of the Proteins of Normal Rats 24 Hours after the Injection of Amino Acid:Analogue Ratios of 1:10 and 1:20.

ratio produced a significant decrease in the C^{14} in the pancreas and plasma proteins at 24 hours compared to the values produced by DL-phenylalanine-3-C^{14} alone (Table 24). The 1:20 ratio produced the largest decrease in all of the protein samples. By 24 hours there was also a slightly increased release of $C^{14}O_2$ with the 1:20 concentration (Table 7). However the general distribution of radioactivity in the proteins of the liver, kidneys and plasma was the same as when only DL-phenylalanine-3-C^{14} was used. In the liver and kidney there was a decrease in the radioactivity at 6 hours and an increase at 24 hours; in the pancreas the radioactivity slowly decreased over the entire 24 hour period. After 24 hours there was little difference in the radioactivity of the proteins of the liver, pancreas and plasma.

The smaller quantities of radioactivity found in the proteins after the addition of p-fluorophenylalanine to the injection material suggested that some of the p-fluorophenylalanine replaced the phenylalanine in the proteins. This would leave a slightly larger quantity of DL-phenylalanine-3-C^{14} available for metabolism. It is also possible that the conversion of p-fluorophenylalanine to tyrosine would ultimately result in higher tyrosine levels and could therefore account for the slight increase in the radioactivity of the CO_2 and the urine produced when the 1:20 ratio was used (Table 7). This conversion could explain the smaller

quantities of radioactivity in the proteins since the resultant non-radioactive tyrosine would dilute the radioactive tyrosine.

The results obtained after the simultaneous injection of non-radioactive BTA and DL-phenylalanine-3-C^{14} are shown in Table 24 and Figure 23. Comparison of these values with those obtained after the injection of DL-phenylalanine-3-C^{14} alone (Table 21) shows that at 2 hours there were no significant differences in the liver and plasma values and little difference in the kidney values. The differences between the pancreas values were much greater. The radioactivity found in the pancreas when BTA was included in the injection material was approximately twice that obtained after the addition of p-fluorophenylalanine (Table 24). While the level of radioactivity in the pancreas (produced by the addition of BTA) indicated that BTA was able to compete with phenylalanine for incorporation into protein, the level of competition between phenylalanine and p-fluorophenylalanine was greater. It should be pointed out that the pancreas was the only organ capable of incorporating the radioactivity of BTA-3-C^{14} into the proteins with some efficiency (Table 23). At 2 hours there was 37% less C^{14} in the pancreas proteins after the injection of phenylalanine-3-C^{14} + BTA than after the injection of the radioactive amino acid alone. The radioactivity in the pancreas proteins decreased rapidly over the 24 hour period.

With one exception, the other organs contained more radioactivity at 24 hours than at 2 hours.

At 6 hours, the C^{14} values for the proteins paralleled those obtained for the total organ samples since both showed an increase at this time. This was different from the results obtained with DL-phenylalanine-3-C^{14} alone (Table 21) or with DL-phenylalanine-3-C^{14} plus p-fluorophenylalanine (Table 24) since, in these cases, there was no increase in the radioactivity at 6 hours. This displacement of the period of maximum incorporation to 6 hours (after BTA was added) may indicate that BTA slowed down the entire process of incorporation of phenylalanine into the proteins without being itself incorporated to any appreciable extent. At 24 hours the 1:20 ratio produced a significant decrease in the radioactivity of the proteins for all but the pancreas samples.

Although in several cases the addition of the nonradioactive analogue produced a significant decrease in the radioactivity of the proteins (Table 23) compared to the levels obtained for DL-phenylalanine-3-C^{14}, this did not necessarily mean that BTA was incorporated into the protein. Instead, it could indicate that the incorporation of the phenylalanine-3-C^{14} was blocked. This argument seems reasonable when one considers the evidence of Wolfe and Hahn (69) which showed that BTA was capable of

becoming activated by the amino acid activating enzyme but was not appreciably used for net protein synthesis in E. coli. At 2 hours the level of radioactivity in the liver and kidney proteins was identical to that found in the protein when only DL-phenylalanine-3-C^{14} was used. However, the radioactivity of the pancreas proteins was lower when the analogue was added. These results could be explained if, as discussed before, the liver and kidney have a special amino acid pool for protein synthesis (205) which BTA was not able to enter freely.

The results obtained after the addition of the third non-radioactive analogue, APBA, to the injection material are also shown in Table 24 and Figures 23 and 24. Since APBA produced a very large increase in $C^{14}O_2$ (Table 7) one could expect a considerable incorporation of APBA into protein and a decrease in the incorporation of phenylalanine-C^{14}. Except for the kidney there was a significant decrease in the radioactivity of all the proteins at 2 and 6 hours (Table 24).

There were two possibilities: either APBA was incorporated into the protein or else it interfered with the incorporation of the radioactive phenylalanine. When BTA was added it is probable that the latter possibility was involved in reducing the level of radioactivity in the protein. The results of amino acid analysis, which will be discussed in section 6, could determine which of these

possibilities occurred with regard to APBA.

By 24 hours only the pancreas proteins did not show a significant decrease in the radioactivity. At 2 hours the value for the pancreas proteins was approximately ½ that obtained when only DL-phenylalanine-3-C^{14} was injected; there was a steady decrease in the C^{14} from 2 to 24 hours. The levels of radioactivity in the proteins of the plasma, liver and brain varied little throughout the experiment.

The high level of radioactivity in the protein of the kidney, observed at 2 and 6 hours, indicated a very active protein incorporation. This level of incorporation seemed to exceed the values obtained for phenylalanine alone. As will be mentioned later in this chapter, this high level of radioactivity in the kidney was also found when DL-phenylalanine-1-C^{14} was used. The addition of APBA to the DL-phenylalanine-3-C^{14} appeared to stimulate the incorporation of phenylalanine into the proteins of the kidney. If, as previously suggested in this chapter, the free amino acid pool in the kidney is different from that in other tissues, it may be that the pool is unable to pick up high concentrations of APBA immediately.

No significant differences were shown between the two ratios of amino acid to analogue indicating that maximum effects on the level of phenylalanine incorporation were obtained at the 1:10 ratio. This was true only for APBA (Figure 24).

A visual comparison of the mean values for DL-phenylalanine-3-C^{14} plus APBA and DL-phenylalanine-3-C^{14} plus p-fluorophenylalanine at 2 hours (Table 24) shows that, in general, the levels of radioactivity were lower when p-fluorophenylalanine was added to the injection. This indicated that this analogue was capable of replacing phenylalanine or inhibiting its incorporation to a greater extent than was the addition of APBA. However, when APBA was added to the injection material, there was a much greater increase in the radioactivity of the CO_2 (Table 7).

The decrease in the level of radioactivity of the proteins at 2 hours, produced by the addition of APBA to the DL-phenylalanine-3-C^{14}, was equal to that obtained when an excess of L-phenylalanine was added to the injection material (Table 22). Both compounds increased the $C^{14}O_2$ production and the radioactivity of the urine (Tables 4 and 7). An excess of APBA affected the level of radioactivity in the same way as did any excess of L-phenylalanine. There was a decrease in the level of incorporation of radioactivity into the proteins as a result of dilution or of competition and an activation of the catabolism of the radioactive phenylalanine. APBA acted both at the level of protein synthesis and at the level of amino acid oxidation. As will be discussed in the next section, APBA was incorporated into the proteins

of the rat.

b) Phenylalanine-1-C^{14} and its Non-radioactive Analogues

The radioactivity in the proteins after the injection of DL-phenylalanine-1-C^{14} plus a non-radioactive analogue is shown in Table 25. Only the 24 hour samples were obtained in this experiment. Since the radioactive carbon would be lost very early along the metabolic pathway, the radioactivity in the protein would largely be accounted for by the presence of phenylalanine or tyrosine rather than any other radioactive amino acid.

There was little significant difference between the values obtained when DL-phenylalanine-1-C^{14} and DL-phenylalanine-1-C^{14} plus a non-radioactive analogue were used (Figure 25). The only exception was the pancreas which showed a considerably higher level of radioactivity when any one of the analogues was added to the injection.

Whenever the analogue was added, the total C^{14} retained in the pancreas was lower (Table 17) and the protein radioactivity was higher (Table 25) than when only radioactive phenylalanine was used. Therefore one could assume that most of the C^{14} retained in the pancreas was incorporated rather than free in the tissues or in the form of some metabolite which might not be incorporated into protein. The increase in the radioactivity in the pancreas proteins caused by the addition of the non-radioactive analogue to

TABLE 25

The Incorporation of C^{14} into the Proteins of Normal Rats after the Injection of DL-phenylalanine-1-C^{14} and a Non-radioactive Analogue

		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-1-C^{14}	24 hr	2.78(1)	6.70	2.73	1.65	5.61
	±SD	0.74	1.69	0.72	0.48	0.18
DL-phe-1-C^{14}	24 hr	3.40	6.31**	5.24	2.07	0.98**
+ p-F-phe 1:10	±SD	1.78	0.45	2.20	0.57	0.26
DL-phe-1-C^{14}	24 hr	3.24*	6.49**	3.87*	1.76*	5.85
+ BTA 1:10	±SD	0.33	0.32	0.73	0.13	0.41
DL-phe-1-C^{14}	24 hr	2.16	8.50	3.59*	1.20	3.70
+ APBA 1:10	±SD	0.69	2.36	0.79	0.38	0.86

(1) expressed as % of administered dose per gm of protein giving mean ±SD

n=8 unless marked * where n=7

**n=6

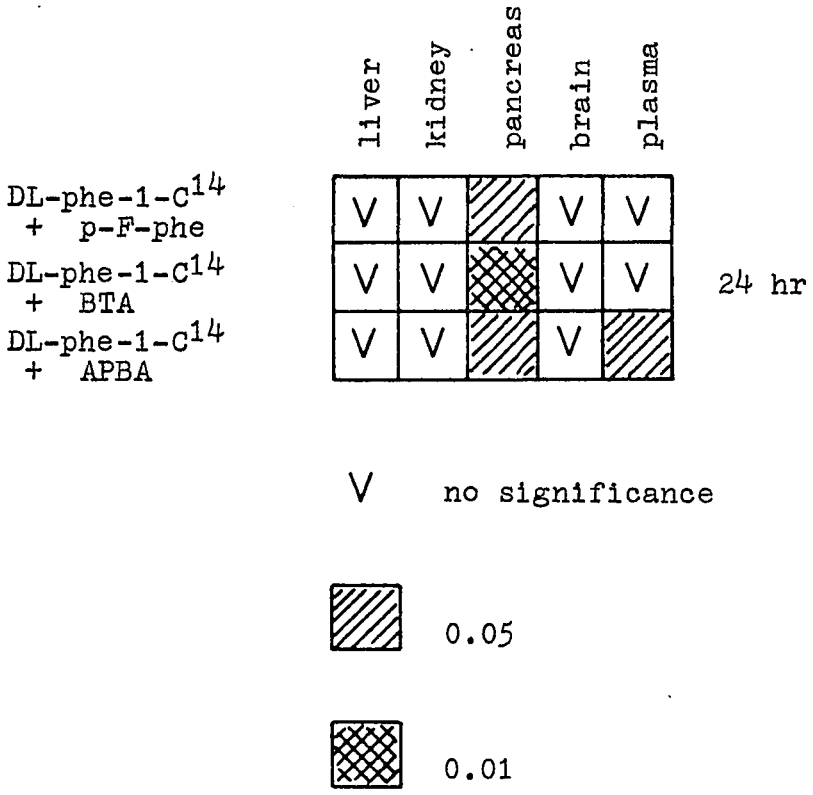


Figure 25

Summary of the Results of the T Test for Significance between Values for C^{14} Content of the Proteins of Normal Rats 24 Hours after the Injection of DL-phenylalanine-1-C^{14} + a Non-radioactive Analogue or DL-phenylalanine-1-C^{14}.

DL-phenylalanine-1-C^{14} differed from the situation produced when the non-radioactive analogue was added to DL-phenylalanine-3-C^{14}. In the latter case (Tables 21 and 24) there was a decrease in the radioactivity in the pancreas after the addition of the analogue. The increase in the radioactivity of the pancreas protein, found when DL-phenylalanine-1-C^{14} plus an analogue was used, could only be explained by an incorporation of radioactive amino acids derived from phenylalanine.

2) PHENYLKETONURIC RATS

A. Injected with DL-phenylalanine-3-C^{14}

The injection of phenylalanine-3-C^{14} into phenylketonuric rats generally produced much lower values (Table 26) than when the same material was injected into normal rats (Table 21). The statistical significance of the results is shown in Figure 26.

With the exception of the brain, all of the values given in Table 26 showed a considerable decrease with time. The lower radioactivity of the proteins of the phenylketonuric rat, compared to that of the normal rat was particularly noticeable in the pancreas. In both the 2 and 24 hour samples the level of C^{14} in the phenylketonuric rat was about 1/3 that in the normal animal. The low C^{14}

TABLE 26

The Level of C^{14} Incorporated into the Proteins of Phenylketonuric Rats after the Injection of Phenylalanine-C^{14} or DL-tryptophan-3-C^{14}

		Liver	Kidney	Pancreas	Brain	Plasma
DL-phe-1-C^{14} PKU rats	2 hr	2.39[1]	3.33	11.85	1.20	4.69
	±SD	0.81	1.83	4.70	0.60	2.80
	24 hr	1.41*	2.94	1.92	1.41	3.04
	±SD	0.23	1.13	0.54	0.57	0.96
DL-phe-1-C^{14} PKU rats	2 hr	1.15	1.47	7.36	0.62	1.96
	±SD	0.38	0.63	0.70	0.19	0.55
	24 hr	1.67	1.98	1.95	1.01	2.20
	±SD	0.40	0.61	0.47	0.32	0.55
L-phe-1-C^{14} PKU rats	24 hr	1.73	2.53	2.41	1.55	2.99
	±SD	0.40	0.84	1.10	0.76	1.25
tryptophan-3-C^{14} normal rats	24 hr	3.15	4.82	4.33	1.65	4.08
	±SD	0.16	0.63	1.12	0.36	0.21
tryptophan-3-C^{14} PKU rats	24 hr	2.91	5.13	5.61	1.51	4.69
	±SD	0.73	1.40	1.47	0.35	0.59

[1] expressed as % of administered dose per gm of protein giving mean ±SD

n=8 unless marked * where n=7

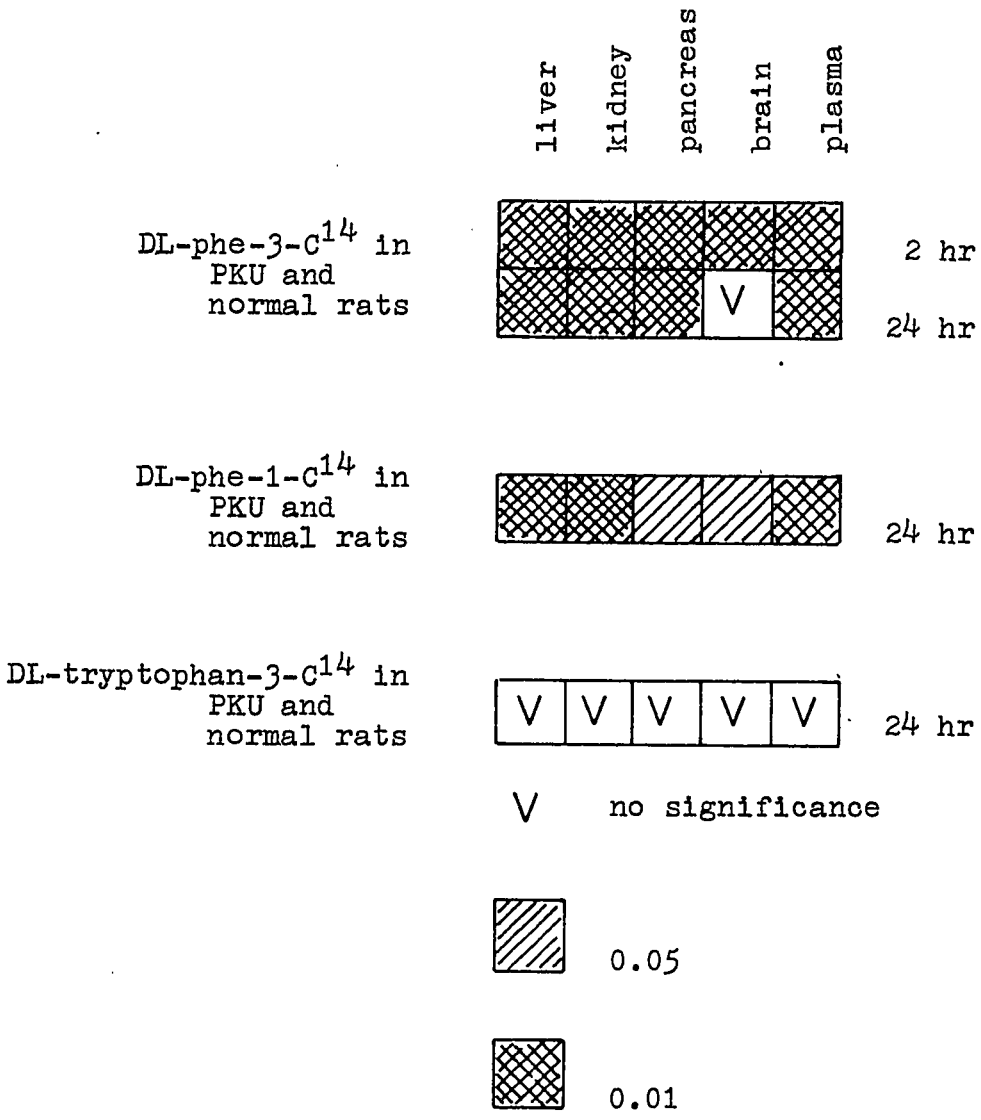


Figure 26

Summary of the Results of the T Test for Significance between Values for C^{14} Content of the Proteins after the Injection of a Radioactive Amino Acid in Normal and Phenylketonuric Rats.

content of the proteins of the phenylketonuric rat was paralleled by the generally small quantity of radioactivity retained in the whole organs (Table 18). However, the radioactivity of the CO_2 and urine was considerably increased (Table 9) over that for normal rats (Table 4). There appeared to be some selectivity in the pathway which the injected phenylalanine took. This was probably caused by the large amount of phenylalanine and tyrosine already in the plasma as a result of the diet (section 1) and may be compared with the alteration in the pattern of distribution of radioactivity in the proteins caused by adding non-radioactive L-phenylalanine to the DL-phenylalanine-3-C^{14} (Table 22). That is, there was a considerable dilution of the radioactive compound therefore less radioactivity was found in the proteins.

B. Injected with DL-phenylalanine-1-C^{14}

When DL-phenylalanine-1-C^{14} was injected into phenylketonuric rats there was less radioactivity incorporated into the proteins than when the same injection was made in normal rats (Table 21). The significance of these differences is shown in Figure 26. By 24 hours the level of radioactivity in all of the proteins of the phenylketonuric rat was significantly lower than for normal rats (Figure 26). This paralleled the lower total C^{14} retained in the phenyl-

ketonuric rat than in the normal rat (Tables 13 and 18).

The quantity of radioactivity in most of the proteins after the injection of DL-phenylalanine-1-C^{14} increased from 2 to 24 hours. This increase in the radioactivity of the proteins did not occur in the phenylketonuric rats injected with DL-phenylalanine-3-C^{14}. The pancreas did not show an increase in the C^{14} content by 24 hours. Instead, the radioactivity decreased with time.

C. Injected with L-phenylalanine-1-C^{14}

When L-phenylalanine-1-C^{14} was injected into phenylketonuric animals there was an increase in the C^{14} content of the protein at 24 hours (Table 26) compared to the levels obtained when the racemic mixture was used. The higher level of C^{14} incorporation may be explained by the fact that all of the phenylalanine had the L-configuration, the form essential for incorporation of the amino acid into protein.

D. Injected with DL-tryptophan-3-C^{14}

No significant differences were found between the radioactivity of the proteins of normal and phenylketonuric rats (Figure 26). There appeared to be a relatively even distribution of the radioactivity among the tissues of the

phenylketonuric rats (Table 18) although there was slightly more C^{14} in the proteins of the plasma, pancreas and kidney than in those of the brain and liver. There appeared to be no significant alteration in the accumulation and incorporation of tryptophan in the phenylketonuric rat.

3) PHENYLALANINE-DEFICIENT RAT

A. Injected with DL-phenylalanine-3-C^{14}

The quantity of radioactivity in the proteins of rats kept on a low phenylalanine diet then injected with DL-phenylalanine-3-C^{14} (Table 27) was surprisingly similar to that of a normal rat (Table 21) following a similar injection.

The similarity of the results was surprising since one would not expect an animal which had been deprived of an essential amino acid for a period of time to incorporate that amino acid into protein at the same rate as a normal animal. Any significant difference which did occur between the two groups of rats (Figure 27) was the result of higher C^{14} levels in the phenylalanine-deficient animals. That the level of radioactivity in the proteins of the phenylalanine-deficient animals was lower than expected could be explained by a possible decrease in the rate of protein synthesis during the interval in which the

TABLE 27

The Level of C^{14} Incorporated into the Proteins of Phenylalanine-deficient Rats after the Injection of DL-phenylalanine-3-C^{14}

	Liver	Kidney	Pancreas	Brain	Plasma
2 hr	4.70[1]	11.24	33.39	2.15	7.47
±SD	0.87	2.12	1.11	0.45	1.51
6 hr	4.66	10.84	3.79	2.93	10.10
±SD	1.32	1.81	5.36	0.44	1.51
24 hr	4.84	9.66	7.63	2.91	8.29
±SD	0.75	1.42	1.00	0.36	2.55

[1] expressed as % of administered dose per gm of protein giving mean ±SD

n=8

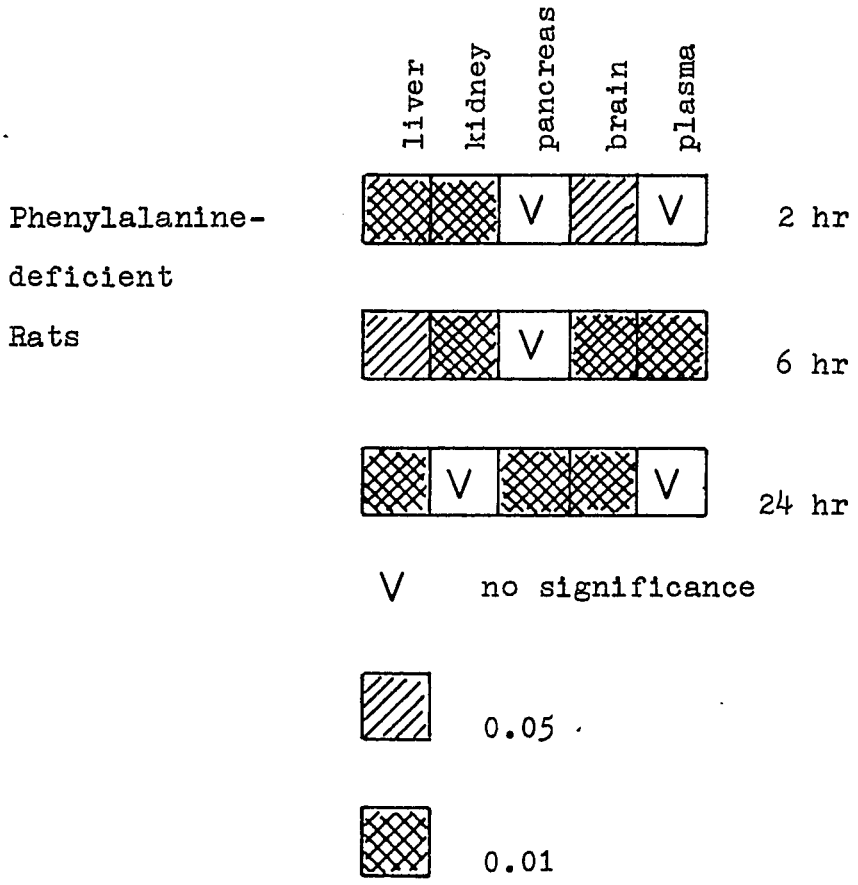


Figure 27

Summary of the Results of T Test for Significance between Values for C^{14} Content of the Proteins after the Injection of DL-phenylalanine-3-C^{14} in Normal and Phenylalanine-deficient Rats.

animals were maintained on the low phenylalanine diet.

6. AMINO ACID ANALYSIS

a. Discussion of Technique

After the injection of a radioactive amino acid or the amino acid analogue some of the proteins were found to be radioactive as was discussed in the previous section. It is known that phenylalanine is converted into tyrosine and that radioactive phenylalanine, injected into animals, will be converted into radioactive tyrosine which can then be incorporated into proteins (205). Udenfriend and Bessman (208) have shown that in dogs, 3 to 24 hours after the injection of phenylalanine-C^{14}, about 20 to 25% of the radioactivity of the proteins was provided by tyrosine which was derived from phenylalanine. In human phenylketonuric patients 24 hours after injection, the ratio of the specific activities of tyrosine and phenylalanine in the plasma proteins was 0.02 compared to a value of 0.22 for control patients. This indicates that there is a much lower conversion of phenylalanine into tyrosine in phenylketonuric patients.

Recently it was shown that the specific activity of the tyrosine-C^{14} (tyrosine-P) derived from phenylalanine-C^{14} and incorporated into plasma albumin increased with time while that of injected tyrosine-C^{14} (tyrosine-T) was constant or else slowly decreased with time (205). As

reported above, the existence of a separate free amino acid pool which could serve for protein synthesis was proposed.

In order to determine the level of incorporation of phenylalanine-3-C^{14} into tyrosine-3-C^{14} in this experiment, plasma and pancreas proteins obtained from 2, 6 and 24 hour samples were hydrolyzed and the radioactivity of the tyrosine and phenylalanine peaks (as shown on the amino acid analyser) was determined. These proteins were chosen because, in most cases, the radioactivity in the pancreas proteins was high at 2 hours then decreased with time, while the high plasma protein values of 2 hours were decreased by 6 hours then increased again at 24 hours.

The results are expressed as the ratio of the total radioactivity in the tyrosine peak over that in the phenylalanine or fluorophenylalanine peak in the same sample. Although Udenfriend et al (205) expressed their results as the ratio of the specific activity of tyrosine to that of phenylalanine the present method of expressing the results seemed preferable since it can safely be assumed that all of the tyrosine-C^{14} was derived from phenylalanine-C^{14} or fluorophenylalanine-C^{14} in this experiment.

The pancreas and plasma proteins were studied after the following injections:

1) In Normal Rats

A. DL-phenylalanine-3-C^{14}

B. DL-p-fluorophenylalanine-3-C^{14}

C. DL-BTA-3-C^{14}

D. DL-phenylalanine-3-C^{14} + p-fluorophenylalanine (1:20)

E. DL-phenylalanine-3-C^{14} + BTA (1:20)

F. DL-phenylalanine-3-C^{14} + APBA (1:20)

2) In Phenylketonuric Rats

A. DL-phenylalanine-3-C^{14}

3) In Phenylalanine-deficient Rats

A. DL-phenylalanine-3-C^{14}

b. Discussion of Results

1) NORMAL RATS

A. Injected with DL-phenylalanine-3-C^{14}

Both radioactive phenylalanine and tyrosine were present in each of the 2, 6 and 24 hour samples of proteins taken from normal rats after the injection of radioactive phenylalanine. A typical curve obtained by determining the radioactivity of the hydrolyzate of the 2 hour pancreas protein sample is shown in Figure 28. The radioactive peaks corresponded to the position of tyrosine and phenylalanine on the chromatogram. The ratio of tyrosine-C^{14} to phenylalanine-C^{14} is given in Table 28.

In the plasma proteins the ratio of tyrosine-C^{14}/

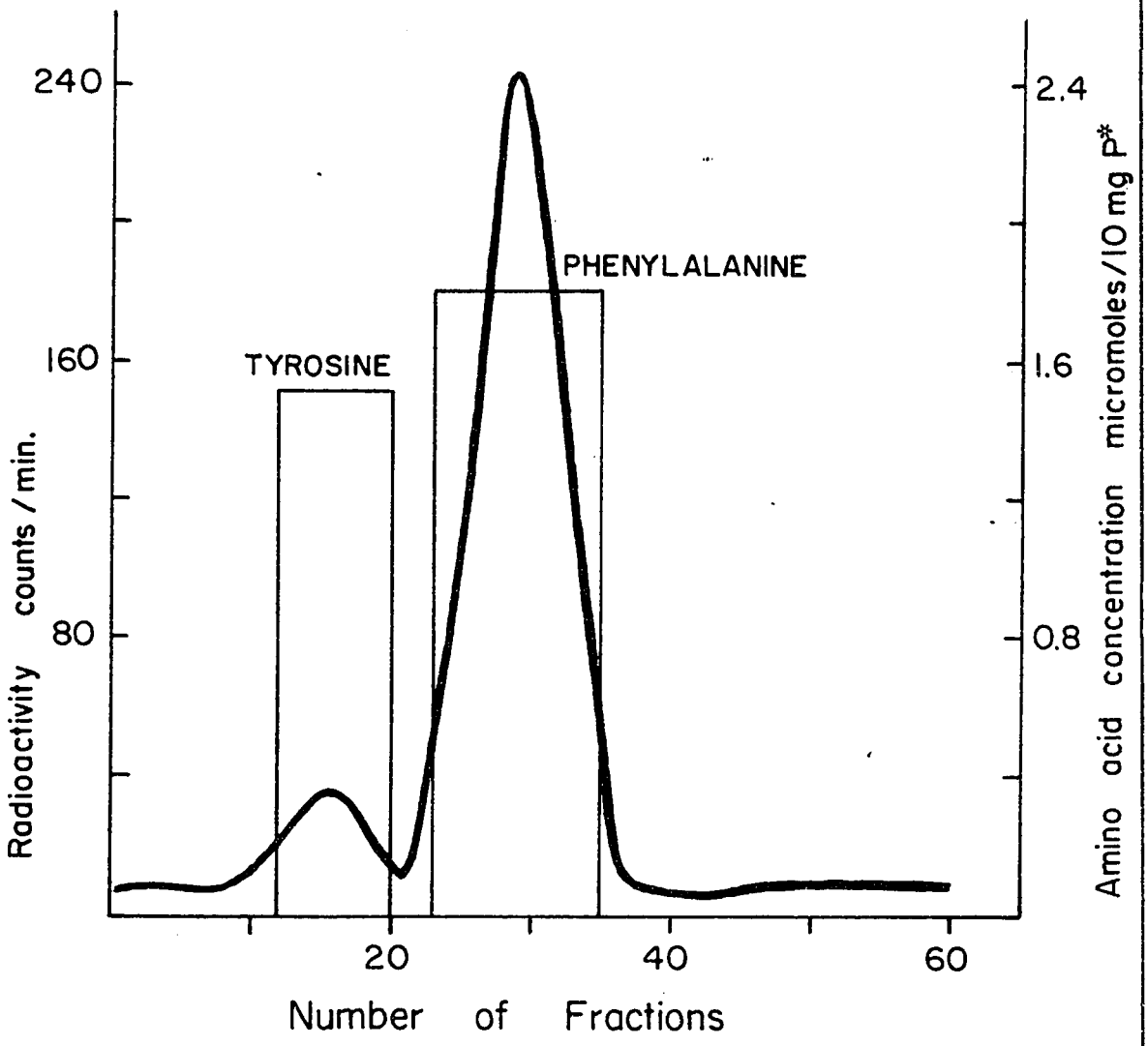


Figure 28

The Radioactivity of a Hydrolyzate of a Pancreas Protein Sample Taken from a Rat 2 Hours after the Injection of DL-phenylalanine-3-C^{14}.

*protein

TABLE 28

Summary of the Ratio of the Quantity of Radioactivity Found in the Tyrosine Peak to that Found in the Phenylalanine or P-fluorophenylalanine Peak after Analysis of Protein Hydrolyzates on the Amino Acid Analyser

Injection	Pancreas			Plasma		
Normal Rats	2 hr	6 hr	24 hr	2 hr	6 hr	24 hr
DL-phe-3-C^{14}	0.15	0.18	0.72	0.28	0.33	0.29
p-F-phe-3-C^{14}	0.32	0.19	0.64	1.42	1.18	0.74
DL-phe-3-C^{14} + p-F-phe (1:20)	0.25	----	0.71	0.34	----	0.55
DL-phe-3-C^{14} + BTA (1:20)	0.14	----	0.60	0.29	----	0.54
DL-phe-3-C^{14} + APBA (1:20)	0.17	0.23	1.36	0.39	0.50	1.77
PKU Rats						
DL-phe-3-C^{14}	0.03	----	0.53	0.13	----	0.25
Phenylalanine-deficient						
DL-phe-3-C^{14}	0.08	0.08	0.67	0.09	0.07	0.38

phenylalanine-C^{14} slowly increased from 2 to 6 hours. Both the 2 and 6 hour ratios for the plasma proteins were twice as high as those for the pancreas proteins. This means that more tyrosine-C^{14} was available at the site of synthesis of some of the plasma proteins, perhaps in the liver as a result of the very active phenylalanine hydroxylase there. By 24 hours, the ratio had slightly decreased from the 6 hour level and was only $\frac{1}{2}$ that found in the pancreas. The ratio in the pancreas at 24 hours was increased over the ratio found at either 2 or 6 hours.

Although the total radioactivity in the pancreas proteins decreased with time (Table 21), the ratio of tyrosine-C^{14}/phenylalanine-C^{14} slowly increased indicating that more and more tyrosine-C^{14}, derived from the injected phenylalanine-C^{14}, was incorporated. That is, as time progressed there was a gradual increase in the percent of the radioactivity in the protein coming from tyrosine-C^{14} derived from injected phenylalanine and a decrease in the percent of the radioactivity coming from injected phenylalanine. As determined from the curves describing the radioactivity of the hydrolyzate, the amount of phenylalanine-C^{14} incorporated into the proteins slowly decreased with time. These curves indicated that, usually, after 24 hours the tyrosine-C^{14} level was higher in the pancreas proteins than in the plasma proteins. At that time, the tyrosine-C^{14} formed in the liver was probably uniformly

distributed throughout the body and the pancreas was able to use the free amino acid from the plasma.

The incorporation of both phenylalanine-3-C^{14} and tyrosine-C^{14} into the proteins was expected since injected phenylalanine-C^{14} is converted to tyrosine-C^{14} and the resultant tyrosine-C^{14} can be incorporated into proteins (205). Udenfriend et al (205) also found that the level of incorporation of the injected phenylalanine-C^{14} decreased with time while that of the tyrosine-C^{14} (produced from the phenylalanine-C^{14}) increased. The ratios (Table 28) indicated that, particularly in the pancreas, as the time progressed the quantity of tyrosine-C^{14} in the proteins became increasingly greater than the quantity of phenylalanine-C^{14}. The level of transformation of phenylalanine to tyrosine in rats, as indicated by the 2 hour plasma ratio in Table 28, was very similar to that observed by Udenfriend and Bessman (208) in dogs. Assuming that the level of tyrosine-C^{14} found in the proteins was directly related to the rate of transformation of phenylalanine to tyrosine the progressively increased ratios would indicate that the level of tyrosine-C^{14} gradually increased as the level of phenylalanine-C^{14} decreased.

B. Injected with P-fluorophenylalanine-3-C^{14}

Analysis of the proteins obtained from rats injected

with p-fluorophenylalanine-3-C^{14} produced two radioactive peaks on the chromatogram. These peaks corresponded to the position of tyrosine and p-fluorophenylalanine (Figure 29). As found *in vitro* (66), no radioactive phenylalanine was detected. It seemed therefore that, *in vivo*, the phenylalanine hydroxylase was able to convert p-fluorophenylalanine to tyrosine directly.

The ratios of radioactive tyrosine/p-fluorophenylalanine (Table 28) indicated that a considerable quantity of p-fluorophenylalanine-C^{14} was converted to tyrosine-C^{14}. Generally, the values obtained when p-fluorophenylalanine-3-C^{14} was injected were much higher than those produced by the injection of DL-phenylalanine-3-C^{14}. The ratios of tyrosine-C^{14}/p-fluorophenylalanine-C^{14} at 2, 6 and 24 hours were very much higher in the plasma proteins than in the pancreas proteins. The ratios for the plasma proteins showed a continual decline over the 24 hour period. This would indicate that there was a decrease in the quantity of tyrosine-C^{14} relative to the quantity of p-fluorophenylalanine-C^{14}. Therefore even if the p-fluorophenylalanine were rapidly transformed into tyrosine, the rate of turnover of the incorporated tyrosine was much faster than that of the incorporated p-fluorophenylalanine.

In the pancreas, where protein synthesis is active, the turnover of tyrosine must have been quite fast since the 2 hour ratio was reduced by 6 hours. That is, either

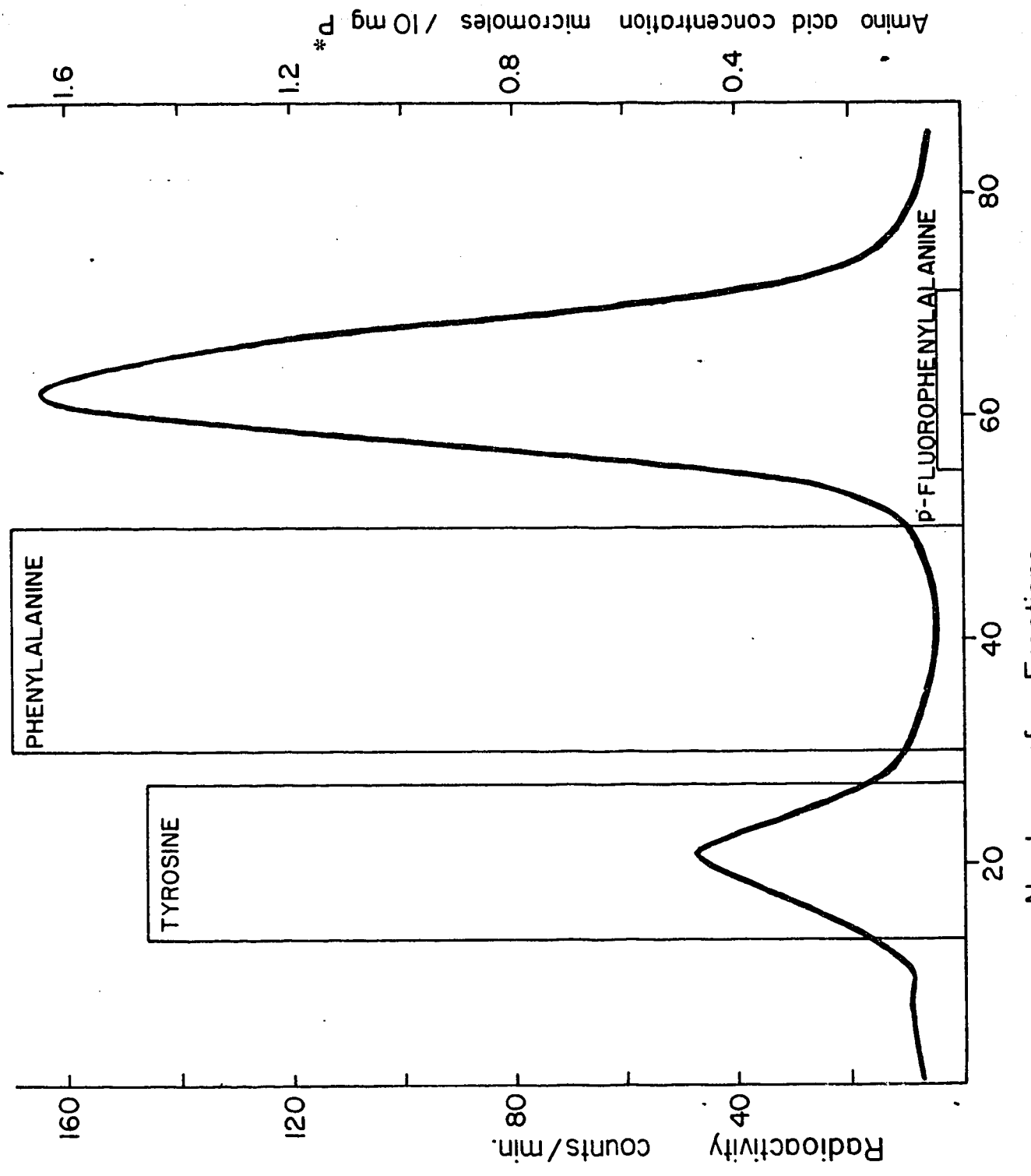


Figure 29

The Radioactivity of a Hydrolyzate of a Pancreas Protein Sample Taken from a Rat 2 Hours after the Injection of p-Fluorophenylalanine-3-C^{14}.

*protein

less tyrosine or more p-fluorophenylalanine was present at 6 than at 2 hours. The subsequent increase in the ratios at 24 hours could indicate that the pancreas used the tyrosine-C^{14} liberated from other proteins, such as the plasma proteins, since the level of the tyrosine-C^{14} in this sample decreased with time.

The chromatograms showed a small peak in the position of p-fluorophenylalanine. Quantitative estimations revealed that in the pancreas proteins after 2 hours 0.13 micromoles of p-fluorophenylalanine were incorporated per 10 mg of protein. In the plasma proteins the level of p-fluorophenylalanine at 2 hours was 0.06 micromoles per 10 mg of protein. That is, less p-fluorophenylalanine was found in the plasma proteins than in the pancreas proteins.

As reviewed in the introduction, p-fluorophenylalanine can be incorporated into animal proteins in vivo (55, 56, 58, 59). Recently, Kaufman (65, 66) found that, in vitro, fluorophenylalanine was transformed directly into tyrosine at about 1/6 the rate of the conversion of phenylalanine to tyrosine. Equal amounts of L-tyrosine and fluoride ions were produced and the reaction was catalyzed by the enzyme phenylalanine hydroxylase. No phenylalanine was formed in this transformation. This work by Kaufman prompted Fruton (209) to write, "The extent to which the incorporation into proteins of label from p-fluorophenylalanine-C^{14} in ex-

periments with E. coli and yeast ... involved prior conversion to a labeled protein amino acid deserves study". The work described in this thesis is probably the first experimental proof of the incorporation into proteins of tyrosine-C^{14} derived from injected p-fluorophenylalanine-C^{14} (210).

That this has never before been reported may be explained by the following facts: 1) Some of the studies of the subject were done on bacterial proteins particularly E. coli which does not contain phenylalanine hydroxylase. 2) Some studies were done on mammalian proteins (in vitro) but most of the systems used were also devoid of phenylalanine hydroxylase. For example, in ovalbumin obtained from the minced oviduct system of a hen, 80% of the radioactivity was recovered as fluorophenylalanine (53). Kruh and Rosa (51) studied the radioactivity in the amino acids of haemoglobin from rabbit reticulocytes using paper chromatography. They found no radioactivity in the tyrosine spot and only a little in the phenylalanine spot. This they attributed to contamination. Using rabbit reticulocyte ribosomes, Arnstein and Richmond (59) reported that p-fluorophenylalanine was incorporated unchanged. 3) In vivo studies in mice, cats and rabbits have been reported. In both mouse pancreas proteins (55) and in cat amylase (56) it was found that, on paper chromatograms, the radioactivity was almost completely confined to the fluoro-

phenylalanine spot. Westhead and Boyer (58) have fed fluorophenylalanine-C^{14} to rabbits for a few weeks and then isolated two muscle enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase. They recovered 87% of the radioactivity of the isolated aldolase in the fluorophenylalanine spot. Non-quantitative elution was blamed for the 13% loss. When amylase was treated by carboxypeptidase, only 10% of the radioactivity which could be expected if fluorophenylalanine had completely replaced the C-terminal tyrosine was obtained. It was concluded the fluorophenylalanine cannot replace tyrosine in the peptide chains. These results could also be taken as proof of incorporation into amylase of tyrosine-C^{14} derived from fluorophenylalanine-C^{14}.

C. Injected with BTA-3-C^{14}

The only proteins which contained much radioactivity after the injection of radioactive BTA were the pancreas and plasma proteins after 2 hours (Table 23).

On chromatograms obtained from the pancreas and plasma protein hydrolyzates there was a small radioactive peak which corresponded to the position of BTA. However, from the chromatograms it was impossible to estimate quantitatively the amount of BTA incorporated into the proteins since the peak was too small for accurate measurements. Never-

theless, these results provided evidence that BTA was incorporated into the animal proteins although the level of incorporation was much lower than that obtained in E. coli (52). A study of the available literature provides no previous indication of the in vivo incorporation of BTA into animal protein. It is probable that this is the first experimental proof that such an incorporation can occur.

D. Injected with DL-phenylalanine-3-C^{14} + P-fluorophenylalanine

When the non-radioactive analogue, p-fluorophenylalanine, was injected at the same time as radioactive phenylalanine the ratios of tyrosine-C^{14}/phenylalanine-C^{14} at 2 hours in both the pancreas and plasma were higher than those obtained when only DL-phenylalanine-3-C^{14} was injected (Table 28). By 24 hours the pancreas values were similar while the plasma ratio was twice as high. At 24 hours the value for the pancreas protein was again higher than that obtained for the plasma. The higher ratios indicated that the quantity of tyrosine-C^{14}, relative to the quantity of phenylalanine-C^{14}, was increased. The excess of p-fluorophenylalanine decreased the radioactivity of the protein (Table 28). The presence of more tyrosine-C^{14} would indicate that the analogue, which was also incorporated into proteins, inhibited the incorporation of some of the phenylalanine-C^{14} and favoured its conversion

to tyrosine-C^{14} which could then be incorporated. Therefore, over the 24 hour period, the conversion to tyrosine was considerably increased by the presence of the analogue. It must also be recalled that in the presence of an excess of p-fluorophenylalanine, there was an increase in the radioactivity of the CO_2 and urine (Table 7). These results indicated that there was a stimulation of the catabolism of phenylalanine-C^{14} by the addition of the p-fluorophenylalanine to the injection.

The partial inhibition, by p-fluorophenylalanine, of the incorporation of phenylalanine into the proteins was expected since the analogue can be incorporated into bacterial and mammalian proteins to replace phenylalanine (55, 56, 58, 59); Cohen and Munier (52) obtained a replacement of 72.5% of the phenylalanine in the proteins of E. coli using p-fluorophenylalanine while Westhead and Boyer (58) obtained a 25% replacement in the rabbit aldolase.

E. Injected with DL-phenylalanine-3-C^{14} + BTA

When non-radioactive BTA was added to the injection, the ratios of tyrosine-C^{14}/phenylalanine-C^{14} in both the pancreas and plasma proteins were the same as those obtained when DL-phenylalanine-3-C^{14} was injected alone (Table 28). However, after 24 hours, there was a big increase in the ratio of tyrosine-C^{14}/phenylalanine-C^{14} in the plasma

proteins comparable to that obtained when p-fluorophenylalanine was added to the injection. In the presence of an excess of BTA there was an increase in the radioactivity of the CO_2 and the urine (Table 7) but no significant changes in the radioactivity of the protein (Table 24). Therefore, it seems that BTA did not inhibit the incorporation of phenylalanine-C^{14} into the proteins to any extent but it did stimulate the catabolism of tyrosine.

That the quantity of phenylalanine incorporated into proteins at 2 hours was unchanged by the addition of BTA was not what one would expect if, as found in E. coli, the BTA were able to combine with the amino acid activating enzyme, as outlined by Wolfe and Hahn (69), or were able to be incorporated into the protein (52). However, the 2 hour pancreas sample contained the only significant quantity of radioactivity in any of the proteins (Table 23).

F. Injected with DL-phenylalanine-3-C^{14} + APBA

APBA, when simultaneously injected with phenylalanine-C^{14}, stimulated the production of radioactive CO_2 and urine (Table 7) and decreased the incorporation of the radioactive amino acid into the proteins (Table 24). When the proteins were hydrolyzed, the ratio of tyrosine-C^{14}/ phenylalanine-C^{14} was higher than that obtained when only phenylalanine-C^{14} was injected - especially after 24 hours

(Table 28). These ratios indicated that much higher levels of tyrosine-C^{14} than phenylalanine-C^{14} were incorporated into the proteins. Again, the plasma proteins always had a higher ratio than did the pancreas proteins. The degree of conversion indicated by the 24 hour ratios was the highest which had been found in this study and indicated a significant stimulation of the transformation of phenylalanine to tyrosine. This confirmed the earlier findings, mentioned in chapter 5, that APBA acted at two different sites. APBA appeared to inhibit the incorporation of phenylalanine and to stimulate the catabolism of tyrosine.

Further evidence that APBA worked at the level of protein synthesis was obtained by studying the chromatograms obtained from protein hydrolyzates. All of the chromatograms had a small peak between the phenylalanine and ammonia peaks. This is the position in which pure APBA is found. When large amounts of the proteins were hydrolyzed, it was possible to estimate quantitatively the APBA which was incorporated. In plasma proteins after 2 hours the APBA represented 0.01 micromoles per 10 mg of dry, TCA insoluble proteins. After 24 hours the level of APBA in the plasma proteins was 0.13 micromoles per 10 mg of protein. In the pancreas proteins APBA represented 0.07 micromoles per 10 mg of protein after 24 hours. Examination of the same protein samples revealed that there

were 1.26 micromoles of tyrosine and 1.70 micromoles of phenylalanine per 10 mg of plasma protein and 1.20 micromoles of tyrosine and 1.74 micromoles of phenylalanine per 10 mg of pancreas protein. It was evident therefore that APBA was incorporated into the proteins to a small extent probably replacing some of the phenylalanine-C^{14}.

2) PHENYLKETONURIC RATS

A. Injected with DL-phenylalanine-3-C^{14}

All of the values obtained for phenylketonuric rats were lower than those obtained for normal rats (Table 28). However, as for normal rats, the plasma ratios were higher than the pancreas ratios. After 2 hours, the ratios were very low indicating that very little tyrosine-C^{14} was present compared to the quantity of phenylalanine-C^{14}. After 24 hours the tyrosine-C^{14} level was considerably increased. The very low ratios obtained at 2 hours and the higher values for 24 hour samples could indicate that initially there was a very slow rate of conversion of phenylalanine-C^{14} to tyrosine-C^{14}. This was probably caused by the dilution of the radioactive phenylalanine by the high levels of plasma phenylalanine. It could also be the result of the dilution of the newly formed tyrosine-C^{14} by the excess tyrosine in the plasma.

The ratios for phenylketonuric rats (Table 28) were considerably higher than those obtained by Udenfriend and Bessman (208) in phenylketonuric patients. However, in phenylketonuric men, the quantity of phenylalanine converted to tyrosine is very low since there is no liver phenylalanine hydroxylase. The results (Table 28) also indicated that in the present experiment the activity of the phenylalanine hydroxylase was not decreased to the same extent as that reported by Waisman (167, 168).

3) PHENYLALANINE-DEFICIENT RATS

A. Injected with DL-phenylalanine-3-C^{14}

The great need for phenylalanine for protein synthesis in the rats maintained on a phenylalanine-deficient diet was indicated by the very low ratios of tyrosine-C^{14}/ phenylalanine-C^{14} in the first 6 hours (Table 28). These ratios indicated that there was much less tyrosine-C^{14} than phenylalanine-C^{14} in the proteins. However, the level of radioactivity in the proteins of these phenylalanine-deficient rats was similar to that in normal rats (Table 27). Therefore, within the first 6 hours, the radioactivity in the proteins must be almost entirely the result of the incorporation of phenylalanine-C^{14}. That is, it is probable that more phenylalanine-C^{14} was incorporated into the

protein of the phenylalanine-deficient animal than was incorporated into the protein of a normal animal. The 24 hour ratios indicated that, after a certain time, because of the protein turnover, the phenylalanine-C^{14} liberated from the proteins was transformed into tyrosine-C^{14} then incorporated into protein as such. Again, the level of tyrosine-C^{14} at 2 hours was higher in the plasma proteins; by 24 hours it was higher in the pancreas proteins.

C. SUMMARY AND CONCLUSIONS

1) NORMAL RATS

A. Injected with Phenylalanine-C^{14}

When DL-phenylalanine-3-C^{14} was injected into normal rats only 3% of the dose was released in the CO_2 after 2 hours. This value steadily increased to a total of 9.55% of the dose by 24 hours. After 6 hours, 2.15% of the dose was found in the urine. This totalled 3.51% of the dose by 24 hours. The relatively small amount of radioactivity in the CO_2 and urine indicated that most of the injected phenylalanine-C^{14} was retained in the body.

Generally, the level of C^{14} retained in the whole organ or plasma decreased from 2 to 6 hours then increased at 24 hours to values which were slightly higher than those obtained at 2 hours. At 2 hours the level of radioactivity was highest in the pancreas (4.35% of the dose per g of tissue) and lowest in the brain (0.24% of the dose per g of tissue). The pancreas values differed from the others since they decreased steadily to a 24 hour value of 1.01% of the dose per g of tissue.

In the proteins the highest level of radioactivity was in the pancreas proteins at 2 hours where 34.31% of the dose was incorporated per g of protein. Next highest

were the plasma and kidney samples with values of approximately 8% per g of protein at 2 hours. The radioactivity of the brain and liver proteins was much lower. Parallel to the situation found for the total C^{14} retention, the radioactivity of the pancreas proteins steadily declined over the 24 hour period. Brain and plasma protein values also decreased with time. However, the C^{14} of the liver and kidney proteins decreased by 6 hours then increased by 24 hours to exceed their 2 hour values. This decrease in the radioactivity at 6 hours may indicate that, at first, the C^{14} was rather equally distributed in all of the proteins. By 6 hours, the protein turnover caused a decrease in the C^{14} in the proteins of the pancreas, brain and plasma but an increase in the C^{14} in the liver and kidney. These last 2 organs are the main sites for amino-acid metabolism.

Determination of the quantity of radioactivity in the TCA soluble fraction of the tissues indicated that, after 2 hours, only about 10% of the total radioactivity of the organs was not incorporated into proteins. By 24 hours this was only 3-5%. Therefore most of the injected phenylalanine was incorporated into protein.

Analysis of the hydrolyzates of the pancreas and plasma proteins showed that the phenylalanine-3-C^{14} was converted into tyrosine-C^{14} and this latter compound was incorporated into the protein. The ratio of tyrosine-C^{14} to phenylalanine-C^{14} at 24 hours indicated that, in the plasma

proteins, tyrosine accounted for 20-25% of the radioactivity in the proteins. At 2 hours about 12% of the radioactivity of the pancreas proteins was in the form of tyrosine-C^{14}. This was increased to 42% by 24 hours.

When DL-phenylalanine-1-C^{14} was injected, 5.66% of the dose was released in the CO_2 after 2 hours. This value steadily increased to 13.78% by 24 hours. Urine values increased from 3.01% at 6 hours to 3.63% by 24 hours.

The 24 hour values for the total C^{14} retention showed that, except for the brain, all of the values were slightly lower than those obtained when DL-phenylalanine-3-C^{14} was used.

The level of radioactivity in the proteins was highest in the kidney and blood. Their values were also very high when DL-phenylalanine-3-C^{14} was injected. All of the protein values were only slightly lower than when DL-phenylalanine-3-C^{14} was used.

The higher level of radioactivity in the CO_2 and in the urine obtained when DL-phenylalanine-1-C^{14} rather than DL-phenylalanine-3-C^{14} was used, is explained by the position of the radioactive carbon. The first carbon on the alanine side chain is lost earlier along the metabolic pathway than is the third carbon. The total quantity of radioactivity excreted in the CO_2 after 24 hours never exceeded 15% of the injected dose when either DL-phenylalanine-3-C^{14} or DL-phenylalanine-1-C^{14} was used. This

value was low considering that Sourkes et al (199) found that 20-30% of the radioactivity of injected tyrosine-2 or -1-C^{14} was found in the CO_2 within 3 hours. However, phenylalanine can enter more metabolic pathways than can tyrosine. Also, since phenylalanine is an essential amino acid it cannot be diluted by phenylalanine synthesized in the cells. Tyrosine, the first important metabolite of phenylalanine, is also incorporated into proteins. The evidence reported by Udenfriend et al (209) indicated that tyrosine derived from phenylalanine (tyrosine-P) is not metabolized as quickly as is injected tyrosine (tyrosine-T).

The radioactivity of the urine over the 24 hour period was quite low in both cases. This was a good indication that very little of the D-phenylalanine-C^{14} or the radioactive phenylpyruvic acid formed during the inversion process was lost in the urine. That is, the normal rat was able to use the D-isomer almost as well as the L-isomer.

Since more of the radioactivity of the DL-phenylalanine-1-C^{14} was lost via the CO_2 and urine it was not surprising that the total C^{14} retained in the organs, and consequently the radioactivity of the proteins, was lower than when DL-phenylalanine-3-C^{14} was injected.

When L-phenylalanine-1-C^{14} was injected, 9.18% of the dose was expired in the CO_2 at 2 hours. This increased to

15.41% by 24 hours. The C^{14} content of the urine totalled only 1.46% of the dose by 24 hours.

After this injection the total C^{14} retention in the organs and plasma was lower than when the racemic mixture was used, although the pattern of distribution was similar. The lowest C^{14} value was found in the brain (0.27% of the dose per g of tissue); the highest was in the liver (1.29% per g of tissue).

However, when L-phenylalanine-1-C^{14} was injected the quantity of radioactivity found in the proteins of the liver, pancreas and brain was comparable to that obtained when DL-phenylalanine-1-C^{14} was injected. The quantity of radioactivity in the kidney was only 1/2 that found after the racemic mixture was used. The plasma protein values were also slightly reduced to 3.95% of the dose per g of protein.

Because only the L-isomer was used, all of the compound could be immediately incorporated into protein or metabolized via the tyrosine pathway. This would increase the quantity of radioactivity in the CO_2 and decrease that in the urine. The higher level of C^{14} in the kidney, found when the racemic mixture was injected, may be explained by the fact that the inversion of the D-isomer occurs in the kidney (157, 158). Therefore, in the kidney, more radioactive amino acid was available for incorporation into protein.

Using L-phenylalanine-1-C^{14} permitted an evaluation of

the contributions of the D and L-isomers of the racemic mixture to the radioactivity of the CO_2 and urine. After 2 hours, the L-isomer accounted for 81% of the $C^{14}O_2$. This value decreased over the 24 hour period to 62.42% at 6 hours, 60.90% at 12 hours and 55.95% at 24 hours. That is, after some time the percent contributions to the $C^{14}O_2$ were nearly equal. However, this was never true for the urine; at 6 hours 80.40% of the radioactivity came from the D-isomer. By 24 hours this was only reduced to 79.89%.

Compared to the values obtained when only DL-phenylalanine-3-C^{14} was injected, the addition of an excess (20X) of the L-isomer increased the 2 hour $C^{14}O_2$ 272% to 10.61% of the dose. By 24 hours, 22.94% of the dose was released in the CO_2. The radioactivity in the urine also increased although not to the same extent. By 24 hours, 5.39% of the dose was excreted in the urine.

The total radioactivity in the organs and plasma was lower than when only the radioactive amino acid was injected but followed the same pattern of distribution. The highest value, 2% of the dose per g of tissue, was found in the kidney at 24 hours.

The C^{14} content of the proteins was also lower when an excess of the L-isomer was injected. This was especially noticeable in the pancreas where values were reduced from 34.31% to 14.58% per g of protein at 2 hours. By 24 hours there was little difference in the values for the two samples.

The pattern of distribution of radioactivity was not altered by the addition of non-radioactive phenylalanine to the injection.

When the D-isomer was added to the DL-phenylalanine, the $C^{14}O_2$ released at 2 hours increased by only 94% over the value obtained when only the radioactive amino acid was injected. By 24 hours, 14.97% of the dose was expired in the CO_2 while 7.87% was excreted in the urine.

In all of the samples, the total C^{14} retained increased at 6 hours then fell to a lower value by 24 hours. The results were generally lower than when the radioactive amino acid was injected alone or with the L-isomer.

When the D-isomer was used, the radioactivity of the proteins was usually lower than when the L-isomer was added to the injection material. Compared to the values obtained when only DL-phenylalanine-3-C^{14} was injected, the brain and plasma values were only half as high - 0.88% and 4.51% per g of protein at 2 hours. The kidney value was much lower - only 2.50% per g of protein at 2 hours. The pancreas level was reduced from 34.31% to 16.56% per g of protein at 2 hours. The relative distribution of C^{14} in the proteins did not differ much after the addition of the D-isomer.

The addition of an excess of either non-radioactive isomer stimulated the metabolism of the radioactive amino acid. Higher $C^{14}O_2$ values were obtained with the L-isomer since the D-isomer must be inverted before it can be

metabolized via the tyrosine pathway. The formation of large quantities of phenylpyruvic acid during this conversion process probably accounted for the increase in the C^{14} in the urine when an excess of D-phenylalanine was used since the excess phenylpyruvic acid would be excreted via the urine.

The increase in the amount of C^{14} retained in the organs at 6 hours, coupled with the decrease in the quantity of C^{14} incorporated into the proteins indicated that the excess D-phenylalanine was responsible for the accumulation of D-phenylalanine-C^{14} in the tissue. Therefore less C^{14} was available for incorporation into the proteins. That the amount of radioactivity in the protein was reduced by the addition of an excess of either the D or L-isomer of phenylalanine was probably the result of a dilution of the radioactive phenylalanine by the non-radioactive phenylalanine and a resultant competition for incorporation.

B. Injected with P-fluorophenylalanine-3-C^{14}

After the injection of p-fluorophenylalanine-3-C^{14} into normal rats, 6.87% of the dose was found in the CO_2 at 2 hours. By 24 hours this had increased to 24.77%. These values were more than twice those obtained for DL-phenylalanine-3-C^{14}. The urine values were also higher when the radioactive analogue was injected. By 24 hours

6.85% of the dose was found in the urine.

The total C^{14} retention in the organs followed the same pattern as that for DL-phenylalanine-3-C^{14}. That is, with the exception of the pancreas proteins, the 2 hour values decreased at 6 hours then increased again at 24 hours. In all cases the results were lower when the analogue was used. The highest value (3.59% of the dose per g of tissue) was found in the 2 hour pancreas samples.

The quantity of C^{14} in all of the proteins was lower than when DL-phenylalanine-3-C^{14} was injected. The value for the pancreas proteins at 2 hours was 19.52% of the dose per g of protein compared to 34.31% per g of protein when radioactive phenylalanine was used.

The amount of radioactivity incorporated into all of the proteins, except the pancreas, increased from 2 to 6 hours. In the pancreas there was only a very small difference between the 2 and 6 hour protein samples compared to that obtained between the 6 and 24 hour samples.

Analysis of the plasma and pancreas proteins revealed that some p-fluorophenylalanine-C^{14} and some tyrosine-C^{14} had been incorporated into the protein. Ratios of tyrosine-C^{14} to p-fluorophenylalanine-C^{14} were very high. The 2 and 6 hour plasma ratios were 1.42 and 1.18 respectively. That is, the quantity of tyrosine-C^{14} exceeded that of p-fluorophenylalanine-C^{14}. The radioactive analogue was the only possible source of the tyrosine-C^{14}. It is known that,

in vitro, the conversion of p-fluorophenylalanine to L-tyrosine (65) occurs at 1/6 the rate of the conversion of phenylalanine to tyrosine (66). Udenfriend and Bessman (205) studied the conversion of phenylalanine-C^{14} to tyrosine-C^{14} and found that about 1/4 of the radioactivity of the protein was provided by tyrosine derived from phenylalanine. Also, in vitro, p-fluorophenylalanine itself can be incorporated into the proteins possibly replacing some of the phenylalanine. The incorporation of tyrosine-C^{14}, derived from p-fluorophenylalanine-C^{14}, into protein has never before been shown (210).

The high ratios of tyrosine-C^{14}/p-fluorophenylalanine-C^{14} in the plasma at 2 and 6 hours were likely the result of a very rapid rate of conversion of p-fluorophenylalanine to tyrosine in the liver - the site of synthesis of many plasma proteins. That these ratios exceeded those of tyrosine-C^{14}/phenylalanine-C^{14}, obtained when DL-phenylalanine-3-C^{14} was injected, indicated that the rate of conversion of p-fluorophenylalanine to tyrosine probably did not proceed at only 1/6 the rate of the phenylalanine to tyrosine reaction. In fact, assuming that the level of incorporation of the amino acid into protein was an indication of the rate of conversion of p-fluorophenylalanine into tyrosine, the rate of conversion seemed to exceed that of phenylalanine.

That a high level of tyrosine-C^{14} was found incorpor-

ated into rat protein may seem surprising since other investigators, working on the incorporation of fluorophenylalanine-C^{14} into animal proteins have not detected the presence of any tyrosine-C^{14}. One possible explanation is that the rat liver contains 12 to 16 times as much phenylalanine hydroxylase as that of other species (190) and this was the first time that the incorporation of p-fluorophenylalanine-C^{14} was studied in rat proteins.

The high levels of radioactivity in the CO_2 and urine, and the low values for the 2 hour protein samples, indicated that, while some of the radioactive analogue was incorporated into proteins, most of it was converted into tyrosine-C^{14}. The incorporation of this tyrosine-C^{14} could explain the increase in the C^{14} content of the proteins at 6 hours. Since less p-fluorophenylalanine than phenylalanine was incorporated, there was more tyrosine-C^{14} available for metabolism in the former case, therefore more $C^{14}O_2$ was produced.

The higher levels of C^{14} in the urine after the analogue was used suggested that some difficulty was involved in using the D-isomer. However, the value was only 6.85% by 24 hours, a figure which represented only a small portion of the total C^{14} injected in the D-form.

It is concluded that the metabolism of p-fluorophenylalanine-3-C^{14} proceeded at a faster rate than that of phenylalanine-3-C^{14} although both reactions followed

the same route (210).

C. Injected with BTA-3-C^{14}

Only trace quantities of radioactivity were found in the CO_2 of rats injected with BTA-3-C^{14}; the total $C^{14}O_2$ expiration after 24 hours was 0.58% of the dose. However, the urine values were very high; the 2 hour value of 29.54% of the dose increased to 38.50% by 24 hours.

The total C^{14} retention in the organs and plasma was also very low. The highest value was 1.47% of the dose per g of pancreas at 2 hours. No C^{14} was found in the brain after 24 hours.

The quantity of radioactivity found in any of the proteins was very small. Most of the values were less than 1% of the dose per g of protein with the exception of the 2 hour plasma protein (1.01%) and the 2 and 6 hour pancreas proteins (3.64% and 1.29% per g of protein).

Amino acid analysis of hydrolyzates of 2 hour samples of pancreas and plasma proteins showed the presence of radioactive BTA in the proteins. However, since the amount of BTA was very small, quantitative measurements were difficult.

This incorporation of BTA into pancreas and plasma proteins in vivo was very low compared to that obtained

by Cohen and Munier (52) in E. coli, but would be expected if the BTA underwent activation by the amino acid activating enzyme without actually being incorporated into proteins in any quantity as was suggested by Wolfe and Hahn (69) after their studies with E. coli.

The large quantities of radioactivity in the urine indicated that this was a major route of excretion of the C^{14}. The investigation of the urine to identify these radioactive compounds would contribute much to the understanding of the pathway of metabolism of BTA.

From the low $C^{14}O_2$ values, it appeared that decarboxylation or oxidative decarboxylation (the normal pathway of phenylalanine and tyrosine metabolism) was of little importance in the metabolism of BTA. By 24 hours the location of up to 50% of the C^{14} was not known.

It can be concluded that the metabolism of BTA-C^{14} proceeded at a very slow rate compared to that of phenylalanine-C^{14}. The route which the analogue followed was not determined.

D. DL-phenylalanine-C^{14} + its Non-radioactive Analogues

Compared to the results obtained when only phenylalanine-3-C^{14} was used, the addition of p-fluorophenylalanine to the injection material increased the radio-

activity of the CO_2 and urine in every sample when the 1:20 ratio was used. Only after 12 hours was there a significant increase in the $C^{14}O_2$ when the 1:10 ratio was used. The increase was always greater when the 1:20 concentration was used. The $C^{14}O_2$ value at 2 hours, with the 1:20 ratio, was 3.51% of the dose. This increased to 11.36% after 24 hours. Each of the urine values with the 1:20 ratio was approximately twice the corresponding figure obtained when only phenylalanine was injected.

The total quantity of C^{14} in all of the samples of the plasma and organs was considerably lower than when only phenylalanine-3-C^{14} was injected. The highest value was 1.60% per g of tissue in the pancreas at 2 hours. The pancreas values decreased with time, however, for all of the other samples, the 24 hour values exceeded the 2 hour values.

The protein samples also contained less radioactivity than when DL-phenylalanine-3-C^{14} was used. Most of the 2 hour values were reduced to 1/2 their former level: the liver contained 2.17% of the dose per g of protein, kidney, 3.43%; brain, 0.91% and blood, 3.61%. The pancreas sample was reduced by 2/3 - from 34.31% to 11.50% per g of protein at 2 hours. Over the 24 hour period the pancreas values decreased while the liver and kidney values decreased at 6 hours then increased at 24 hours to values exceeding the 2 hour values. The brain and

plasma values steadily increased with time. By 24 hours the 1:20 concentration resulted in less radioactivity in the proteins than did the 1:10 ratio.

Amino acid analysis of the pancreas and plasma protein hydrolyzates produced higher ratios of tyrosine-C^{14}/ phenylalanine-C^{14} for all except the 24 hour pancreas proteins compared to the ratios for only DL-phenylalanine-3-C^{14}. That is, the incorporation of phenylalanine-C^{14} was probably inhibited by the presence of the analogue although the conversion to tyrosine-C^{14} was increased.

Including BTA in the injection also increased the values for CO_2 and urine compared to the values for phenylalanine-3-C^{14}. This increase occurred in all of the samples and was larger when the 1:20 ratio was used. All of the increases were larger than when p-fluorophenylalanine was added to the injection. The 2 hour $C^{14}O_2$ value for the 1:20 concentration was 4.94%; this increased to 11.67% by 24 hours. Urine values rose from 6.04% of the dose at 6 hours to a total of 8.40% by 24 hours.

The total C^{14} retention was lower than when only phenylalanine was injected but not so low as when p-fluorophenylalanine was added. All of the values were highest at 6 hours. The 1:10 ratio produced higher C^{14} levels than did the 1:20 ratio at 24 hours.

Some of the 2 hour protein results were lower than those found when DL-phenylalanine-3-C^{14} was used, however,

the liver and kidney values were actually higher. Also, while the pancreas protein value was reduced (from 34.31% to 21.56%) the difference was not so great after the addition of p-fluorophenylalanine or APBA. Brain, plasma, kidney and liver proteins had their highest values at 6 hours. Pancreas values decreased with time. Again, the 1:10 ratio gave higher values than did the 1:20 ratio at 24 hours.

The protein hydrolyzates had the same ratios of tyrosine-C^{14}/phenylalanine-C^{14} as those obtained when only DL-phenylalanine-C^{14} was used. BTA did not inhibit the incorporation of the radioactive amino acid, although it did increase the catabolism of it.

The highest values for the radioactivity in the CO_2 and urine were produced by the addition of APBA to the injection. The 2 hour value at the 1:10 ratio was 8.47%. This increased to 21.68% of the dose by 24 hours. Relative values for the 1:20 ratio were 11.45% and 27.53% of the dose. The total C^{14} excreted in the urine at 24 hours was 6.27% (1:10) and 5.96% (1:20).

The total C^{14} retention was lower in all cases; the 2 hour pancreas value was reduced from 4.35% when DL-phenylalanine-3-C^{14} was used, to 2.26% when APBA was added. Not much difference was noted between the 1:10 and 1:20 ratios at 24 hours.

The amount of C^{14} in the proteins was reduced by the

addition of APBA. After 2 hours, the decrease was similar to that obtained by adding an excess of the L-isomer to the injection material.

Analysis of the protein hydrolyzates revealed that some APBA was incorporated into the proteins. Compared to the values obtained when only DL-phenylalanine-3-C^{14} was used, there was a slight increase in the ratio of tyrosine-C^{14}/phenylalanine-C^{14} in both the pancreas and plasma proteins. By 24 hours, the increases were very large indicating relatively high levels of tyrosine-C^{14}, possibly because some of the phenylalanine-C^{14} was replaced by the APBA. This analogue inhibited the incorporation but stimulated the catabolism of phenylalanine.

When any one of these analogues was injected simultaneously with DL-phenylalanine-1-C^{14}, the changes in the C^{14} distribution, compared to the values obtained when only DL-phenylalanine-1-C^{14} was injected, were similar to the differences produced by adding any one of the analogues to the DL-phenylalanine-3-C^{14} before injection.

To conclude, the decrease in the quantity of C^{14} found in the protein as a result of the addition of a non-radioactive analogue to the phenylalanine-C^{14}, indicated that the analogue was capable of replacing some of the radioactive amino acid in the proteins. In fact, quantities of each analogue were incorporated into

the proteins. Since less phenylalanine-C^{14} was incorporated, more was available to be transformed into tyrosine-C^{14} and metabolized to produce $C^{14}O_2$. An exception occurred when p-fluorophenylalanine was added since the tyrosine formed from the analogue probably diluted the tyrosine-C^{14}.

2) PHENYLKETONURIC RATS

According to Woolley's method (173) for inducing experimental phenylketonuria, infant rats were fed several drops of a 7% L-phenylalanine solution each day. This produced signs of toxicity and frequently the animals died. Analysis of crystals taken from the bladder and ureters showed very high tyrosine and phenylalanine levels. The tyrosine/leucine ratio in the crystals was 41.90, and the phenylalanine/leucine ratio was 14.20. Since it is known that tyrosine is toxic (76) and since the symptoms of tyrosine toxicity, as described by Schweizer (80), accurately describe the condition found in these rats, it was concluded that the high plasma tyrosine level, produced by the high phenylalanine diet, caused the toxicity. This disagreed with the findings of Harper (82) since he claimed that an excess of dietary phenylalanine did not elicit any signs of toxicity. It appears that this is the first experi-

mental proof of phenylalanine toxicity (211).

Using Waisman's method (167, 172), dietary treatment was begun on 21 day old rats. By 35 days all of the animals gave positive tests for phenylketonuria. Although the amino acid levels of the plasma proteins did not vary greatly from those of normal rats, the free amino acid levels of phenylalanine and tyrosine increased rapidly after the initiation of dietary treatment. Prolonged treatment (over 40-45 days) produced higher levels of tyrosine than phenylalanine in the plasma, however, the $FeCl_3$ test was no longer positive.

Experimentally induced phenylketonuria appeared to be a very temporary situation, since even short periods of fasting produced great differences in the free tyrosine and phenylalanine levels in the plasma. In phenylketonuric rats, fasting for 24 hours lowered the tyrosine/leucine ratio from 1.50 per ml of plasma to 0.07 while the tyrosine/phenylalanine ratio fell from 0.90 to 0.03 per ml of plasma. These ratios were 0.49 and 0.09 after a 6 hour period of fasting. The ratios were 0.10 and 0.19 in normal rats.

A. Injected with DL-phenylalanine-3-C^{14}

Compared to the values obtained in normal rats, the injection of DL-phenylalanine-3-C^{14} in phenylketonuric

rats generally produced higher levels of radioactivity in the CO_2 and urine. By 6 hours the level was 17.33% of the dose - an increase of 3 X the normal value. This increase continued to 24 hours, when the $C^{14}O_2$ totalled 25.77% of the dose. The radioactivity of the urine was increased over normal although only a slight increase was noted at 6 hours. By 24 hours 7.37% of the dose was found in the urine.

Total C^{14} retention in the 2 hour plasma samples of the phenylketonuric rats was higher than in normal rats; the liver values were equal and the other organs had lower C^{14} values. All of the 24 hour values were lower than the 2 hour samples.

The radioactivity in the proteins of phenylketonuric rats was lower at 2 hours than in the normal rats. Pancreas samples contained 11.85% of the dose compared to 34.31% in the normal rats. The plasma value was 4.69% - 1/2 of the normal value. By 24 hours all but the brain values were reduced and were considerably lower than those in normal rats.

Amino acid analysis of the protein hydrolyzates showed that the ratio of tyrosine-C^{14}/phenylalanine-C^{14} in the pancreas proteins was very low (0.03). The plasma value was 0.13, which was approximately 1/2 that found in normal rats. By 24 hours these values had increased to 0.53 and 0.25 - ratios which were only slightly below

normal.

The 2 hour lag period in $C^{14}O_2$ production may have been caused by a dilution of the radioactive compound by the high plasma levels of free phenylalanine and tyrosine. Higher than normal C^{14} excretion seemed to indicate an acceleration of phenylalanine metabolism in these phenylketonuric rats.

Lower C^{14} levels in the proteins indicated that there was a considerable dilution of the radioactive amino acid therefore less of the C^{14} compound was incorporated into protein.

The lag in C^{14} utilization at 2 hours was exemplified in the lower tyrosine-C^{14}/phenylalanine-C^{14} ratios in the proteins at 2 hours. Almost no tyrosine-C^{14} was incorporated into proteins at 2 hours. By 24 hours some conversion of phenylalanine-C^{14} to tyrosine-C^{14} had occurred since the ratios were comparable to those found in normal rats.

B. Injected with Phenylalanine-1-C^{14}

There was no lag period in the $C^{14}O_2$ production. By 2 hours 9.58% of the dose was excreted in the CO_2. By 24 hours this value had increased to 42.20%, compared to 13.78% in normal rats. The radioactivity of the urine was also increased above normal. 8.50% of the dose was

found in the urine of the phenylketonuric rat at 2 hours. At 24 hours this totalled 11.86%.

C^{14} retention in all of the samples was much lower than in normal rats. Values decreased from 2 to 24 hours.

All of the protein values were lower than for normal rats. All but the pancreas values were higher at 24 hours than at 2 hours.

Decarboxylation and oxidative decarboxylation appeared to be greatly increased in the phenylketonuric rat. Coupled with results of analysis of free amino acids, the high $C^{14}O_2$ values indicated that the conversion of phenylalanine to tyrosine still occurred in these phenylketonuric animals.

Dilution of the radioactive amino acid by high plasma levels of phenylalanine and tyrosine accounted for the lower C^{14} values in the proteins. The high urine values may have been the result of the formation of radioactive urea utilizing the large quantities of $C^{14}O_2$.

As found in normal rats, more radioactivity was contained in the CO_2, urine and proteins when the L-isomer was used instead of the racemic mixture. This also permitted evaluation of the percent of the radioactivity from DL-phenylalanine-1-C^{14} contributed by each isomer. In the 2 hour $C^{14}O_2$ sample each isomer formed approximately 1/2 of the radioactivity. By 24 hours, 65.94% came from the L-isomer. Urine values showed higher C^{14} contribu-

tions from the D-isomer - 73.53% by 6 hours. Only 8.50% of the dose was excreted in the 6 hour urine sample. This represented 6.45% of the total dose indicating that much of the D-isomer was converted to the L-isomer and metabolized. This accounted for the higher percent contribution of the L-isomer by 24 hours.

Alpha-methyldopa produced a 37% decrease in the radioactivity of the CO_2 after 2 hours. Since this level of inhibition was not very high, it seemed that most of the $C^{14}O_2$ produced by the phenylketonuric rat involved a conversion of phenylalanine to tyrosine and an eventual oxidative decarboxylation.

Injection of parnate, a monoamine oxidase inhibitor, produced a decrease of 50% in the radioactivity of the urine and of 32% in the $C^{14}O_2$. Both of these decreases should not have been caused by the specific action of the drug; rather they must have been the result of a general reduction in the rate of phenylalanine metabolism.

C. Injected with DL-tryptophan-3-C^{14}

There were differences in the level of radioactivity in the CO_2 or proteins of the phenylketonuric rats compared to that of normal rats. However, there was a very large increase in the C^{14} excreted in the urine of the phenylketonuric rats. This was possibly caused by the

excretion of normal tryptophan metabolites other than 5-hydroxyindoleacetic acid (107) or of indoleacetic acid and indolelactic acid, compounds which are found in the urine of human phenylketonurics (106).

From this series of experiments on phenylketonuric rats it was concluded that the experimentally induced condition of phenylketonuria in Wistar rats was of a rather transient nature, and, because of the very high levels of tyrosine found in the blood plasma, did not really simulate the disease as it is found in humans. Under certain conditions, the induction of phenylketonuria produced evidence of a toxic effect from dietary phenylalanine. Generally the radioactivity of the CO_2 and urine was greatly increased over that found in normal rats when DL-phenylalanine-C^{14} was injected. Therefore, some stimulation of the metabolism of this amino acid must have occurred as a result of the high phenylalanine diet. That the C^{14} content of the proteins was lower was probably another result of the high plasma levels of phenylalanine. Tryptophan metabolism differed from normal only in an increase in the C^{14} of the urine, possibly caused by certain abnormal metabolites of tryptophan which are found in the urine of human phenylketonurics.

3) PHENYLALANINE-DEFICIENT RATS

A. Injected with DL-phenylalanine-3-C^{14}

Injection of DL-phenylalanine-3-C^{14} produced slightly lower than normal values for the $C^{14}O_2$ and the radioactivity in the urine although 2 hour $C^{14}O_2$ values were not significantly different. The decrease in the radioactivity of the urine was possibly caused by a more efficient use of the D-isomer (165).

In all but the 2 hour pancreas sample, the C^{14} retention was higher than that of normal animals.

However, little difference was noted between the levels of C^{14} incorporated into the protein in normal and phenylalanine-deficient animals.

Amino acid analysis of the protein hydrolyzates produced very low ratios of tyrosine-C^{14}/phenylalanine-C^{14} at 2 hours. The pancreas ratio was 0.08 while that of the plasma was 0.09. Therefore most of the radioactivity of the proteins was the result of the incorporation of radioactive phenylalanine. That is, very little tyrosine-C^{14} was formed thus indicating the need of the tissues for the essential amino acid and demonstrating once again the remarkable sensitivity and adaptability of the animal organism.

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