

1949

Oxidation of L-tyrosine by liver tissue

Ralph K. Barclay
Iowa State College

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OXIDATION OF L-TYROSINE BY LIVER TISSUE

by

Ralph K. Barclay

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological and Nutritional Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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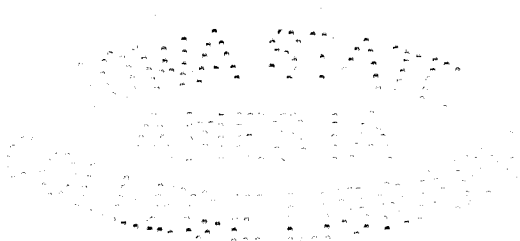
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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENT	ii
I. INTRODUCTION	1
II. HISTORICAL	4
A. The Metabolism of Tyrosine and Phenylalanine in Alcaptonuria	4
B. The Metabolism of Tyrosine <u>in Vitro</u>	33
C. The Relation of Vitamins and Other Dietary Factors to Tyrosine Metabolism	42
D. The Application of Tracer Techniques to Tyrosine Metabolism	56
III. EXPERIMENTAL	64
A. Synthesis of N ¹⁵ -DL-Tyrosine	64
1. 2-Methyl-4-(4'-acetoxybenzal)-5- oxazolone	65
2. p-Acetoxy- α -acetaminocinnamic acid	66
3. p-Hydroxyphenylpyruvic acid	69
4. N ¹⁵ -DL-Tyrosine	70
B. Resolution of N ¹⁵ -DL-Tyrosine	77
1. Preparation of N ¹⁵ -acetyl-DL-tyrosine	78
2. Attempted resolution with brucine	80
3. Development of resolution method with optically-active α -phenylethylamine.	80
a. Synthesis of <u>dl</u> - α -phenylethylamine.	81
b. Resolution of <u>dl</u> - α -phenylethylamine	82
c. Salt formation with α -phenylethyl- amine and acetyltyrosine	83

	Page
d. Resolution with <u>d-α</u> -phenyl-ethylamine	89
C. N^{15} -L-Tyrosine Oxidation <u>in Vitro</u>	91
1. Manometric method	94
2. Fractionation methods	97
3. Isotope analysis	103
4. Experimental observations	109
D. Feeding Experiments with N^{15} -L-Tyrosine.	131
1. Methods	133
2. Results with excess ascorbic acid	136
3. Results of control experiment	138
4. Results with excess pteroylglutamic acid.	140
IV. DISCUSSION	145
V. SUMMARY	153
VI. BIBLIOGRAPHY	155
VII. APPENDIX	166

I. INTRODUCTION

The amino acid, tyrosine, has been shown to be a constituent of many important and necessary protein molecules. The activity of many of these protein molecules, such as enzymes, hormones, and antibodies, has been correlated with the presence and availability of free hydroxyl groups of tyrosine. It is natural, therefore, that the biochemist should wish to know as completely as possible the metabolic handling of this amino acid by the normal animal organism. That he does not know more completely the intermediary metabolism of tyrosine is not due to lack of experimentation, but rather to the extreme difficulty encountered in isolating the several components of the system or systems involved. Neither the enzyme systems participating in the oxidation of tyrosine nor many of the normal intermediate compounds have been isolated in sufficient purity.

It has been shown that tyrosine is oxidatively deaminated by the L-amino acid oxidase systems of rat liver and kidney. On the other hand, there has also been ample evidence obtained to prove that ammonia is not liberated in the oxidation of tyrosine by rat and guinea pig liver and kidney. Since no conclusive evidence has been obtained as to the ability of tyrosine to participate in the transaminase system, the question has arisen and is still unanswered: what happens to the nitrogen of the amino acid when oxidation

takes place in normal organs?

The metabolism of tyrosine has been studied under a great variety of conditions. The discovery that complete oxidation of tyrosine is prevented or deranged in an hereditary abnormality called alcaptonuria provided the first general point of attack on the problem. The discovery that the oxidation of tyrosine in a normal organism is dependent upon the presence of an adequate amount of Vitamin C has been a second major point of attack. Application of these two relationships to feeding experiments, nitrogen balance experiments, and manometric experiments has supplied many of the answers sought.

There is no doubt but that the use of isotopic atoms incorporated into the amino acid molecule constitutes a third major point of attack. Experiments with radioactive carbon in the tyrosine molecule have already been reported, and it is now known that the alpha- and beta-carbon atoms of the side chain and two adjacent carbon atoms of the benzene ring of the tyrosine molecule form acetoacetic acid when this amino acid is incubated with liver slices. Further, one is enabled to give a fairly accurate hypothesis as to the mechanism of such a transformation.

No such catabolic experiments have been reported with the use of isotopic nitrogen, N¹⁵. We have therefore undertaken to synthesize tyrosine containing isotopic nitrogen, resolve it to obtain the natural isomer, and conduct experi-

ments designed to indicate the fate of the nitrogen of the amino acid when it is oxidized by animal tissues.

It is felt that application of all three of the general points enumerated above should furnish information which will go far to elucidate the probable physiological pathways of tyrosine metabolism.

II. HISTORICAL

A. The Metabolism of Tyrosine and Phenylalanine in Alcaptonuria.

Although tyrosine was first isolated in 1846, it was almost fifty years before any clues were obtained as to its fate in the animal organism. Wolkow and Baumann (1), in 1891, isolated homogentisic acid from the urine of an alcaptonuric patient and proved that it arose from tyrosine.

The history of alcaptonuria dates back to 1861, when Bodeker (2) found a reducing substance in the urine of one of his patients which was shown not to be sugar. The urine of this patient, when made alkaline and shaken with air, turned a dark brown color analogously to tannic acid, pyrogallie acid, or hydroquinone. Bodeker isolated the substance responsible for these properties in somewhat impure form by precipitating with basic lead acetate, decomposing the precipitate with hydrogen sulfide, concentrating the filtrate and extracting with ether. He was unable to identify the substance chemically, and named it "alkapton".

Fürbringer (3), working at Heidelberg, reported the qualitative examination of urine from an alcaptonuric, and with no experimental proof decided the substance in the urine was pyrocatechol. Fleischer (4) examined the urine of a patient who had been administered large amounts of sali-

cylic acid, and found the properties of it to be very similar to Bödeker's and Fürbringer's alkapton urine. Thereupon he used Bödeker's isolation procedure to isolate a small amount of a substance which he declared was pyrocatechol from qualitative comparison with a known sample of this substance. Ebstein and Müller (5), working with alcaptone urine, isolated a substance by a slightly different procedure which they decided was also pyrocatechol. They evaporated the urine to a small volume, added absolute alcohol and filtered off the inorganic residue. The filtrate was concentrated and extracted with ether. The ether was evaporated off, leaving a yellow, syrupy mass. Addition of a little water to this left a small amount of white residue (hippuric acid) which was filtered off. The filtrate was acidified with sulfuric acid and allowed to crystallize.

These reports, plus Baumann's article (6) concerning the isolation of pyrocatechol from normal horse urine, prompted Salkowski and Leube (7) to declare "Wahr-scheinlich identisch mit Brenzcatechin ist das Alkapton von Bödeker und Fürbringer". Smith (8), in Dublin, reported on a three-year old alcaptonuric, using the isolation procedure of Bödeker. He decided the substance was not pyrocatechol, but protocatechuic acid (oxyphenic acid). Interestingly enough, he found that the browning action of the urine was more noticeable whenever the

child had a cold or some other slight ailment. It might be well to point out that pyrocatechol, protocatechuic acid, and oxyphenic acid are all the same compound--the compound now known as catechol.

Moving over to England, Kirk (9), in 1886, reported results of his observations on three young children in one family with alcaptonuria; an older brother and the parents were all apparently normal. Using the isolation procedure of Ebstein and Müller (5) he obtained a substance from the urine similar to protocatechuic acid in its properties. However, comparison with authentic samples of both pyrocatechol and protocatechuic acid made Kirk decide that he had something entirely different. So he tried another method of isolation. He acidified the urine with hydrochloric acid and extracted with ether. The ether was evaporated off, leaving large stellate groups of crystals. This acid was very soluble in water and ether, giving yellow solutions. It reduced alkaline copper solutions, darkened in the presence of alkalies, and absorbed moisture when exposed to air. A solution of it gave a very transitory green color with ferric chloride. Since he could not identify this acid with any known to him, he names it "urrrhodinic acid".

Brune (10), attempting to repeat Smith's work, isolated a substance which he could not identify as protocatechuic acid. He considered the substances described

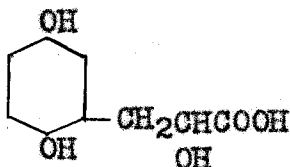
by Bödeker, Ebstein and Müller, Smith, and Kirk were all identical. Since the subject furnishing the urine was and had been in excellent health, Brune considered the excretion of this compound had no pathological significance.

Marshall (11), at the University of Pennsylvania, isolated this reducing substance from the urine of an essentially well man as the lead salt. He found this substance to have about five times the reducing properties of glucose. Although it resembled Kirk's urrhodinic acid, he felt that it was not the same. He suggested as a name for this acid, "glycosuric acid".

Kirk (12) later found that his urrhodinic acid was a mixture of two or more compounds. From this mixture he isolated a substance melting at 133° , colorless crystals, analyzing $C_9H_{10}O_5$. He named this compound "uroleucine acid".

With this background Wolkow and Baumann (1) finally obtained what we know now to be the correct answer to the problem. They isolated a substance from alcaptone urine which they identified as hydroquinone acetic acid. Their isolation procedure was similar to Kirk's and Marshall's. They acidified the urine with sulfuric acid and extracted with ether. The oil left on evaporation of the ether was dissolved in water and lead acetate added. The lead salt of the acid was filtered and washed, suspended in water and decomposed with hydrogen sulfide. The filtrate was boiled to expel the hydrogen sulfide and the solution concentrated to

crystallization. They did an elementary analysis of the compound, finding $C_8H_8O_4 \cdot H_2O$. It melted at $146.5-147^\circ$, gave a blue color with ferric chloride, reduced silver nitrate but not bismuth salts, and gave a dark brown color with alkali. They showed it to be a mono-basic acid, and to have two hydroxyl groups. Of the possibilities allowed from the foregoing facts, they eliminated methylgentisic acid by a potassium hydroxide fusion, from which they obtained gentisic acid and a little hydroquinone. Thus they concluded that they had hydroquinoneacetic acid. Since this compound is the next higher homologue of gentisic acid, they named the compound homogentisic acid. They made the lead salt, the ethyl ester, and the lactone of the acid, and proved the structure of the acid by synthesis. They then showed by feeding tyrosine to the alcaptonuric that this amino acid was the source of the homogentisic acid. They considered Kirk's uroleucine acid to be a trihydroxyphenyl propionic acid. Huppert (13) showed later by qualitative tests and degradation studies that uroleucine acid was actually hydroquinone lactic acid:



Meyer (14), in 1901, reported some metabolic experiments on an alcaptonuric, feeding tyrosine and analyzing quantitatively for homogentisic acid, total acidity, total sulfate,

inorganic sulfate, ethereal sulfate, and ammonia in the urine. He was surprised to find that although hydroquinone and gentisic acid are excreted as conjugated ethereal sulfates, homogentisic acid is excreted as the sodium salt. As a result of his investigations and observations, he concluded that alcaptonuria is not a harmful condition to the organism.

In 1903 Falta and Langstein (15) characterized alcaptonuria as the excretion of homogentisic acid and, in rare cases, also uroleucine acid. They reported that phenylalanine also is converted to homogentisic acid in the alcaptonuric. From L-phenylalanine they obtained, on a weight basis, 90% conversion to homogentisic acid (i.e., 5 g. of L-phenylalanine gave 4.47 g. of homogentisic acid in the urine). From DL-phenylalanine they obtained a 50% conversion to homogentisic acid. Apparently, although it is known that D-phenylalanine may replace the natural L-isomer for growth purposes in the normal organism, the D-isomer is not handled in the same way as the L-isomer in the alcaptonuric.

Neubauer and Falta (16), in 1904, undertook a systematic approach to the alcaptonuria problem. They divided aromatic acids into the following groups for testing purposes in an alcaptonuric: non-oxidized aromatic acids (phenylacetic acid, phenylpropionic acid, and cinnamic acid); mono-phenol aromatic acids (p-hydroxycinnamic acid, o-hydroxycinnamic acid, and coumarin, the lactone of o-hydroxycinnamic acid); aromatic acids hydroxylated in the side chain (phenyl-α-

lactic acid, phenyl- β -lactic acid, phenylglyceric acid, and phenylpyruvic acid); and aromatic acids hydroxylated twice in the aromatic nucleus (gentisic acid, 2,4-dihydroxybenzoic acid, protocatechuic acid, and caffeic acid). Of all these, the only compounds yielding homogentisic acid were found to be phenyl- α -lactic acid and phenylpyruvic acid. These results led them to some interesting conclusions on the catabolism of aromatic amino acids. Inasmuch as phenyl- α -lactic acid was converted to homogentisic acid, they assumed that these α -hydroxy acids occurred in the organism of alcaptonurics as intermediate products of degradation of aromatic amino acids derived from proteins. This being accepted, the phenylpyruvic acid was considered to undergo reduction to the corresponding lactic acid in order to be converted further into homogentisic acid. In the case of tyrosine, then, they had to assume that either it underwent a reduction to phenylalanine followed by oxidation in the 2- and 5- positions in order to produce homogentisic acid, or, what is more likely, that a simple displacement of the hydroxyl group or of the side chain takes place. Applying the principle that the most simple sufficient explanation is accurate, they believed that the oxidation of aromatic amino acids in the normal organism follows the path of the alcaptonic acids, and that the disturbance in alcaptonuria consists only in a hindrance of metabolism

which stops the degradation at this point. Also, they theorized that normally the further degradation of these alcaptone acids does not start in the side chain; they thought that the process which altered the benzene ring by the introduction of two hydroxyl groups in the 2,5-positions progresses further to the final rupturing of the ring.

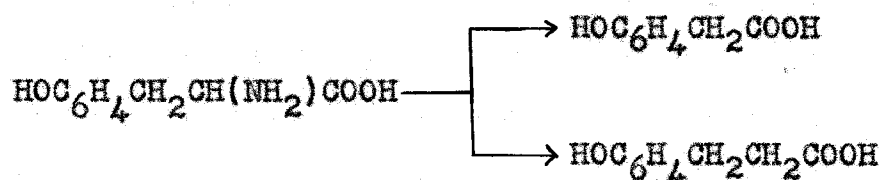
Later, Neubauer (17) found that p-hydroxyphenyl- α -lactic acid, in contrast to phenyl- α -lactic acid, does not increase homogentisic acid production in the alcaptonuric. He also found (18) that the p-hydroxyphenyl- α -lactic acid, when perfused through a surviving dog liver, does not produce acetone bodies, whereas p-hydroxyphenyl pyruvic acid does; he therefore formed the opinion that the first step in amino acid degradation is oxidative deamination to the corresponding keto acid, and not hydrolytic deamination to the corresponding hydroxy acid.

In 1906, Embden, Salomon and Schmidt (19) introduced a new technique for studying the alcaptone problem. They were investigating the ability of a great variety of substances to form acetone when mixed with blood and perfused through an isolated dog liver. They found that tyrosine, phenylalanine, phenyl- α -lactic acid, and homogentisic acid all increased significantly the acetone content of the effluent. On the other hand, phenylacetic acid, phenylpropionic acid, cinnamic acid, and phenyl- β -lactic

acid do not produce acetone under similar conditions. They were inclined to support the view then held by Neubauer, that the catabolism of amino acids proceeded through deamination with production of the corresponding hydroxy acid, followed by further oxidation to produce acetone bodies, and ultimately carbon dioxide and water.

Further proof of the ketogenic nature of phenylalanine and tyrosine was provided by Baer and Blum (20), who fed phenylalanine and tyrosine to a diabetic, and found increased ketone body excretion in the urine.

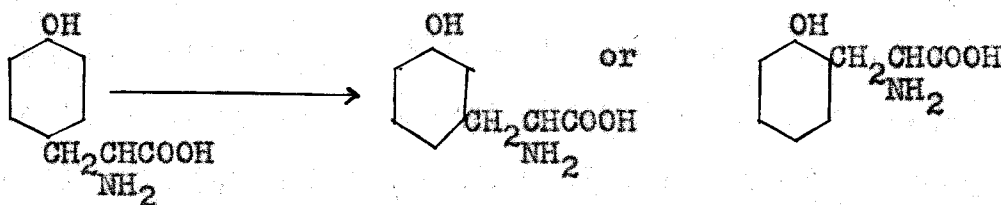
Blum (21), in 1908, discussed the possible mechanisms involved in the production of homogentisic acid from tyrosine. He considered three changes to be involved: 1) degradation of the side chain $-\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH} \longrightarrow -\text{CH}_2\text{COOH}$; 2) a shift in the position of the side chain relative to the hydroxyl group; and 3) oxidation of the nucleus by introduction of an hydroxyl group. In the first instance, tyrosine could conceivably be degraded to p-hydroxyphenylacetic acid or p-hydroxyphenylpropionic acid:



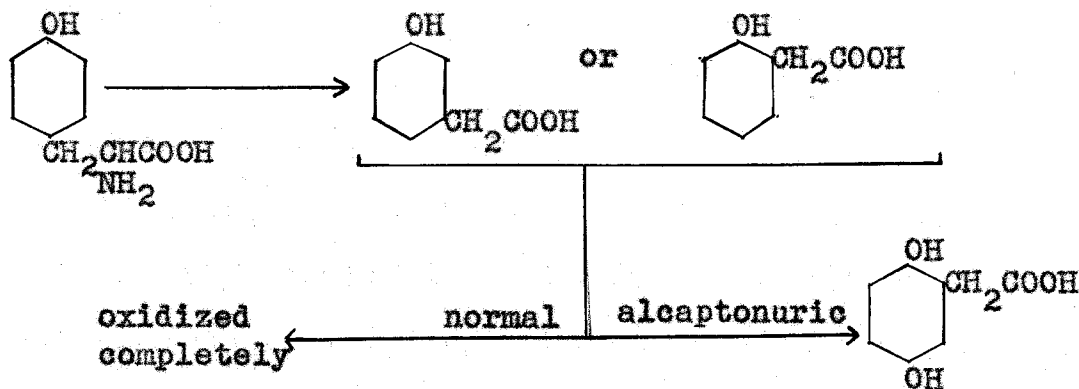
He disposed of these possibilities by showing that the normal organism excretes 13% of a test dose of hydropara-coumaric acid unchanged and 13% of it as p-hydroxybenzoic

acid (conjugated with glycine); *p*-hydroxyphenylacetic acid is excreted about 79% unchanged. In the alcaptonuric, neither gives rise to homogentisic acid.

In the second change considered, there was the possibility of tyrosine being converted to *o*-tyrosine or *m*-tyrosine, with oxidation and degradation of the side-chain following:



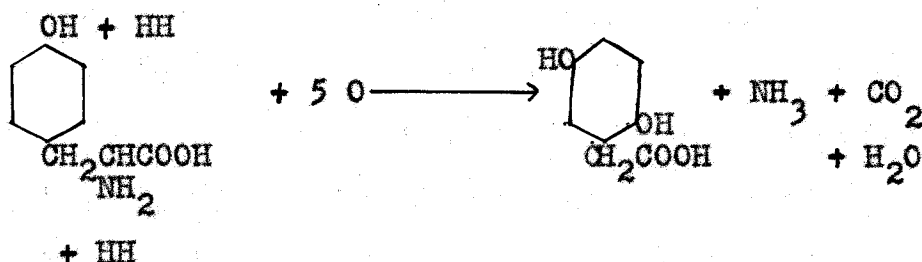
However, Blum showed that neither *o*- nor *m*-tyrosine gives homogentisic acid in the alcaptonuric; in the normal organism they both are converted to the corresponding hydroxyphenylacetic acid. The third consideration was degradation of the side chain and simultaneous shifting of position on the ring, followed by oxidation of the nucleus in the para- position:



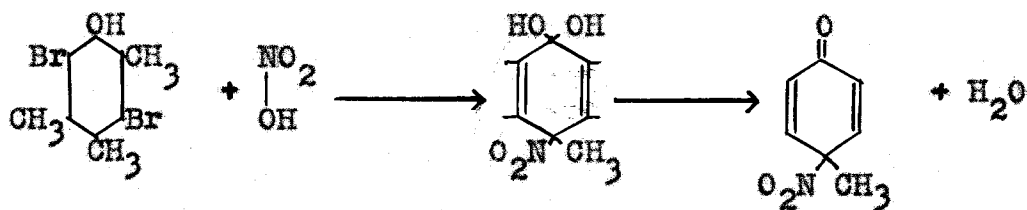
But neither o- nor m-hydroxyphenylacetic acid gave homogentisic acid in the alcaptonuric. In the normal organism, the two acetic acids were excreted unchanged almost quantitatively.

Blum thought the catabolism of both phenylalanine and tyrosine in the normal organism proceeded through homogentisic acid; the final proof--isolation of homogentisic acid--was admittedly still lacking.

An interesting theory for the conversion of tyrosine to homogentisic acid was proposed by Friedmann (22). He formulated the change chemically, thus:

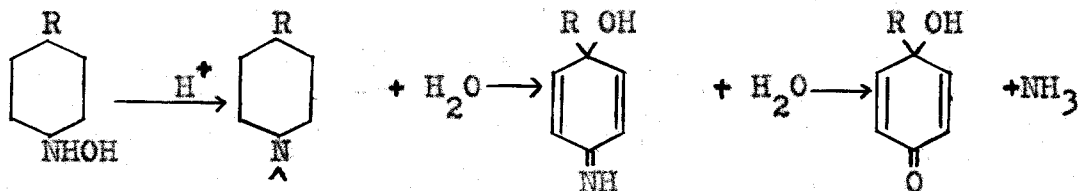


and was reminded that Zinke (23) and Auwers (24) had prepared a class of compounds called "quinols" by nitric acid oxidation of para-alkylated phenols, thus:

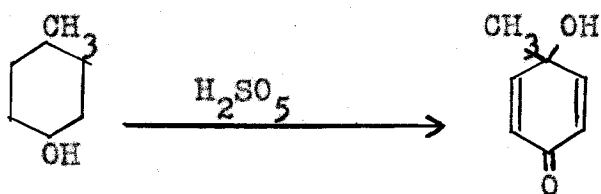


Bamberger (25) has contributed even more to this field, preparing the quinols by treating para-alkylated phenyl-

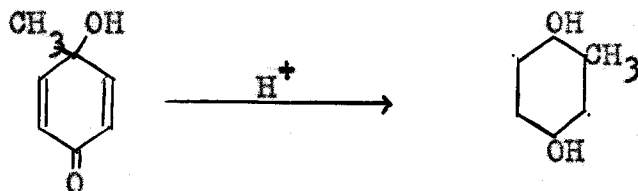
hydroxylamines with dilute sulfuric acid, thus:



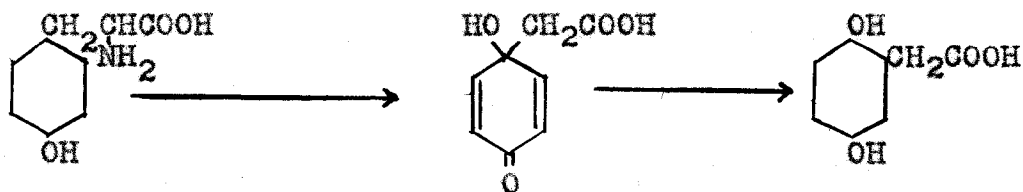
Direct oxidation of substituted phenols by monopersulfuric acid (Caro's acid) also produces quinols:



Quinols have been found to undergo rearrangement quite easily (treatment with dilute acid or alkali) to di-hydroxy compounds,

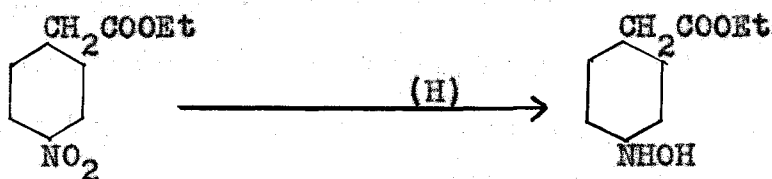


Friedmann saw the possibility that homogentisic acid might be formed similarly:



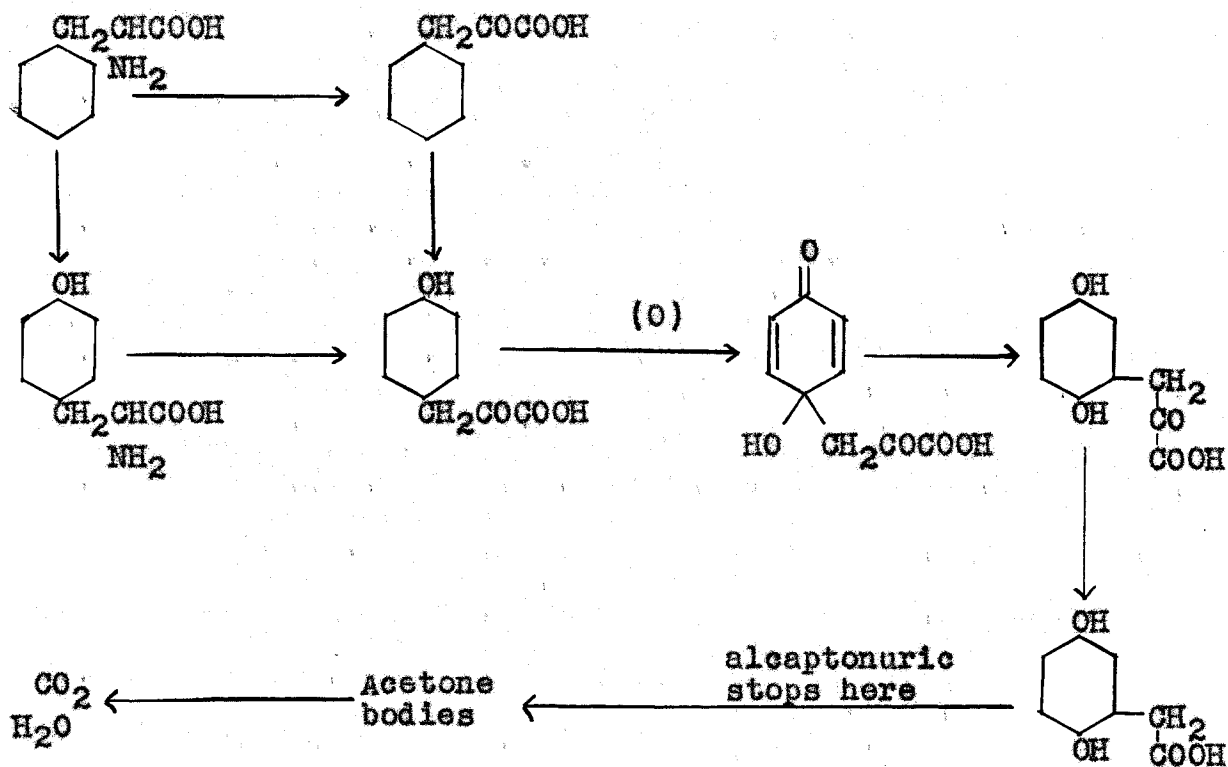
To investigate this theory, he prepared an arylhydroxylamine

with an acid side chain:



and announced his intention of preparing the quinol from this if possible, and of testing the quinol in vitro and in vivo. Unfortunately, there has been no further report of such work.

By this time Neubauer (17) had formulated further views on the process of amino acid catabolism, and a theory on the mechanism of oxidation of tyrosine which took into account Friedmann's suggestion. He considered the first step in amino acid catabolism to be oxidative deamination to the corresponding keto acid. If the amino acid is non-aromatic, the keto acids are decarboxylated and then follow the regular metabolic route of fatty acids. In the case of tyrosine and phenylalanine, he proposed the following scheme:



The two intermediates between p-hydroxyphenylpyruvic acid and homogentisic acid have of course never been isolated, and thus are highly theoretical. Yet on the surface it seemed to Neubauer a most logical sequence. This plan means that Neubauer considered homogentisic acid an intermediate in tyrosine catabolism in the normal organism as well as in the alcaptonuric.

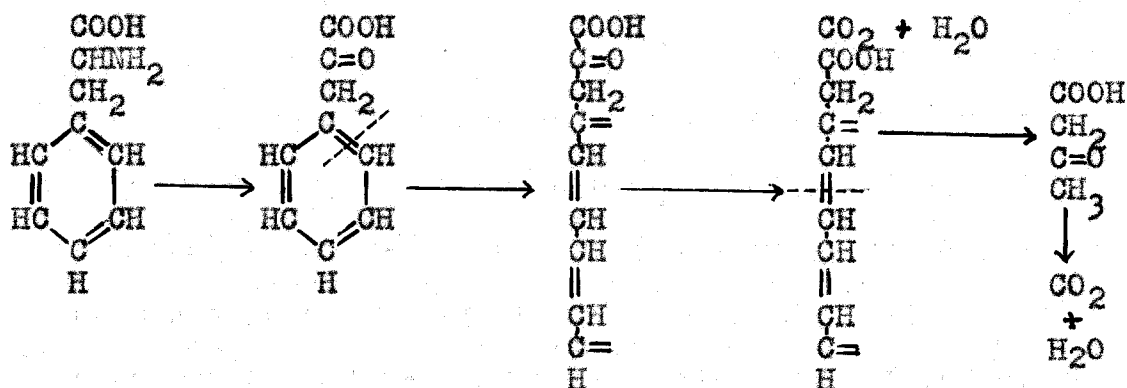
Dakin (26) immediately took exception to Neubauer's conception of the mechanism outlined above. If the formation of a quinonoid-type intermediate could be inhibited, and the compound could still be oxidized in the animal organism Dakin

reasoned, the formation of a quinol intermediate would not be a necessary condition for combustion. Therefore, he fed p-methoxyphenylalanine to a cat; apparently it was oxidized satisfactorily, since no end products could be isolated in the urine except a small amount (8%) of p-methoxyphenylacetic acid. A similar amount of DL-tyrosine fed to the same animal was followed by a small amount of tyrosine in the urine and a notable increase in p-hydroxyphenylacetic acid. The justifiable conclusion to Dakin was that a para-quinonoid intermediate was not necessary for combustion of tyrosine. Further arguments cited, which he admitted were inconclusive, were: the administration of phenylalanine and tyrosine in such large quantities that much appears unchanged in the urine is not followed by any appearance of homogentisic acid in the urine; nor in case of phenylalanine could an increase in phenolic substances be observed, so that at that time there was no evidence available of conversion of phenylalanine into tyrosine, as Neubauer pictured. Further, the discovery that benzene administered to a dog leads to muconic acid in the urine suggested the possibility that oxidation of the aromatic nucleus may not necessarily be preceded by introduction of hydroxyl groups in the ring.

Wakeman and Dakin (27) extended these conclusions further by observing the fate of p-methylphenylalanine, p-methoxyphenylpyruvic acid, and p-methylphenylpyruvic acid in the normal organism. They found that these compounds were

practically completely oxidized in precisely the same fashion as phenylalanine and tyrosine. These substances also yield acetoacetic acid and acetone when perfused through a surviving liver of a dog. Also p-methylphenylalanine and p-methoxyphenylalanine, when fed to an alcaptonuric, are, "within reasonable limits", completely oxidized. Therefore it follows that even the alcaptonuric is provided with a mechanism for oxidation of an aromatic nucleus of amino acids, provided that their conversion into homogentisic acid is prevented by suitable substitution in the para-position.

Dakin then postulated a possible pathway of oxidation of aromatic amino acids in a normal organism, taking into account his belief that cleavage of the ring does not require prior introduction of an hydroxyl group:



Thus Dakin felt (28) that alcaptonuria represents a condition in which the formation of homogentisic acid is abnormal as well as the failure to catabolize it when formed.

Dakin (28,29) contributed two more facts to the cata-

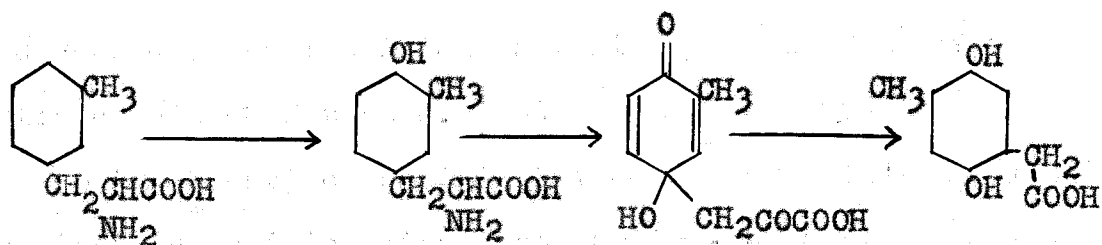
bolism of tyrosine which have a bearing on the metabolic handling of this amino acid. When quantities of the order of 5 gm. of DL-p-methylphenylalanine were fed to an alcaptonuric, a significant amount (1.1 gm.) of the D-acetyl derivative of p-methylphenylalanine was separated from the urine. When a larger amount (8 gm.) of DL-tyrosine was fed to a normal animal (rabbit), 1.7 gm. of tyrosine were recovered in the urine, of which 75% was the dextro isomer. However, when smaller amounts were fed, no unchanged tyrosine was found in the urine, indicating that the rates of decomposition of the D- and the L- isomers cannot be widely different. In no case under the conditions of this experiment could homogentisic acid be detected.

Abderhalden and Massini (30) added to Dakin's analogue technique by administering monopalmityl-L-tyrosine and distearyl-L-tyrosine to an alcaptonuric. They found a slight, if any, increase in excretion of homogentisic acid and a "disturbed metabolism in general". p-Aminophenylalanine when administered to the same patient led to a marked increase in the output of homogentisic acid, measured by the increased reduction ability of the urine.

The disagreement between Neubauer's theory and Dakin's theory of normal tyrosine catabolism could of course have been resolved if it could be shown that homogentisic acid is produced from tyrosine in a normal organism, as well as in an alcaptonuric. Abderhalden (31) observed this qualitatively

after a laboratory assistant ingested 50 gm. of L-tyrosine during 24 hours. However, attempts to duplicate these results, in which Abderhalden himself ingested 150 gm. of L-tyrosine within 3 hours, were entirely unsuccessful.

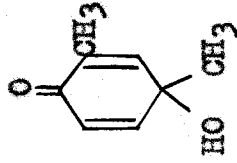
Fromherz and Hermanns (32,33) extended Dakin's analogue experiments further. They found that m-methylphenylalanine is oxidized in the normal organism just as Dakin had shown for the para-isomer. They then tried the meta-isomer in an alcaptonuric. If it is handled according to Neubauer's theory, methylhomogentisic acid should result:



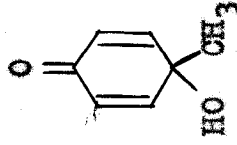
They found that no homogentisic acid derivative was formed, and apparently the administered substance was completely oxidized. The same results--that is, no homogentisic acid formation--were observed upon administration of m-methyl-tyrosine. Apparently the first oxidation of such a compound does not involve the para-position necessarily. Apparently also the meta-methyl group inhibits homogentisic acid formation, or is catabolized through a different pathway than is tyrosine.

These two workers then decided it would be worthwhile to observe the effect of quinol administration to normal and

alcaptonuric organisms. If homogentisic acid were formed from tyrosine through a quinol-type intermediate, then possibly simple quinols would also rearrange to the corresponding hydroquinone compounds. Therefore they tried the two simplest quinols, m-dimethylquinol and toluquinol:



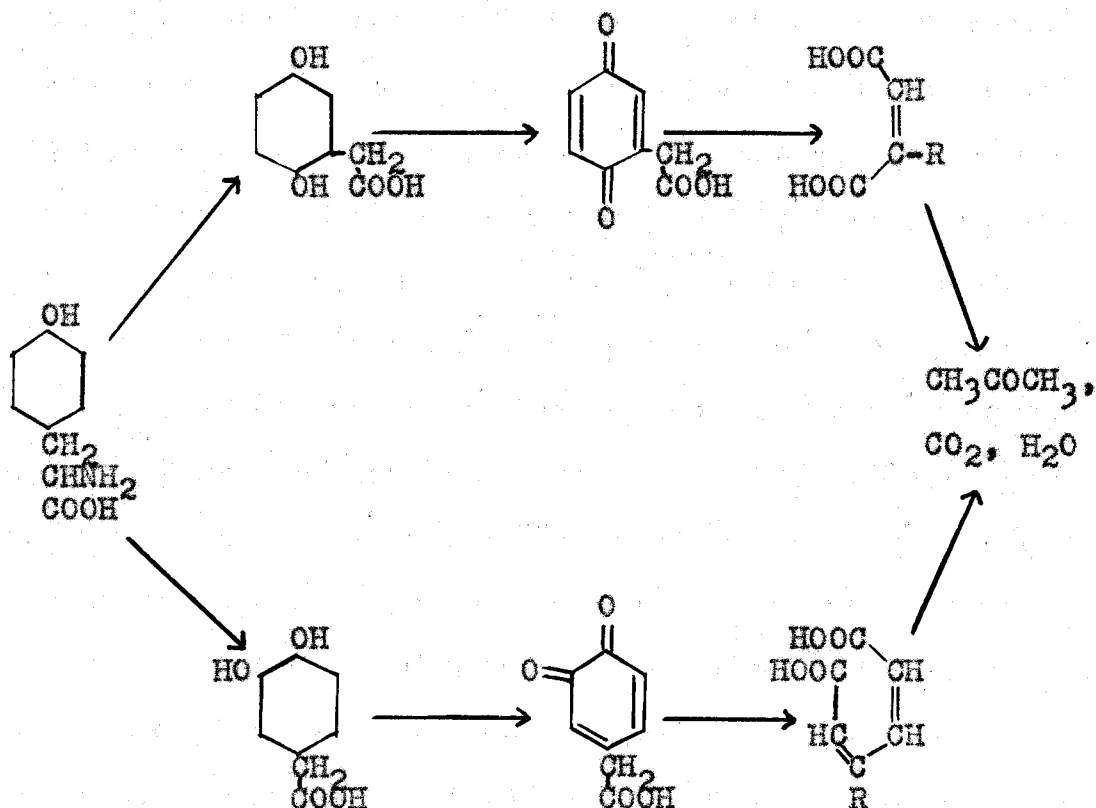
m-dimethylquinol



toluquinol

in both a normal animal (dog) and an alcaptonuric patient (human). Neither of the compounds gave detectable formation of hydroquinone derivatives in either of the test animals. This of course was not at all proof that quinol formation does not take place in tyrosine catabolism.

These men presented as their conception of the oxidation of tyrosine the following:



Embden and Baldes (34), by means of liver perfusion experiments, showed the probable conversion of phenylalanine into tyrosine. Phenylpyruvic acid, the keto acid corresponding to phenylalanine, did not give acetoacetic acid in the perfusion experiments, as did *p*-hydroxyphenylpyruvic acid, the keto acid corresponding to tyrosine. Yet phenylpyruvic acid did give homogentisic acid in the alcaptonuric. Therefore Embden and Baldes considered that the primary pathway of phenylalanine oxidation was not through the keto acid by deamination, but through nuclear oxidation to tyrosine.

Kotake (35) fed 10 gm. of L-tyrosine daily for five days to a rabbit, and isolated a small amount of L-hydroxyphenyllactic acid from the urine. Later, he and his colleagues (36)

repeated this and isolated *p*-hydroxyphenylpyruvic acid as well as a small amount of the lactic acid. When DL-tyrosine was fed a portion of that administered was excreted as the dextro-isomer.

Rapport and Beard (37), in 1927, investigated the effect of phenylalanine and tyrosine on specific dynamic action in the animal organism. They found that phenylalanine has a greater effect than glycine, and tyrosine has an effect comparable to alanine. This rendered untenable the theory current at that time that the specific dynamic action of amino acids is proportional to their power of increasing the amino nitrogen content of the blood, since glycine has a far greater effect than phenylalanine in regard to the increase of blood amino nitrogen content.

Along these lines, Shambaugh, Lewis and Tourtellotte (38) have shown that administration of either tyrosine or phenylalanine to rabbits causes increased blood urea nitrogen content, but no increase of the amino acid nitrogen content. The creatine and amino nitrogen content of the urine both increased slightly. When phenylalanine was fed they found evidence of the presence of a small amount of phenylpyruvic acid in the urine; however, when tyrosine was fed, no *p*-hydroxyphenylpyruvic acid could be noted.

Continuing the dietary aspects of tyrosine metabolism, Reinwein (39) noted that when an alcaptonuric is fed on a minimum nitrogen diet, the ratio of homogentisic acid to

nitrogen excreted stayed remarkably constant. In this respect he confirmed the work of Garrod and Hele (40). These latter two were struck with this fact from study of five different cases of alcaptonuria; in spite of the fact that no simple standard diet was used, the ratio varied only within narrow limits.

Important in evaluating the fate of the tyrosine nitrogen was a report by King, Simonds and Aisner (41), who investigated the fate of tyrosine upon intravenous injection into a dog. After injecting 5 gm., there was a rapid disappearance of the amino acid from the blood. The slight, if any, rise in blood urea nitrogen did not account for any appreciable amount of deamination of the tyrosine.

Butts, Dunn and Hallman (42) compared the metabolism of DL-phenylalanine and DL-tyrosine in the normal rat. Since Shambaugh and his co-workers (38) had found phenylpyruvic acid in urine after feeding phenylalanine, but no *p*-hydroxyphenylpyruvic acid after feeding tyrosine, and since Womack and Rose (43) had shown that phenylalanine was an essential amino acid in the diet and tyrosine could not replace it, Butts and his colleagues felt that the two amino acids might not have the same metabolic pathway. They fed phenylalanine and tyrosine to rats and analyzed the livers for glycogen and the urine for ketone bodies. They found that phenylalanine gave significant increases in liver glycogen content, whereas tyrosine gave only slight increases. After inducing acetone

body excretion by feeding sodium butyrate, addition of tyrosine to the diet did not increase this acetone body excretion. Phenylalanine actually caused a decrease in the acetonuria. Phenylalanine gave significantly higher urinary nitrogen values, whereas tyrosine gave no increase. These workers feel that they had strong evidence for concluding that sugar formation is one fate of phenylalanine in metabolism.

In a later publication, Butts, Sinnhuber and Dunn (44) fed L-tyrosine to rats fasted 48 hours. They found a significant increase in the liver glycogen content. DL-Tyrosine did not increase the glycogen content. They also found that the urine from these rats, when fed the L-isomer, reduced ammonium phosphomolybdate, whereas the urine from the rats fed the DL-mixture did not.

Kriss and Marcy (45) studied the metabolism of tyrosine in regard to respiratory exchange and heat production. The amino acid was fed to rats as a supplement to a mixed maintenance ration, in the quantity to supply 7.5 kg.-cal./day, and apparently about 97% of the fed tyrosine was absorbed. Nitrogen-balance experiments indicated that none of the tyrosine was retained in the body. In light of the isotopic experiments of Schoenheimer and his colleagues, to be discussed in Section D, this is difficult to explain, and gives added emphasis to the question: what happens to the nitrogen of tyrosine when it is catabolized?

This ever-present question arises again upon reviewing

the report of Zorn (46) , who studied the degradation of tyrosine by liver, kidney, and muscle tissue. He reported that the oxidative degradation of L-tyrosine proceeded neither by oxidative deamination nor by transamination. The pathway Zorn and his co-workers believe predominant is discussed in Section B of the Historical.

Zorn's criterion for the occurrence or non-occurrence of transamination seems a little weak. Since he could not isolate any glutamic acid (which would be formed from α -ketoglutaric acid if transamination took place), he concluded that transamination was out of the question with tyrosine. It is pointed out in the Discussion that transamination could probably occur in the presence of only catalytic amounts of α -ketoglutaric and glutamic acids, provided there is another keto acid present (pyruvic acid) to accept the amino group from any glutamic acid which might be formed.

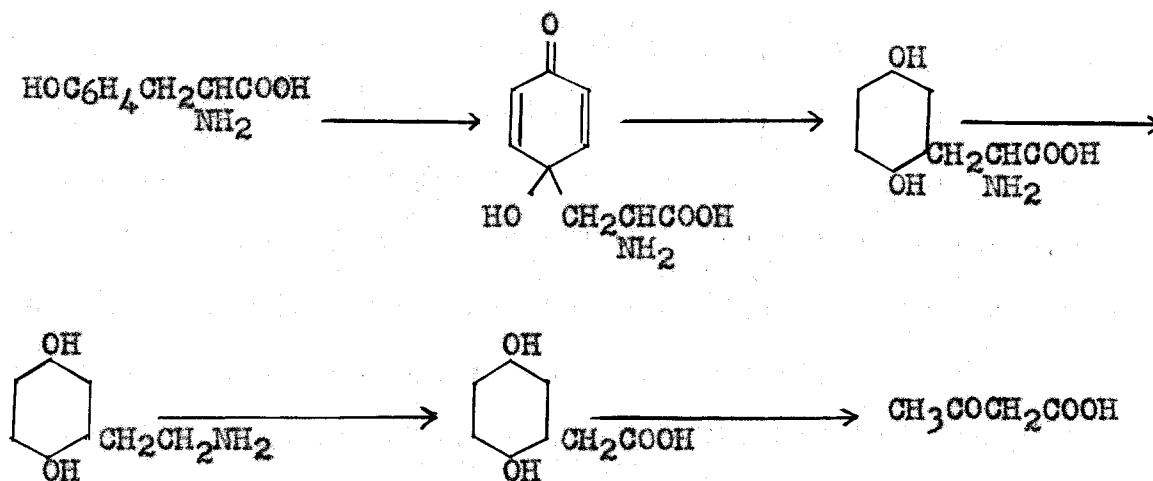
Lanyar (47) reported a comparison between the handling of the natural and unnatural isomers of tyrosine and phenylalanine by the alcaptonuric. The L-isomers of both amino acids when fed by mouth were converted quantitatively into homogentisic acid. D-Phenylalanine was only 40-45% converted into homogentisic acid, whereas DL-tyrosine was converted to the extent of 68%. This supports the view that the oxidation of D-amino acids takes another pathway than the L-amino acids in the animal organism.

A more recent report (1947) on experiments conducted on

a human alcaptonuric is that of Neuberger, Rimington and Wilson (48). They determined that, apart from homogentisic acid, no other aromatic substance likely to arise from tyrosine or phenylalanine metabolism is excreted in the urine. By a statistical evaluation, they showed that even on a constant diet, both the daily output of homogentisic acid and the H:N ratio in the urine varied considerably. According to their figures, tyrosine and phenylalanine are converted to homogentisic acid to the extent of about 80-85%. Catabolism by other pathways, which no doubt exist, accounts for from 5-20% of these amino acids. In attempting to find a clue as to the site of formation of the homogentisic acid in the body, they found a very low concentration of this acid in the plasma. This in conjunction with high renal clearance volumes suggested to them that homogentisic acid is either formed in the kidney or is actively excreted by the tubules at a very high rate. There thus exists the possibility that alcaptonuria may be due to an abnormality in secretory function of the kidney.

Leading to the mechanism of oxidation of tyrosine, they found that ingestion of DL-2,5-dihydroxyphenylalanine by the alcaptonuric produced an increased excretion of homogentisic acid over the basal level. This led them to postulate that tyrosine normally may first be oxidized to the 2,5-dihydroxy compound, and this may then be converted to homogentisic acid through the keto acid.

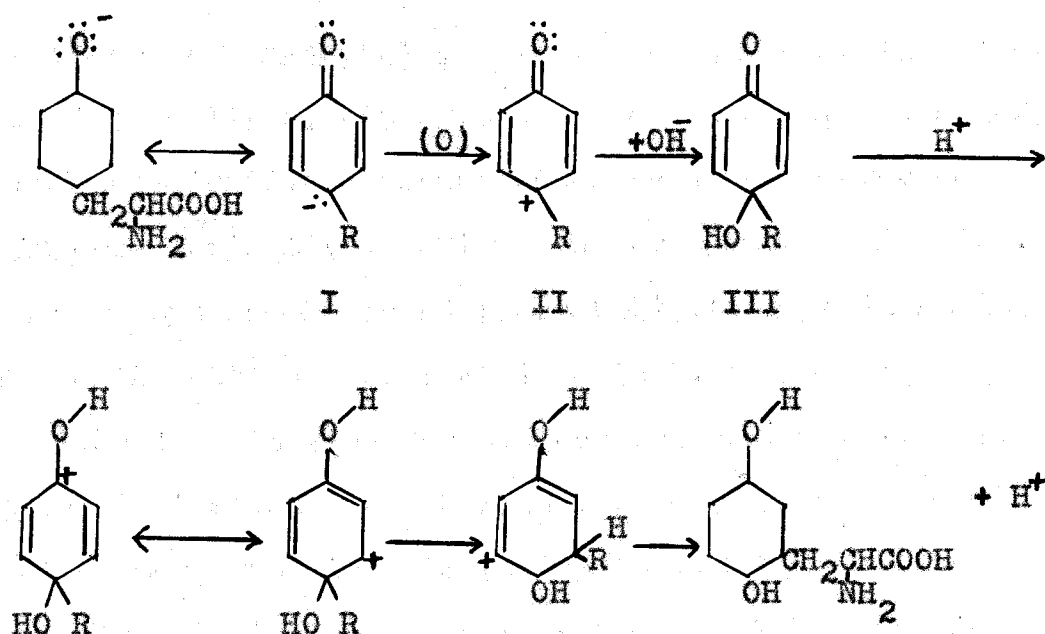
Later, Neuberger (49) synthesized and resolved DL-2,5-dihydroxyphenylalanine and fed each isomer to a rat. He found that the L-isomer was readily metabolized, a supplement as high as 1.2 gm. producing no reducing substance in the urine. The D-isomer was metabolized completely if the amount fed did not exceed 200 mg./100 gm. of body weight. When the L-isomer was fed to a normal human, it again was apparently metabolized completely. So also was 2,5-dihydroxyphenylethylamine, the substance resulting from decarboxylation of the amino acid. Since there exists a mammalian enzyme capable of decarboxylating L-2,5-dihydroxyphenylalanine to produce the amine, and since mammalian kidney tissue contains an amine oxidase, Neuberger postulated that the normal metabolism of tyrosine may go partly through the above two intermediates:



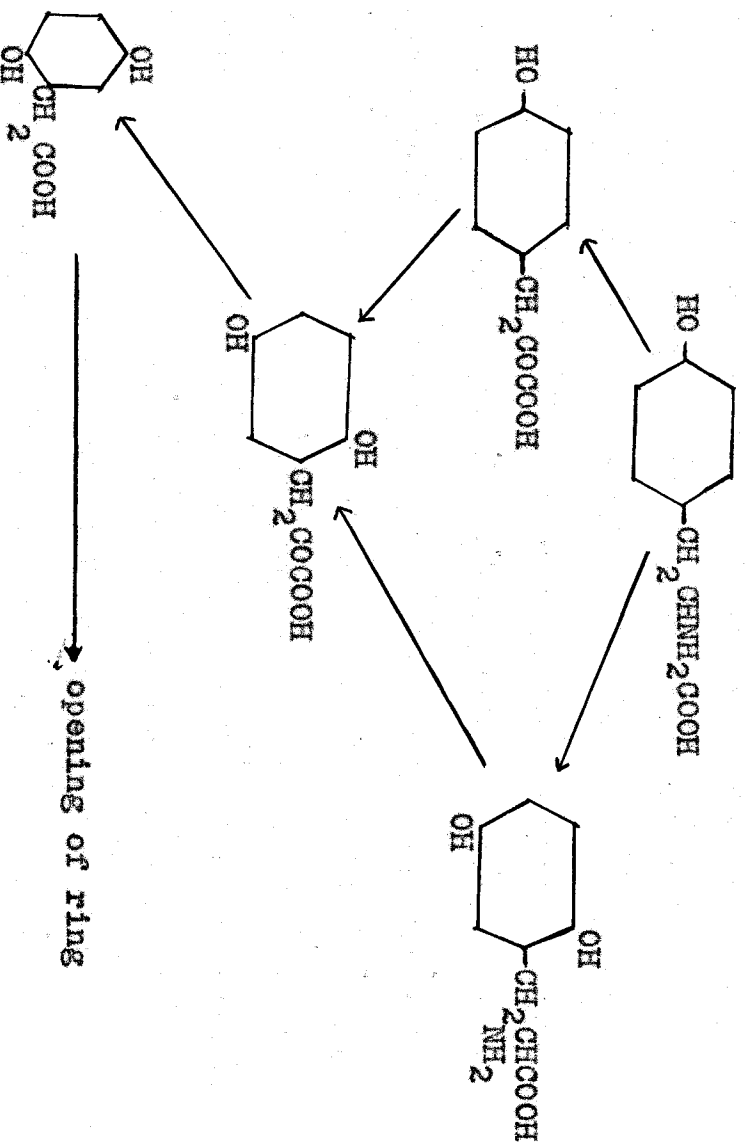
Added weight to the postulation of 2,5-dihydroxyphenylalanine as an intermediate is the fact found by Neubauer (17) and

Fromherz and Hermanns (32) that *p*-hydroxyphenylpyruvic acid is far less effective than tyrosine as a precursor of homogentisic acid in the alcaptonuric. This suggests that oxidation of the ring precedes that of the side chain.

Neuberger (50) has advanced a likely mechanism for the formation of a quinol from tyrosine. The oxidation involves an attack by an electrophilic reagent, and the three positions in the ring with the highest electron densities are the two ortho and the para positions. Preferential attack in the para position might be facilitated by suitable substitution in the side chain or by steric factors in the enzyme. The most reasonable picture of such an oxidation is pictured to be the removal of two electrons from one of the resonating structures of the phenoxide ion leading to a carbonium ion, II. The latter then would combine with an hydroxide ion to give an hydroxydienone (quinol), III. This then rearranges according to the mechanism pictured for dienone-phenol rearrangements by Arnold and co-workers (51), forming 2,5-dihydroxyphenylalanine as a product:



Although this section is concerned with tyrosine metabolism as studied by means of its aberration in alcaptonuria, it would not be complete without presenting the facts on another error of tyrosine metabolism: tyrosinosis. In 1932, Medes (52) reported the results of a prolonged study undertaken on a patient whose illness had been diagnosed as myasthenia gravis. Although he unmistakably had this, it was noted that he also excreted in the urine p-hydroxyphenylpyruvic acid. On a starvation diet or a non-protein diet the excretion was constant at 1.7 gm. daily. The feeding of tyrosine or phenylalanine increased this amount to as much as 3.2 gm. The subject could apparently metabolize homogentisic acid completely, since feeding this acid caused no detectable amount of homogentisic acid in the urine. Neubauer (53) had proposed two alternate pathways for tyrosine metabolism:



It will be recalled that Dakin theorized that the only pathway of normal tyrosine oxidation was to *p*-hydroxyphenylpyruvic acid, in which compound opening of the ring occurs. Medes considers the above fate of homogentisic acid in her patient corroborative evidence of Neubauer's mechanism, i.e., normal oxidation of tyrosine includes introduction of a second hydroxyl group in a position leading to production of homogentisic acid as a preliminary to disruption of the ring.

When tyrosine was introduced into the diet--3 gm. daily--some appeared in the urine. Evidently the capacity of the body for converting tyrosine to *p*-hydroxyphenylpyruvic acid had been exceeded. However, this limitation of the conversion capacity was not absolute, but was dependent upon the concentration of the tyrosine, since continuous increase of

keto-acid output was shown under such conditions. Under this high tyrosine feeding, 3,4-dihydroxyphenylalanine also appeared in the urine. This was notable in that it established the fact that 3,4-oxidation of the benzene ring takes place in the body (showing the possibility of tyrosine serving as precursors of indole derivatives and melanin in its anabolic role). Oxidation of 2,5-dihydroxyphenyl compounds proceeded in the body much easier than oxidation of 3,4-derivatives, since homogentisic acid was completely oxidized both by a normal subject and by the patient, whereas 3,4-dihydroxyphenylalanine administration led to the appearance of some of the unchanged substance in the urine. Medes' data also provided clear-cut evidence for the conversion of phenylalanine into tyrosine in the animal organism.

Medes concluded that tyrosinosis is a complete stoppage of oxidation of tyrosine at the stage of *p*-hydroxyphenylpyruvic acid; also every step theretofore is slowed, as evidenced by excretion of *p*-hydroxyphenyllactic acid, tyrosine, and L-3,4-dihydroxyphenylalanine. Unfortunately for the intermediary metabolism of tyrosine this error of metabolism is so rare that no other cases of it have ever been reported.

B. The Metabolism of Tyrosine in Vitro.

The advent of Warburg's technique of following oxidation in isolated tissue slices and suspensions by manometric

means provided a great impetus to the study of metabolism in general. That it provided a valuable tool for the study of amino acid oxidation was realized almost immediately.

Kisch (54), in 1931, reported manometric studies of several amino acid oxidations by rat, guinea pig, rabbit, cat, dog, and pig kidney and liver tissue. Upon incubation of the amino acids with the tissues, he noted an increase in the respiratory activity which could be accounted for only by the presence of the added amino acids. The increase was particularly great with rat kidney tissue. In a second paper (55), he noted greater activity at a pH of 7.4 and 8.0 than at 6.9.

Krebs (56) used the Warburg technique to study the site and mechanism of deamination of amino acids in the body. Among the facts which he noted were that liver and kidney were the principle sites of deamination; that amino acids were the principle sources of urinary ammonia; that optically-active unnatural isomers were deaminated faster by kidney tissue than the natural isomers; and that deamination proceeded oxidatively to the keto acids in most cases.

Bernheim and Bernheim (57) prepared "broken cell suspensions" by grinding liver and kidney tissue in a mortar with sand and phosphate buffer and studied the oxidation of tyrosine and phenylalanine by such preparations. The Warburg respirometer was used to follow the uptake of oxygen. They found that, with liver tissue, provided the tissue concentration was

sufficiently high, tyrosine oxidation proceeded with the uptake of four atoms of oxygen per mole of tyrosine. This oxidation was found to be independent of salt concentration and presence of fluoride. However, cyanide did inhibit the oxidation completely.

If 0.5 mg. of L-tyrosine were used, the "theoretical" uptake of four atoms of oxygen per mole occurred; if the tyrosine concentration were doubled, less oxygen was used. The authors felt that the enzyme system involved was inhibited by excess substrate. As is pointed out later (page 49, reference (84)) what these authors thought was inhibition was the result of using insufficient liver tissue for oxidizing 1 mg. of tyrosine. In order to obtain the theoretical uptake of 4 atoms of oxygen per mole of tyrosine, the ratio of liver tissue to tyrosine must be 2 grams per milligram.

Tyrosine was found to be oxidized slowly or not at all by kidney tissue of rat, guinea pig, cat or dog; rabbit kidney tissue, on the other hand, oxidized the tyrosine faster than did the rabbit liver tissue. The most active of all the preparations was that from rat liver.

Phenylalanine, in contrast to tyrosine, was oxidized by both liver and kidney, with the uptake of one atom of oxygen per mole of amino acid. In the case of phenylalanine, the theoretical amount of ammonia was produced from the L-isomer. Phenylalanine, in contrast to tyrosine, showed no substrate inhibition in amounts up to 8 mg. of amino acid, and its

oxidation was not inhibited by cyanide. These differences between the two amino acids suggest of course that two different enzyme systems are operating. To the Bernheims these differences "proved definitely that phenylalanine is not converted to tyrosine".

Since Krebs had shown that the oxidation of tyrosine by intact cells was always accompanied by deamination, it was evident that the deaminizing system was destroyed by the grinding treatment, whereas the oxidizing system was not.

Edson (58) studied the formation of ketone bodies from amino acids incubated with rat liver slices. He found the most strongly ketogenic ones to be leucine, tyrosine, and phenylalanine. In contrast to Bernheim's results with broken-cell suspensions, Edson was not able to obtain an oxygen to tyrosine ratio of more than two atoms per mole with the slices. As a result of his findings, Edson considered that there were two possible pathways for phenylalanine catabolism: conversion to tyrosine and then through *p*-hydroxyphenylpyruvic acid and homogentisic acid, and oxidative deamination with formation of phenylpyruvic acid.

Bernheim (59) continued his studies of the oxidation of tyrosine by liver and kidney suspensions, including observations upon the DL-amino acid as well as the natural isomer. By running parallel experiments with DL-tyrosine and L-tyrosine, differences can be observed which may be attributable to the D-isomer. In this manner he found that

the D-tyrosine is oxidized and deaminated by kidney suspensions, whereas the L-isomer is not attacked. With liver suspensions both isomers are oxidized. The D-isomer undergoes deamination, as evidenced by the production of ammonia equivalent to the amount of D-tyrosine present, and its oxidation is not inhibited by cyanide. By dilution of the liver suspension, it is possible to effect oxidation of the D-isomer but not the L-isomer. It was quite evident to Bernheim that the enzyme systems responsible for the oxidation of the two isomers were entirely different.

Evidence for the stepwise degradation of L-tyrosine by liver "brei" was obtained by Felix, Zorn and Dirr-Kaltenbach (60). These investigators found the oxygen consumption of L-tyrosine to be dependant on hydrogen ion concentration. At a pH of 6.8 only one atom of oxygen per mole of tyrosine was consumed, at pH 7.2 two atoms per mole, at pH 7.6-7.8 four atoms per mole, and at pH 8.2 again only one atom of oxygen was taken up for each mole of tyrosine added.

After the first atom of oxygen had been taken up analysis of the flask contents gave a Millon's value still equivalent in intensity to the amount of L-tyrosine originally used as substrate. Apparently the primary oxidation product was still closely related to tyrosine, and rupture or loss of the benzene ring or removal of the hydroxyl group had not occurred. After the uptake of two atoms of oxygen at pH 7.2 the color difference indicated about 75% of the tyrosine

added was no longer present. With an oxygen uptake of four atoms the Millon's test was negative. The end products of the oxidation were acetone and carbon dioxide. Ammonia was not split off in the course of the oxidation.

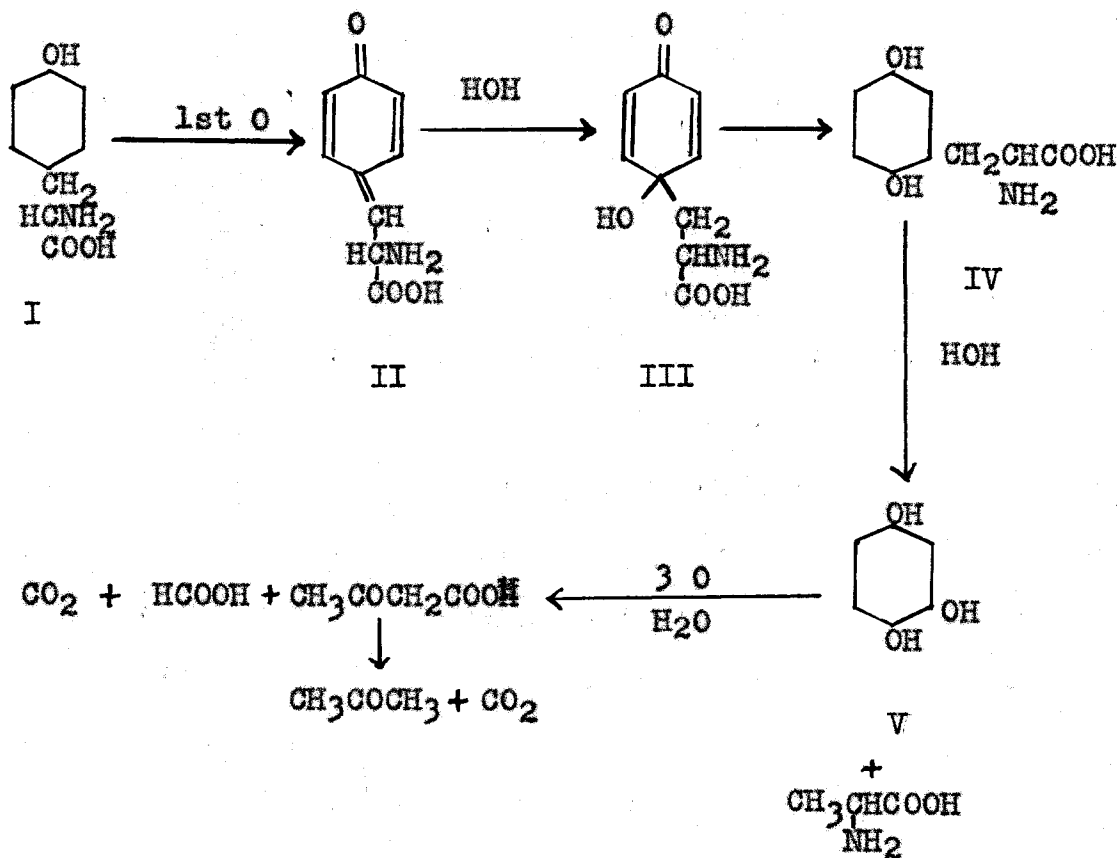
DL-Tyrosine was also oxidized by liver suspension with the uptake of four atoms of oxygen per mole of substrate. In this case, however, p-hydroxyphenylpyruvic acid and ammonia were formed equivalent to the amount of the D-isomer present. With kidney suspension, both L-tyrosine and DL-tyrosine were oxidized with the uptake of one atom of oxygen per mole of substrate. The L-isomer was not deaminized, whereas the DL-isomer formed p-hydroxyphenylpyruvic acid and ammonia equivalent to the amount of D-isomer present.

These authors also found that p-hydroxyphenylpyruvic acid was oxidized by liver brei with the uptake of three atoms of oxygen per mole of substrate, giving as end products acetone and carbon dioxide. Homogentisic acid was oxidized by liver brei with the uptake of two atoms of oxygen per mole of substrate, also giving as end products acetone and carbon dioxide.

Since no ammonia was formed during the oxidation of L-tyrosine, and previous experiments had indicated that transamination does not occur, Felix and Zorn (61) postulated that the only other course for the reaction to take is the splitting off of the tyrosine side chain to form alanine. To confirm this hypothesis, they incubated L-tyrosine with

pig liver brei and analyzed the deproteinized reaction mixture for alanine. This was done by deamination of the mixture with nitrous acid, and oxidation of the lactic acid formed to acetaldehyde with potassium permanganate. The acetaldehyde was then determined by titration with a standard iodine solution. A refinement of the method was introduced by carrying out the oxidation in the presence of mercuric acetate. This salt prevents the formation of acetaldehyde from serine and aspartic acid. Their results indicated that for every mole of tyrosine approximately one mole of alanine was produced.

To explain the production of the identified end products and the oxygen uptake observed, Felix and Zorn postulated the following oxidation scheme:



Lang and Westphal (62), in 1942, attempted to determine whether L-phenylalanine and L-tyrosine were oxidized by a group-specific type of enzyme, as was found for the D-amino acids by Krebs, or whether a specific oxidase was responsible for the degradation of these two amino acids. They believed they discovered a specific L-phenylalanine oxidase in rat liver which carried out aerobic oxidation of both L-phenylalanine and L-tyrosine by the same mechanism. In the case of phenylalanine, one atom of oxygen per mole of substrate was used; the proportion for tyrosine is not given. Neither amino acid was deaminated. Some characterization of the enzyme was given. Thus, it was water-soluble, present in the rat liver but not the kidney, was quite unstable at room temperature but fairly stable at 0°. The optimum pH for its action was 7.8; it was inactivated by dialysis, but could be reactivated by liver and yeast juices. Hydrogen cyanide did not inhibit the enzyme action, showing that it was not identical with the L-amino acid oxidase described by Krebs. Copper, manganese, zinc, and magnesium ions had no influence on the enzyme, but cobalt inhibited it at a concentration of 10^{-4} moles. Although no end products of the oxidation were isolated or identified, they concluded that oxidation of the benzene ring took place.

An examination of their experimental methods makes it appear quite obvious that they did not have a specific L-phenylalanine oxidase, but the same enzyme system as de-

scribed by Bernheim and Bernheim (57,59,63) and by Sealock and Goodland (84). Thus they found activity only in the liver, and not in the kidney. The tissues were ground in a mortar with three portions of M/10 or M/5 phosphate buffer, each of equal weight to the tissue. This extract was centrifuged to obtain the cell-free enzyme preparation. They operated their incubations at a pH of 8.00. Thus they had an enzyme preparation three times as dilute as those of the above-mentioned investigators, but obtained by the same method. Although Lang and Westphal do not give the oxygen uptake with tyrosine as a substrate, it is safe to assume that they did not observe anywhere near the theoretical uptake of 4 atoms of oxygen per mole of tyrosine. In addition, as Felix, Zorn and Dirr-Kaltenbach (60) had observed, the uptake of oxygen with rat liver brei and tyrosine substrate at a pH of 8.0 is limited to one atom per mole.

Their conclusion that oxidation of the benzene ring probably occurred is entirely unfounded, since no end products were isolated or even identified, and there was nothing cited to substantiate such a conclusion.

In 1944, Bernheim and Bernheim (63) investigated the action of various muscular tissues, as well as liver and kidney, upon L-tyrosine, tyramine and phenol in vitro. They found that skeletal muscle, smooth muscle, liver, and kidney slices all were capable of breaking the ring of the tyrosine molecule, as shown by disappearance of "estimatable hydroxy

groups". Heart muscle was incapable of oxidizing tyrosine. With the possible exception of the experiment with liver, no deamination occurred, and deamination with the liver tissue was somewhat doubtful.

C. The Relation of Vitamins and Other Dietary Factors to Tyrosine Metabolism

Since the occurrence of hereditary alcaptonuria is comparatively rare, it is natural that biochemists should turn their efforts to the production of induced alcaptonuria in experimental animals, as an aid to the study of the metabolism of the aromatic amino acids. Papageorge and Lewis (64), in 1938, showed that an experimental alcaptonuria could be induced in white rats by the feeding of large amounts of phenylalanine. Butts, Dunn and Hallman (42) substantiated this fact soon afterward. Sealock, Zeigler and Driver (65), while working on the precursors of melanin pigment in the guinea pig, discovered that the feeding of L-tyrosine to the animals increased its requirement for Vitamin C.

In 1939, Sealock and Silberstein (66) made the significant observation that the experimental alcaptonuria induced in the guinea pig could be controlled by Vitamin C. The excretion of homogentisic acid was at a maximum in a Vitamin C-deficient animal. Administration of only 0.5 mg. of ascorbic acid resulted in excretion of 20-50% of the theoretical amount of homogentisic acid from the 0.5 gm. of L-

tyrosine fed; administration of 5.0 mg. caused complete disappearance of this tyrosine derivative in the urine. Furthermore, withdrawal of the ascorbic acid supplement caused almost immediate reappearance of the homogentisic acid. They then tried the effects of Vitamin C on tyrosine metabolism in two human subjects. Daily feeding of L-tyrosine along with a Vitamin-C-free diet resulted in the excretion of significant amounts of homogentisic acid. This excretion was completely prevented by the ingestion of reasonably large doses of ascorbic acid. Another interesting fact reported by these investigators was the non-relation of Vitamin C to the handling of D-tyrosine. Thus, D-tyrosine also caused the excretion of homogentisic acid, though not in as large amounts as the L-amino acid. But the same amount of Vitamin C which prevented homogentisic acid excretion with the L-tyrosine supplementation had no effect on the homogentisic acid excretion with the D-tyrosine feeding.

That ascorbic acid is concerned in the metabolism of tyrosine was immediately confirmed by Levine, Marples and Gordon (67), who reported on a defect in the metabolism of the aromatic amino acids in premature infants. When such infants are fed a fairly high protein food (a cow's milk formula as opposed to human milk), they excreted L-p-hydroxyphenyllactic acid and some p-hydroxyphenylpyruvic acid in the urine. After administering from 50 to 200 mg. of ascorbic acid parenterally, the excretion of these intermediate metabolites of tyrosine and

phenylalanine disappeared within 48-72 hours.

In a later paper, Sealock and Silberstein (68) reported evidence indicating the importance of the degree of Vitamin C saturation in the tissues in producing or preventing the experimental alcaptonuria.

They also investigated the question of whether the effect of Vitamin C in tyrosine metabolism was due to the anti-scorbutic property of the vitamin or to a non-specific reducing property of the ascorbic acid by testing the diastereoisomer of ascorbic acid, d-isoascorbic acid. This compound possesses identical reducing properties but only one-twentieth as much antiscorbutic activity as the natural l-ascorbic acid. On an equal weight basis, d-isoascorbic acid was completely ineffective in preventing the excretion of tyrosine metabolites by scorbutic guinea pigs. However, on increasing the dose of the unnatural isomer twenty times, the characteristic metabolite excretion was completely eliminated.

The possibility of controlling hereditary alcaptonuria by Vitamin C was shown to be out of the question by Sealock, Galdston and Steele (69). They found that large doses of ascorbic acid (1-4 gm.) had no effect whatever on the excretion of homogentisic acid. Thus it would appear that Vitamin C is not the factor at fault in human alcaptonuria, although it would be of great interest to find a scorbutic alcaptonuric upon whom to repeat these experiments.

Other reports of producing experimental alcaptonuria in-

clude those of Lanyar with white mice (70), and white rats (71), and Abbot and Salmon (72) with white rats by the feeding of phenylalanine or tyrosine. Glynn, Himsworth and Neuberger (73) induced the excretion of homogentisic acid in rats by feeding a diet deficient in methionine and cystine and containing only relatively small amounts of tyrosine and phenylalanine. If cystine is added to the diet, no alcaptonuria occurs unless the tyrosine intake is raised appreciably. Neuberger and Webster (74) reported the production of alcaptonuria by feeding a diet practically free of protein and adding a small amount of tyrosine. Under these conditions the excretion of homogentisic acid was not affected by the administration of methionine or cystine. These investigators reported that the threshold amount of phenylalanine or tyrosine necessary to produce alcaptonuria was significantly reduced by a deficiency of tryptophane and, to a lesser extent, lysine. They suggest that any deficiency of an essential amino acid may impair the capacity of the organism to deal in a normal manner with phenylalanine and tyrosine.

This, of course, follows from the fact that deficiency of an essential amino acid impairs the organism's ability to carry on normal processes of tissue synthesis. This compels the organism to turn more of the tyrosine and phenylalanine than usual into catabolic channels. From this it might be deduced that homogentisic acid is a normal intermediate in the oxidation of tyrosine, and appearance of it in the urine

under the above conditions means that the kidney threshold concentration is exceeded under the forced conditions brought about by the deficiency of essential amino acids.

Demole (75) has reported a drastic way to induce alcaptonuria in rats and mice. He found that feeding these animals as much as 3-4 gm. of ascorbic acid per kilogram of body weight was harmless, but lethal or sub-lethal doses (8-9 gm./kg. of body weight) produced a severe alcaptonuria.

With the connection of Vitamin C to tyrosine metabolism established, most of the experiments that followed made use of this relationship. Sealock, Perkinson and Basinski (76) investigated the problem further by feeding L-phenylalanine to Vitamin C-deficient guinea pigs. They found that such feeding gave rise to the excretion of tyrosine metabolites (p-hydroxyphenylpyruvic acid) as well as homogentisic acid and phenylpyruvic acid. Since Womack and Rose (43) had shown that phenylalanine is an essential amino acid, whereas tyrosine is not, and Moss and Schoenheimer (77) had demonstrated the conversion of phenylalanine to tyrosine by means of deuterium tracer experiments, these results were not surprising. The unpredictable fact was that these metabolites occurred only in an animal which was Vitamin C-deficient, and were abolished when adequate amounts of the vitamin were fed.

Further evidence that the vitamin effect is related to the concentration of Vitamin C in the tissues was furnished

by Sealock (78) in a report of the effect of dicarboxylic acids on the metabolite excretion. Glutamic acid was found to decrease the excretion of the tyrosine metabolites, but continued administrations had a decreasing effect. The activity was found to be due to acidification. Hawley and associates (79,80) had previously shown that administration of ammonium chloride in amounts sufficient to produce a highly acid urine increases the excretion of Vitamin C. In this case glutamic acid, as well as other acids such as fumaric, succinic, malic, and aspartic, acting as an acid, mobilized tissue reserves of Vitamin C, and this newly mobilized vitamin then exerted its usual action of the tyrosine metabolite excretion.

The oxidation of tyrosine by normal and lack of oxidation by scorbutic tissues was correlated with in vivo results by Lan and Sealock (81) in 1944, by studying the oxygen consumption of liver and kidney slices from normal and scorbutic guinea pigs. These investigators found that liver slices of normal animals oxidized L-tyrosine with the uptake of one atom of oxygen per mole of tyrosine present, whereas liver slices from scorbutic animals did not oxidize the amino acid. However, the scorbutic liver regained its ability to oxidize tyrosine by the administration of ascorbic acid either in vivo or in vitro.

Basinski and Sealock (82) showed that the ascorbic acid effect exhibits a structural specificity for tyrosine, phenyl-

alanine and phenylpyruvic acid by studying a long list of structural analogues. The compounds tested were D-tyrosine, D-phenylalanine, N-acetyl-L-phenylalanine, N-acetyl-L-tyrosine, diacetyl-L-tyrosine, L-p-methoxyphenylalanine, DL-phenylaminobutyric acid, and L-S-benzylcysteine. These were fed to scorbutic guinea pigs with and without ascorbic acid supplementation. An analysis of the daily urinary excretion for keto acid and phenolic (tyrosyl) values showed that each is metabolized by the guinea pig independently of ascorbic acid.

Darby, DeMeio, Bernheim and Bernheim (83) reported a study of the metabolism of phenolic compounds by normal and scorbutic guinea pig liver slices in vitro. Liver slices from scorbutic guinea pigs were incubated with either 6 mg. of DL-phenylalanine or 1 mg. of L-tyrosine. After 4 hours incubation at 37° the flask contents were deproteinized with trichloroacetic acid, and estimatable hydroxyl groups determined by the method of Theis and Benedict. Their data showed that liver slices from scorbutic guinea pigs produced an hydroxyphenyl compound from phenylalanine, and apparently metabolized tyrosine to the same extent as the normal guinea pig liver. The scorbutic liver slices also conjugated phenols to the same extent as the normal liver slices. Their disagreement with Lan and Sealock's results is apparently due to the different method used in following the oxidation. Lan and Sealock followed the oxidation by oxygen consumption de-

terminated manometrically; Darby et al. determined hydroxyphenyl compounds colorimetrically. In any event, these authors conclude that the main defect in the metabolism of aromatic compounds by scorbutic guinea pigs is an inability of the deficient liver to oxidize the side chain of tyrosine, rather than an inability to oxidize the ring or conjugate the phenolic group.

In a very recent paper, Sealock and Goodland (84) reported an extensive study of cell-free homogenates in the oxidation of tyrosine by liver from normal guinea pigs. First they duplicated Bernheim and Bernheim's results with "broken cell suspensions", obtaining an oxygen uptake of 4 atoms per mole of tyrosine with normal liver homogenate, provided an adequate quantity of liver per unit of tyrosine is employed. They found that a ratio of 2 gm. of liver per mg. of tyrosine was necessary for maximum oxidation with this species. In an investigation of the enzyme system involved, they found that the tyrosine-oxidizing system of the guinea pig liver exhibited maximum oxidation at a pH of 7.2 to 7.4 and a remarkable stability upon storage in the cold. As much as 87% of the original activity remained after 4 days of storage in the refrigerator. The activity was found to be soluble in aqueous phosphate buffer solution and was not a part of the particulate matter which separated with high speed centrifugation. Dialysis reduced the ability to oxidize tyrosine rapidly; addition of boiled liver extract restored the

original activity. From this it may be concluded that thermostable dialyzable components constitute a portion of the enzyme system. Sealock and Goodland point out that though the enzyme system exhibited a low order of activity, this should not be regarded as being indicative of lack of physiological importance. For,

when one considers the ease with which phenylalanine may be converted to tyrosine in the animal body, it is apparent that a more active destruction of tyrosine in oxidative degradation would decrease the quantity of the two amino acids available for protein syntheses and other anabolic functions. At the same time, operation of this system, even at reduced velocity, must decrease the quantity of tyrosine available for formation of undesirable products such as the pressor amines, tyramine and 3-hydroxytyramine. Thus it can be concluded that this tyrosine-oxidation system plays a significant role in the total economy of the animal body.

Fishberg (85,86) has reported experiments which also demonstrate the close relationship between Vitamin C and tyrosine metabolism. She found that urine of scorbutic animals is capable of inducing methemoglobin formation in vitro. Further investigation proved that the causative agent of this effect is benzoquinoneacetic acid, the oxidized phase of the oxidation-reduction system homogentisic acid-benzoquinoneacetic acid. She found this chemical in the urine of human scurvy and rheumatic fever patients, as well as in the urine of scorbutic guinea pigs. The assumption was made that it is an obligate intermediate in the normal catabolism of tyrosine and phenylalanine, and that normally its existence is so short as to be undemonstrable chemically except for

the appearance of characteristic absorption bands in the ultra-violet region on spectrophotometric examination of blood serum and urine. In rheumatic fever and scurvy, however, this quinone persists for a longer time, owing to the interruption of the normal tyrosine catabolism at the quinoid stage as a sequel to lack of sufficient available ascorbic acid, a key component of the enzyme system necessary for complete tyrosine oxidation.

The possible role of another vitamin, thiamine, upon the metabolism of phenylalanine and tyrosine has been investigated with rather inconclusive results. Closs and Fölling (87) reported that phenylpyruvic acid was excreted in the urine of thiamine-deficient rats when extra phenylalanine was fed to them. Their evidence was not conclusive because it was deduced from differences which they observed upon qualitative tests applied to urines of different concentrations, but it is consistent with the known role of thiamine in the decarboxylation of α -keto acids. Also, evidence has been presented by Sure, Theis and Harrelson (88) and Govier and Greig (89) that ascorbic acid synthesis in the rat and dog is dependent upon an adequate supply of thiamine. Thus it is possible that thiamine deficiency leads to a secondary Vitamin C deficiency in these animals. Kaser and Darby (90) fed phenylalanine and tyrosine to rats maintained on a thiamine-free diet, but could detect no increase in the excretion of abnormal metabolic products. Following adminis-

tration of DL-phenylalanine by stomach tube to pair-fed deficient and control rats, the observed increases in the excretion of all metabolites tested for were statistically comparable. They measured the keto acid excretion after feeding phenylalanine by a colorimetric method for the determination of phosphorus: reduction of phosphomolybdic acid by the urine. The keto acid values were thus expressed as "phosphorus equivalents". One cannot help but feel that this is an odd way of determining the amount of keto acid metabolites from phenylalanine; a more specific method for such metabolites would have made these results more reliable.

DeLollis (91), however, reported the results of feeding 250 gm. of a cheese containing 4.2 gm. of tyrosine per 100 gm. to a human subject. He found that a 20-68% increase in urinary elimination of Millon-reactive substances was obtained. When 25 mg. of thiamine was administered intravenously at the same time as the feeding, this increase did not occur.

Evidence has been presented by Swendseid and co-workers (92,93) to the effect that tyrosine metabolism is altered in pernicious anemia. Since a deficiency of pteroylglutamic acid has also been found in pernicious anemia, Rodney, Swendseid and Swanson (94) investigated the possible effect of pteroylglutamic acid on the oxidation of tyrosine. Rats were made folic-acid-deficient by feeding sulfasuxidine. Liver homogenate from deficient and normal rats was then tested for tyrosine oxidation in the Warburg respirometer. The oxi-

dation of 0.5 mg. of tyrosine by 0.5 gm. of liver tissue was followed over a two-hour period. In other experiments 10% of crystalline pteroylglutamic acid were added to the liver suspensions from the deficient rats. All experiments showed decreased oxidation of tyrosine by livers from the pteroylglutamic acid-deficient rats, and a restoration of oxidation upon addition of the crystalline pteroylglutamic acid.

This report prompted Woodruff and Darby (95) to investigate the effect of pteroylglutamic acid in vivo upon tyrosine metabolism in the scorbutic guinea pig. A scorbutigenic diet plus 5% of L-tyrosine was fed to the guinea pigs. Tyrosine and p-hydroxyphenylpyruvic acid were determined in the urine; after stabilization at a high level, ascorbic acid and pteroylglutamic acid were fed orally. The excretion of tyrosine and keto acid before adding the vitamin supplement was 45% and 21% of added tyrosine, respectively; corresponding values in the group receiving ascorbic acid were 8.2 and 1.6%, and in the group receiving pteroylglutamic acid 19.8% and 5.4%, respectively.

Sealock and Lepow (96) have reported similar results upon administration of antipernicious anemia liver extracts to scorbutic guinea pigs. That the reduction in tyrosine metabolites found after injection of the liver extracts may be due to the pteroylglutamic acid contained in the extract is very unlikely. Liver extract, as contrasted with pteroylglutamic acid, does not increase the in vitro oxidation of tyrosine by liver

tissue.

In a later paper Woodruff and co-workers (97), report that not only does pteroylglutamic acid abolish the "hydroxyphenyluria" of scorbutic guinea pigs fed extra tyrosine, but so also does the pteroyltriglutamic acid. Antipernicious anemia liver extract did not abolish the excretion of hydroxyphenyl compounds by these same animals. One animal which was excreting homogentisic acid was given 5 mg. of pteroylglutamic acid daily for one week with no effect on the homogentisic acid excretion.

Essentially similar results were found by Rodney, Swendsen and Swanson (98) in in vitro experiments with pteroylglutamic acid and liver tissue. Liver tissue from rats deficient in pteroylglutamic acid gave a lowered rate of tyrosine oxidation; this rate was increased to nearer the normal rate upon addition of pteroylglutamic acid in vitro. However, liver extract and pteroylheptaglutamic acid had no effect upon the oxidation rate.

It may be well at this point to take stock of the tyrosine problem as it appears at the present. It is quite evident from the mass of experimental data accumulated that the aromatic amino acids, phenylalanine and tyrosine, may have two or even more paths of catabolism in the normal animal organism. Whether one of the paths is the main one, and the others less important, has not been definitely determined. It is probable that the mechanism predominating at any one time is decided by the particular needs of the organism at

that time. Garrod (99), in 1923, summarized the facts known about tyrosine oxidation up to that time in excellent fashion, and concluded that evidence for homogentisic acid being a normal intermediate of tyrosine degradation far outweighed that which could be brought against it. Thus, as reported by Abderhalden, a man, apparently normal, undoubtedly excreted homogentisic acid after ingestion of a large dose of tyrosine. Also reports of temporary or intermittent alcaptonuria favor such a view. The recent work on the experimental production of alcaptonuria in laboratory animals by dietary means also strengthens such a view. It is reasonable to assume that these dietary deficiencies produce alcaptonuria by affecting the cellular concentration of an enzyme or other factor necessary for the normal metabolism of tyrosine and phenylalanine rather than by endowing the organism with a new type of metabolic reaction not normally found. Yet it may be that the production of homogentisic acid is an emergency route which is not normally used or needed.

What is definitely known is that tyrosine and phenylalanine produce acetoacetic acid and eventually carbon dioxide and water in the normal organism. Strong evidence by means of isotope experiments (to be cited in the next section) indicates that the acetoacetic acid is formed, at least from phenylalanine, by the intermediate formation of a quinol. Beyond this everything is conjecture. Whether tyrosine is first oxidatively deaminated, whether it is oxidized to 3,4-

dihydroxyphenylalanine, whether it forms first a quinonoid structure as Felix suggests, remain to be proven. It is quite possible that the determination of the fate of the nitrogen or amino radical of the tyrosine will be the clue which will point more definitely to one or the other of the suggested pathways of normal oxidation.

D. The Application of Tracer Techniques
to Tyrosine Metabolism

In 1939 Schoenheimer and his collaborators (100-103) instituted a comprehensive study of protein and amino acid metabolism by the application of isotopic nitrogen, N^{15} . The theoretical considerations involved in the use of this isotope were discussed, and it was pointed out that a lower isotope concentration and smaller quantities of isotopic nitrogen are needed than is the case with metabolic studies with deuterium. The chance for dilution of N^{15} with ordinary nitrogen is much smaller, since the living cell contains only one-fiftieth as many nitrogen atoms as hydrogen atoms. They pointed out that a loss or transfer of isotopic nitrogen from an amino acid in the animal body is to be expected only if chemical reactions occur at the amino group.

They investigated the problems of synthesis involved in the introduction of the nitrogen isotope into amino acids. Since at that time the isotope was extremely rare and costly, it was necessary to consider only those reactions which were

most economical (i.e., reactions which give the best yields in the step which incorporates the isotope into the molecule). They made use of two general methods of synthesis: 1) catalytic hydrogenation of the corresponding α -keto acids in the presence of isotopic ammonia (104); and 2) coupling of the corresponding α -bromesters with isotopic potassium phthalimide (105). By method one they synthesized isotopic alanine, norleucine, phenylalanine, tyrosine, glutamic acid, and aspartic acid; by method two they synthesized glycine, deuteroleucine, and lysine.

Their experimental investigation of the metabolism of tyrosine consisted in feeding to a rat isotopic tyrosine as a supplement to a casein diet. The diet contained 15% casein, which in turn contained 4.5% of tyrosine. Tyrosine was then added to equal twice the amount furnished by the casein, i.e., 1.35 gm. of N^{15} -DL-tyrosine per 100 gm. of diet. At the end of the 10-day period, the animal had consumed 1.867 gm. of N^{15} -DL-tyrosine.

The animal was then sacrificed, and the collected urine, blood, liver, and carcass were analyzed for isotope content. They recovered about 50-60% of the administered N^{15} from the urine, the rest in tissues, mostly in proteins. From 25-30% of the isotope in the proteins was present in tyrosine, showing that some dietary tyrosine was directly deposited in the tissue proteins. Apart from tyrosine, the isotope was found in four different places: 1) in the α -amino group of dicar-

boxylic amino acids (transamination?); 2) in histidine isolated from the liver; 3) in arginine isolated from the liver (in that part of the guanido group which represents "potential urea"); and 4) in the "amide nitrogen" in proteins from liver and carcass, liberated as ammonia during proteolysis. Lysine was the only amino acid recovered which contained no isotope. They pointed out that a considerable portion of the isotope was used for the formation of other nitrogenous compounds, even of such amino acid molecules as were abundant in the diet.

Further isotope experiments were reported by Moss and Schoenheimer (77) who proved conclusively the conversion of phenylalanine to tyrosine in the normal animal body with the use of deuterio-DL-phenylalanine. This compound they prepared by an interesting method: an exchange reaction between DL-phenylalanine and 85% deuteriosulfuric acid. Upon feeding this isotopically-marked amino acid to rats, they found that both growing and mature animals converted phenylalanine into tyrosine. They found a continuous formation of tyrosine from the dietary amino acid even when the rats were receiving abundant supplies of tyrosine in the diet. This suggested to them that this conversion is an essential step in the oxidative degradation of phenylalanine.

Moss (106) found that tyrosine was also formed from β -phenyllactic acid in the rat by feeding the deuterium-marked lactic acid. This conversion took place despite the presence

of abundant supplies of tyrosine and phenylalanine in the diet. The conversion was found to be "automatic non-specific", i.e., if the organism has the ability to oxidize phenyllactic acid in the α -position, the reaction will always take place when the hydroxy acid is present, irrespective of the composition of the diet and the nutritional state of the organism.

The availability of radioactive isotopes resulting from the rapid development of atomic and nuclear chemistry provided another very useful isotope for metabolic studies: C^{14} . Friedberg (107) has shown the activity of protein synthesis by the intestine by means of radioactive isotopes. When methionine labeled with S^{35} , and tyrosine labeled with C^{14} , were injected into the jugular vein of an experimental animal, the protein of the intestinal mucosa showed the highest concentration of isotope. It was postulated that the intestinal wall is more active in protein synthesis in order to compensate for the large amounts lost as enzymes and mucous-proteins.

Winnick, Friedberg and Greenberg (108) fed tyrosine labeled with C^{14} in the β -position of the side chain to a normal and tumor-bearing rat. They found that the carbon chain of the tyrosine, unlike the nitrogen, does not contribute significantly to the formation of other amino acids. The isotope was rapidly incorporated into proteins of various organs following the administration, which was by injection. Intestinal mucosa, kidney, and plasma had the highest con-

centration of C^{14} , followed by liver and spleen. The tumor proteins had high activity and accounted for 9.75% of the administered dose. Amino acid isolations showed that almost all of the radioactivity of the proteins was due to tyrosine itself. In the urine, isotope was present in tyrosine, urea, creatinine, hippuric acid, and ketone bodies. The presence of C^{14} in hippuric acid and creatinine was postulated to be due to the following conversions:

tyrosine \longrightarrow acetoacetic acid \longrightarrow acetic acid \longrightarrow glycine

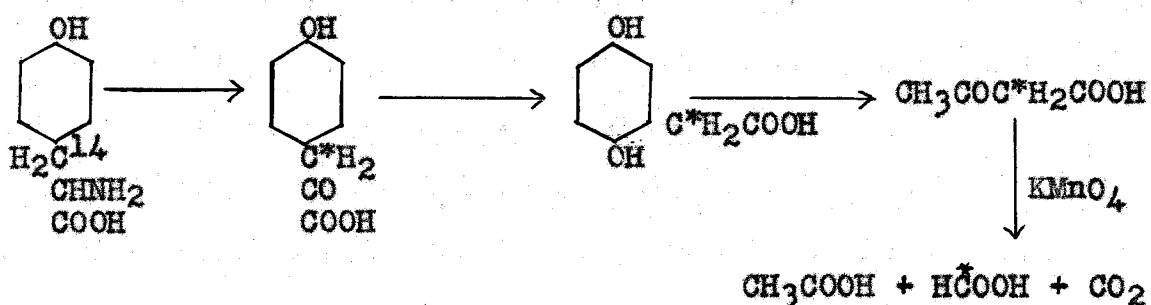
Reid and Jones (109) reported a similar study of administration of radioactive tyrosine to mice bearing a melanosarcoma. After 72 hours, 30% of the administered dose appeared in the respiratory carbon dioxide, 40% in the urine and feces, and 30% in the body. Although radioactivity was found in every tissue of the body, the adrenals (epinephrine), thyroids (thyroxine), intestines (protein anabolism), kidneys, liver, plasma, spleen, and tumor showed the highest specific activities. Erythrocytes had the lowest, with bone the next lowest.

All of the foregoing researches have been of the same type, i.e., metabolic turnover studies. Although much can be deduced from such experiments, clues as to oxidative and catabolic mechanisms must needs be indirect. In addition, they all suffer from the same error: they used racemic tyrosine instead of the natural isomer. It is well known that

many, if not all, of the enzyme systems concerned in protein and amino acid catabolism have a stereochemical specificity for the naturally-occurring isomers. In fact, some evidence has been given to show that D-amino acids may even be toxic to the organism, although admittedly in extremely high doses. On the other hand, Albanese and co-workers (110) and Bubl and Butts (111) have shown the possibility of a species-difference in the handling of D-tyrosine. Humans evidently cannot utilize the unnatural isomer, whereas the white rat can. Nevertheless it is felt that results in metabolic experiments are more valid and more correctly interpreted when the natural isomers of the amino acids are used.

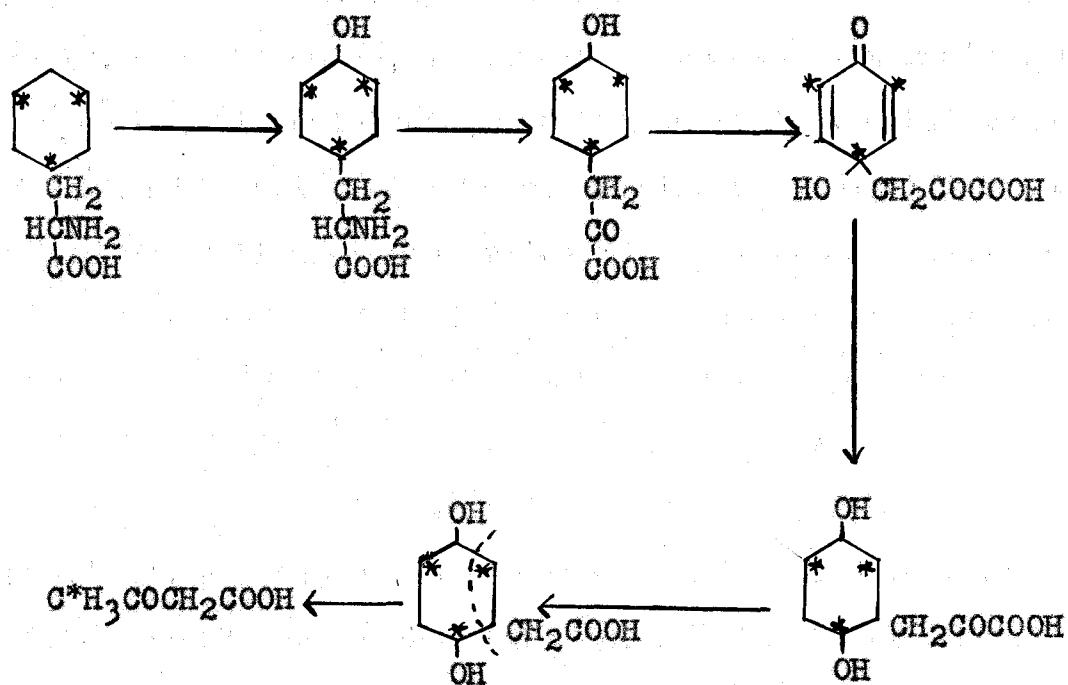
Weinhouse and Millington (112), with the use of L-tyrosine containing C^{14} in the β -position of the side chain, have substantiated the conversion of this amino acid to acetoacetate by rat liver slices, and provided information concerning the mechanism of this transformation. The liver slices were incubated for 2 hours with the tyrosine; the deproteinized mixture was then treated by the Van Slyke procedure for conversion of acetoacetate to acetone and carbon dioxide. The acetone had a high specific activity, the carbon dioxide had a very low activity. They then investigated the position of the C^{14} in the acetoacetate molecule. They found that when acetoacetate is treated with an excess of potassium permanganate in the cold it is rapidly and quantitatively converted into one molecule each of acetic, formic and carbonic

acids. The reasonable assumption was made that these three acids are derived, respectively, from the β -, γ -, the α -, and the carboxyl carbons of the acetoacetate. Upon performing this oxidation and isolating the acids, they found almost all of the activity in the formic acid, or originally, therefore, in the α -carbon atom of the acetoacetate. Thus the acetoacetate had its origin as in the following scheme:



Schepartz and Gurin (113) have recently reported experiments on the intermediary metabolism of phenylalanine labeled with C^{14} . When DL-phenylalanine labeled with radioactive carbon in the carboxyl and α -carbon of the side chain is fed to phlorhizinized rats, the respiratory carbon dioxide and urinary ketone bodies had high activity. Incubation of this same phenylalanine with liver slices gives, in the deproteinized mixture, $\text{CH}_3\text{COCH}_2\text{C}^*\text{OOH}$. They then used phenylalanine labeled in the 1, 3, and 5 positions of the benzene ring. When this compound was incubated with liver slices, they isolated C^*O_2 and $\text{C}^*\text{H}_3\text{COCH}_2\text{COOH}$. This is direct proof that the side chain of phenylalanine shifts to an adjacent ring carbon during formation of the ketone bodies. Although they do not

give any formulation for this change, this writer feels that the quinol intermediate is the most logical explanation of this shift, as shown:



III. EXPERIMENTAL

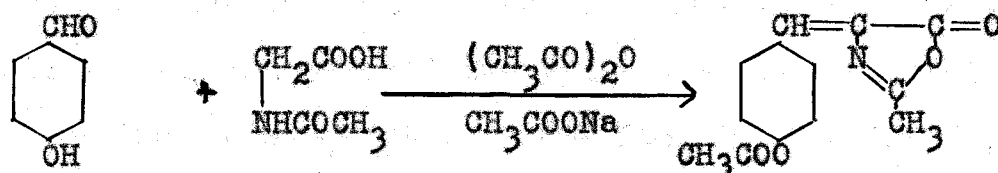
A. Synthesis of N¹⁵-DL-Tyrosine

As has been mentioned in the Historical section, Schoenheimer and his associates (102) found the Knoop reaction the desirable method of introducing isotopic nitrogen into the tyrosine molecule. Since this method was found convenient and afforded an excellent yield of product, it was adopted for our study.

The primary intermediate for the Knoop reaction is p-hydroxyphenylpyruvic acid. The method adopted for its production is an adaptation of the synthesis of phenylpyruvic acid as given by Herbst and Shemin (114), with modifications where necessary. This synthesis was used by Sealock, Parkinson and Basinski (76), and many helpful points were acquired from their experience.

The method may be summarized as follows: condensation of p-hydroxybenzaldehyde with acetylglycine to give the "azlactone" of p-acetoxy-α-acetaminocinnamic acid (Beilstein designation, 2-methyl-4-(4'-acetoxybenzal)-5-oxazolone); opening of the azlactone ring with water to give p-acetoxy-α-acetaminocinnamic acid; and hydrolysis of this to give p-hydroxyphenylpyruvic acid.

1. 2-Methyl-4-(4'-acetoxybenzal)-5-oxazolone



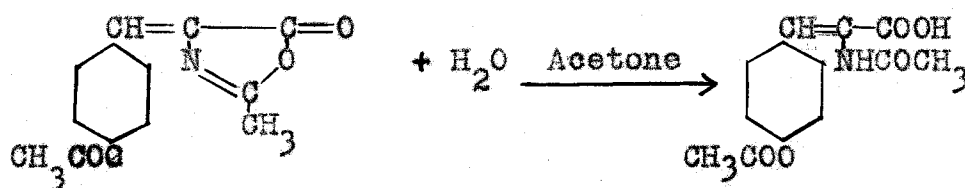
Herbst and Shemin's directions for the corresponding benzal oxazolone require 0.25 moles of acetylglycine, 0.37 moles of benzaldehyde, 0.185 moles of sodium acetate, and 0.625 moles of acetic anhydride, and refluxing for one hour on the steam bath. Previous work, both by Sealock, Perkinson and Basinski and in this laboratory¹, had shown that the azlactone formation with substituted benzaldehydes usually required much longer refluxing time. So the first run of azlactone was made using the above proportions (*p*-hydroxybenzaldehyde of course substituted for benzaldehyde) and a five hour heating time on a boiling water bath, with an air condenser attached to the reaction flask. After overnight refrigeration, the solid brown-yellow mass was broken up and stirred well with 125 ml. of ice-cold water and returned to the refrigerator for another 24 hours. The product was then filtered, washed well on the funnel with ice-cold water, and dried in a vacuum desiccator over KOH and P₂O₅. The resultant yield was only 32.6% of the theoretical.

In modifying the above conditions, it was found that

¹ Sealock and Speeter, unpublished.

increasing the amount of acetic anhydride to 1.1 moles raised the yield of azlactone to 59.6% of the theoretical. A further adjustment of the proportions of all the reactants increased the yield to 66%, and this was used for all subsequent runs. This was: 0.4 moles of acetylglycine, 0.3 moles of anhydrous sodium acetate, 0.37 moles of *p*-hydroxybenzaldehyde, and 1.33 moles of acetic anhydride (preferably redistilled). These proportions with the above procedure gave consistent yields of 66-70% of crude azlactone. The crude product was found sufficiently pure to use in the second step. If desired it may be recrystallized from ethyl acetate with the addition of a little petroleum ether after solution is effected. After recrystallization, the azlactone melts at 133.5-134.5^o (uncorr.).

2. *p*-Acetoxy- α -acetaminocinnamic acid.



Here again Herbst and Shemin's directions for the preparation of the ring-unsubstituted cinnamic acid were followed first. This procedure calls for solution of 0.25 moles of the azlactone in 450 ml. of acetone and 175 ml. of water, and completing the hydrolysis by refluxing for four hours. Most of the acetone is then

distilled off, boiling water added to dissolve the cinnamic acid, the solution filtered, treated with Norite and filtered again, and refrigerated. This procedure was most unsatisfactory with the more highly-substituted azlactone. The yield was poor, 47.7%, and a large amount of orange-brown insoluble solid was left after distillation of the acetone and addition of the boiling water.

The modification which was made and adopted for all succeeding batches was as follows: 0.12 moles of the azlactone were dissolved in 260 ml. of acetone and heated to reflux temperature in an oil bath. When the acetone was refluxing, 100 ml. of water were added dropwise through the top of the reflux condenser, and refluxing was continued for 4 hours. The acetone-water solution was then refrigerated. After filtering off the product, a second crop could be obtained by concentrating the filtrate in vacuo. By this procedure the yields varied from 72-94% of crude substituted cinnamic acid.

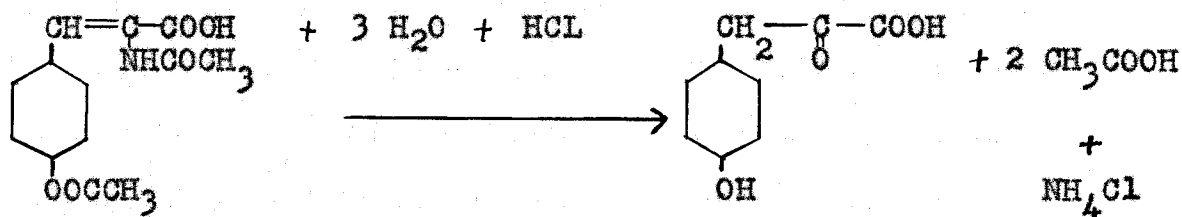
Since large losses occurred in recrystallization of p-hydroxyphenylpyruvic acid, due to the general instability of α -keto acids, it was deemed advisable to effect purification at this stage in the stepwise synthesis. The substituted cinnamic acid could be recrystallized from water, but large amounts of water were required, and the solubility of the acid dropped sharply on slight cooling, so that filtering of the Norited solution was difficult without having the pro-

duct crystallize out on the funnel. Recovery was also poor, due probably to hydrolysis of the substituted cinnamic acid to the corresponding pyruvic acid. Dioxane was found to be fairly suitable, giving a pure product melting at 213-213.5° (uncorr.). However, here again, large volumes of solvent were necessary, 1 gm. of acid requiring 60 ml. of dioxane, and recovery from this dilute a solution was generally poor, varying from 37-70%.

Glacial acetic acid was found to be most satisfactory as a solvent for recrystallization. One gm. of acid required 15 ml. of solvent, Norite could be used effectively to decolorize the product, and recovery varied from 82-88%. The melting point of the substituted cinnamic acid from glacial acetic acid was a little low, 208.5-209° (uncorr.), but the product was found to be very satisfactory for the production of pure p-hydroxyphenylpyruvic acid.

Early in the work reported here, another method of opening the azlactone ring was tried, that used by Bergmann and Stern (115) and by Dakin (116). Their procedure involved dissolving the azlactone in normal or half-normal alkali at 60°, cooling, and acidifying with dilute hydrochloric acid. The product obtained by this method is p-hydroxy- α -acetaminocinnamic acid. However, we obtained a deep-red crystalline crop which had to be purified extensively by recrystallization from boiling water. The final yield of pure product by this method was 17-28%, so this method was rejected.

3. p-Hydroxyphenylpyruvic acid.

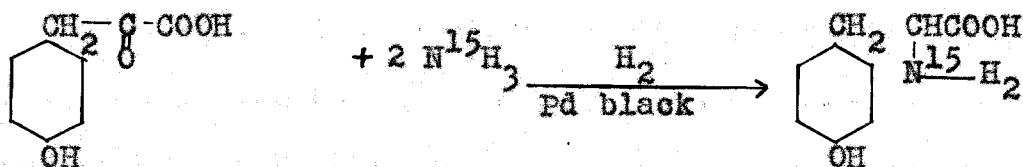


Herbst and Shemin's directions for preparation of phenylpyruvic acid were followed for this reaction. The substituted cinnamic acid (0.05 mole) was refluxed for three hours with 200 ml. of 1 N hydrochloric acid. Refrigeration of the resulting solution gave 72-78% of slightly yellow crystals, melting at 202-203° (uncorr.). Since it was desired to use pure p-hydroxyphenylpyruvic acid for conversion to isotopic tyrosine, the procedure was modified by adding Norite to the hot solution immediately after refluxing was ended, filtering while hot, and refrigerating for 24-48 hours. This gave yields of from 48-53% of pure white product, melting at 204.5-205°. This modification was found to be more satisfactory than recrystallizing the product originally obtained; such a procedure resulted in a pure product, but in only 20-30% final yield.

p-Hydroxyphenylpyruvic acid, like all α-keto acids, slowly decomposes upon standing, turning a light- to dark-brown color. Therefore this step was not performed until we were ready to use the intermediate in the next step. Thus, stockpiles of p-acetoxy-α-acetaminocinnamic acid were accumulated, and p-hydroxyphenylpyruvic acid synthesized when

needed in amounts adequate for the next reaction.

4. N¹⁵-DL-Tyrosine.



The reaction used to incorporate isotopic nitrogen into the tyrosine molecule was first reported by Knoop and Oesterlin (104). They used this reaction as an analogy or model for a possible method of formation of amino acids in the animal body from carbohydrate and other non-protein precursors. Schoenheimer and Ratner (102) later adapted it for synthesis of N¹⁵-DL-tyrosine, improving the reaction by using palladium black, instead of platinum black, as the hydrogenation catalyst.

The isotopic ammonia used in the reaction was generated from ammonium nitrate containing 7.5 atoms% N¹⁵ in the ammonium radical. This was done by dropping a concentrated water solution of the appropriate amount of enriched ammonium nitrate into boiling 40% sodium hydroxide. Nitrogen gas was bubbled through the mixture to minimize bumping, and the isotopic ammonia was swept directly into the reaction bottle containing the palladium catalyst suspended in 95% ethyl alcohol. A "cold-finger" condenser was placed between the generating flask and the hydrogenation bottle to prevent

water distilling over. The hydrogenation reaction bottle, an ordinary citrate bottle, was cooled in a mixture of alcohol and dry-ice to further the solution of the ammonia in the alcohol. To save any isotopic ammonia which was not absorbed in the reaction bottle, a lead-off tube from the hydrogenation bottle was immersed in dilute sulfuric acid, designated Trap 1.

It was found that two moles of ammonia were needed for one mole of p-hydroxyphenylpyruvic acid: one mole for the amino group of the tyrosine, and one mole to form the ammonium salt of the acid. This latter amount is recovered from the reaction mixture at the end of the hydrogenation, as will be described later.

The isotopic ammonium nitrate was obtained from Eastman Kodak Company, and contained 7.5 atoms% N^{15} , or 7.12 atoms% excess N^{15} over normal abundance. Such an enrichment allows a little over a 1:200 dilution with normal nitrogen, since the mass spectrometer can detect a 1% difference in the normal ratio of N^{15}/N^{14} of 0.00379 (see Appendix A). On an absolute basis, the amount of isotope used in excess of the normal amount may be calculated easily. Thus, 6 gm. of enriched ammonium nitrate are used in a single synthesis of N^{15} -DL-tyrosine. Since this salt contains 7.12 atoms% excess N^{15} , the amount used is 0.427 gm. of excess N^{15} .

In a typical run, 6 gm. (0.074 moles) of ammonium nitrate were dissolved in 20 ml. of water and dropped into

40 ml. of 10 N sodium hydroxide heated to boiling with a microburner. The ammonia generated was swept over by a stream of nitrogen into the hydrogenation bottle containing 2 gm. of catalyst suspended in 35 ml. of 95% ethyl alcohol. It was found that from 2½-3½ hours were required to sweep over this amount of ammonia; the long time necessary was probably due to a too-efficient condenser in the generating system.

A suspension of 6.63 gm. (0.037 moles) of *p*-hydroxyphenylpyruvic acid in 35 ml. of water was then added quickly to the hydrogenation bottle. The bottle was placed on a Parr low-pressure hydrogenation apparatus and connected to the hydrogen reservoir tank.

The reaction bottle was then evacuated until the alcohol just started to boil, then filled with hydrogen, evacuated and filled with hydrogen again, evacuated and filled with hydrogen a third time. Three such evacuations and fillings with hydrogen were found to be sufficient to remove practically all of the air. With the bottle filled with hydrogen and opened to the hydrogen reservoir, the shaker mechanism was started, the temperature read and the hydrogen pressure in the system noted.

The hydrogenator had previously been standardized by the method of Adams and Voorhees (117), in which 0.1 mole of pure maleic acid, melting at 134.0-134.5°, was quantitatively reduced to succinic acid. By noting the dial

pressures at the beginning and end of the reaction, it was ascertained that the uptake of 0.1 mole of hydrogen corresponded to a pressure drop of 9.9 pounds per square inch at 25°C. Thus the theoretical uptake of hydrogen for the production of tyrosine as pictured on page 70 would be the pressure drop corresponding to 0.037 moles, i.e., 3.7 pounds per square inch. The shaking was continued until this pressure drop was obtained. In several trial runs with normal ammonia, this uptake was usually observed to occur in from 2-4 hours with platinum black catalyst, and from 0.5-3 hours with palladium black. In a few instances this pressure drop was not realized; inasmuch as these instances coincided with the third or fourth run with the same catalyst, it was concluded that the catalyst needed regeneration every two or three runs for this particular reaction.

At the end of the reaction, the bottle was removed from the apparatus, and excess ammonia was distilled into dilute sulfuric acid (Trap 2). For this distillation, which was carried out over a boiling water bath, it was found advantageous from a time standpoint to add about 5 ml. of N sodium hydroxide to the reaction mixture. Nitrogen gas was bubbled through to prevent bumping and to sweep the ammonia gas over into the receiver; it was also found necessary to add alcohol to the distillation mixture periodically to replace that which distilled. This distillation usually required two to three hours in order to recover all of the

ammonia; the end of the time was determined by testing a drop of the distillate with litmus.

Traps 1 and 2 were analyzed by the Kjeldahl method for nitrogen, and, in the case of runs with isotopic ammonia, saved for recovery of the isotope. From the nitrogen determinations it was found that the reaction to form tyrosine usually consumed from 0.03 to 0.04 moles of ammonia when 0.037 moles of the hydroxyphenylpyruvic acid were used.

After the distillation of ammonia, a small amount of hot water and about 2-3 ml. of concentrated hydrochloric acid were added to dissolve the tyrosine, which precipitates as it is formed on the hydrogenator. The catalyst was filtered off, washed well with alcohol, and stored under alcohol for re-use. The tyrosine was precipitated from the filtrate by neutralization with sodium hydroxide, plus glacial acetic acid dropwise until slightly acid to litmus, followed by refrigeration. After a minimum time of 24 hours in the refrigerator, the tyrosine was filtered off, washed with absolute ethyl alcohol and ether, and dried.

As has been pointed out already, it was found that a particular amount of catalyst could be used only two or three times. The third use usually gave variable results; therefore in all runs with isotope, the catalyst was never used more than twice. Schoenheimer has pointed out that this type of reaction requires a rather large amount of

catalyst. This was confirmed by our experiments; the optimum amount was found to be about 2.1-2.25 gm. of catalyst for 0.037 moles of hydroxyphenylpyruvic acid. We have also confirmed another fact which Schoenheimer reported, namely, that palladium black is much better than platinum black as a catalyst for this reaction. This is amply illustrated by reference to Table 1, which shows a comparison of the two catalysts used in the reaction.

A summary of the reactions used and the yields obtained in the synthesis of N¹⁵-DL-tyrosine is given in Figure 1, page 76.

Table 1
Comparison of Time and Yield of Tyrosine Production
with Different Catalysts

Run	Catalyst	Time for theoretical uptake of hydrogen	Yield ¹
1	Platinum black	4 hours	15%
2	" "	7	22.4
3	" "	3½	52.5
4	Palladium black	3	75
5	" "	5½	89.4
7	" "	1	72.8
9	" "	¾	88.2
10	" "	1½	90.1

¹Yield based on 0.037 moles of p-hydroxyphenylpyruvic acid.

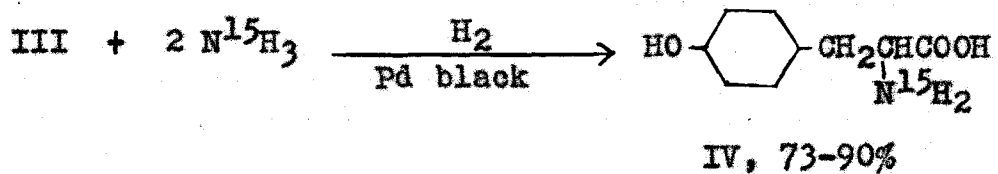
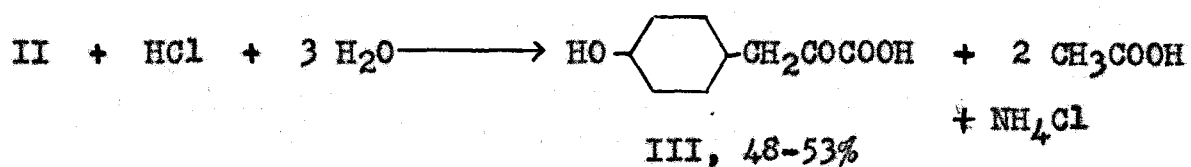
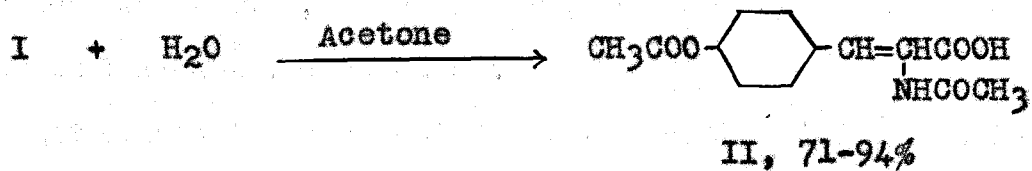
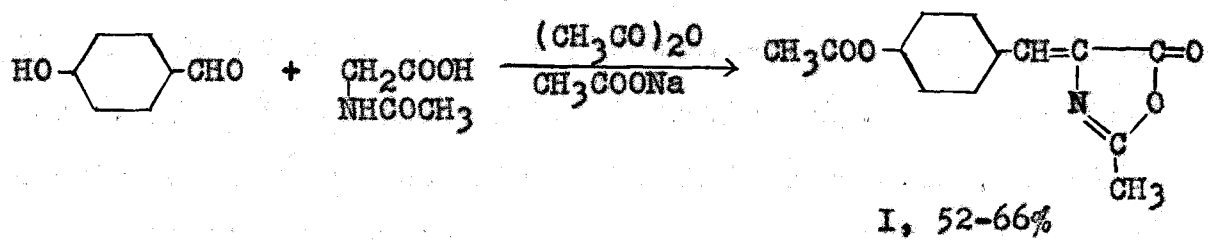


Figure I. Reactions and Yields in the Synthesis of N¹⁵-DL-Tyrosine

B. Resolution of N¹⁵-DL-Tyrosine

In view of the difference exhibited by the animal organism in the metabolic handling of L-tyrosine and D-tyrosine, as cited in the Historical section, it was considered of prime importance to use the L-isomer in our investigations. Therefore the resolution of the synthetic N¹⁵-DL-tyrosine to obtain N¹⁵-L-tyrosine was our next concern.

A survey of resolution methods already published shows surprisingly few good methods of obtaining the natural isomer from the racemate. This is probably due to the fact that optically-pure L-tyrosine is easily isolated from natural materials without racemization occurring. Until the recent use of isotopes, therefore, there has been little need of a practical and satisfactory resolution procedure. Fischer (118) in 1899 resolved the benzoyl derivative of DL-tyrosine with brucine to obtain 44% yield of benzoyl-L-tyrosine. The entire procedure, including benzylation, resolution, and hydrolysis, was felt to be too long and give too low a yield to use with the isotopically-marked amino acid. Abderhalden and Sickel (119) used brucine to resolve formyl-DL-tyrosine, but were able to obtain only the formyl-D-tyrosine. Triem (120), using cholestanone sulfonic acid, also obtained only the D-isomer. Sealock (121), in a paper on the preparation of D-tyrosine, has reported the isolation of the L-isomer. He used the catalytic racemization procedure of du Vigneaud and Meyer (122), in which the diacetyl-DL-tyrosine is prepared

from the natural tyrosine. Upon the hydrolysis of this to the N-monoacetyl-DL-tyrosine, a good yield of the D-isomer is obtained with brucine. The mother liquors from this give the L-isomer by distillation of the alcohol and recrystallization of the residue from water until optically pure. Although this is admittedly an indirect method, it seemed the most satisfactory one at hand, especially since the acetyl-DL-tyrosine can be prepared in excellent yields.

1. Preparation of N¹⁵-acetyl-DL-tyrosine

The procedure used for the preparation of the monoacetyl derivative of tyrosine was essentially that used by Sealock and du Vigneaud (123) for the preparation of acetyl-DL-tryptophane. All steps in this and in subsequent sections were tried thoroughly with normal (i.e., non-isotopic) compounds before proceeding with the N¹⁵-containing substance.

In a typical run, 4 gm. (0.022 moles) of DL-tyrosine were dissolved in 8 ml. of water and 11.2 ml. 2 N sodium hydroxide, and cooled in ice-water. The solution was stirred mechanically, and 53.5 ml. of 2 N sodium hydroxide and 5.35 ml. of redistilled acetic anhydride were added in ten portions over a period of one-half hour. Care was taken to keep the solution alkaline to phenolphthalein at all times. The mixture was stirred for one hour at room temperature, and then the calculated amount of 6 N sulfuric acid to neutralize the sodium hydroxide exactly was added. This amount was determined by direct titration of the sulfuric

acid with the alkali used in the reaction, using phenolphthalein as indicator.

The neutralized mixture was concentrated in vacuo, water added and concentrated again, and again a third time water added and concentrated. The resulting mixture of brown oil and white solid was extracted with about 50 ml. of acetone. This was filtered, and the solid on the funnel (sodium sulfate) was washed well with more acetone and filtered. The acetone solution was concentrated in vacuo, water added and concentrated again; this was repeated until all traces of acetone were absent. The resulting oil was then transferred to a test tube with a transfer pipette and diluted to 15 ml. volume with water. The sides of the test tube were scratched well with a glass stirring rod until crystallization commenced, and then refrigerated for 24-36 hours.

The precipitated acetyltirosine was filtered, and the filtrate was concentrated to obtain a second crop. Yield, 4.1 gm. of first crop and 0.2 gm. of second crop, 80% of theoretical. The acetyltirosine was the monohydrate, melting at 89-90°C., uncorrected. Sealock (121) gives as the melting point 94-95°C., corrected.

It was found that the acetyltirosine thus obtained was satisfactory to use without further purification. The yields varied between 74 to 85% in all preparations with the N¹⁵-tyrosine. In all, from 35 gm. of N¹⁵-DL-tyrosine, 36 gm. of N¹⁵-acetyl-DL-tyrosine were prepared, giving an average

yield of 77% of the theoretical.

2. Attempted resolution with brucine

According to the procedure of Sealock (121), 4.05 gm. (0.0168 moles) of N-acetyl-DL-tyrosine monohydrate and 6.62 gm. (0.0168 moles) of anhydrous brucine (melting 173-174° C.) were dissolved in 42.7 ml. (4 volumes) of absolute ethanol plus 0.3 ml. of water by warming in a boiling water bath. This solution was allowed to stand at room temperature until crystallization commenced, after which it was refrigerated.

The resulting crystalline precipitate was recrystallized three times from 4 volumes of 95% ethanol to give a 79% yield of optically-pure brucine-acetyl-D-tyrosine. However, we were able to recover only 0.7 gm. (13% of theoretical) of pure brucine-acetyl-L-tyrosine from the mother liquors, plus 1.16 gm. of racemic mixture.

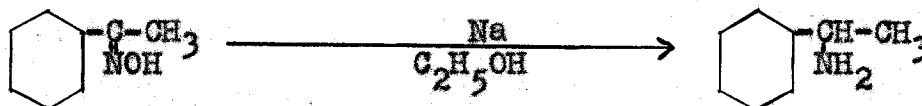
A repetition of this procedure necessitated seven recrystallizations in order to purify the brucine-acetyl-D-tyrosine. At this point it was decided that this method was going to be unsuitable for our purposes, and other means of obtaining the L-isomer were sought.

3. Development of a resolution method by means of optically-active α -phenylethylamine

Sealock and du Vigneaud (123), in 1932, had reported the preparation of D-tryptophane in good yield by the use of d- α -phenylethylamine and acetyl-DL-tryptophane. Later, in this

laboratory¹, it was found that l- α -phenylethylamine gives a good yield of acetyl-L-tryptophane from the racemate. Therefore it was decided to try d- and l- α -phenylethylamine with acetyl-DL-tyrosine, in the hopes that one of these optically-active bases would yield a sparingly-soluble salt with acetyl-L-tyrosine and thus enable us to obtain the desired isomer directly. This necessitated the synthesis and resolution of the amine.

a. Synthesis of dl- α -phenylethylamine. dl- α -Phenyl-ethylamine is best made by catalytic reduction of acetophenone in the presence of ammonia (124). This, however, requires quite high pressures (in the neighborhood of 150 atmospheres), and a suitable high-pressure hydrogenation apparatus was not available to us. Mohr (125) has reported a synthesis of this amine by reduction of acetophenone oxime, thus:



He did not report a yield for this reaction. Several runs by this method gave a maximum yield of 21% of amine in our hands.

The amine was therefore synthesized by the procedure of Ingersoll (126). Since no modifications were made in his

¹Sealock and Barclay, unpublished experiments

The yield of this salt was 66% of the theoretical.

The amine from the mother liquors of the above crystallizations, containing a large excess of the l-form, was recovered from the malate salt and precipitated as l-phenylethylamine-d-tartrate. This salt was recrystallized until it gave a constant specific rotation of -13.6° (Ingersoll gives -13.2° for the salt). This fraction was obtained in a 62% yield.

One run of the above salts was stored as such and the amine recovered in the amount desired for use. From another run the l-amine and the d-amine were recovered from their respective salts and stored in the pure free state.

Figure 2, page 84, shows a schematic diagram of such a resolution.

c. Salt formation with α -phenylethylamine and acetyltyrosine. The first point to investigate was whether either isomer of the amine would form a crystallizable or sparingly-soluble salt with acetyltyrosine. Accordingly two solutions of the amino acid, each containing 0.5 gm. in 5 ml. of 95% ethanol, were made. To one was added an ether solution of 0.25 gm. of l-phenylethylamine, and to the other an ether solution of 0.25 gm. of d-phenylethylamine. These ether solutions were made by dissolving 0.56 gm. of l-phenylethylamine-d-tartrate for the one, and 0.53 gm. of d-phenylethylamine-l-malate for

109 gm. (0.9 mole) dl- α -phenylethylamine
 91 gm. (0.68 mole) l-malic acid

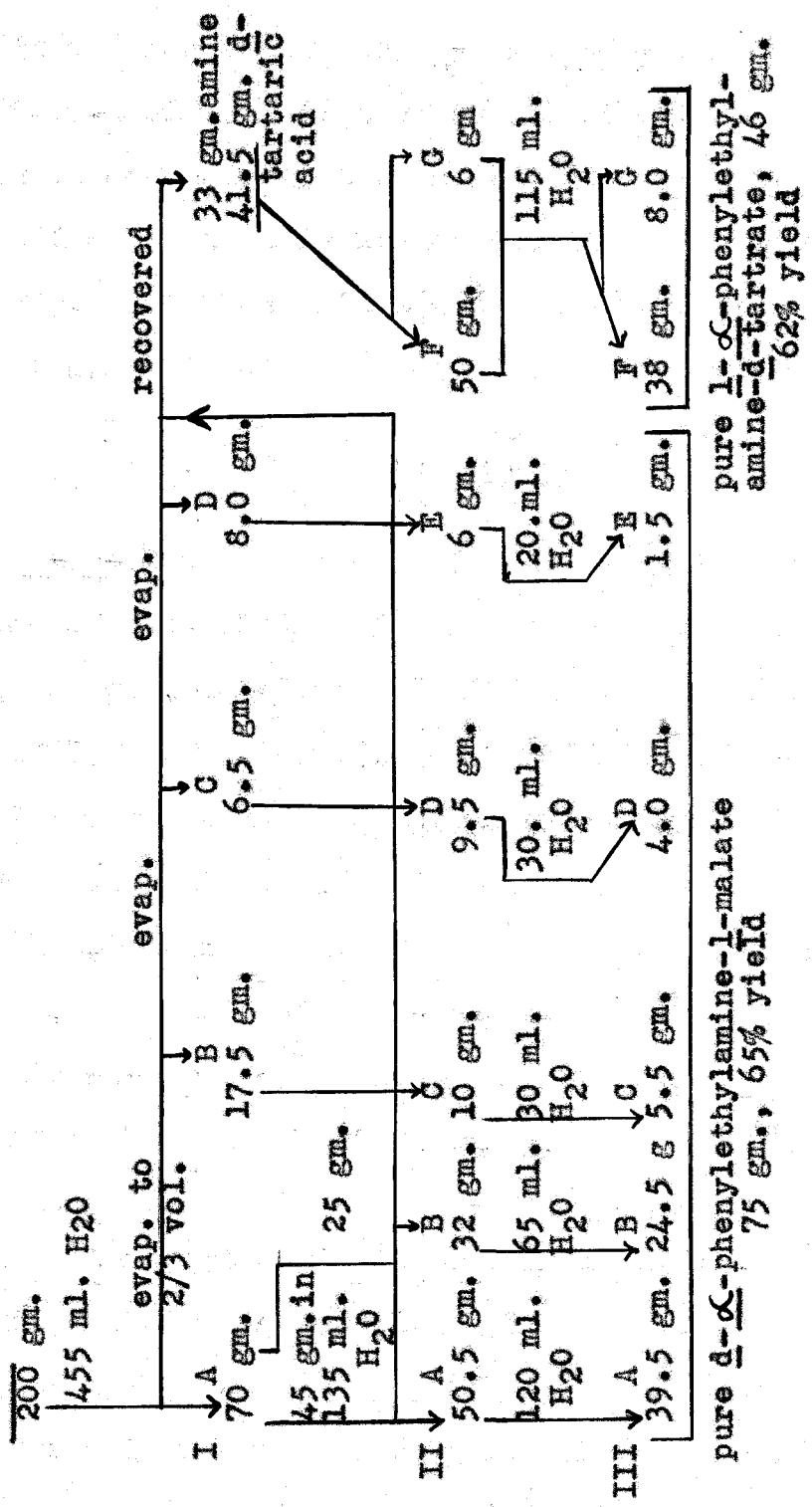


Figure 2. Resolution of dl-phenylethylamine

the other, in 5 ml. of water, adding 2 ml. of 20% sodium hydroxide and extracting four times with 5 ml. portions of ether. The resulting ether solution of the appropriate amine was dried with anhydrous sodium sulfate and filtered into the alcohol solution of the amino acid. The ether was evaporated off by aeration, and the alcohol solution cooled in ice-salt mixture.

Both tubes gave a small amount of white crystalline solid. It was found that changing the solvent to absolute ethanol gave a larger amount of precipitate. The l-amine gave more salt formation with the acetyltyrosine than did the d-amine. Remembering that with acetyltryptophane the l-phenylethylamine gave a more insoluble salt with acetyl-L-tryptophane, it was decided to investigate the possibility of the same type of salt formation with the l-phenylethylamine and acetyltyrosine.

Before proceeding further, the salt formed with the l-amine and acetyltyrosine was investigated for degree of hydration. Thus, 0.1514 gm. of the salt was dried in a Fischer dryer over phosphorus pentoxide; the weight of the dry salt was 0.1372 gm. The loss in weight, due to the removal of water of hydration, amounted to 0.0142 gm., or 9.3%. This value may be compared with the theoretical value of 9.45% water for two molecules of water of hydration.

Then 4.0 gm. (0.0164 moles) of acetyl-DL-tyrosine mono-

hydrate and 1.98 gm. (0.0164 moles) of pure l-phenylethylamine were dissolved in 24 ml. (4 volumes) of absolute ethanol plus 0.3 ml. of water with warming. The sides of the tube were scratched with a glass rod, and the mixture refrigerated.

The solid which was filtered off after 24 hours amounted to 1.0 gm. when dry. In a capillary tube it softened at 84° and melted at $95-98^{\circ}$ with decomposition. To investigate which isomer of the acetyl-DL-tyrosine had been precipitated in the salt, 0.213 gm., equivalent to 0.125 gm. of acetyl-tyrosine, were dissolved in 5 ml. of water, 5 ml. of 5 N sodium hydroxide added and the mixture extracted three times with 4 ml. portions of ether. The extracted water layer containing free acetyl-tyrosine was neutralized and made up to 25 ml. with water, and the optical rotation of this solution determined in the polarimeter. The specific rotation was -30.1° (0.5% solution, 2 dm. tube).

This first crop of salt was recrystallized further from three volumes of absolute ethanol, and the resulting precipitate investigated for evidence of further optical purification by the above method. The specific rotation of this fraction, after extraction of the amine, was -35.2° . Sealock (121) gives as specific rotations, $+47.3^{\circ}$ for acetyl-L-tyrosine (0.5% in water), and -48.3° for acetyl-D-tyrosine. Thus it was evident that the particular isomer involved in the salt formation with l-phenylethylamine was the acetyl-D-tyrosine.

Since the acetyl-L-tyrosine was the isomer desired, it was decided to try d-phenylethylamine with acetyl-DL-tyrosine.

Four grams (0.0164 moles) of acetyl-DL-tyrosine and 2.0 gm. (0.0164 moles) of d-phenylethylamine were dissolved in 18 ml. (3 volumes) of absolute ethanol plus 0.3 ml. of water with warming. The lowered ratio of salt to solvent was tried in hopes that a larger yield of salt would be obtained.

The first crop of salt amounted to 5.0 gm., had a specific rotation of $+ 4.97^{\circ}$, and a melting point of $95-102^{\circ}\text{d}$. Upon going through the procedure for splitting the salt as given on page 86 in order to observe the specific rotation of the acetyltyrosine involved in the salt formation, a value of $+ 4.3^{\circ}$ was obtained. Further recrystallization and investigation of the acetyltyrosine precipitated as the amine salt gave specific rotation values of $+ 15.5^{\circ}$, $+ 35.4^{\circ}$, and $+ 51.4^{\circ}$. The specific rotation of the acetyltyrosine-d-phenylethylamine salt from the last crystallization was found to be $+ 36.87^{\circ}$. Thus it was obvious that optically-pure acetyl-L-tyrosine can be obtained by the use of d-phenylethylamine in relatively few recrystallizations. The yield in this first attempt was 33% of the theoretical.

The details of this resolution are summarized in Figure 3, which served as a guide for subsequent resolutions of the N^{15} -acetyl-DL-tyrosine by the use of d-phenylethylamine.

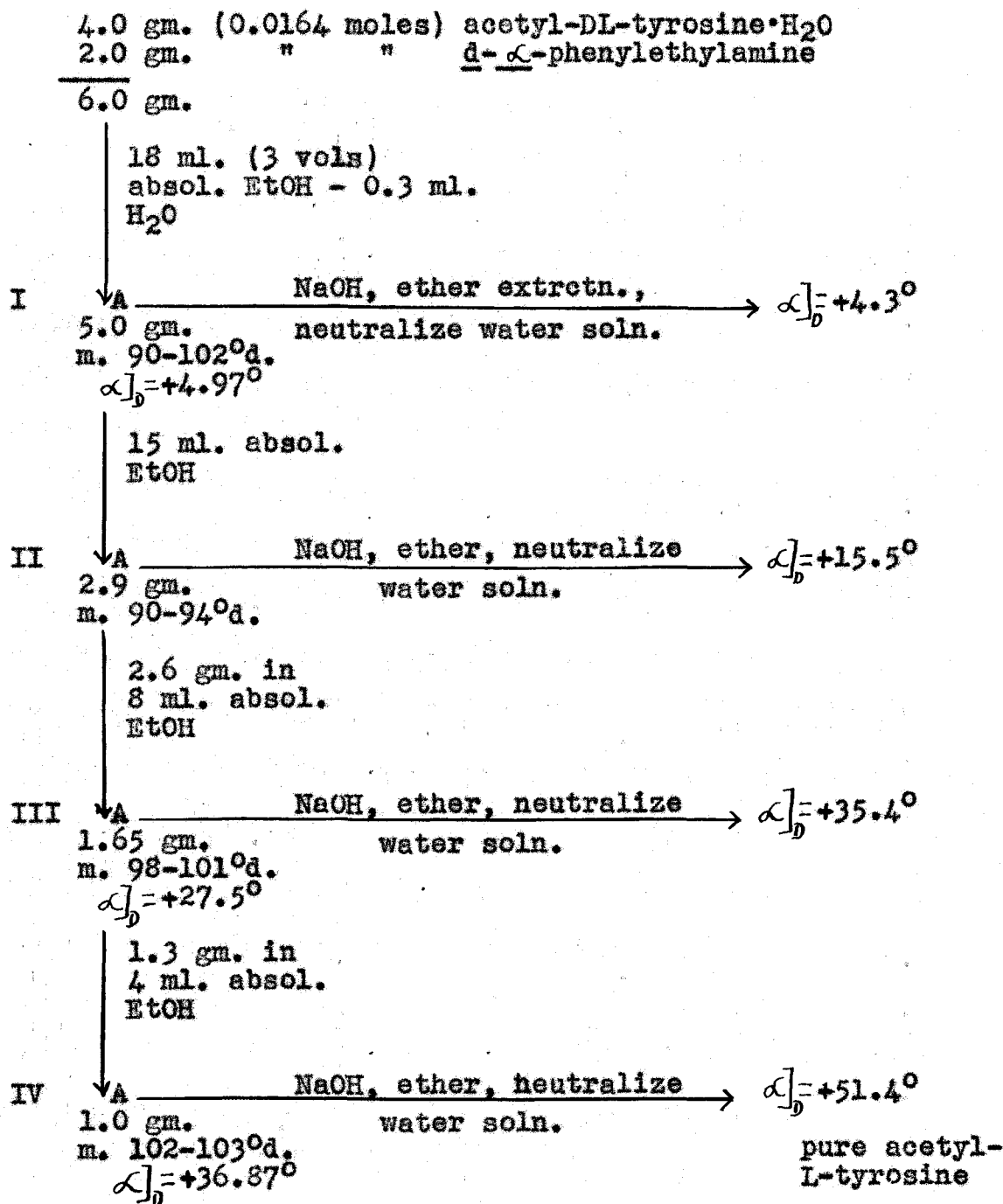


Figure 3. Resolution of acetyl-DL-tyrosine with d-α-phenylethylamine

d. Resolution with d- α -phenylethylamine. Having developed a rapid and simple method for obtaining acetyl-L-tyrosine, the isotopic acetyl-DL-tyrosine was resolved with the use of d- α -phenylethylamine, in batches of 0.0228 mole to 0.0318 mole. Of the seven batches resolved, two of them required only one recrystallization to obtain optical purity, while the other five batches required only two recrystallizations. The yields varied from 36-70% of the theoretical, with an average of 50% for the seven runs.

The criterion of optical purity of the N¹⁵-acetyl-L-tyrosine-d- α -phenylethylamine was a specific rotation of +36.87° (0.5% in water), as shown in the previous section. A deviation of $\pm 10\%$ in the rotation was deemed close enough for acceptance for our work.

The mother liquors from these resolutions were saved and combined. From this combined solution the acetyltyrosine was recovered, by first replacing the alcohol by water as solvent, making alkaline and extracting the phenylethylamine away with ether, and then performing the racemization procedure of Sealock (121). This yielded the monoacetyl-DL-tyrosine. The amount of acetyltyrosine in the mother liquors at any one time was calculated from the known amount of acetyl-DL-tyrosine used minus the amount recovered as acetyl-L-tyrosine. From one batch containing 13.9 gm. of acetyltyrosine, 11.95 gm. of acetyl-DL-tyrosine were re-

covered, for an 85.5% yield. This amount was then resolved by the above procedure.

The next step was the isolation of N^{15} -L-tyrosine from the resolved salt. The following procedure illustrates the isolation of the desired compound.

Three and eight-tenths grams (0.01 mole) of the N^{15} -acetyl-L-tyrosine- d - α -phenylethylamine dihydrate were dissolved in 25 ml. of water with warming. To this solution was added 10 ml. of 5 N sodium hydroxide, and the resulting mixture was extracted four times with 10 ml. portions of ether. The aqueous layer was neutralized with 4.2 ml. of concentrated hydrochloric acid, and 30 ml. more of the concentrated acid were added to make the final solution 5 N in hydrochloric acid. The solution was then refluxed for 3 hours and concentrated to dryness. Thirty ml. of water were added to the residue and distilled off; this was repeated again to drive off excess acid.

The final residue was dissolved in 30 ml. of water, the solution treated with Norite and filtered, and the N^{15} -L-tyrosine precipitated by neutralizing the filtrate with sodium hydroxide and adding two drops of glacial acetic acid to make very slightly acid. After 24-36 hours of refrigeration the tyrosine was filtered, washed with absolute alcohol and ether, and dried. The resulting yield of N^{15} -L-tyrosine was 1.4 gm., 76.8% of the theoretical. The specific rotation of the tyrosine was -7.2° (2% in 1 N HCl). Sealock (121) lists

a specific rotation of -10.6° for a 4% solution of L-tyrosine in 1 N HCl. $C_9H_{11}O_3N$, N calc. 7.74%

found 7.69%
7.72%

Table 2 gives a summary of resolutions and isolations of the N^{15} -L-tyrosine performed for this work.

Table 2. Resolution of N^{15} -Acetyl-DL-Tyrosine by Use of d- α -Phenylethylamine

Run	Yield of salt ¹	Rotation of salt	Yield of N^{15} -L-tyrosine
I	35.0%	36.4 ⁰	
II	43.5	34.0	
III	55.0	33.7	
IV	47.0	37.1	76.8% ²
V	58.0	33.0	
VI	40.0	33.3	76.8
VII	60.0	36.2	76.5

¹ N^{15} -acetyl-L-tyrosine-d- α -phenylethylamine

² Runs I-IV were combined for isolation of N^{15} -L-tyrosine; Runs V and VI were likewise combined.

C. N^{15} -L-Tyrosine Oxidation in Vitro

The general plan of our experiments was to incubate tyrosine with a cell-free extract of liver, and determine if possible the fate of the nitrogen upon oxidation of the amino acid.

A suitable method for carrying out such a procedure is the use of the Warburg respirometer, since the oxidation of the substrate may be followed by manometric measurements, and the incubation stopped at a definite stage of the process.

Several investigators have used this technique in following the oxidation of amino acids by animal tissues, and the conditions have been well studied in the case of tyrosine. Sealock and Goodland (84), in their recent paper, have given the optimum conditions for oxidation by guinea pig liver. Their results were made use of as far as possible in the present work.

As in all of the previous steps, runs were made with non-isotopic substrate before using the "marked" tyrosine. In this way conditions were standardized for the fractionation procedure to be performed on the incubation mixtures in which isotope was used. Both the dummy runs and the runs using isotopic compound will be described in this and the next section.

After performing the incubations in the respirometer, the fractionation of this incubation mixture offered two general alternatives. One could either attempt to isolate any compound possible by usual protein hydrolysate fractionation procedures and then investigate such compounds for isotope content; or one could start from a definite hypothesis and prove or disprove that hypothesis by isotope determination. The small solubility of tyrosine in solutions of the proper

pH for enzymatic action limited drastically the size of the incubation runs. This in turn made the first alternative exceedingly difficult from a manipulative standpoint.

On the other hand, there has been stated an hypothesis of tyrosine oxidation which lends itself admirably to proof or disproof by isotope determination. This is the scheme of Felix and Zorn (61) outlined on page 39. It will be recalled that they presented evidence that the oxidation of tyrosine by liver "brei" produced a mole of alanine for each mole of tyrosine oxidized. It seemed to us that their analytical methods were not specific enough for alanine to accept their conclusions without further substantiation. Therefore the first goal which we set was to prove or disprove the presence of alanine in the incubation mixture by means of isotopic content.

Fortunately there has been reported a precipitant for alanine which is quite specific. Bergmann and his co-workers (128) have investigated the solubility product of salts of amino acids with many aromatic sulfonic acids. They found that azobenzene-*p*-sulfonic acid formed a salt with alanine with a smaller solubility product than any other amino acid. Operating within the correct concentration range, they were able to isolate alanine from silk fibroin hydrolyzate in excellent yield. The only restrictions found necessary for the precipitation of alanine were freedom from certain organic bases which form insoluble salts with the azo dye

even in acid solution piperidine, aniline, pyridine, hydroxylamine, ethanolamine, creatine, and urea), and cooling to 0° for the precipitation.

It was planned therefore to add a certain amount of normal L-alanine to the incubation mixture as a carrier (since the amount of isotopic alanine derived from tyrosine, if any, would necessarily be very small) and isolate the alanine salt formed with azobenzene-p-sulfonic acid.

1. Manometric method

The sources of liver tissue were young healthy guinea pigs maintained on a stock diet of Purina chow (Checkers) with supplementation of green leafy vegetables and carrots. To make sure that satisfactory oxidation would take place, the animals chosen were given 50 mg. of ascorbic acid by mouth twenty-four hours before use. The animals were killed by stunning and severing of the jugular veins. The livers were removed as rapidly and completely as possible and weighed. The livers were cut into small pieces with scissors and homogenized in a Waring Blender with about an equal weight of 0.2 M phosphate buffer of pH 7.4. The Blender jar was cooled in ice-water before use, and the homogenization was carried out in three one and one-half-minute periods with intermittent cooling. The resulting mixture was centrifuged at about 2000 r.p.m., and the residue extracted twice with small amounts of phosphate buffer.

The resulting supernatant layers were made up to such a volume that 1 ml. of cell-free extract was equivalent to 0.5 gm. of liver. The pH of the extract was adjusted to 7.4.

The incubation flasks were usual Warburg vessels with side-arm and center well. Twelve flasks were used for each run. In each flask 2 ml. of liver extract were put in the main compartment, and 0.3 ml. of 20% potassium hydroxide and a roll of filter paper in the center well. In six of the flasks 1 ml. of phosphate buffer was placed in the side arm; this series served as the control. In the other six flasks 1 ml. of tyrosine solution in phosphate buffer, containing 1 mg. of tyrosine per ml., was placed in each side arm. These six constituted the experimental series. In addition zero time tubes were set up for each run. For instance, in tube 15 was placed 2 ml. of 10% metaphosphoric acid, 2 ml. of liver extract, 1 ml. of phosphate buffer, and water to 10 ml. In tube 16, 1 ml. of tyrosine solution replaced the 1 ml. of phosphate buffer. In tube 17, 2 ml. of phosphate buffer replaced the liver extract, otherwise it was like tube 16. Thus, tube 17 was a standard for the colorimetric determination of tyrosine carried out on the control and experimental flasks at the end of the incubation. Also, the tyrosine value found in tube 15 subtracted from the tyrosine value found in tube 16 serves as a check on the standard and thus a check on the accuracy of the colorimetric method for determining tyrosine. The use of these zero time tubes will

be illustrated below in the case of the first run.

The flasks were placed on individual manometers and mounted on the respirometer so that the flasks and contents were immersed in a 37° constant-temperature water bath. The shaking mechanism was started, and after a 15 minute equilibration period the contents of the side arms were mixed into the main compartment and mixed thoroughly with the liver extract. The oxidation of the substrate was then followed by periodic reading of the manometers.

At the appropriate time the incubation was stopped by transferring the contents of the flasks as quickly as possible to a centrifuge tube containing 2 ml. of 10% metaphosphoric acid per flask. The contents of the six control flasks were combined with 12 ml. of the deproteinizing acid, and the contents of the six experimental flasks were likewise combined. Both mixtures were made up to 60 ml. with water, stirred well and allowed to stand $\frac{1}{2}$ hour, after which they were centrifuged for 10 minutes at about 2500 r.p.m. Each residue was washed well with 10 ml. of "synthetic mother liquor" (3 ml. of phosphate buffer, 2 ml. of 10% metaphosphoric acid, and 5 ml. of water), and the combined centrifugates of each series were refrigerated.

As an illustration of the use of the zero time tubes, tyrosine determinations were made on 1 ml. of the control series, the experimental series, and of each of the zero time tubes listed on page 95. The colorimetric method used

was that of Folin and Ciocalteu (127). The calculation is as follows:

$$\frac{E - C}{\text{tube 16} - \text{tube 15}} \times 0.552 \times 70 = \mu \text{ moles tyrosine present in E}$$

This value is subtracted from the μ moles of tyrosine used in the incubations to give the amount oxidized in the enzymatic reactions.

2. Fractionation methods

Many preliminary tests and experiments were performed in order to standardize conditions as much as possible. The precipitant used, azobenzene-*p*-sulfonic acid, was prepared some time previous to this work by a former member of this laboratory (M. E. Speeter) by sulfonation of commercial azobenzene with fuming sulfuric acid. It was recrystallized twice from 1.5% hydrochloric acid after treating the solution with Norite and filtering. It had a melting point of 125-126°d. in a capillary tube, and 133-134°d. on the Fisher-Johns micro hot stage¹. Drying a sample in the Fischer dryer over phosphorus pentoxide in vacuo showed the dye to have two molecules of water of hydration. Thus, 0.1482 gm. of compound lost 0.0201 gm. upon drying, or 13.56% water. The calculated value is 12.10% for two molecules of water of hydration.

The azobenzene sulfonate salt of DL-alanine and the

¹All melting points given are uncorrected.

corresponding salt of L-alanine were prepared to serve as reference compounds. In both cases they were prepared by dissolving 0.5 gm. of the amino acid in 25 ml. of 1 N HCl, adding 0.75 gm. of the sulfonic acid and refrigerating. The precipitated solid was filtered and recrystallized from the minimum amount of hot water. From DL-alanine was obtained 0.70 gm. of salt, m. 235-237^od. in a capillary tube, 255-256.5^od. on the Fischer-Johns block. From L-alanine was obtained 0.55 gm. of salt, m. 262^od. on the micro hot-stage.

It was found that alanine formed a salt with the sulfonic acid dye in a wide range of acidity, from a pH of about 0.1 to 6.5. On a qualitative basis the amount of precipitate appeared greater for the same amount of alanine at a pH of 3.0 to 3.5. The precipitation seemed to be unaffected by small amounts of tyrosine added to the mixtures. Thus, 1 mg. of alanine and 0.5 mg. of tyrosine in 2 ml. of 0.1 N HCl and 75 mg. of dye added gave about the same amount of precipitate as the same mixture without the tyrosine. Further, investigation of the precipitate showed that very little of the tyrosine precipitated with the alanine. The precipitate was dissolved in about 5 ml. of hot water and the dye removed as the barium salt by adding 1 ml. of barium acetate solution containing 50 mg. of barium acetate. The filtrate was treated with sulfuric acid until no more barium sulfate precipitated. The filtrate from this precipitate

was analyzed colorimetrically for tyrosine by the Folin-Ciocalteu method. Therefore it was concluded that the original amino acid-sulfonate salt had contained 0.086 mg. of tyrosine.

All attempts to form a tyrosine-azobenzene-*p*-sulfonate salt by the same procedure as that used in the case of alanine were unsuccessful; the precipitate from such a procedure melted at 120-122^od., and proved to be the free azobenzene-*p*-sulfonic acid. Also, Bergmann (128) reported no salt of tyrosine with this precipitant. Therefore it appeared likely that any tyrosine which appeared with the alanine precipitate would be adsorbed to the precipitate and not present as an independent salt. Careful washing and recrystallization of the alanine precipitate should free it entirely of any adsorbed tyrosine.

Although the alanine-dye salt requires refrigeration for precipitation, it was found that a precipitate invariably formed at room temperature on standing. Thus, a solution of 5 mg. of alanine, 2 mg. of tyrosine, and 5 ml. of 0.2 M phosphate buffer (pH 7.4) was adjusted to a pH of 3.5, and 375 mg. of azobenzene-*p*-sulfonic acid added. It was found that from 5 to 10 ml. of methyl cellosolve or dioxane were needed to effect a complete solution of the dye. Upon standing 24 hours at room temperature, about 90-100 mg. of precipitate had formed. Investigation of this precipitate, after removing the dye, showed the presence of 0.31 mg. of tyrosine, colori-

metrically.

Refrigeration of the filtrate from this precipitate resulted in 153 mg. of solid. This solid was centrifuged, washed four times with 2 ml. of ice-cold water, and recrystallized four times from 6-10 ml. of water, leaving a final amount of 4.5 mg., m. 235-238^od. in a capillary tube.

We then set up a fractionation procedure for the combined incubations described on pages 95 and 96 of the preceding sub-section. Combined filtrates "C" (controls, 178 ml.) and combined filtrates "E" (176 ml.) were adjusted to a pH of 3.5, and to both were added 1.0 gm. of azobenzene-p-sulfonic acid dissolved in 12 ml. of water. Ten ml. of dioxane were added to each to effect complete solution of the dye, and the mixtures were allowed to stand at room temperature for 36 hours. At the end of this time the precipitates--E-1 and C-1--which had formed were collected by centrifugation and dried. The filtrates were refrigerated at 0^o for 36 hours.

Precipitate C-1 amounted to 200 mg., and precipitate E-1 weighed 253 mg. The dye was removed from these two solids in the manner previously described. These final solutions were made to 10 ml., and analyzed for nitrogen by the Kjeldahl method. It was found that the solution from precipitate E-1 contained 0.12 mg. of nitrogen, and the solution from precipitate C-1 contained 0.17 mg. of nitrogen after removal of the dye. If all of the nitrogen

in E-1 were due to tyrosine, there would be $0.12 \times \frac{181}{14} = 1.55$ mg. of tyrosine. However, a colorimetric determination of tyrosine on these same solutions showed 0.027 mg. of tyrosine in the E-1 solution and 0.014 mg. of tyrosine in the C-1 solution, indicating an insignificant loss of tyrosine. The solutions were then evaporated to dryness by aeration, leaving residues of 51 mg. in the case of E-1 and 28 mg. in the case of C-1. These residues were dissolved in 1 ml. of water and 4 ml. of absolute ethanol added. A white precipitate flocced down, and after refrigeration was centrifuged and dried. From E-1 was obtained 13.6 mg.; from C-1, 6.4 mg. Solution of these in 1 ml. of water gave negative Millon's and biuret tests. Attempts to make a p-toluene sulfonyl derivative (assuming an amino acid present) were unsuccessful in the case of C-1; with E-1, shaking it with an ether solution of 15 mg. of p-toluene sulfonyl chloride for four hours, separating the ether and acidifying the water layer gave 6.1 mg. of a white solid. In a capillary tube this solid softened at 117° and melted gradually from $180-200^{\circ}$. It was then recrystallized from 3 ml. of 60% ethanol; 1.9 mg. of solid were recovered. This solid softened at 115° , part of it melted at 139° , the rest charred and sublimed at 200° . It was concluded that no identifiable amino acid was present in the original room-temperature precipitates E-1 and C-1.

The original filtrates which had been refrigerated were

centrifuged after thirty-six hours at 0°, giving precipitates E-2, 288 mg., and C-2, 430 mg. These precipitates obviously had a large amount of free dye in them. They were washed four times with ice-cold water and recrystallized four times from hot water, leaving 25 mg. of E-2 and 56 mg. of C-2. These were dissolved in the minimum amount of hot water and the dye removed as previously described with barium acetate. The filtrates from the removal of the excess barium were evaporated to dryness by aeration, leaving 10 mg. of residue from E-2 and 16 mg. of residue from C-2. Attempts to make a p-toluenesulfonyl derivative from C-2 were unsuccessful; with E-2, 20 mg. of a white solid were obtained as a first crop which proved to be inorganic material. From the mother liquor was obtained about 2 mg. of a solid which melted at 137°, which is close to the recorded melting point for the p-toluenesulfonyl derivative of L-alanine (139°, Shriner and Fuson (129)). Thus it was concluded that precipitate E-2 had been the alanine salt of azobenzene-p-sulfonic acid.

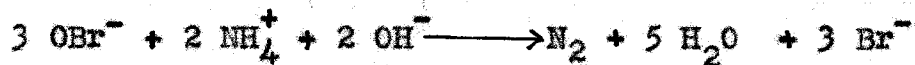
From the foregoing preliminaries, the standardized conditions for fractionation of the incubation mixtures derived from the isotopic experiment were set up as follows:

Adjust the pH of the mixture to 3.5, and add the calculated amount of azobenzene-p-sulfonic acid to precipitate the amount of alanine to be added plus the amount of alanine which might be present from the incubation. Since the room-temperature precipitate obtained after this treatment was

found not to contain alanine, filter or centrifuge, add normal L-alanine to the filtrate and refrigerate at 0° in order to precipitate the alanine-dye salt. The room-temperature precipitate should of course be investigated for nitrogen content and isotope content, and identified if possible. After 24-36 hours at 0°, collect the alanine-dye precipitate, purify by washing and recrystallization, and analyze for nitrogen and isotope content. Then isolate any other nitrogenous constituent from the solution left after removal of the alanine salt. This would involve removal of the dye as the insoluble barium salt, precipitation of the barium salts of dicarboxylic amino acids with the addition of alcohol, and finally extraction of the aqueous solution with butyl alcohol.

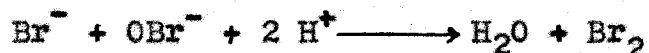
3. Isotope analysis

Since the mass spectrometer analyzes gaseous samples, the next step was to convert our nitrogen samples into gaseous elemental nitrogen. The simplest procedure for doing this is the method used by Rittenberg (131). This involves oxidizing the ammonium salts obtained from a Kjeldahl distillation with alkaline hypobromite solution, according to the following reaction:



Thus the samples used for determining the nitrogen

content were also used as a source of the gas for isotope analysis. They were made just acid to Congo red, and stored for later oxidation. Care must be taken not to have too much acid in the Kjeldahl distillates, otherwise a side reaction occurs which produces bromine, thus:



This of course would contaminate the sample and introduce errors in the measurement.

The apparatus for oxidizing and collecting the nitrogen is shown in Figure 5, page 105. "A" is a 50-ml. Erlenmeyer flask with a bent neck fused on and a ground-glass joint on the neck, "B" a round-bottom 50-ml. flask fused to a trap, "C", other end of which has a ground-glass joint for connection to the rest of the apparatus. "E" is a ground-glass stopcock and the lead-off to a mercury-diffusion vacuum pump which, with a mechanical fore-pump, is used to evacuate the entire system. The right side of the apparatus is a Toepler pump, consisting of a 250-ml. bulb with 10-mm. glass tubing fused on opposite sides. The bottom tubing extends down into "H", a 500-ml. filter flask containing about 350 ml. of redistilled mercury and a three-way stopcock, "D", fused onto the side-arm. The upper half of the tubing from the bulb has a three-way stopcock, "F", and a ground-glass joint into which the gas-bulb, "G", fits. The gas bulb is simply a 15-ml. round

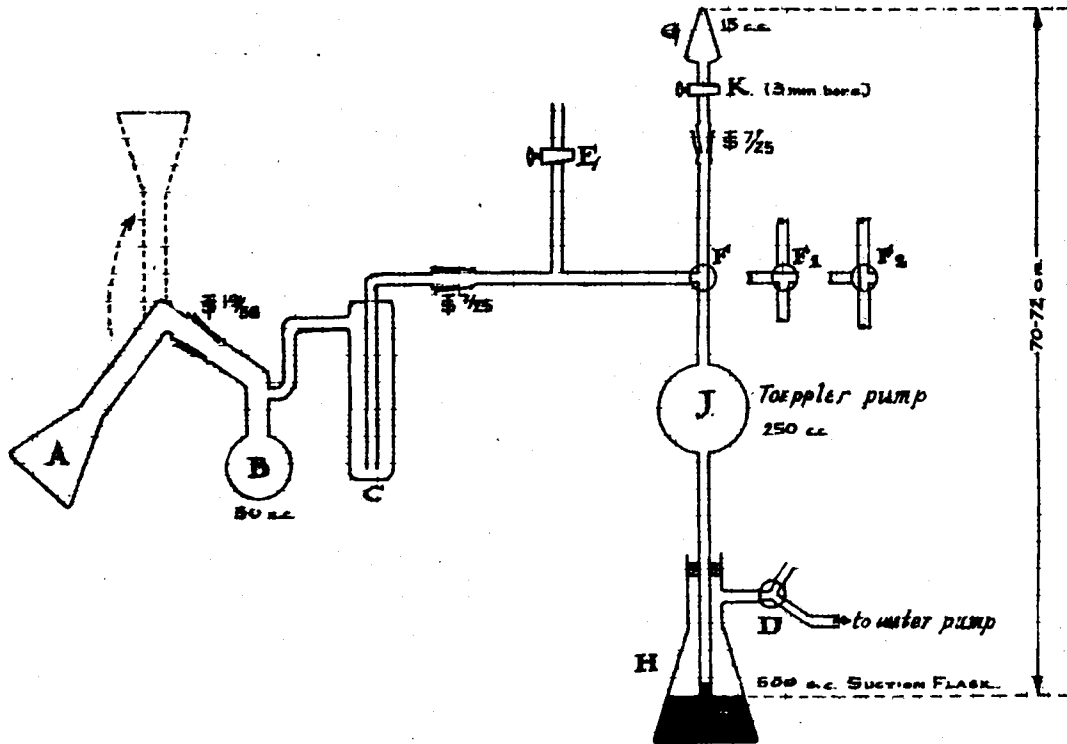


Figure 5. Apparatus for Collection of Nitrogen Gas

bulb fused to a stopcock which has a 7/25 standard taper ground-glass joint on the other end. From the bottom of the Toepler pump to the top of the gas bulb is a distance of 70 cm., so that when the system is fully evacuated, the mercury will rise to the top of gas bulb "G" when air is admitted into "H". A Dewar flask containing alcohol and dry-ice is placed around the trap "C".

The following is the procedure used for operating the apparatus. The gas bulb "G" was lubricated at the stopcock and the joint and put in place on top of the Toepler pump¹. The Kjeldahl distillate is boiled down to a volume of about 3-4 ml. on a sand bath, and transferred to "B" by means of a transfer pipette. This transfer need not be quantitative, since it is the ratio of N²⁹ to N²⁸ which is being determined. The ground glass joint at "C" is then greased and put in place on the apparatus. The alcohol-dry ice bath is placed around "C".

Five ml. of diluted sodium hypobromite solution (stock solution diluted 1:1 with water) are placed in "A", and this piece is placed onto "B" in the downward position. Stopcock "E" is opened and the whole system evacuated by means of a mercury-diffusion pump with a Cenco Hi-vac mechanical pump as a fore-pump. The mercury in "H" is prevented from

¹A special lubricating grease, Apiezon N, was recommended for use on the gas bulb by H. J. Svec of the Institute for Atomic Research, Iowa State College. This lubricant is obtained from James G. Biddle Co., 1316 Arch Street, Philadelphia 7, Pennsylvania.

rising in the Toepler pump by means of a water pump connected to "H" with the stopcock "D" closed. As the system slowly evacuates, the solution in "B" first boils and then freezes. Immediately thereafter the hypobromite solution in "A" de-gasses. The vigor with which these two changes occur is controlled by closing and opening stopcock "E".

After the solution in "A" has been de-gassed, the extent of evacuation of the system is determined. This is done by turning the three-way stopcock "F" to position "F₂" and admitting air into "H" by opening stopcock "D". If the system is fully evacuated, the mercury will rise in the Toepler pump all the way to the top of the gas bulb "G". In practice, the best vacuum attainable by us left a small disc of air about the size of the head of a thumb-tack in the gas bulb upon such an operation. This amount of air was found not to affect the accuracy of isotope measurement.

When the system has been adequately evacuated as shown by this test, the mercury is drawn back down into "H" by suitable manipulation of stopcock "D", stopcock "F" is left at position "F₂", stopcock "E" is closed, and the hypobromite solution is poured into "B" by turning the flask to position shown by the dotted lines.

The mixture in "B" is heated gently with a micro-burner to melt the solidified sample. As soon as the reaction has

ceased, the gas is introduced into "J" by turning stopcock "F" to position "F₁". The stopcock is then turned to position "F₂", and the gas is pumped into the gas bulb "G" by introducing air into "H" (with stopcock "D") and allowing the mercury to rise in the Toepler pump. One stroke of the mercury is sufficient. The stopcock on "G" is closed, the mercury is drawn back down into "H", and the system opened to the air by opening "E" and "D" simultaneously.

The operator of the mass spectrometer customarily introduced the sample into his apparatus at a pressure of 5 cm. It was found that between 0.5-1.0 mg. of nitrogen was a satisfactory sample for analysis under these conditions.

The stock solution of hypobromite was made as follows: 200 gm. of sodium hydroxide pellets were dissolved in 300 ml. of water. To one-half of this solution, cooled in ice and with vigorous stirring, were added 60 ml. of bromine over a period of ten minutes. The other one-half of the solution of alkali was added, and the whole solution refrigerated. After 2-3 days a copious amount of sodium bromide had precipitated and was filtered off through an asbestos mat (the solution disintegrates filter paper). The filtrate served as the stock solution and was stable for months when kept in the refrigerator. The stock solution was diluted 1:1 with water just before use.

Care must be taken to admit air to all parts of the apparatus between collection of samples, to prevent a carry-

over of isotope from one sample to the next. Bulb "G" was removed, and then flask "A" and piece "B-C". "A", "B", and "G" were rinsed thoroughly with distilled water and allowed to drain before re-use. It was not found necessary to dry them thoroughly; allowing them to drain while the next sample was being concentrated on the sand-bath was sufficient.

After considerable experience with the apparatus, a rate of one sample every twenty minutes could be attained.

4. Experimental observations

Three separate incubations were performed by the method given in Part 1 of this Section. The oxidation of the tyrosine was followed by averaging the uptake of the six control flasks and the uptake of the six experimental flasks and graphing the values as shown in Figure 4a, page 110. The curves shown are typical of a normal oxidation of tyrosine by guinea pig liver extract. In Figure 4b is shown the average uptake of oxygen by the experimental flasks in excess of that used by the control flasks. The incubation in the respirometer was stopped when the curve in Figure 4b approached its asymptote, i.e., when the "excess oxygen uptake" approached a maximum constant value. This point was usually reached between three and four hours.

The three incubations were performed with the same liver extract. The experimental series were all combined, and the control series were all combined. These two com-

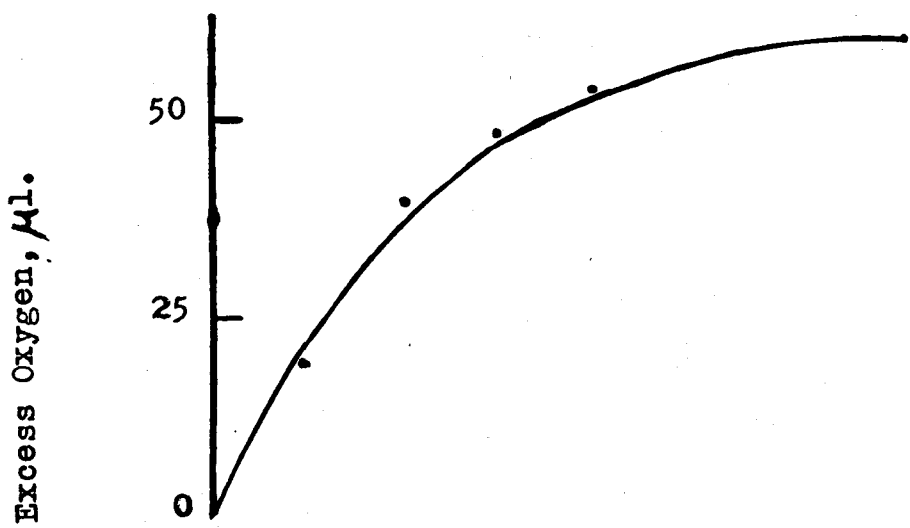


Figure 4b

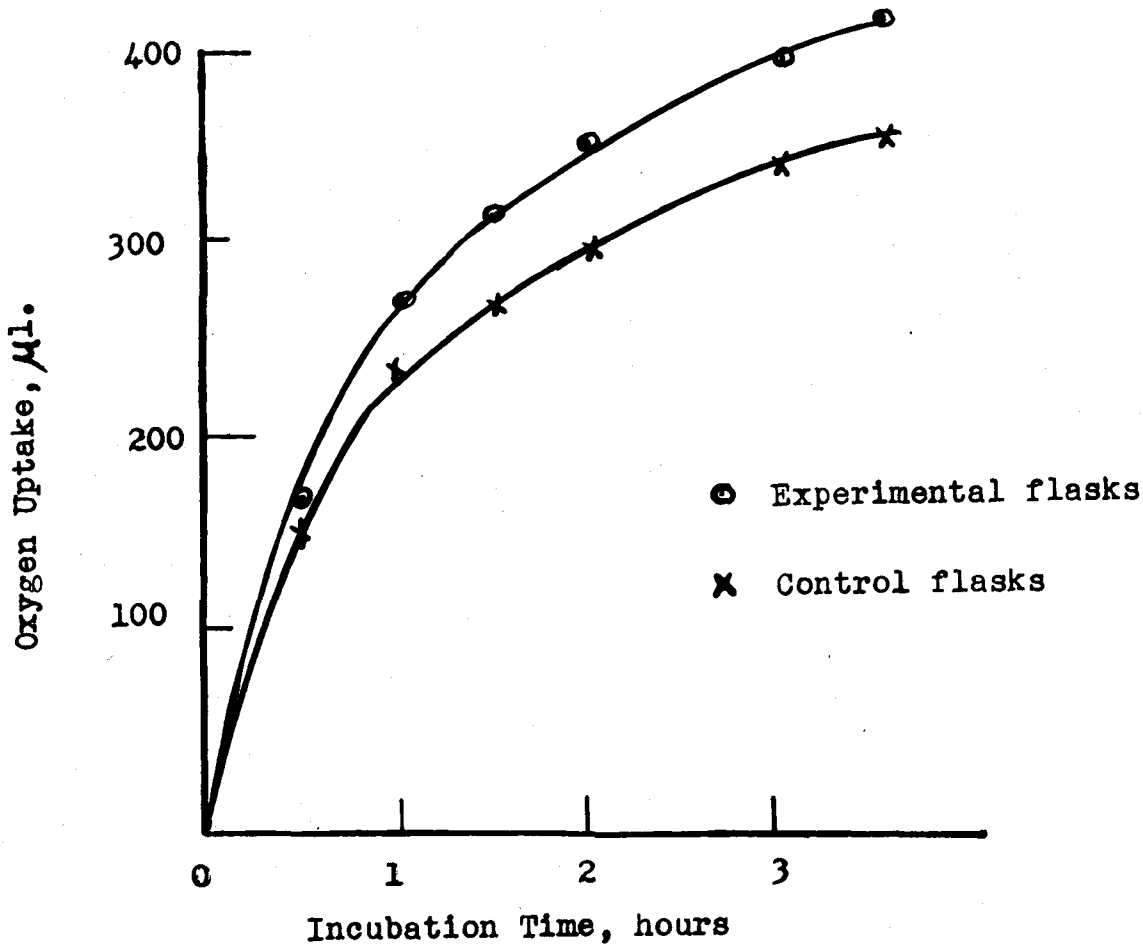


Figure 4a. Oxygen Uptake of Normal Guinea Pig Liver
Brei with N¹⁵ -L-Tyrosine

bined solutions were used in preliminary experiments for developing fractionating procedures, as described in Part 2 of this Section.

A summary of the results of these three incubations is given in Table 3. The column O/T present represents the ratio of the oxygen consumed to the amount of tyrosine added, in atoms per mole. By making use of the tyrosine determinations at the end of the incubations, the ratio O/T calculated is obtained. Thus, 69.6 μ atoms of oxygen consumed divided by 23.19 μ moles of tyrosine oxidized gives a calculated ratio of 3.00. This figure is probably more useful in evaluating the extent of oxidation, since it can be compared to the theoretical oxygen uptake of 4 atoms per mole of tyrosine oxidized, the figure obtained by Bernheim and Bernheim (57) and confirmed by subsequent investigators. There are also two ways of calculating the per cent of tyrosine oxidation. The one, giving 60.33% oxidation, is calculated from the amount of tyrosine oxidized--59.95 μ moles--as shown colorimetrically, and the amount of tyrosine added--99.36 μ moles. Since this method includes possible colorimetric errors (phenolic compounds other than tyrosine may contribute to the color), the method based on the amount of oxygen consumed represents more nearly the true state of oxidation.

Table 3. Tyrosine Oxidation by Guinea Pig Liver

Run	O ₂ Consumed		Excess O ₂	Tyrosine oxidized	O/T _{pres}	O/T _{calc.}
	C	E				
I	4296µl.	5076µl.	780µl. 69.6µatoms	23.19µmoles	2.10	3.00
II	5010	5802	792 70.8	19.32	2.13	3.66
III	5070	5700	630 56.76 ¹ 197.16 ¹	17.44 59.95 ²	1.71 1.98 ³	3.25 3.30 ³

¹On basis of oxygen consumed: $\frac{197.16}{397.44} \times 100 = 49.60\%$ oxidation

²On basis of tyrosine oxidized: $\frac{59.95}{99.36} \times 100 = 60.33\%$ oxidation

³Average values

Using the same procedure, three incubations were performed using a solution of N¹⁵-L-tyrosine in phosphate buffer--1 mg./ml.--as substrate for the experimental series. Three different guinea pigs were used for the three runs, the amount of liver from each animal being just slightly more than was needed for one complete incubation.

The results of these three experiments are presented in Table 4, page-113. The combined incubation filtrates, "C" for the controls, and "E" for the experimentals, were fractionated as described in the following pages.

Table 4. N¹⁵-L-Tyrosine Oxidation by Guinea Pig Liver

Run	O ₂ Consumed		Excess O ₂	Tyrosine oxidized	O/T pres. calc.	O/T
	C	E				
V	2238μl.	2598μl.	360μl. 32.10 atoms	17.55μ moles	0.97	1.83
VI	2133	2490	357 31.86	9.37	0.96	3.40
VIII	2130	2460	330 29.46 93.42 ¹	10.68 37.60 ²	0.89 0.94 ³	2.76 2.66 ³

¹On basis of oxygen consumed: $\frac{93.42}{397.44} \times 100 = 23.50\%$ oxidation

²On basis of tyrosine oxidized: $\frac{37.60}{99.36} \times 100 = 37.80\%$ oxidation

³Average values

Since we were concerned with isotope recoveries, it was decided to investigate every fraction obtained for nitrogen content and isotope content.

The volumes of both "C" and "E" from these incubations were 143 ml., the pH 3.16. The following reasoning was used in determining the amount of precipitant to add. Taking the value of 37.80% oxidation obtained in the three incubations, and assuming alanine was produced mole per mole of tyrosine oxidized: 18 mg. of tyrosine would produce 9 mg. of alanine if there had been 100% oxidation; 37.80% oxidation would then produce 3.4 mg. of alanine. Since the N¹⁵-L-tyrosine used had an isotopic enrichment of 5.635 atoms per cent excess N¹⁵, a

dilution of 1:100 would still be well within the accuracy of determination of the mass spectrometer. A 1:100 dilution of the 3.4 mg. of alanine theoretically produced would mean addition of 340 mg. of normal L-alanine. To be on the conservative side, it was decided to add 150 mg. of normal L-alanine. The solubility product of alanine-azobenzene-*p*-sulfonate, as determined by Bergmann (128), is 4×10^{-4} .

$$150 \text{ mg./}143 \text{ ml.} = 1.06 \text{ gm./liter} = 0.012 \text{ moles/l.}$$

$$(\text{dye}) = \frac{0.0004}{0.012} = 0.033 \text{ moles/liter}$$

The molecular weight of the dye is 262; therefore 0.033 moles of precipitant, 8.65 gm. per liter, or 1.24 gm. per 143 ml., should be added to obtain precipitation of the added alanine.

So 1.24 gm. of azobenzene-*p*-sulfonic acid dissolved in 5 ml. of water was added to both C and E, plus 15 ml. of dioxane to each to retain complete solution. The mixtures were then allowed to stand for 24 hours at room temperature.

At the end of this time the mixtures were centrifuged in 15 ml. weighed tubes, giving precipitates E-1 and C-1, and filtrates E-1f and C-1f. The precipitates were dried in vacuo over phosphorus pentoxide; 150 mg. of L-alanine were added to both E-1f and C-1f, after which they were refrigerated at 0° for 75 hours.

The weight of E-1 was 575 mg., that of C-1 259 mg., when dry. The fact that twice as much precipitate was ob-

tained from the experimental mixture led us to suspect a combination of the dye with some unknown intermediate compound in the tyrosine oxidation scheme. Accordingly these two room temperature precipitates were investigated. They were both dissolved in 30 ml. of water with warming. To E-1 was added 561 mg. of barium acetate in 11 ml. of water (assuming the precipitate to be mostly free dye, this is a mole of barium acetate per mole of precipitate); after cooling well in ice the precipitated barium salt of the sulfonic acid was filtered off. The calculated amount of 6 N sulfuric acid was added to precipitate the excess barium in the filtrate, Norite was added, and the mixture filtered. The filtrate was diluted to 50 ml. volume. The same procedure was performed with C-1, using 253 mg. of barium acetate in 5 ml. of water. The first point of interest to investigate was the ultra-violet absorption of the filtrate; accordingly 2 ml. of the 50 ml. filtrates were diluted to 25 ml., and the absorption determined with the Beckman spectrophotometer. As can be seen from Table 5, the absorption was essentially negative from a wave length of 350 mu to 220 mu. Thus it was apparent that no aromatic compound was present in the solution made with the room-temperature precipitates E-1 and C-1.

Table 5. Ultra-violet Absorption of Room Temperature Precipitates

Wave Length	Slit	C		E	
		Per cent T	D	Per cent T	D
350 m μ	0.52	99.0	0.005	99.8	0.001
325	"	99.0	.005	99.7	.002
310	"	98.8	.006	99.7	.002
300	"	99.0	.005	99.8	.001
280	"	98.2	.008	99.5	.003
260	"	97.3	.012	98.7	.006
240	"	94.8	.024	97.0	.013
230	1.575	92.3	.035	93.9	.028
225	"	89.0	.050	89.0	.050
220	"	84.1	.075	82.6	.084
215	"	77.5	.110	74.1	.130

A Folin-Ciocalteu colorimetric determination for tyrosine on these solutions was negative for C-1, and gave a value of 0.88 mg. of tyrosine in E-1. Both the biuret and Ninhydrin tests were negative on both solutions (the ninhydrin used was quite old and impure, and the results of this test are not entirely dependable). Results of Kjeldahl nitrogen determinations showed 0.51 mg. of nitrogen in filtrate C-1 and 0.82 mg. of nitrogen in filtrate E-1. Accordingly the entire filtrates were concentrated to about 3 ml.,

digested and distilled by the Kjeldahl procedure, and the distillates saved for isotope determinations.

The refrigerated filtrates E-1f and C-1f were centrifuged in 15 ml. weighed tubes and the solid dried, giving precipitates E-2 and C-2 and filtrates E-2f and C-2f. The weights of the impure alanine-dye fractions were: E-2, 805 mg., and C-2, 928 mg. These precipitates were washed four times with 5 ml. of ice-cold water, and recrystallized from hot water three times, leaving a final yield of 326 mg. of C-2 and 372 mg. of E-2. Both of these solids melted at 260° d. on the micro hot-stage, leaving one or two crystals of unmelted residue. Mixed melting point determinations with equal amounts of pure L-alanine-azobenzene-p-sulfonate gave sharp melting at 261-262° d.



The filtrates from the removal of the alanine-dye precipitates, C-2f and E-2f, were fractionated further for possible nitrogen-containing substances. It was decided that the next step should be investigation for the presence of glutamic or aspartic acids, which might conceivably be present if the metabolism of tyrosine involved transamination reactions. These two dibasic acids may be removed from solution by precipitating their barium salts from aqueous solution with the addition of alcohol. Therefore the sulfonic acid left in C-2f and E-2f was precipitated

by addition of barium acetate solution until no more precipitate formed, followed by neutralization of the solution with 3% barium hydroxide. This gave about 3 gm. of a cream-colored precipitate which was obviously not the barium salt of the azobenzene sulfonate. Investigation of these precipitates proved them to be one of the barium phosphates. Kjeldahl determinations on hot water extracts of these precipitates showed no nitrogen-containing substance had adsorbed to the barium phosphate precipitates, so they were discarded. In order to precipitate all of the dye from C-2f and E-2f, it was necessary to add more barium acetate and Norite the mixture. The filtrates from these were concentrated to about 40 ml., and 5 volumes of 95% ethanol added. Copious white, feathery precipitates were formed immediately. After allowing these mixtures to stand at room temperature for a few hours, they were filtered, giving precipitates C-3 and E-3, and filtrates C-3f and E-3f. The precipitates were dried, and the filtrates were refrigerated.

The precipitates amounted to 8.8 gm. of C-3 and 8.9 gm. of E-3. These proved to be mostly barium acetate; on dissolving each in hot water and adding sulfuric acid to precipitate the barium, the filtrate smelled strongly of acetic acid. Each filtrate was concentrated in vacuo to dryness, water added and again concentrated to dryness to get rid of most of the acetic acid. The residues were then dissolved

in 3 ml. of water and Kjeldahl determinations made to see if any nitrogen-containing substance had co-precipitated with the barium acetate. If any had, it would be quite likely that it was glutamic or aspartic acid. The residue from C-3 contained 1.25 mg. of nitrogen, that from E-3 contained 1.66 mg. of nitrogen. The Kjeldahl distillation samples were therefore saved for isotope determination.

The filtrates C-3f and E-3f did not afford any precipitate upon refrigeration. Therefore sulfuric acid was added to both to precipitate the barium remaining in the solutions. The barium sulfate precipitates were filtered off; extraction of these precipitates with hot water and Kjeldahl determinations on the extracts showed an appreciable amount of nitrogen-containing substance had adsorbed to the barium sulfate precipitates. Thus, adsorbed to C-4 was 1.13 mg. of nitrogen, and adsorbed to E-4 was 0.752 mg. of nitrogen. Accordingly these samples were saved for isotope determination. It is likely that this fraction and the fraction adsorbed to the barium acetate, E-3 and C-3, are the same or very similar substances.

The filtrates from the barium sulfate filtration, C-4f and E-4f, were concentrated to about 15 ml. in vacuo, and neutralized with alkali. They were then extracted 10 times with 10 ml. of water-saturated n-butanol, giving fractions C-5 and E-f, and fractions C-6 and E-6. These are, of course, the butanol-soluble and the butanol-insoluble sub-

stances left in the incubation mixture. The butanol may be expected to contain any simple monoamino-monocarboxylic acids present; the butanol-insoluble fraction probably contains, among other things, most of the tyrosine unoxidized in the incubations. Although tyrosine is a monoamino-monocarboxylic acid, it was found on preliminary extraction procedures that very little tyrosine dissolved in the butanol upon such an extraction.

The two final fractions listed above were then concentrated to dryness in vacuo, each residue dissolved in 5 ml. of water, and Kjeldahl digestions and distillations performed on each. Of the butanol-soluble fractions, C-5 contained 3.01 mg. of nitrogen, E-5 contained 2.03 mg. of nitrogen. The butanol-insoluble fractions contained 18.6 mg. of nitrogen in C-6, and 25.75 mg. of nitrogen in E-6. These samples were then all saved for isotope determinations.

Figure 6, page 121, summarizes the fractionation procedure performed on the isotope incubation mixture, giving the various fractions isolated and analyzed for isotope content. The numbers in parentheses refer to the isotope sample number as given in the list of isotope determinations, page 123.

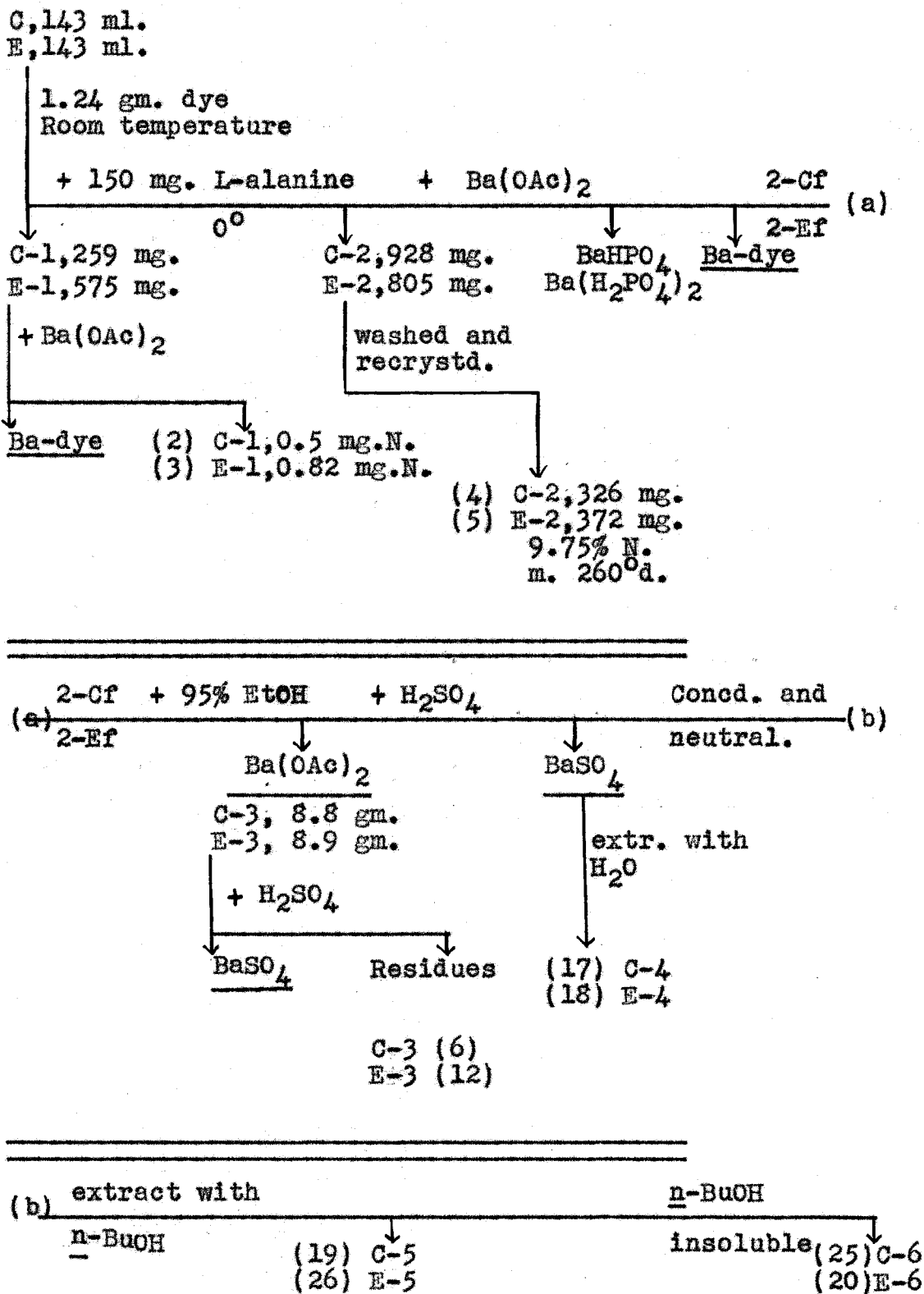


Figure 6. Chart of Fractionation of Isotope Incubation Mixture

The gas samples collected in marked gas bulbs, by the procedure already described, were turned over to H. J. Svec of the Institute for Atomic Research, Iowa State College, for analysis. Mr. Svec built the mass spectrometer on which the determinations were run, and we are greatly indebted to him for his cooperation in this problem.

Table 6 gives a complete list of all the samples collected for analysis. Column 3 gives the isotope determination number by which the results were reported.

Ten out of the thirty-two samples were found to have varying amounts of air contamination. In order to calculate the N_2^{29} and N_2^{28} peaks due to the sample alone, both with and without air contamination, the following values were reported:

$$\frac{N_2^{29}}{N_2^{28} \text{ Total}}, (N_2^{28})_{\text{Total}}, (N_2^{28})_{\text{Background}}, \text{ and } \left[(O_2^{32})_T - (O_2^{32})_B \right].$$

An illustration of the form of report of analytical data is given in Table 7, page 124. An illustration of the calculation involved will be given for sample (5), which was the only sample with an appreciable amount of air in it.

Table 6. Isotope Determinations

Sample	Fraction	Isotope determination number
(2)	From ppt. C-1	HS-5-115a
(3)	From ppt. E-1	HS-5-114b
(4)	Ppt. C-2	HS-5-114a
(5)	Ppt. E-2	HS-5-113b
(6)	Res. C-3	HS-5-113a
(12)	Res. E-3	HS-5-121b
(15)	N ¹⁵ -L-tyrosine, I	HS-5-122b
(17)	Res. C-4	HS-5-123b
(18)	Res. E-4	HS-5-124a
(19)	Res. C-5	HS-5-124b
(20)	Res. E-6	HS-5-130a
(25)	Res. C-6	HS-5-132b
(26)	Res. E-5	HS-5-133a

The total N_2^{29}/N_2^{28} ratio is a resultant of three terms, thus:

$$(1) \quad \left(\frac{N_2^{29}}{N_2^{28}} \right)_T = \frac{(N_2^{29})_S + (N_2^{29})_B + (N_2^{29})_A}{(N_2^{28})_S + (N_2^{28})_B + (N_2^{28})_A}$$

where S stands for sample, B stands for background, and A

Table 7. Analytical Data Reported

1	2	3	4	5	6
Determination Number	$\left(\frac{N_2^{29}}{N_2^{28}}\right)_T$	$(N_2^{28})_T$ peak in volts	$(N_2^{28})_B$ volts	$[(O_2^{32})_T - (O_2^{32})_B]$ volts	Corr. atom% excess N^{15} over stand.
HS-5-115b	0.00873	2.10	0.050	0.0005	0.069
HS-5-115a	.05500	1.91	.050	.0030	2.413
HS-5-114b	.02795	0.58	.050	.0010	1.143
HS-5-114a	.00820	2.00	.050	.0015	0.044
HS-5-113b	.00736	2.50	.050	.1240	0.214
HS-5-113a	.00820	2.00	.050	.0015	0.044
HS-5-109 ¹	.00728	----	----	-----	-----

¹Tank N₂

stands for air (i.e., the nitrogen contained in the contaminating air). Referring to Table 7, the peak value for $(N_2^{29})_T$ can be calculated by multiplying column 2 and column 3. In order to calculate $(N_2^{29})_S$, we then need to evaluate $(N_2^{29})_B$ and $(N_2^{29})_A$, and similarly for the N_2^{28} peaks. The normal ratio of nitrogen 29 to nitrogen 28 in air has been determined and reported by Rittenberg (131) to be 0.00378. Thus,

$$(2) \quad \left(\frac{N_2^{29}}{N_2^{28}}\right)_B = 0.00378 = \left(\frac{N_2^{29}}{N_2^{28}}\right)_A \quad \text{So,}$$

$$(3) \quad (N_2^{29})_B = 0.00378(N_2^{28})_B$$

Similarly, $(N_2^{29})_A = 0.00378(N_2^{28})_A$

In order to evaluate $(N_2^{29})_A$, the ratio $\left(\frac{N_2^{28}}{O_2^{32}}\right)_A$ has been de-

termined to be 10.73 for this particular mass spectrometer. This ratio differs from 4 because of the different rates of ionization of nitrogen and oxygen, and also because of individual variations in different mass spectrometers. The term $(O_2^{32})_A$ is equal to the term reported in column 5 of Table 7. So,

$$(4) \quad (N_2^{28})_A = 10.73 \left[(O_2^{32})_T - (O_2^{32})_B \right]$$

Then

$$(5) \quad (N_2^{29})_A = 0.00378 \times 10.73 \left[(O_2^{32})_T - (O_2^{32})_B \right]$$

With the values reported in Table 7, we then can calculate the correct concentrations of N_2^{29} and N_2^{28} in the sample.

Thus,

$$(6) \quad (N_2^{29})_S = (N_2^{29})_T - (N_2^{29})_B - (N_2^{29})_A$$

$$(7) \quad (N_2^{29})_S = (N_2^{29})_T - 0.00378(N_2^{28})_B - 0.00378 \times 10.73 \times \left[(O_2^{32})_T - (O_2^{32})_B \right] \quad \text{and}$$

$$(8) \quad (N_2^{28})_S = (N_2^{28})_T - (N_2^{28})_B - 10.73 \left[(O_2^{32})_T - (O_2^{32})_B \right]$$

The corrected atoms per cent N^{15} is then calculated by a formula developed by Rittenberg (131):

$$\text{Atoms\%N}^{15} = \frac{100}{2R+1} \quad \text{where } R = \frac{N_2^{28}}{N_2^{29}}$$

By calculating the term R and the atoms% N^{15} for the standard, we then can obtain the atoms% excess N^{15} over the standard for each sample.

To illustrate the procedure, sample (5) will be calculated.

$$(N_2^{29})_T = 0.00736 \times 2.5 = 0.0184$$

$$(N_2^{29})_S = 0.0184 - 0.000189 - 0.0406 \times 0.124 = 0.0132$$

$$(N_2^{28})_S = 2.5 - 0.05 - 10.73 \times 0.124 = 1.12$$

Then

$$R = \frac{N_2^{28}}{N_2^{29}} = \frac{1.12}{0.0132} = 84.8$$

$$\text{Atoms\% } N^{15} = \frac{100}{2 \times 84.8 + 1} = 0.586$$

For the standard, the atoms% N^{15} content is calculated to be 0.372. Then,

$$\begin{array}{r} \text{Atoms\% excess } N^{15} \\ \text{over standard} \end{array} = \begin{array}{r} 0.586 \\ -0.372 \\ \hline 0.214 \end{array}$$

It may be seen by inspecting formulae (7) and (8) on page 125, that when there is no air contamination in the sample, the last term in each formula drops out. Then the values for the sample are calculated simply by correcting the observed reading for the small amount of "background" nitrogen left in the spectrometer. This background value was constant for all the determinations.

By the above method all thirty-two samples were calculated in terms of atoms% excess N^{15} over standard. The re-

sults are given in the following tables. Table 8, page 128, lists the results of the tissue incubation experiment, in which each experimental sample is paralleled by a control sample. The control samples should have no excess N^{15} , of course; within the accuracy of the instrument this was true except for samples (2) and (17). We are at a loss to explain the high enrichment of N^{15} found in sample (2), since no isotope was used in the control series.

From a qualitative standpoint, it would appear that the room temperature precipitate had the most excess N^{15} in it. It does have the highest enrichment, but the small amount of nitrogen contained in it means it has a small amount of the total isotope used. A more accurate diagnosis of the fate of the isotope may be made by calculating the yields of excess N^{15} obtained in the different fractions. The milligrams of nitrogen contained in the sample times the atoms% excess N^{15} in that sample should give the milligrams of excess N^{15} in that fraction. This value can then be compared to the total milligrams of excess N^{15} used in the incubations. In this way we can see in what fraction most of the isotope was found. Table 9, page 129, gives such a summary for the experimental fractions of the tissue experiment.

Examination of the Table shows that almost 100 per cent of the excess isotope present in the original incubation mixtures was recovered in the alanine fraction. The only other fractions with appreciable amounts of isotope are the room

Table 8. Results of Tissue Incubation Experiment

Sample	Fraction	Atom% excess N ¹⁵ over standard
(2)	From room temp. ppt. C-1	2.413
(3)	From room temp. ppt. E-1	1.143
(4)	Alanine-dye, ppt. C-2	0.044
(5)	Alanine-dye, ppt. E-2	0.214
(6)	Res. C-3, from Ba(OAc) ₂	0.044
(12)	Res. E-3, from Ba(OAc) ₂	0.347
(17)	Res. C-4, from BaSO ₄	0.121
(18)	Res. E-4, from BaSO ₄	0.200
(19)	Res. C-5, BuOH-soluble	0.061
(26)	Res. E-5, BuOH-soluble	0.250
(25)	Res. C-6, BuOH-insoluble	0.025
(20)	Res. E-6, BuOH-insoluble	0.064

tem perature precipitate, E-1, and the butanol-insoluble residue, E-6.

The high amount of nitrogen in the butanol-insoluble residue, in conjunction with the quite low isotope enrichment, undoubtedly means that the majority of the nitrogen is contained in compounds which did not enter into the tyrosine oxidation scheme. Thus the isotope is probably present in this fraction in a very small amount of a compound with quite high enrichment. It is likely that a small amount of

Table 9. Recovery of Isotope Used in Incubations

Used 18 mg. N^{15} -L-tyrosine containing 1.393 mg. of nitrogen. 5.635 atoms% excess N^{15} , or 0.0785 mg. excess N^{15} .

Sample	Total N in Sample	Atoms% excess N^{15} over standard	Mg. excess N^{15}	Per Cent Yield
(3) E-1, from room temp. ppt.	0.82 mg.	1.143	0.00937	11.93
(5) E-2, alanine-dye	36.62	0.214	0.07837	99.84
(12) E-3, adsorbed to $Ba(OAc)_2$	1.66	0.347	0.00576	7.34
(18) E-4, adsorbed to $BaSO_4$	0.752	0.200	0.0015	1.91
(26) E-5, BuOH-soluble	2.03	0.250	0.00507	6.46
(20) E-6, BuOH-insoluble	25.75	0.064	0.01648	20.99

unoxidized isotopic tyrosine is present in the butanol-insoluble residue, since none of the fractionation steps preceding the extraction with butanol will precipitate tyrosine. In addition, it has already been mentioned that in practice extraction runs, the tyrosine was practically insoluble in butanol.

It is possible that fraction E-5, the butanol-soluble substance or substances, contains a small amount of alanine formed by oxidation of the isotopic tyrosine. The presence

of other mono-amino mono-carboxylic acids containing isotope is not absolutely excluded, although their formation under the experimental conditions used is not readily explainable.

The further disposal of alanine by the liver tissue, may involve the introduction of the amino group into the "metabolic pool" where it is available for amino acid and protein synthesis. One would not expect such extensive syntheses to be carried out in the relatively short incubation time used, but they may have occurred to the small extent indicated by the recovery of 6.46% of the isotope in this fraction.

Fraction E-3, which was adsorbed to barium acetate, may well be, in part, glutamic or aspartic acid. These acids would probably be present if the tyrosine nitrogen was disposed of by means of transamination reactions. Since we were trying to precipitate the barium salts of these acids in the step which gave us the barium acetate, barium glutamate or aspartate may have co-precipitated with the inorganic salt. The presence of 7.34% of the isotope in this fraction may thus be indicative of transamination having occurred in the course of tyrosine oxidation.

Fraction E-4, adsorbed to the barium sulfate, obviously indicates incomplete washing of this precipitate. It is well known that barium sulfate adsorbs other ions and molecules which may be present in such a medium. This fraction belongs with one of the following two, E-5 or E-6; which one it is

impossible to say.

We can speculate only on the meaning of the occurrence of almost 12% of the isotope in the room temperature precipitate, E-1. As mentioned earlier, we examined it for the presence of a possible intermediate of tyrosine oxidation with no success. Since the spectrophotometric examination showed the presence of no aromatic nuclei, it is definite that no unchanged isotopic tyrosine had precipitated in this fraction. It may be possible that a small amount of alanine precipitated as the dye salt even at room temperature, and thus accounted for the isotope content of this fraction.

On the basis of the above results, we concluded that the oxidation of tyrosine by guinea pig liver tissue results in the formation of alanine mole for mole. This fact, plus the results from the other fractions, in conjunction with previous work reported in the literature enables us to present a tentative hypothesis as to the mechanism of oxidation of tyrosine by liver tissue.

D. Feeding Experiments with N¹⁵-L-Tyrosine

The influence of Vitamin C on the metabolic disposal of tyrosine has been discussed in the Historical Section. Just where in the tyrosine catabolic scheme this vitamin exerts its influence, and just what kind of influence it exerts are still open questions. Having isotopically-marked tyrosine at our disposal, it occurred to us that valuable information

might be obtained from feeding experiments with tyrosine and Vitamin C. When guinea pigs are fed a scorbutigenic diet supplemented with tyrosine, a large per centage of the fed tyrosine is excreted as such (or at least as a compound giving a "tyrosyl" value with the Folin-Ciocalteu phenol reagent); when Vitamin C is then added to this same diet, the tyrosyl value, as well as the tyrosine metabolites value (keto acids), drops sharply. Apparently the added Vitamin C enables the animal to dispose of the added tyrosine in a more nearly normal fashion. Questions which might be answered by using isotopic tyrosine in such a feeding experiment are: does Vitamin C promote the catabolism of tyrosine in the liver with the resultant formation of urea? or does the vitamin produce deamination of the amino acid in the kidney with perhaps an increase in urinary ammonia? or does the vitamin act as part of a system whereby the amino group of the tyrosine is transferred to some other compound in the metabolic pool? It was felt that we would be better able to answer these questions if we could ascertain the location of the amino acid nitrogen when tyrosine is oxidized in vivo. Since we were more interested in the catabolic phase than the anabolic phase of tyrosine metabolism, we decided to examine the urine and feces for isotope content and save the carcass and blood for future investigation.

It was planned therefore to feed a guinea pig a scorbutigenic diet, add isotopic tyrosine and Vitamin C, and ana-

lyze the urine and feces for isotope. A second animal would be handled the same except for no added vitamin, thus serving as a control. Since pteroylglutamic acid has also been found to exert an influence on tyrosine oxidation, a third feeding experiment was planned with this vitamin as a supplement instead of ascorbic acid.

1. Methods¹

The usual scorbutigenic diet used in this laboratory is ground Purina rabbit chow which has been spread out in a thin layer exposed to the air for about ten days. It was felt, however, that a stricter control of the intake could be achieved if a synthetic diet could be used. Accordingly a diet containing all of the essential amino acids, salts, and vitamins except Vitamin C, was prepared. It was found that adding about 5% of the ground Purina chow to this synthetic mixture increased the palatability of the diet and promoted more complete ingestion of the food by the guinea pig.

A 300-gm. guinea pig was fed on this synthetic diet plus added tyrosine--200 mg./100 gm. body weight--until it was excreting 35% of the added tyrosine in the urine as keto acids. Then the animal was fed 500 mg.--200 mg./100 gm. body weight--of N¹⁵-L-tyrosine over a twenty-four hour period. In experi-

¹These feeding experiments were performed in cooperation with R. S. Schweet of this laboratory, who composed the diet and performed the Kjeldahl nitrogen determinations.

ment 1, the animal was given 25 mg. of ascorbic acid by mouth, at the beginning of this twenty-four hour period, another 25 mg. of ascorbic acid three hours later, and another 25 mg. six hours after the start of the period, making a total supplementation of 75 mg. of ascorbic acid.

In experiment 2, no supplementary vitamin was given. This animal thus served as a control for the preceding and following experiments.

In experiment 3, two intraperitoneal injections of pteroylglutamic acid were given; 10 mg. were injected twenty-four hours before isotopic tyrosine feeding, and 15 mg. at the beginning of the isotope feeding period.

The urine was collected over this twenty-four hour period in about 10 ml. of dilute sulfuric acid, diluted to 100 ml., and analyzed for total tyrosine value, keto acids, total nitrogen, ammonia nitrogen, urea nitrogen, and residual nitrogen.

Tyrosine was determined colorimetrically by the method of Folin and Ciocalteu (127); the keto acids were determined colorimetrically by the method of Penrose and Quastel (130). Ammonia was tested for by making an aliquot of the urine alkaline and connecting to an aeration train. Air was bubbled through the sample and then into a tube containing 4% boric acid. In all cases there was not enough ammonia to turn the methyl red-methylene blue indicator. Urea was then determined on the same sample by adding two urease tablets crushed with

a little water, adjusting the pH to about 6.5, and incubating at 38° C. for 4 hours. The ammonia formed was then aerated out of the sample by adding alkali and bubbling air through and into 4% boric acid containing the above mixed indicator. The amount was determined by titration with standard hydrochloric acid, and the titration samples saved for isotope determinations. Residual nitrogen was then determined on the same sample by digesting with concentrated sulfuric acid and distilling in a micro-Kjeldahl apparatus. The total nitrogen of the urine, the food and feces nitrogen, and the intestinal contents nitrogen were all determined by the Kjeldahl procedure.

The animal was sacrificed at the end of the twenty-four hour period by stunning and decapitation. The blood was collected in a beaker containing oxalate. The volume of blood collected was so small--about 10-15 ml.--that 10 ml. of 0.9% sodium chloride solution were used to help rinse the blood into a centrifuge tube. The plasma was separated off after centrifugation and diluted to 50 ml. volume; the cells were laked and also diluted to a volume of 50 ml. Two drops of concentrated hydrochloric acid were added to each fraction, and they were stored in the refrigerator for possible future analysis.

The entire gastro-intestinal tract from the esophagus to the anus was dissected out, slit open and the contents washed into a beaker with distilled water. These intestinal contents

were then digested with concentrated sulfuric acid and the nitrogen content determined by the Kjeldahl procedure. The distillates were saved for isotope determinations. The washed gastro-intestinal tract was then put back into the animal and the carcass stored in the freezing compartment of the refrigerator for possible future investigation.

The uneaten food at the end of the twenty-four hour period, plus any scattered about the metabolism cage, plus the feces of the twenty-four hour period were combined and digested by the Kjeldahl procedure, and the distillates saved for isotope determination. This fraction, plus the intestinal contents fraction, represents the part of the tyrosine which was not absorbed into the metabolic system of the animal.

There were thus six fractions of immediate interest which were collected and investigated for isotope content: urea, ammonia, residual nitrogen, and total nitrogen of the urine, intestinal contents, and food and feces. It was hoped that at least a partial answer to the question of the fate of the tyrosine in the presence of Vitamin C could be obtained by an analysis of the isotope distribution in the urinary fractions listed above.

2. Results with excess ascorbic acid

Experiment 1 of the feeding experiments consisted in supplementation with excess ascorbic acid at the time of

feeding the isotopic tyrosine. As was described in the Methods, 75 mg. of ascorbic acid were fed to the animal. The six fractions mentioned were then collected and analyzed. Tables 10 and 11 summarize the results of these analyses.

Table 10. Isotope Determination Samples, Run 1

Sample	Fraction	Isotope determination number
(1)	Intestinal contents,	HS-5-115b
(7)	Urea	HS-5-119a
(8)	Residual nitrogen	HS-5-119b
(9)	Food and feces	HS-5-120a
(10)	Intestinal contents	HS-5-120b
(13)	Total nitrogen	HS-5-122a

In calculating the amount of isotopic tyrosine absorbed and the yields, the amount of excess N^{15} fed was calculated from the amount of tyrosine--500 mg., containing 38.7 mg. of nitrogen--and the enrichment of the tyrosine--5.635 atoms% excess N^{15} . This amount fed to 2.181 mg. of excess N^{15} fed in all three of the feeding experiments. To obtain the amount of isotope absorbed, the food and feces and intestinal contents values were subtracted from the 2.181 mg. fed. This gave a value of 1.989 mg. of excess N^{15}

Table 11. Results of Isotope Analyses on Feeding Experiment 1
 Vitamin C + 500 mg. N¹⁵-L-tyrosine supplementation. 2.181 mg.
 excess N¹⁵ fed, 1.989 mg. excess N¹⁵ absorbed. Tyrosyl value,
 22.20%; keto acid value, 10.40%

Sample	Total N in sample mg.	Atoms% excess N ¹⁵ over standard	Mg. excess N ¹⁵	Per cent yield
Urea	185.50	0.320	0.5936	29.84
Residual N ¹	141.00	0.120	0.1692	8.50
Total N	219.90	0.322	0.7081	35.60
Food and feces	46.80	0.110	0.0515	} = 0.1917 mg. not absorbed
Intestinal con- tents	203.20	0.069	0.1402	

¹It is obvious that the urea nitrogen plus the residual nitrogen should equal the total nitrogen. The large value of the residual nitrogen is due to the nitrogen added in the urease tablets for the urea determination. The value, when corrected for this addition, is 56.7 mg.

absorbed in the first experiment. The tyrosyl value and keto acid value were calculated upon the basis of 500 mg. of N¹⁵-L-tyrosine fed.

3. Results of control experiment

The same procedure as in Part 2, above, but without added Vitamin C, was followed to furnish a control to the above and to the following experiment.

The animal weighed 300 gm. and was given 500 mg. of

N^{15} -L-tyrosine, but no Vitamin C. The urine was collected over the twenty-four hour period, after which the animal was sacrificed and handled exactly as described previously.

An unexpected feature of both experiment 1 and this one was the absence of any detectable urinary ammonia. Since guinea pigs are entirely herbivorous, a low urinary ammonia is to be expected. The lack of any (within the limits of the analytical method used; aeration of an aliquot made alkaline) was attributed to the synthetic diet employed. Evidently the mixture of amino acids does not have as pronounced an effect on the acid-base balance as a diet of natural protein does.

The results of the control experiment are summarized in the following tables.

Table 12. Isotope Determination Samples, Run 2

Sample	Fraction	Isotope determination number
(21)	Total nitrogen	HS-5-130b
(22)	Urea	HS-5-131a
(23)	Residual nitrogen	HS-5-131b
(24)	Intestinal contents	HS-5-132a
(32)	Food and feces	HS-5-135b

Table 13. Results of Isotope Analysis on Feeding Experiment 2

Control, 500 mg. N¹⁵-L-tyrosine supplementation. 2.181 mg. excess N¹⁵ fed, 1.455 mg. excess N¹⁵ absorbed. Tyrosyl value, 45.70%; keto acid value, 33.40%

Sample	Total N in sample mg.	Atoms% excess N ¹⁵ over standard	Mg. excess N ¹⁵	Per cent yield
Urea	154.3	0.239	0.3688	25.34
Residual N ¹	150.0	0.075	0.1125	7.73
Total N	195.0	0.239	0.4661	32.03
Food and feces	267.2	0.226	0.6039	} = 0.7262 mg. not absorbed
Intestinal contents	163.0	0.075	0.1223	

¹Corrected to 57 mg. N, (see footnote 1, Table 11).

In view of the fact that not all of the tyrosine fed is absorbed, as shown by these isotope experiments, the metabolite excretion values, tyrosyl and keto acids, should probably be quite a bit higher than the figures given.

4. Results with excess pteroylglutamic acid

In the case of Vitamin C supplementation to a diet containing added tyrosine, the keto acid value (tyrosine metabolites, evidence of incomplete tyrosine oxidation) is immediately lowered. However, when pteroylglutamic acid is

added to such a diet instead of ascorbic acid, the lowering of the keto acid value is delayed somewhat, and it usually requires two supplements of this vitamin, twenty-four hours apart, to produce a significant lowering of the tyrosine metabolite excretion. This fact was taken into account in this feeding experiment.

A guinea pig of about 300 gm. weight was fed on the synthetic diet plus added tyrosine until the keto acid value was around 35% of the added amino acid. Pteroylglutamic acid was then injected intraperitoneally. Twenty-four hours later a second injection of the vitamin was given, 15 mg., and 500 mg. of N¹⁵-L-tyrosine added to the diet at that time. The urine was then collected over the next twenty-four hour period, after which the animal was sacrificed and handled exactly as in the previous two experiments.

The results are summarized in the following two tables, page 142.

Table 16, page 143, gives a comparison of the isotope content of the different urinary fractions for the three experiments just described.

The slight increase in urinary content of isotope after feeding ascorbic acid is not significant enough to warrant definite conclusions. These experiments have been preliminary in nature, and must be followed by more extensive work. It is extremely interesting to note that pteroylglutamic acid supplementation did increase the urea and total nitrogen iso-

Table 14. Isotope Determination Samples, Run 3

Sample	Fraction	Isotope determination number
(27)	Total nitrogen	HS-5-133c
(28)	Food and feces	HS-5-134a
(29)	Intestinal contents	HS-5-134b
(30)	Residual nitrogen	HS-5-134c
(31)	Urea	HS-5-135a

Table 15. Results of Isotope Analysis on Feeding Experiment 3

Pteroylglutamic acid + 500 mg. N¹⁵-L-tyrosine supplementation.
 2.181 mg. excess N¹⁵ fed, 1.584 mg. excess N¹⁵ absorbed. Tyrosyl value, 11.4%; keto acid value, 3.47%

Sample	Total N in sample mg.	Atoms% excess N ¹⁵ over standard	Mg. excess N ¹⁵	Per cent yield
Urea	325.0	0.164	0.5330	33.65
Residual N ¹	155.0	0.083	0.1287	8.12
Total N	390.0	0.181	0.7059	44.56
Food and feces	275.6	0.136	0.3748	} = 0.5969 mg. not absorbed
Intestinal contents	347.0	0.064	0.2221	

¹Corrected nitrogen value, 61.7 mg.

Table 16. Isotope Recoveries in Urine after Feeding N¹⁵-L-Tyrosine

Urinary Constituent	Per cent yield of isotope		
	control	ascorbic acid added	pteroylglu- tamic acid added
Total nitrogen	32.03	35.60	44.56
Urea	25.34	29.84	33.65
Residual nitrogen	7.73	8.50	8.12

tope content appreciably. Also, as Tables 11, 13, and 15 show, the tyrosyl and keto acid values were lowered more by this vitamin than by ascorbic acid. These results tend to corroborate the belief held by several workers that the two vitamins do not exert their influence on tyrosine oxidation by the same mechanism.

It would seem from these results that Vitamin C takes part in tyrosine metabolism either by influencing the disposal of the carbon skeleton of the amino acid after the nitrogen has been removed, or, if its action is directed toward the amino group, by aiding in the anabolic reactions whereby the tyrosine nitrogen is incorporated in tissue proteins. The former alternative would seem more plausible. At any rate, the question of the disposal of the nitrogen when ascorbic acid is given to scorbutic animals requires more experiments and a more complete investigation of tissue

constituents.

It may be tentatively concluded that pteroylglutamic acid does affect the disposal of the amino nitrogen to some extent. The fact that the increase in isotope content is mostly in the urea fraction would indicate a more complete use of the tyrosine nitrogen in the Krebs-Henseleit urea cycle. This effect may be brought about either by a direct action in the tyrosine oxidation system or by an indirect action from the acceleration of the general metabolic processes.

It may be of significance that ascorbic acid seemed to promote more complete absorption of the amino acid than was shown by the control animal. In this connection, the varying amounts of absorption shown in the three cases means that experiments based on tyrosyl and keto acid values in urine excretion should be re-examined, since such values have been based on the amount of tyrosine fed to the animal. Actually, the values should probably be higher in some instances than they appeared to be, since relating them to the amount of amino acid absorbed would represent the more accurate picture of the metabolic processes taking place.

IV. DISCUSSION

There seems to be no doubt but that the normal oxidation of tyrosine by guinea pig liver tissue results in the formation of alanine, either as an intermediate substance or a final product of the particular enzyme system concerned. If this fact can be reconciled with other known facts concerning tyrosine oxidation, any mechanism proposed to account for this finding will have added validity.

The confirmation of Felix and Zorn's finding of alanine in a tyrosine oxidation mixture necessitates a re-examination of their proposed oxidation scheme. A reference to page 39 shows that they postulated, first, the uptake of one atom of oxygen to form a quinonoid intermediate, II. This then adds water to produce a quinol, which re-arranges to form 2,5-dihydroxyphenylalanine, IV. It is from this compound that they propose that the alanine is split off by hydrolysis, forming, in addition to alanine, 1,2,4-trihydroxybenzene, V. The action of three more atoms of oxygen plus one molecule of water then ruptures the benzene ring to produce acetoacetic acid, carbon dioxide, and formic acid.

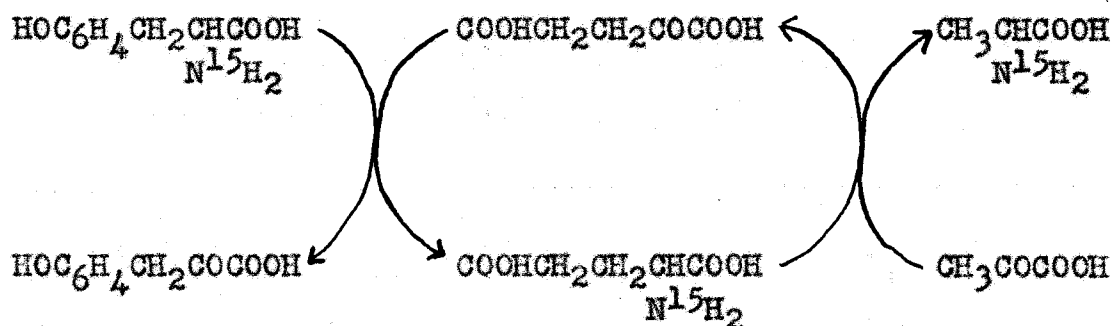
There are two powerful arguments against this mechanism, however. Most of the evidence cited in the Historical Section tends to prove that homogentisic acid is a normal intermediate in the catabolism of tyrosine. Felix and Zorn's mechanism excludes the possibility of formation of homogentisic acid as

a normal catabolite. But even stronger refutation is provided by Weinhouse and Millington's results (112) with tyrosine labeled in the β -position of the side chain with C^{14} , as well as Schepartz and Gurin's results (113) with phenylalanine labeled in the same place with the same isotope. After incubation of either of these amino acids with rat liver slices, almost all of the radioactivity was found in the α -position of acetoacetic acid, proving that the side chain of tyrosine is involved in the formation of this keto acid, as pictured on pages 62 and 63.

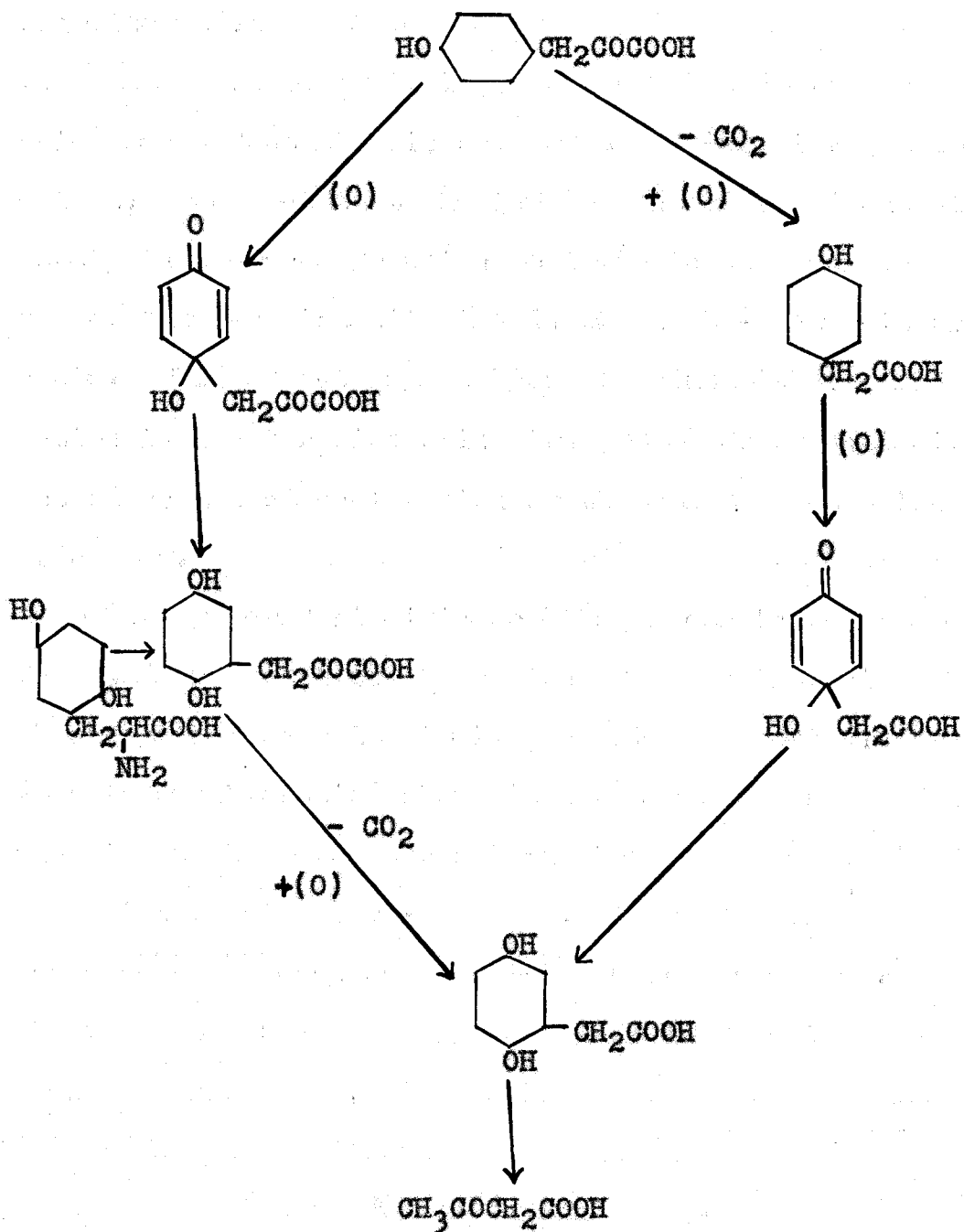
There are two possible hypotheses by which the results of Weinhouse and Millington and of Schepartz and Gurin can be reconciled to the findings of Felix and Zorn and those reported here. It is possible that the first step in the oxidation of tyrosine might be oxidative deamination, with the production of *p*-hydroxyphenylpyruvic acid and ammonia. The ammonia thus formed may then react with pyruvic acid to form alanine. Wiss (132) has reported the isolation of alanine (as the β -naphthalene sulfonate derivative) from a guinea pig liver homogenate incubated with pyruvic acid and ammonium chloride. The *p*-hydroxyphenylpyruvic acid then could be further oxidized to acetoacetic acid through the intermediate formation of homogentisic acid. It is universally conceded, however, that tyrosine is not oxidatively deaminated by liver tissue, since no trace of ammonia production has ever been found in liver incubations with tyrosine. Even though the

ammonia may be used to form alanine, there should be at least detectable amounts present, unless the formation of alanine from ammonia and pyruvic acid proceeds much more rapidly than the deamination of tyrosine.

A second possibility for explaining the observed facts might be transamination according to the following scheme:



Thus, the amino group of tyrosine may be transferred to α -ketoglutaric acid to form glutamic acid. This may then pass the amino group on to pyruvic acid to form alanine. Under such a scheme, only catalytic amounts of α -ketoglutaric acid (and thus of glutamic acid) need be present. This may explain why incubation mixtures of tyrosine and α -ketoglutaric acid with liver tissue yield no detectable amounts of glutamic acid, as Zorn reported (46). The p-hydroxyphenylpyruvic acid could then be further oxidized to acetoacetic acid through the intermediate formation of homogentisic acid, according to either one of two possible pathways:



This would also explain Neuberger's results (48) in feeding 2,5-dihydroxyphenylalanine to an alcaptonuric and obtaining increased production of homogentisic acid in the urine. Oxidative deamination or transamination of the dihydroxyphenyl-

alanine followed by oxidative decarboxylation would produce homogentisic acid.

Although the feeding experiments cited in this paper were not designed to add to the problem discussed above, it is interesting to note that in Feeding Experiments I and III, (Table 16, page 143), the recovery of isotope in the urea fractions were higher than in the control, Experiment II. Alanine, of course, is capable of undergoing oxidative deamination, and the ammonia formed ultimately produces urea by means of the Krebs-Henseleit urea cycle. In other words, in the experiments where the oxidation of tyrosine may be expected to proceed in a more nearly normal manner, more alanine is produced, and thus ultimately more urea excreted.

The production of alanine from tyrosine oxidation might well explain several facts discussed in the Historical Section. Thus, Rapport and Beard's report (37) that tyrosine exerts a specific dynamic action comparable to alanine becomes understandable. The finding by Shambaugh, Lewis and Tournellotte (38) that feeding phenylalanine and tyrosine to rabbits caused an increased blood urea nitrogen may be explained as above, i.e., by oxidative deamination of the alanine and the formation of urea from the ammonia thus produced.

The rather surprising evidence reported by Butts, Dunn, and Hallman (42) that phenylalanine and tyrosine increased the glycogen content of livers of animals fed these amino acids

may be explained by the known fact that alanine is a "glycogenic amino acid". In other words, alanine is oxidatively deaminated to yield pyruvic acid, which then may enter the carbohydrate metabolic cycle to form glucose or glycogen.

The facts reported in this paper do not warrant a definite conclusion as to the complete mechanism of oxidation of tyrosine. The production of alanine in an incubation mixture of tyrosine and liver homogenate has been shown. In conjunction with the prior experiments with radioactive tyrosine, the facts tend to indicate that transamination occurs. The incubation of doubly-labeled tyrosine, that is, with N^{15} in the amino group and C^{14} in the β -position of the side chain in the same molecule, would be a very informative experiment, and would help to substantiate one of the two possibilities discussed here.

Another experiment which would help establish the oxidation mechanism would be to dialyze the liver brei, and then determine the tyrosine oxidation manometrically upon addition of the transaminase co-enzyme, pyridoxal phosphate. This may not yield the whole picture, however, since it is quite possible that the system which disposes of the *p*-hydroxyphenylpyruvic acid with the uptake of oxygen is a different system than the one which carries out the transamination. Isolation of alanine from such an experiment, however, would confirm the occurrence of transamination as pictured in this Section.

Still another informative experiment would be to feed a guinea pig glucose marked with radioactive carbon, sacrifice the animal and use the liver tissue to oxidize tyrosine marked with N^{15} . Since carbohydrate is one of the main sources of pyruvic acid in the organism, alanine isolated from such an experiment should have both heavy nitrogen and radioactive carbon in its molecule if transamination had occurred.

The results of the feeding experiments, though preliminary in nature, may be used to draw some general conclusions worth noting. If the urinary content of isotope had been appreciably higher for the ascorbic acid supplementation than for the control, one could have concluded that the vitamin is concerned in the step or steps which remove the nitrogen from the tyrosine molecule. Since such was not the case, it may be that the ascorbic acid operates upon the carbon residue of the amino acid after the amino group is removed. This is logical considering that a Vitamin C deficiency in the guinea pig leads to excretion of *p*-hydroxyphenylpyruvic acid and homogentisic acid. This supposition also agrees with Fishberg's belief (86) that, on the basis of her finding benzoquinoneacetic acid in the blood and urine of scorbutic and rheumatic fever patients, Vitamin C operates in the oxidation mechanism after homogentisic acid formation.

A direct comparison of our feeding experiments with those of Schoenheimer, Ratner and Rittenberg (103) cannot be

made, since they used a normal rat to which isotopic tyrosine was fed for 10 days, and in addition they used N¹⁵-DL-tyrosine. However, it is interesting to note that, aside from tyrosine itself which was deposited in the tissues, one of the four main fractions isolated from tissues which contained N¹⁵ was the dicarboxylic amino acid fraction, that is, glutamic and aspartic acids.

Under the conditions of their experiment they recovered 50-60% of the administered isotope in the urine. Only in the case of pteroylglutamic acid supplementation did our recovery of isotope in the urine approach 50%. Since our supplementation was over a 24-hour period, after which the animal was immediately sacrificed, it would appear that the vitamin has a positive effect on the urinary excretion of nitrogen, mostly as urea. The question still remains as to the fate of the amino nitrogen when ascorbic acid is added to enable a more complete oxidation of tyrosine by a scorbutic animal. If, as has been mentioned above, ascorbic acid affects the carbon skeleton only, then the transamination reaction would probably occur in the scorbutic animal as well as the normal animal, although probably at a somewhat lesser rate. In this case, the recovery of similar amounts of nitrogen in the urine of the control and ascorbic acid-supplemented animals is to be expected. Such was the case in our experiments.

V. SUMMARY

1. The synthesis of DL-tyrosine containing isotopic nitrogen has been effected in good yields by a four-step process: the Erlenmeyer azlactone synthesis as modified by Herbst and Shemin, in 52-66% yield; breaking the ring by mild hydrolysis with acetone and water, in 71-94% yield; acid hydrolysis to obtain the hydroxyphenylpyruvic acid, in 48-53% yield; and catalytic hydrogenation of the substituted pyruvic acid in the presence of isotopic ammonia, in 73-90% yield.

2. N^{15} -acetyl-DL-tyrosine has been resolved by the use of d - α -phenylethylamine, to give yields of 35-60% of the salt of the L-isomer, from which N^{15} -L-tyrosine was isolated in 77% yield. This new method of resolution involves less manipulation, thus is less time-consuming, and gives slightly better yields than the methods reported heretofore.

3. The isotopic tyrosine was oxidized in vitro by guinea pig liver homogenate, and alanine subsequently isolated from the reaction mixture as the azobenzene-p-sulfonate salt.

4. Isotopic analysis of the isolated compound showed almost all of the N^{15} from tyrosine to be contained in the alanine derivative.

5. Isotopic tyrosine was fed to guinea pigs on scorbutigenic diets with and without supplements of ascorbic

acid and pteroylglutamic acid, and the urine and feces examined for isotope content. These experiments indicated that the vitamins apparently have little effect on the disposal or fate of the amino-nitrogen of tyrosine.

6. A simple but satisfactory method has been described for collecting isotopic nitrogen gas for analysis on the mass spectrometer.

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VII. APPENDIX

The normal abundance of N^{15} in nitrogen is 0.378%. Thus, one molecule of nitrogen contains 0.00378 atoms of N^{15} and 0.99622 atoms of N^{14} . This gives an N^{15}/N^{14} ratio of 0.00379. The mass spectrometer can detect a 1% difference in this ratio, or a difference of 0.00004.

Assuming we have a compound with 7.5 atoms% N^{15} , it can be shown that a 1:200 dilution of this isotopic content with normal nitrogen does not decrease the N^{15}/N^{14} ratio below this limit of accuracy, thus:

	$\frac{N^{14}}{N^{14}}$	$\frac{N^{15}}{N^{15}}$	Ratio $\frac{N^{15}}{N^{14}}$
1 mole enriched nitrogen	0.925	0.075	0.0810
200 moles normal nitrogen	$\frac{199.244}{200.169}$	$\frac{0.756}{0.831}$	0.0041