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The synthesis, resolution, and metabolism of tyrosine isomers and analogues

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NOTE TO USERS

THE SYNTHESIS, RESOLUTION, AND METABOLISM OF TYROSINE ISOMERS AND ANALOGUES

by

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I. INTRODUCTION

The metabolism of the aromatic amino acids, phenylalanine and tyrosine, has from the early infancy of physiological chemistry attracted the interest of many of the outstanding workers in the field. The literature presents a variety of papers on the subject and although much has been accomplished the major intermediate steps involved in the anabolic and catabolic metabolism of these substances can only be partially postulated. The scarcity of complete and satisfactory evidence and conclusions is in marked contrast to the extensive need for more detailed information fundamental to the solution of a large number of major biochemical problems.

The recent evidence that vitamin C is essential for the proper utilization of phenylalanine and tyrosine has given another point of attack in the elucidation of metabolic mechanism. As one phase of this new approach it has been shown that simple alteration in the structure of tyrosine through the formation of simple derivatives causes these compounds to be no longer dependent on an adequate intake of vitamin C for their metabolism. The derivatives studied thus far have usually had some substituent added to the molecule such as an N-acetyl group or a substituent on the phenolic hydroxyl group. The further extension of this method involves rearrangement of the constituent parts of the tyrosine structure so that from an analysis of the analogue metabolism, particularly in

relation to vitamin C, additional evidence can be obtained in regard to the probable physiological pathways of the natural compound. Of first importance in this objective is the relocation of the phenolic hydroxyl group from the para to the ortho or meta position. For the sake of completeness it is also necessary to study the analogues in which two or more of these positions contain hydroxyl groups.

Metabolic studies of the scope contemplated required fairly adequate quantities of the pure compounds. The principle goal in this study has been the synthesis in reasonable quantity of the desired meta-tyrosine, ortho-tyrosine, and related compounds. The metabolic studies required the optically active isomers instead of the racemic mixtures obtained in synthesis, therefore methods of resolution had to be developed. Contingent on the successful resolution procedure was the necessity for proof of configuration of the isomers isolated prior to their intelligent application in metabolic studies. The proof of configuration procedure would establish which isomer possessed the natural configuration and was thus related to the amino acids which are normally encountered physiologically.

With the synthesis and resolution accomplished, metabolic study as a guide to the more complete evaluation could be undertaken. These screening preliminary tests would likewise be of value in establishing the quantity needed, and determine

the necessity for further improvement in the synthetic procedures.

II. HISTORICAL

A. The Synthesis of Phenylalanine and Tyrosine

In undertaking the preparation of the tyrosine isomers and related compounds with resolution and metabolic studies in mind, a complete survey of the literature methods for the synthesis of such substances was essential. Thus, to provide background for our experimental studies, a critical chronological review of the methods of synthesis which have been applied in the preparation of phenylalanine, tyrosine, and their analogues has been included.

Tyrosine was first isolated, in 1846, by Liebig (1,2). He obtained the compound through fusion of casein with potassium hydroxide, followed by acidification with acetic acid. The elemental constitution of the compound, $C_9H_{11}O_3N$, was established through the analyses of De La Rue (3) and also Hinterberger (4). The physical and chemical properties of the molecule were extensively studied by Strecker (5) and Städeler (6). These workers brought out the amino acid nature of tyrosine, and laid the ground work for the complete picturing of the structure.

Schmitt and Nasse (7) sought, somewhat prematurely, to synthesize tyrosine, but were foredoomed to failure as they postulated an ethylaminosalicylic acid structure for the molecule. The work of Barth (8), in 1865, showed the relation

which existed between tyrosine and p-hydroxybenzoic acid rather than salicylic acid. Hüfner's (9) studies proved the amino group was primary in nature, in contrast to the early belief that it was secondary.

As a consequence of these early studies, Beilstein and Kuhlberg (10) sought to synthesize tyrosine, in 1872, as an amino-p-hydroxyphenylpropionic acid. They did not decide whether the amino group were in the α or the β position of the propionic acid chain, but attempted to establish this point by synthesis. Their starting material was p-hydroxy-cinnamic acid, which they intended to convert to tyrosine through the following series of reactions. After preliminary

$$HO\cdot C_6H_4\cdot CH{=}CH\cdot COOH \xrightarrow{HOCl} HO\cdot C_6H_4\cdot \underset{Cl}{CH}\cdot \underset{OH}{CH}\cdot COOH$$

$$\xrightarrow{H_2} HO\cdot C_6H_4CH_2\cdot \underset{OH}{CH}\cdot COOH \xrightarrow{HCl} HO\cdot C_6H_4CH_2\cdot \underset{Cl}{CH}\cdot COOH$$

$$\xrightarrow{NH_3} HO\cdot C_6H_4CH_2\cdot \underset{NH_2}{CH}\cdot COOH$$

experiments they discontinued their studies when Barth (11) claimed he had already carried out the proposed synthesis. Barth obtained a small yield of a product which gave a positive Piria's test, a test which involved the reaction of ferric chloride with the compound, after digestion with sulfuric acid and neutralization with calcium carbonate. The violet color produced due to the formation of the ferric salt of tyrosine

sulfuric acid was considered good evidence for the success of the synthesis. The quantity of material obtained, however, was insufficient for any further characterization.

A year later in 1873, Ladenburg (12), evidently not satisfied with the structure for tyrosine postulated by earlier workers, sought to synthesize the molecule through a condensation of ethylene oxide with p-aminobenzoic acid. His product was shown not to be tyrosine. Ossikovsky (13) considered tyrosine to be a mixture of the ortho-, meta- and para-amino derivatives of the α and β-phenyllactic acids. His efforts to synthesize these compounds likewise were unsuccessful.

The tyrosine problem was simplified somewhat by concurrent work with phenylalanine. The discovery and synthesis of this amino acid was reported in 1879. In that year Posen (14) claimed to have made α-amino-β-phenylpropionic acid from α-bromohydrocinnamic acid. However, his melting point of 121° for the product obtained showed he was not successful. His error confused the issue of the actual structure of phenylalanine for some time. Thus, the actual isolation of phenylalanine was credited to Schulze and Barbieri on the basis of work published later in the same year (15,16). They isolated an amino acid from sprouted Lupinus lutens, an herb belonging to the pea family, through ethyl alcohol extraction and lead acetate precipitation. The acid isolated was shown to be an amino derivative of phenylpropionic acid. This they estab-

lished from the fact it oxidized to benzoic acid with potassium dichromate and sulfuric acid and analyzed for an empirical formula $C_9H_{11}NO_2$. The properties of the substance agreed with those given by Schutzenberger (17) for a component of the substance "tyroleucine", which he isolated as a decomposition product of albumin. Schulze and Barbieri believed their amino acid to be the source of the benzoic acid Guchelberger (18) isolated after oxidation of protein materials.

Although Schulze and Barbieri isolated β-phenylethylamine on decarboxylation of their amino acid, they did not postulate an α-amino-β-phenylpropionic acid structure for the molecule in view of Posen's statement that the amino acid with that structure melted at 121°, which was about 150° lower than the melting point of the product from Lupinus lutens. Later, when Erlenmeyer and Lipp (19) synthesized phenylalanine, samples were sent to Schulze and Barbieri (20), and these proved identical, in most respects, with the natural product. Erlenmeyer and his coworker used the cyanhydrin amino acid synthesis which involved the condensation of hydrogen cyanide with phenylacetaldehyde. The cyanhydrin intermediate yielded phenylalanine after reaction with ammonia followed by an acid hydrolysis.

$$C_6H_5 \cdot CH_2 \cdot CHO \xrightarrow{HCN} C_6H_5 \cdot CH_2 \cdot \underset{\displaystyle OH}{CH} \cdot CN \xrightarrow{NH_3} C_6H_5 \cdot CH_2 \underset{\displaystyle NH_2}{CH} \cdot CN$$

$$\xrightarrow[NaOH]{HX} \quad C_6H_5 \cdot CH_2 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH$$

After consideration of the work accomplished with phenylalanine, Erlenmeyer and Lipp (21) published an analysis of the quite extensive research on tyrosine which had been completed before 1883. On the basis of the evidence which we have outlined they concluded that this amino acid was most likely β-(p-hydroxyphenyl)-α-aminopropionic acid. To support their postulate they synthesized this molecule by two methods and were able to show the identity of the two synthetic and natural products. They nitrated phenylalanine in the para position (22), converted this to the amine by reduction, and then, through the usual diazotization procedure, replaced the amino group by a phenolic hydroxyl group to give tyrosine.

$$C_6H_5 \cdot CH_2 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH \xrightarrow{HNO_3} O_2N \cdot C_6H_4 \cdot CH_2 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH \xrightarrow{SnCl_2}$$

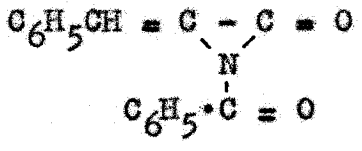
$$H_2N \cdot C_6H_4 \cdot CH_2 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH \xrightarrow[Boil]{HNO_2} HO \cdot C_6H_4 \cdot CH_2 \cdot \underset{\displaystyle NH_2}{CH} \cdot COOH$$

In studies of other methods for the preparation of tyrosine, these investigators were unable to convert phenylalanine-p-sulfonic acid to the desired amino acid through fusion with alkali. The p-nitro-α-nitrocinnamic acid prepared earlier by Friedländer (23) was also investigated. Erlenmeyer and Lipp were able to convert this molecule to tyrosine by

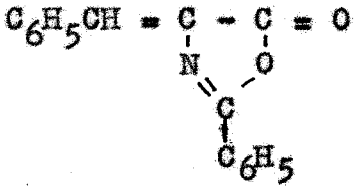
reduction to p-aminophenylalanine, and subsequent application of the previously used diazotization reaction. Schulze and Nagli (24), a few years later, studied the conversion of phenylalanine to tyrosine but outside of confirmation of the results of Erlenmeyer and Lipp nothing of value was accomplished.

Plöchl, in 1883 and 1884, (25,26) published the results of his studies of the condensation of benzaldehyde with hippuric acid. He was able to obtain phenylalanine by a series of reactions involving the primary condensation product. His interpretation of the course of the reaction, however, was so completely in error that the only correctly assigned formula in the entire synthesis was that of the final product, phenylalanine.

It was 1899 before Erlenmeyer and Kunlin (27) were able to explain the actual course of Plöchl's synthesis. The product of the condensation of bensaldehyde with hippuric acid was first considered to be a "lactimid" shown as (I) below. Later Erlenmeyer (28) considered a five membered ring structure (II) more probable. This molecule, with acid or

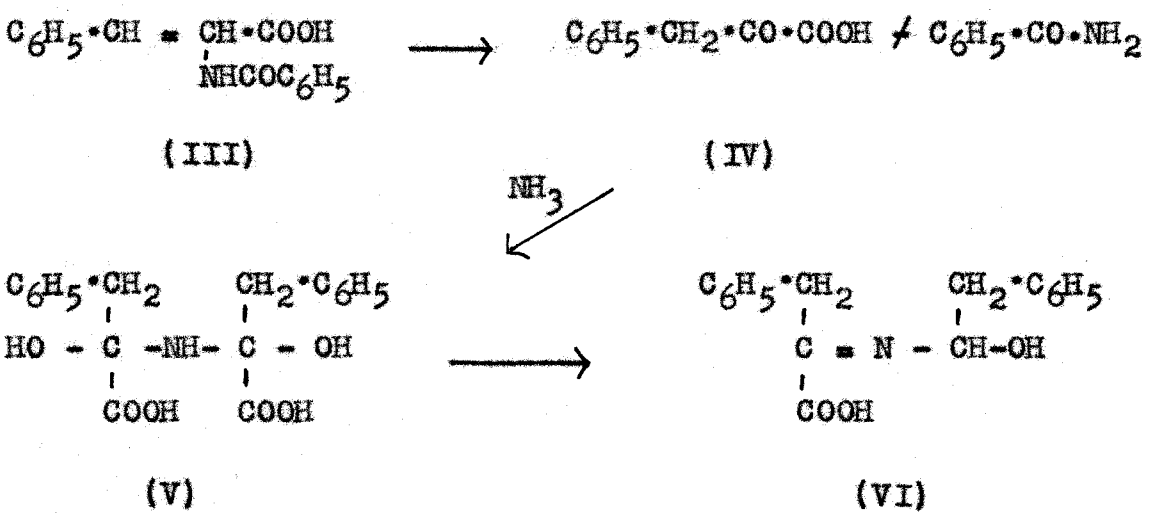


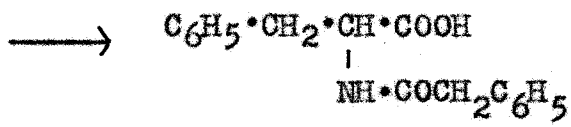
(I)



(II)

alkali, gave the substituted cinnamic acid (III). Erlenmeyer and Kunlin's exhaustive investigation then proved that the cinnamic acid reacted with concentrated ammonium hydroxide, when heated in a sealed tube, to yield as the first intermediate phenylpyruvic acid (IV). The phenylpyruvic acid, in turn, condensed with the ammonia to give the postulated intermediate (V). This molecule lost carbon dioxide and water to give another theoretical intermediate (VI). Through a one-three shift of the hydrogen linked to oxygen, together with an alteration in the position of the double bond, N-phenylacetylphenylalanine was formed. Acid hydrolysis of this molecule yielded phenylalanine and phenylacetic acid, both of which were isolated and characterized. In addition, a nearly theoretical yield of benzamide was obtained, which established the nature of the reaction in the conversion of the α-benzoylamidocinnamic acid to the phenylpyruvic acid.



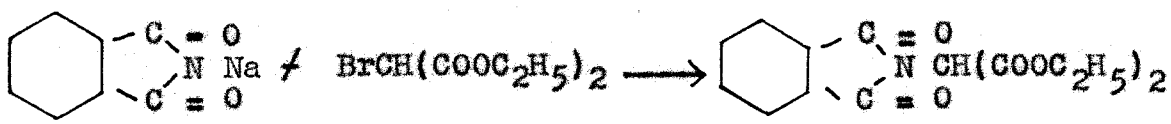


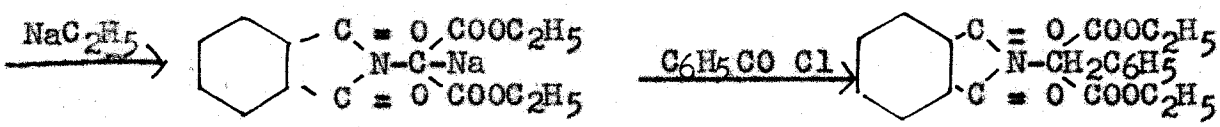
(VII)

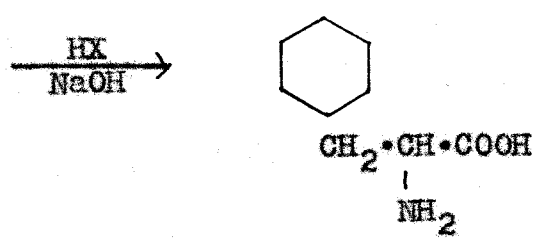
Erlenmeyer (29,30) was able to convert compound (III) to phenylalanine by a more direct method. A sodium amalgam reduction reduced the double bond of the substituted cinnamic acid to give N-benzoylphenylalanine, which was readily hydrolized to phenylalanine with acid. The conversion of an azlactone to a substituted cinnamic acid, followed by the reduction of the double bond and subsequent hydrolysis of the benzoyl group, became a general procedure for the synthesis of amino acids as the widely used Erlenmeyer synthesis. The method was used successfully to prepare aliphatic, aromatic and heterocyclic amino acids. However, the first extension of the method was to the synthesis of tyrosine, in 1897, by Erlenmeyer and Halsey (31,32). The yield, however, was quite low. Another method of amino acid synthesis was developed in Erlenmeyer's laboratories during this period. In 1892 (33) he prepared the oxime of phenylpyruvic acid and reduced it to phenylalanine with tin and hydrochloric acid.

The method used by Erlenmeyer and Halsey for tyrosine was studied by Fisher (34,35) in detail. He improved the yield through increasing the amount of sodium amalgam used in the reduction of the substituted cinnamic acid.

Sörensen applied his general synthesis of amino acids, which made use of phthalimidomalonic ester, to the preparation of phenylalanine in 1905 (36,37). The starting material, ethyl phthalimidomalonate, was readily prepared through the condensation of ethyl bromomalonate with potassium phthalimid. The phthalimid derivative, with sodium ethoxide, gave a sodio derivative which condensed with benzyl chloride to yield ethyl phthalimidobenzylmalonate. An over-all yield of 64% was obtained through this procedure, including the final step of basic and acidic hydrolysis to yield the free amino acid.







Stephen and Weizmann (38) adapted the Sörensen method to the synthesis of tyrosine. They were able to convert the condensation product formed by reacting p-methoxybenzyl bromide with ethyl sodiophthalimidomalonate to tyrosine in one step, in contrast to the two step hydrolytic method used by Sörensen. This was accomplished through hydrolysis with concentrated hydrochloric acid in a sealed tube at 170°.

Fischer applied malonic ester in another manner to the synthesis of phenylalanine. His general method, published in 1904 (39), is still extensively used with little modification (40). The starting point of this method involved the condensation of benzyl chloride with ethyl sodiomalonate to give ethyl benzylmalonate. Halogenation of the benzylmalonic acid derived from the ester was followed by decarboxylation to yield α-bromo-β-phenylpropionic acid. Reaction of the halogen intermediate with ammonia produced phenylalanine.

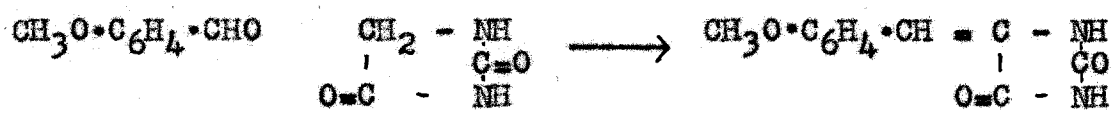
$$C_6H_5 \cdot CH_2 \cdot Cl + Na \cdot CH(COOC_2H_5)_2 \longrightarrow C_6H_5 \cdot CH_2 \cdot CH(COOC_2H_5)_2$$

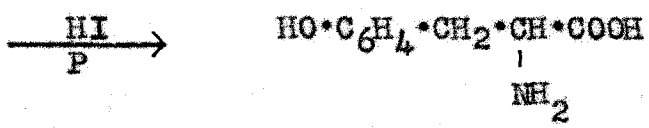
$$\xrightarrow[HCl]{KOH} C_6H_5 \cdot CH_2 \cdot CH(COOH)_2 \xrightarrow{Br_2} C_6H_5 \cdot CH_2 \cdot \underset{Br}{C}(COOH)_2$$

$$\xrightarrow{heat} C_6H_5 \cdot CH_2 \cdot \underset{Br}{CH} \cdot COOH \longrightarrow C_6H_5 \cdot CH_2 \cdot \underset{NH_2}{CH} \cdot COOH$$

Another excellent amino acid synthesis was brought forth as a result of investigations by Wheeler and Hoffman (41).

They studied the synthesis of various benzalhydantoins, and methods to convert these compounds to amino acids. Hydantoin was found to condense with benzaldehyde in 85% yield. In but slightly smaller yield the double bond was reduced to give a benzylhydantoin. Hydrolysis of this compound to phenylalanine was accomplished in 71% yield. The direct reductive hydrolysis of benzalhydantoins to amino acids with hydriodic acid also was found to give excellent yields. This method is illustrated below in the synthesis of tyrosine; accomplished by condensation of anisaldehyde with hydantoin followed by hydrolysis with hydriodic acid and red phosphorus. The synthesis of the hydantoin intermediate proceded best when the



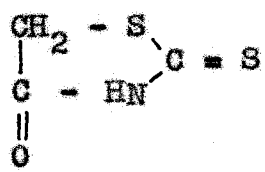


phenolic hydroxyl group was methylated. The methoxy group was readily converted to a hydroxyl through the hydriodic acid reaction procedure.

Some years later Boyd and Robson (42) reinvestigated the hydantoin amino acid synthesis and were able to improve the yield in the first step by substitution of piperidine or diethylamine for the sodium acetate and acetic acid Wheeler

and Hoffman used as a condensation catalyst. However, in a number of cases the use of these amines actually gave a lower yield than the original method. The reduction step was also modified to allow synthesis of compounds unstable to hydriodic acid (43). Various sulphide reagents were found to reduce the double bond, but, as the yields were low and the method time-consuming, the procedures offered no advantage in the synthesis of tyrosine or phenylalanine.

The related heterocyclic compound rhodanine (VIII) was used by Gränacher to prepare phenylalanine and tyrosine (44).

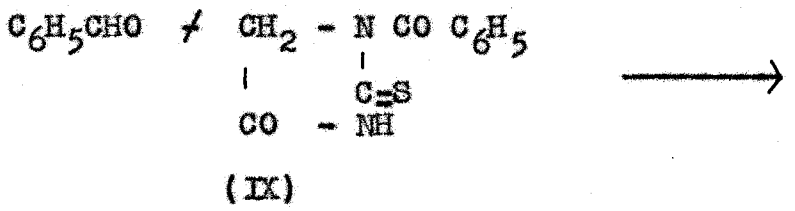


(VIII)

Reaction of the benzalrhodanine with hydroxylamine yielded the oxime of the appropriate phenylpyruvic acid, which, in turn, was reduced to the amino acid.

Johnson and O'Brien (45) sought to improve upon the hydantoin method of amino acid synthesis through the substitution of the more readily available 2-thio-3-benzoylhydantoin for hydantoin itself. They were motivated by the labor and cost factors involved in the preparation of hydantoin from glycine ethyl ester hydrochloride and potassium cyanate. As the 2-thio-3-benzoylhydantoin was readily made from the much

cheaper hippuric acid and potassium thiocyanate, they applied this compound to phenylalanine synthesis. The three step reaction series, which involved condensation of the hydantoin derivative (IX) with benzaldehyde, acidification to yield the substituted benzalhydantoin (X), and finally hydrolytic reduction to give the amino acid, gave a 94% over-all yield. In spite of the excellent yield claimed the method appears to have found little application.



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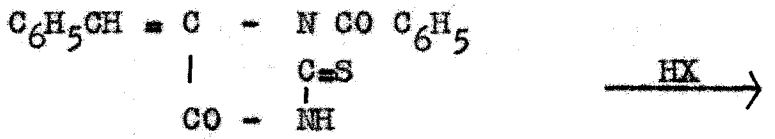
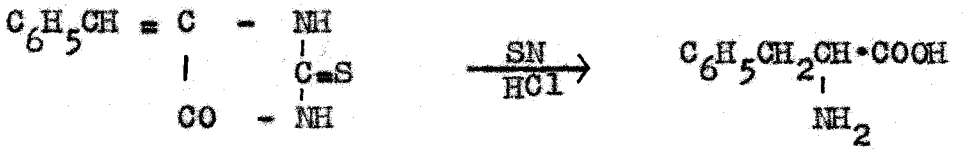
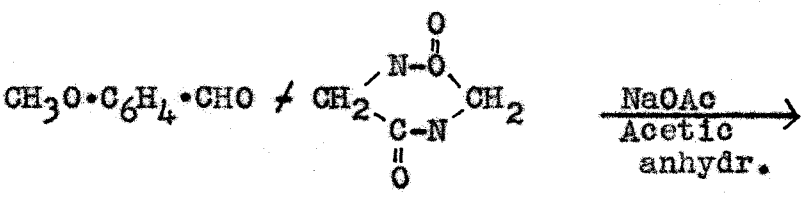


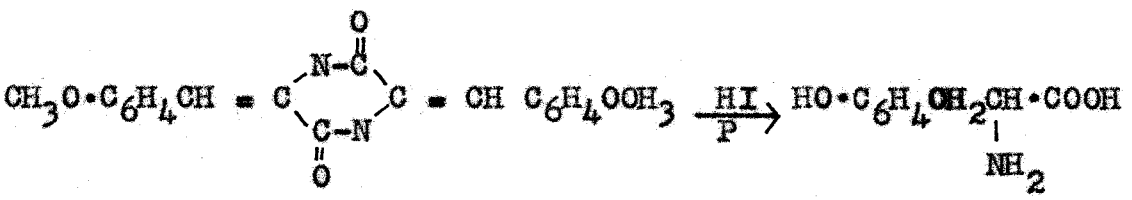


(X)

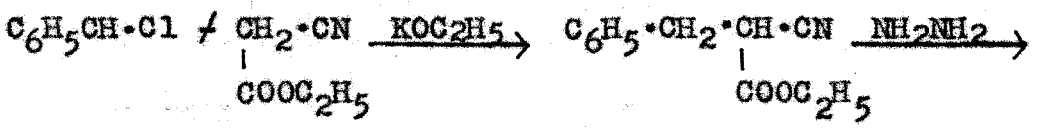
Another modification of the original Erlenmeyer amino acid synthesis was published in 1921. Sasaki (46) condensed 2,5-diketopiperazine with benzaldehyde to give 3,6-dibenzal-2,5-diketopiperazine in 62% yield. Hydrolysis of this intermediate with hydriodic acid yielded phenylalanine in 83% yield. With anisaldehyde as the starting material, tyrosine

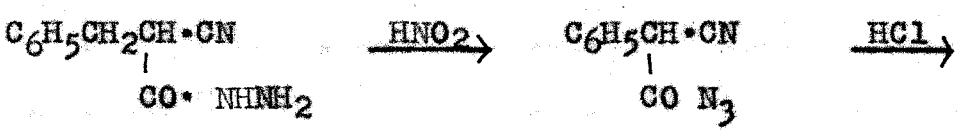
was obtained in an over-all yield of 58%. The steps were the same as those involved in the phenylalanine synthesis and are illustrated in the equation





In 1922 Curtius (47) applied the series of reactions which bears his name to the preparation of phenylalanine. The preparation, which involved five steps, was of theoretical interest only as the reactions did not lend themselves to even moderate scale operation. A yield of 36% for the total synthesis was claimed. In the synthesis by Curtius, malonic ester was used in the primary step. Gagnon's (48) application of the same general method, which is shown in equations below, used ethyl cyanoacetate.





$$C_6H_5CH_2 \cdot \underset{\displaystyle NH_2}{\underset{|}{CH}} \cdot COOH$$

Schmidt (49) made use of a Curtius type rearrangement to prepare phenylalanine. The substituted acetoacetic ester (XI) was reacted with hydrazoic acid in the presence of concentrated sulfuric acid. The intermediate isolated was N-acetylphenylalanine (XII) which was readily hydrolyzable to phenyl-

$$\underset{\displaystyle CH_3C{=}O}{\underset{|}{C_6H_5CH_2CH} \cdot COOC_2H_5} \xrightarrow{N_3H} \underset{\displaystyle NH\ CO\ CH_3}{\underset{|}{C_6H_5CH_2 \cdot CH}} \cdot COOC_2H_5$$

(XI) (XII)

alanine. Although the rearrangement involved was of theoretical interest, manipulations with hydrazoic acid do not allow large scale operation because of the explosive hazard involved.

In their studies of amino acid syntheses <u>in vivo</u>, Knoop and Oesterlin (50) believed a reductive amination of pyruvic acid derivatives was involved. To support this view they found a mixture of phenylpyruvic acid and ammonia, on catalytic hydrogenation, yielded phenylalanine in 72% yield. They also showed that very mild reducing conditions, such as the presence of ferrous sulphate or cystein, converted a mixture of phenylpyruvic acid and ammonia to phenylalanine (51).

Catalytic methods were also applied by Bergmann, Stern

and Witte (52). These workers claimed catalytic reduction of the corresponding acetamidocinnamic acids yielded excellent returns of N-acetylphenylalanine and N-acetyltyrosine. The experimental details for the preparation of phenylalanine, not given in detail in the paper by Bergmann and coworkers, have been presented by Herbst and Shemin (53).

Harington and Barger (54), in their work on the synthesis of thyroxine, reinvestigated agents for the reduction of cinnamic acid derivatives, as the classical sodium amalgam method was not applicable to their iodine containing molecule. After much experimentation they found constant-boiling hydriodic acid satisfactory. In the application of this reducing agent to the synthesis of phenylalanine and tyrosine, Harington and McCartney (55) found a mixture of hydriodic acid and acetic anhydride more suitable. By this method α-benzoylamidocinnamic acid was simultaneously reduced and hydrolyzed to phenylalanine in 88% yield. Tyrosine was best prepared from the ethyl ester of α-benzoylamido-p-methoxy-cinnamic acid in which case the yield was 60%.

Soon after the work of Harington and Barger on the reaction of hydriodic acid with substituted cinnamic acids, Hoffmann-LaRoche and Company (56) obtained a patent for the direct synthesis of amino acids, such as phenylalanine and thyroxine, from the corresponding azlactones through a hydriodic acid reductive hydrolysis. Lamb and Robson (57)

studied this reaction, using a variety of conditions, in the preparation of tyrosine and phenylalanine. They substituted acetic acid for the acetic anhydride used by earlier workers, however, and discovered that limiting the amount of hydriodic acid used, limited the extent of hydrolytic cleavage of the molecule. For example, the cinnamic acid derivatives could be converted to the corresponding N-benzoyl amino acid if the amount of hydriodic acid added was limited to the amount theoretically needed for the reduction only. In contrast, to remove the benzoyl substituent, eight volumes of a mixture of equal quantities of hydriodic acid and acetic acid was needed for each volume of reactant. In table 1 the yields in the various steps in the synthesis of tyrosine are shown.

Cerchez and Locquin (58) synthesized phenylalanine in 1928 through a condensation of benzyl chloride with ethyl sodioaminomalonate, followed by hydrolysis and decarboxylation. These workers gave no yields or experimental details in their synthesis. Redemann and Dunn (59) protected the amino group through benzoylation of the ethyl aminomalonate. The sodio derivative of ethyl benzoylamidomalonate condensed with benzyl chloride to give 75% of the theoretical yield. Hydrobromic acid hydrolysis of the intermediate (XIII) gave the amino acid in 90% yield.

Table 1

Yields of Tyrosine and Intermediates

Using Hydriodic Acid

Oxazolone	N-Benzoyl p-methoxycinnamic Acid	N-Benzoyl p-methoxyphenylalanine	Tyrosine
Concentrated hydriodic acid-acetic[1] →			55%
	Concentrated hydriodic-acetic acid[1] →		63%
		Conc. acid[2] →	75%
Minimal hydriodic acid[2] →		57%	
	Minimal acid[2] →	65%	

1. These reactions used 80 ml. of a mixture of equal parts of 1.7 sp. g. hydriodic acid and glacial acetic with 10 gm. of reactant.

2. In this case 1.4 ml. of hydriodic acid and 2.5 gm. of red phosphorus was used for 10 gm. of reactant.

$$C_6H_5{\cdot}CH_2Cl + C_6H_5CO{\cdot}NH{\cdot}CH\,(COOC_2H_5)_2 \xrightarrow{NaOC_2H_5}$$

$$\underset{\displaystyle NH{\cdot}CO{\cdot}C_6H_5}{C_6H_5{\cdot}CH_2{\cdot}C(COOC_2H_5)_2} \xrightarrow{HBr} \underset{\displaystyle NH_2}{C_6H_5CH_2CH{\cdot}COOH}$$

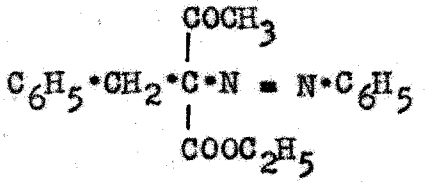
(XIII)

Ethyl acetamidomalonate and ethyl acetamidocyanoacetate have recently been used in the synthesis of a number of amino acids including phenylalanine (60,61,62). By choice of the proper hydrolytic conditions, either the free amino acid or the N-acetyl compound was obtained.

Substituted acetoacetic esters have been converted to phenylalanine and tyrosine through a method proposed by Hamlin and Hartung (63). They converted benzylacetoacetic ester to the oxime of ethyl phenylpyruvate through the action of an alkyl nitrite. Catalytic reduction of the oxime gave the ethyl ester of phenylalanine.

Phenylmalonic acid treated with hydrazoic acid gave Briggs and coworkers (64) a 16% yield of phenylalanine. The explosive danger involved negates any advantage gained due to the simplicity of the reaction.

Feofilactove (65) also made use of substituted acetoacetic esters in the synthesis of a series of amino acids. He was able to condense ethyl benzylacetoacetate with potassium phenyldiazoate to give the intermediate (XIV). This was readily hydrolyzed to the phenylhydrazone of phenylpyruvic acid which was hydrogenated to phenylalanine quantitatively.



(XIV)

Modifications of this method were published later by the Russian investigators (66,67). In one case benzylmalonic ester, or benzylcyanoacetic ester, was substituted for the acetoacetic acid derivative first used. It was also found that nitrosyl chloride reacted with benzylmalonic acid to give the oxime of phenylpyruvic acid. This was reduced, by classical methods, to the amino acid.

One of the most recent methods published for the synthesis of phenylalanine and tyrosine resembled the early Beilstein and Kuhlberg (10) method as it involved the preparation of a substituted α-halogen acid, and the subsequent amination of this derivative. Gaudry (68) prepared the halogen acid through a condensation of acrylonitrile with benzendiazonium chloride. The product was α-chloro-β-phenylpropionitrile. Through hydrolysis of the nitrile, and reaction of the resulting chloro-acid with ammonia, phenylalanine was obtained. Tyrosine was obtained in an analogous procedure but the yield, in both syntheses, was less than 10%.

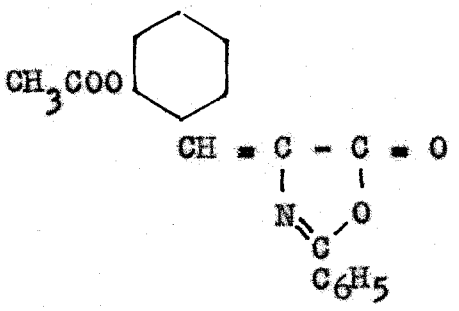
Of the tyrosine and phenylalanine syntheses discussed in this review, a number were of theoretical interest only. Those which involved a number of steps, extensive manipulation or poor yields were readily eliminated from consideration in the study of methods for the moderate scale preparation of tyrosine isomers and analogues.

The more recent modifications of the Erlenmeyer syn-

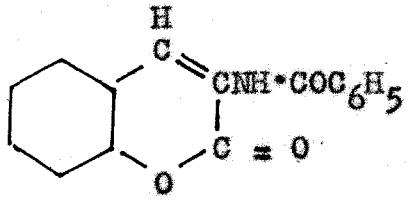
thesis offered attractive yields and simple manipulative procedures. Also the starting materials needed could be purchased or prepared readily. Although the use of acetamidomalonic ester in amino acid synthesis has given good yields with reasonable labor involved neither the malonic ester derivative or the substituted benzyl halides could be considered readily available.

B. The Synthesis of Tyrosine Isomers

The general methods applied to the synthesis of phenylalanine and tyrosine have been used by various investigators in the preparation of ortho- and meta-tyrosine with little modification. However, Blum, who first prepared the two isomers of tyrosine which differ only in the position of the phenolic hydroxyl group, encountered some difficulties in the preparation of the ortho isomer (69). In the preparation of this amino acid through the original Erlenmeyer procedure he found the initial condensation of salicylaldehyde and hippuric acid gave a mixture of products, because of the tendency of ortho-hydroxyaldehydes to give coumarins in a Perkin's condensation. Thus in addition to the expected azlactone (XV), Blum isolated the coumarin (XVI).



(XV)



(XVI)

Also, when the azlactone was treated with base to yield the substitute cinnamic acid, additional quantities of coumarin were obtained. In basic solution the coumarin ring opened, thus the mixture of cinnamic acid and coumarin was dissolved in alkali, and reduced with sodium amalgam according to the classical Erlenmeyer procedure. meta-Tyrosine was prepared by the same method using meta-hydroxybenzaldehyde as the starting material. In this case, however, there was no difficulty with coumarin formation. Although Blum does not give his over-all yields Abderhalden (73) prepared meta-tyrosine in 15% over-all yield by the Blum method. Considerable difficulty was experienced in obtaining a pure product.

In 1915 Johnson and Scott (70) applied a modified hydantoin procedure to the synthesis of o-tyrosine. The condensation product formed in a reaction between salicylaldehyde and benzoylthiohydantoin was converted to o-tyrosine by a three step procedure which involved desulfurization, reduc-

tion and hydrolysis. Although actual yields were not given, each step was described as good or excellent. However, experimental manipulation was quite laborious and time consuming, 24 hours being required for the final hydrolysis.

Some time later Ueda (71) prepared ortho and meta-tyrosine through the diketopiperazine procedure of Sasaki. Overall yields of 45% and 68% were reported for ortho and meta-tyrosine respectively. However, Dickenson and Marshall (72) could not duplicate Ueda's results in the preparation of ortho-tyrosine from salicylaldehyde. When the methylether of salicylaldehyde was used, however, he was able to duplicate Ueda's yield. On the other hand, Abderhalden and co-workers (73) obtained but an 8% yield on the synthesis of o-tyrosine through the diketopiperazine procedure. Also the applications of this method in the literature were carried out on such a scale that the final yield of amino acid obtained was always small.

Ueda (74) prepared meta-tyrosine by an interesting modification of the original diketopiperazine method. In this case m-nitrobenzaldehyde was condensed with diketopiperazine. The condensation product, on reductive hydrolysis with hydriodic acid and red phosphorus gave meta-aminophenylalanine. Thus the nitro group was reduced in addition to the usually observed reactions involved in the synthesis. The m-aminophenylalanine, through diazotizing and subsequent boiling

with dilute sulfuric acid was converted to ortho-tyrosine.

Methods of synthesis for these compounds involving the use of the corresponding hydroxybenzyl halides with phthalimido malonic ester, benzoylamidomalonic ester and related intermediates have not found application because of the difficulties involved in the preparation of o-hydroxy and m-hydroxybenzyl chloride.

Thus, although a moderate amount of work has been done on the synthesis of o- and m-tyrosine none of the methods seemed to be suitable for synthesis of the amounts needed for resolution studies. The original Blum method involved four steps and from the studies of other workers it can be concluded that the yields were not good. In particular, few workers have found the sodium amalgam reduction procedure satisfactory for the synthesis of amino acids in any quantity. The diketopiperazine synthesis, which involved only two steps, had several disadvantages. The starting material was the most difficult to prepare, or costly to purchase, of the starting materials for an Erlenmeyer type of amino acid synthesis. In addition the behavior of the reaction has been erratic in the hands of different investigators. Also, it has not been established that the method lends itself to even moderate scale synthesis. The procedure of Johnson and Scott (70) has already been discussed and the principal points of difficulty outlined.

It thus appeared that two alternatives were left. The first was the possibility one of these syntheses just discussed could be so modified as to serve our needs. The second was to apply to the synthesis of o- and m-tyrosine some of the more recently developed methods used on tyrosine, which have been discussed. In particular, the procedure of Herbst and Shemin, which involved the catalytic reduction of substituted cinnamic acids to N-acetyl amino acids seemed particularly suitable. This procedure appeared to be the most promising of the possible methods which would give N-acetyl-o-tyrosine and N-acetyl-m-tyrosine in but three steps.

C. The Synthesis of Polyhydroxyphenylalanines

The naturally occuring 3,4-dihydroxyphenylalanine has been the most extensively studied dihydroxyphenylalanine. This compound was first isolated by Torquati (75) from the pods and sprouts of Vicia faba, the velvet bean. Guggenheim (76) studied the compound, and through analyses and degradation studies was able to establish the correct structure. Miller's (77) investigations showed the amino acid to be characteristic of the seeds of all plants of the genus Stizolobium, of the leguminous plants.

A number of workers have prepared 3,4-dihydroxyphenylalanine using the various modifications of the Erlenmeyer method. The results obtained are shown in table 2.

Waser and Lewandowski (85) made use of an entirely different approach in order to prepare l-3,4-dihydroxyphenylalanine from l-tyrosine. They nitrated tyrosine in a position ortho to the hydroxyl group. This nitro group was then converted to a hydroxyl group through the classical steps involving reduction to the amine, diazotization and hydrolysis.

Postulated as an intermediate in tyrosine catabolism, 2,5-dihydroxyphenylalanine was the most interesting of the dihydroxyphenylalanines from the viewpoint of our studies. This substance was prepared by several workers, and sought unsuccessfully by others, for the purpose of biochemical investigation.

The 2,5-dihydroxyphenylalanine was first sought by Johnson and Scott (86). They successfully prepared 2,5-dihydroxybenzylhydantoin but could not convert this intermediate to the amino acid by any of the usual procedures. The necessary substituted hydantoin was prepared from 2-hydroxy-5-nitrobenzaldehyde through a difficult six-step series of reactions. Hirai (87) and Freedman (88) both claimed to have prepared this amino acid, in about 6% over-all yield, using the diketopiperazine procedure. Both claimed the amino acid melted at 203-204°. However, Schaaf and Labouchere (89), using hippuric acid in the Erlenmeyer procedure obtained a product melting at 242°. Although they gave no experimental details or analyses they stated the synthesis was carried out through

Table 2

Syntheses of 3,4-Dihydroxyphenylalanine

Aldehyde (reference)	Other[a] Reactant (cond. yield)[b]	Intermediate Steps (yields)[c]		Amino acid (yield)	Overall (yield)
		%	%	%	%
3,4-carbonydioxybenzaldehyde (78)	H. a. 74	75	32	47	8
vanillin (79)	G. a. 79	—	—	66	47
vanillin (80)	H. a. —	70	50	—	—
vanillin (81,82)	H. a. 75	65	—	50	24
protocatechuic aldehyde (83)	H. a. 65	50	—	61	24
vanillin (83)	G. a. 71	—	—	70	50
protocatechuic aldehyde (84)	A. g.	27	72	—	—

a. H. a. represents hippuric acid while G. a. and A. g. represent glycine anhydride and acetylglycine respectively.
b. The yield given was that obtained on condensation of the aldehyde with the reactant listed.
c. In this reaction the cinnamic acid was isolated directly on recrystallization of the crude azlactone.

the reduction of the benzoylamidocinnamic acid followed by hydrolysis. Thus, the synthesis of the 2,5-dihydroxyphenylalanine seemed to yield two substances by two different synthetic procedures. Obviously, before any metabolic study with the compound could be contemplated the establishment of one or the other of these compounds as 2,5-dihydroxyphenylalanine must be undertaken.

The only other dihydroxyphenylalanine reported in the literature was the 2,4-isomer. Hirai (90) claimed a 40% yield of this compound starting with 2,4-dimethoxybenzaldehyde and using glycine anhydride. Deulofeu (91,92) later prepared the compound through the original Erlenmeyer procedure and also through the hydantoin method. In neither case was the yield satisfactory. The Hirai method had the disadvantage of a tedious separation of the amino acid from the excess hydriodic acid through a lead acetate precipitation.

Of the five possible trihydroxyphenylalanines only two have been synthesized. The 2,3,4- and 3,4,5-isomers were prepared by Schaaf and Labouchère (89) through condensation of the corresponding aldehydes with hydantoin followed by reduction and a two stage hydrolysis. The over-all yields, after four steps which involved extensive manipulation were 28% in the case of the 2,3,4-isomer and 17% for the 3,4,5-trihydroxyphenylalanine. The l-isomer of 3,4,5-trihydroxyphenylalanine was later prepared by Waser, Labouchere, and Sommer (93). These workers dinitrated tyrosine and reduced this compound to the diamino derivative. Tetrazotization and hydrolysis in a complex apparatus, so that all operations could be carried out in an inert atmosphere, yielded a product which they believed to be the one desired. The properties listed for the compound, however, offered definite contrast

with those given for the racemic mixture prepared by the hydantoin method. Analyses could not be obtained because of the hygroscopic nature of the product. It remains to be established that 3,4,5-trihydroxyphenylalanine was actually obtained through the tetrazotization method.

Although much work has been done on the di- and polyhydroxyphenylalanines it cannot be said that any general method for their synthesis exists. Each seemed to offer a special problem in preparation or isolation. In addition, a number of the di- and polyhydroxyphenylalanines have never been prepared.

D. The Resolution and Proof of Configuration of Aromatic Amino Acids

The resolution of the aromatic amino acids, phenylalanine and tyrosine, has been accomplished through the use of several methods of general application. As we were contemplating the resolution of tyrosine isomers and related compounds a critical study of the methods previously applied was made.

Fischer (94,95) was the first to resolve a racemic aromatic amino acid to obtain the optically active forms. Through the use of the brucine salt of the benzoyl derivative of dl-tyrosine he obtained, after several recrystallizations from water, that diasterioisomer which could be converted to

l-tyrosine. The recrystallization of the cinchonine salt of N-benzoyltyrosine from water enabled him to prepare the unnatural isomer of tyrosine which Lippmann (96) had earlier claimed to have isolated from beet sprouts. The work of Lippmann was not supported by any proof of identity of the compound, thus Fischer was credited with the first characterization of d-tyrosine.

Phenylalanine resolution studies were undertaken by Fischer and Mouneyrat (97). They sought to use the cinchonine salt of the N-benzoyl derivative and although the unnatural isomer was obtained in 75% yield, the l-phenylalanine was not obtained in reasonable purity. Fischer and Schöller (98), using the N-formyl derivative of phenylalanine with brucine successfully obtained both d and l isomers in good yield. The yields of l-formylphenylalanine and d-formylphenylalanine were approximately 70%. On the other hand, brucine with the formyl derivative of tyrosine gave Abderhalden and Sickel (99) only a 39% yield of d-tyrosine. The other isomer retained considerable amounts of the racemic mixture.

The procedures just outlined for tyrosine and phenylalanine resolution have all made use of N-acyl derivatives. Holmes and Adams (100) considered it possible to shorten the classical resolution procedure through acylation of the amino acid with an optically active acyl halide. To test this possibility experimentally they reacted l-menthoxyacetyl chloride

with several amino acids including phenylalanine. The method was successful in several cases and optically active amino acids were obtained on hydrolysis of the N-l-menthoxyacetyl-derivatives. The hydrolysis of the recrystallized N-l-menthoxyacetylphenylalanine however yielded an inactive product. Holmes and Adams believed a partial racemate was formed.

Freim (101) claimed cholestenone sulfonic acid excellent as an agent for the resolution of small amounts of tyrosine in a short time. One or two recrystallizations, involving only an hour, served to separate the d-isomer. The l- form could not be obtained pure by this method.

Recently Sealock (102) published a resolution procedure for tyrosine using brucine and N-acetyl-dl-tyrosine. The over-all yield of d-tyrosine was 70%. Similar studies using brucine and diacetyl-dl-tyrosine were not successful as the compound did not give satisfactory salts.

Several biochemical methods have been used to obtain the isomeric forms of the aromatic amino acids. In 1923 Abderhalden and coworkers (103) studied the action of esterase on dl-tyrosine ethylester. The l-isomer was hydrolyzed while the d-ester was untouched and could be extracted from the reaction mixture with chloroform. He also studied the action of bacteria on dl-tyrosine as had Tsudji (104) in 1917.

Bergmann and his coworkers (105) studied the reaction of benzoyl-dl-phenylalanine with aniline in the presence of

papain-cystein. Under controlled conditions, 66% of the theoretical yield of benzoyl-l-phenylalanine anilide was obtained. Enzyme specificity was also made use of in the preparation of acetyl-l-phenylalanine phenylhydrazide from the racemic acetyl derivative and phenylhydrazine in the presence of papain. It was also found (106) that acetyl-dl-phenylalanylglycine reacted with aniline in the presence of papain to give the l-peptide anilide. A yield of 11.2 gm. of l-phenylalanine was isolated, on hydrolysis, from a reaction involving 64 gm. of the acetylated peptide.

Of the di- and polyhydroxyphenylalanines only the resolution of the 3,4-dihydroxyphenylalanine has been reported. This was accomplished by Harington and Randall (84). These investigators sought to resolve the triacetylderivative of the amino acid with brucine but found the O-acetyl groups were slowly hydrolyzed so that the brucine salt obtained was that of N-acetyl-3,4-dihydroxyphenylalanine. The salts were quite slow in crystallization but very few recrystallizations were necessary before a constant rotation was obtained. Yields, however, were not given.

Of great importance to anyone carrying out biochemical investigations with amino acids of synthetic source is the establishment of configuration. The metabolic significance of many experiments is lost when one does not know whether the natural or unnatural configuration is being used. Obviously,

synthetic compounds which cannot be compared with an isolated naturally occurring sample must be analysed by entirely satisfactory means.

Clough (107) in 1915 published a method for the correlating of the stereochemical configurations of similarly constituted optically active compounds. His procedure involved the study of the changes in rotations produced on the introduction of various substituents into the optically active molecule. These substituents were usually N-derivatives. The method was very well illustrated in the work of Karrer and Veer (108) with valine. The naturally occurring dextrorotatory amino acid and its ethyl ester on conversion to the benzoyl, benzenesulfonyl and similar derivatives showed optical displacements of similar magnitude and in the same direction as the displacements observed when the same substituents were introduced into dextrorotatory alanine. Thus these two amino acids were assumed to possess the same configuration. The method does not appear to be general as introduction of acyl derivatives on the amino group of some aromatic amino acids causes inversion in sign of rotation. It is obvious also that the method would be quite laborious.

Goldschmidt and Fryss (109), through degradative studies, were able to establish the configurational relationship between the aromatic and aliphatic amino acids, which supported the view that all naturally occurring amino acids were con-

figurationally of the same family. They oxidized benzoyl-l-tyrosine in alkaline solution with permanganate. From the reaction mixture they were able to isolate N-benzoylaspartic acid which hydrolyzed to l-aspartic acid. As the conditions were such that no inversion would be expected, because the asymetric carbon atom was not involved in the reaction, the evidence appeared conclusive that l-tyrosine and l-aspartic acid had the same configuration. Earlier studies had shown the relationship of l-aspartic acid to the other aliphatic amino acids. This method was of obvious theoretical value but the establishment of configuration of new compounds by this method would involve destruction of moderate amounts of valuable material, a procedure not necessary in view of other methods to be discussed.

Possibly the most convenient criterion to apply in the establishment of configuration is the rule of Lutz and Jirgensons (110,111) which was established empirically by actual measurement of optical activity in relation to differing concentrations of acid and base. This rule states that the specific rotations of l-amino acids become more positive, or less negative, with increasing molecular equivalents of hydrochloric acid. du Vigneaud and Irish (112) have applied this rule to α-amino-γ-phenylbutyric acid. They resolved this amino acid, and plotted specific rotation, in terms of molecular equivalents of acid present, for both isomers. The

isomer which showed an increase in rotation from +25° to +45° as acidity was increased was considered to have the natural or levo configuration.

Of the literature methods the Lutz and Jirgenson procedure appeared to be by far the simplest in application. The ready recovery of all amino acid used in application of the method was yet another point in favor of its use in preference to others mentioned.

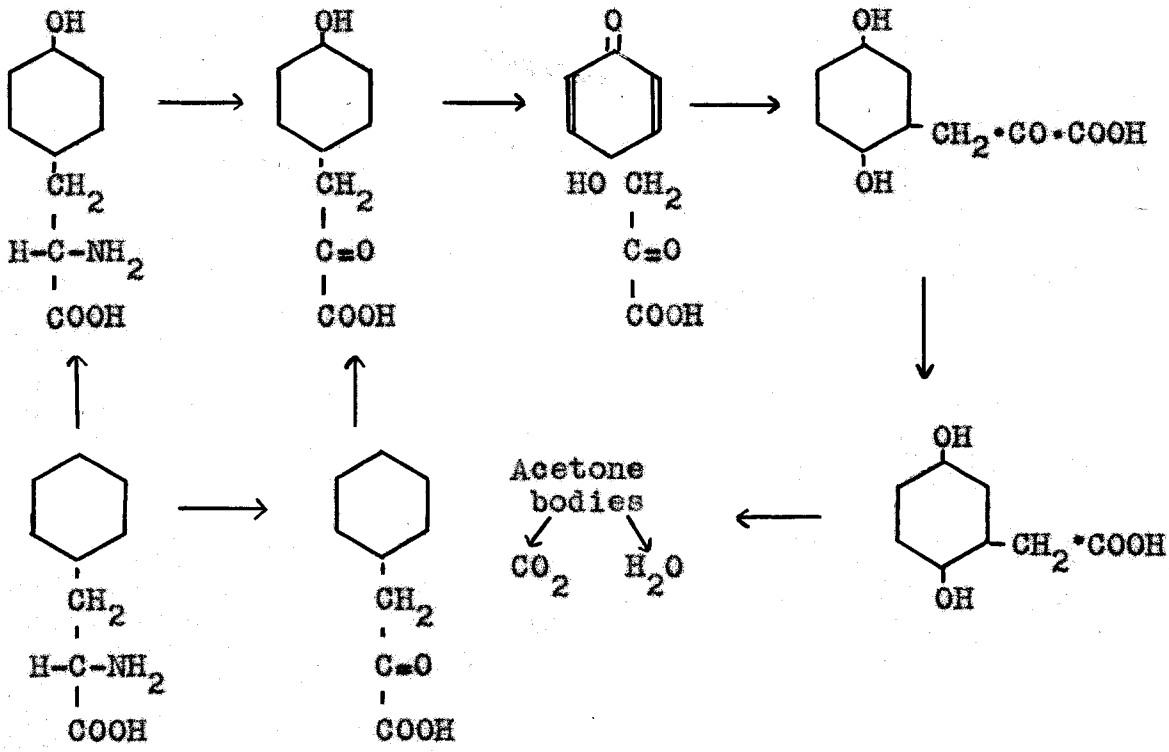
E. The Metabolism of Phenylalanine, Tyrosine, and Related Compounds

When Bödecker (113), in 1859, published a report that the urine of one of his diabetic patients contained a reducing substance in addition to glucose, he little realized the impetus this information would give to the study of the metabolism of the aromatic amino acids. The compound responsible for this reducing action was isolated by Bödecker in an impure state. He was not able to identify the material, but named the condition which was marked by excretion of the compound, alcaptonuria. Some years later Wolkow and Baumann (114) were able to establish the composition of the reducing substance as that of hydroquinone acetic acid. Homogentisic acid was the common name they assigned the molecule since it was the next higher homologue of gentisic acid or 2,5-dihydroxybenzoic acid.

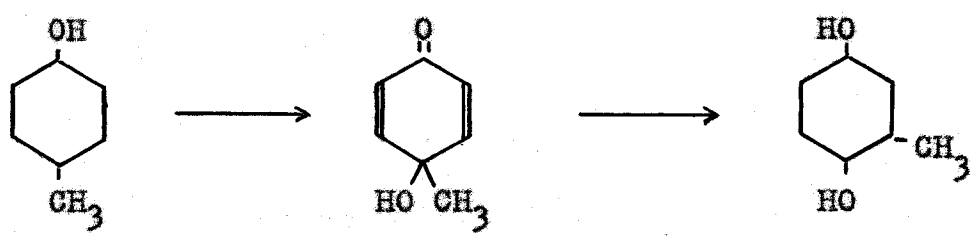
On investigation of the source of this compound in experiments with persons exhibiting alcaptonuria, they found that tyrosine when fed was converted almost quantitatively to homogentisic acid. Later, Falta and Langstein (115) found phenylalanine was also a precursor of homogentisic acid.

The peculiar type of rearrangement involved in the conversion of tyrosine to homogentisic acid caught the interest of a number of investigators. Many projects were undertaken to determine what molecules were converted to homogentisic acid by an alcaptonuric. Compounds so handled could then be considered possible intermediates in the metabolism of aromatic amino acids.

From the fact that phenylalanine, tyrosine, p-hydroxyphenylpyruvic acid and a substance he believed to be 2,5-dihydroxyphenylpyruvic acid yielded homogentisic acid in these *in vivo* studies, Neubauer (116) devised the following scheme for the metabolism of the substances.



The rearrangement postulated in this mechanism, although not frequently encountered, does have <u>in vitro</u> analogies. Bamberger (117) found <u>p</u>-tolylhydroxylamine rearranged with hot dilute sulfuric acid to give methylhydroquinone. Also <u>p</u>-cresol on oxidation with persulfuric or Caro's acid rearranged according to the equation following. The intermediate postulated was not isolated.

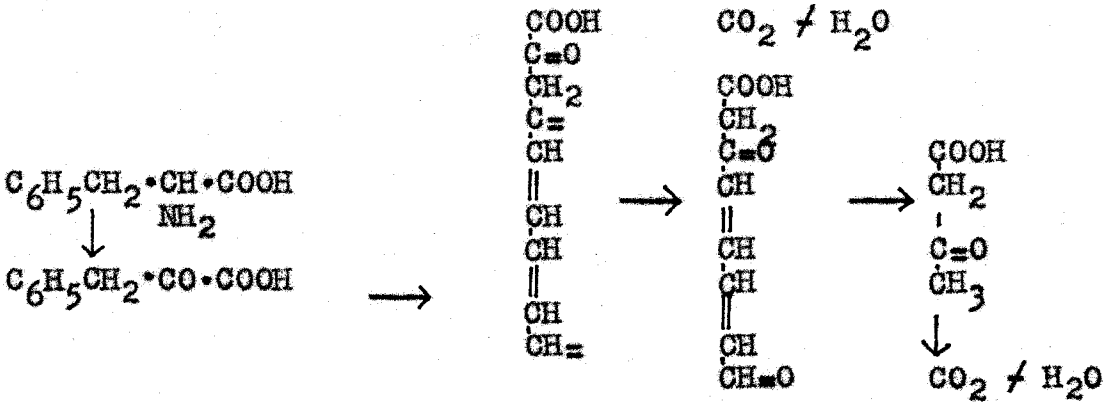


A point difficult to explain in the Neubauer scheme was found in the fact that the increase in homogentisic acid excretion on feeding 2,5-dihydroxyphenylpyruvic acid was less than that observed when an equal amount of tyrosine or p-hydroxyphenylpyruvic acid was added to the diet. This did not appear consistent with the fact 2,5-dihydroxyphenylpyruvic acid was only one step removed from the final product, a step which involved relatively simple decarboxylation and oxidation reactions. It can be considered possible that Neubauer did not have the substance 2,5-dihydroxyphenylpyruvic acid, as he gave no analyses for it. He appears to be the only investigator who has ever prepared an ortho-hydroxyphenylpyruvic acid, as these compounds are commonly obtained in the anhydride or coumarin form. If he did have 2,5-dihydroxyphenylpyruvic acid, it can be postulated that some was converted to the corresponding 3-keto-5-hydroxycoumarin which may have been eliminated through some detoxification mechanism.

Additional support for the Neubauer scheme was given when the liver perfusion experiments of Emden, Salomon and Schmidt (119) showed phenylalanine, tyrosine and homogentisic acid yielded acetone bodies. Later investigations of Embden and Baldes (120) using the same technique also gave evidence of conversion of phenylalanine to tyrosine. More recent work by Moss and Schoenheimer (121), which involved the feeding of deuterium containing phenylalanine, proved this point as

deuterium containing tyrosine was isolated from the tissues.

The Neubauer scheme postulated homogentisic acid as a normal intermediate in the metabolism of phenylalanine and tyrosine. Dakin (122) has been unwilling to accept the view that the pathway of metabolism for these amino acids must involve homogentisic acid as an intermediate. As Jaffe (123) had isolated muconic acid from animals fed a small amount of benzene, Dakin believed that oxidation of the aromatic nucleus in phenylalanine and tyrosine did not require the prior introduction of hydroxyl groups, consequently, the scheme proposed by Wakemann and Dakin (124) shown in the following flow sheet was presented.



Dakin (125) found that when p-methylphenylalanine and p-methoxyphenylalanine were administered to alcaptonurics no homogentisic acid derivative was excreted and the compounds underwent complete oxidation. These results were supported by Fromherz and Hermanns (126) who found 3-methyltyrosine was

not capable of increasing the homogentisic acid excretion of an alcaptonuric. Dakin (122) summarized these results stating,

> These results may be taken as evidence in favor of the view that a quinoid substance is a necessary precursor of homogentisic acid, and furthermore that alcaptonurics have not lost the power to effect the complete oxidation of phenylalanine and tyrosine derivatives provided their structure is such that homogentisic acid formation is prevented. Alcaptonuria, according to this view, represents a condition in which there is not only an abnormal formation of homogentisic acid but also an abnormal failure to catabolize it when formed.

Another source of information in regard to the mechanism of tyrosine and phenylalanine metabolism has been the extensive in vitro experiments using various tissue materials, and these amino acids as substrates. Early investigations along these lines were carried out by Kisch (127) and Krebs (128). However, the important contributions toward elucidation of mechanism are found in the contributions of the Bernheims and those of Felix and coworkers.

In their first communication in this series Bernheim and Bernheim (129) studied phenylalanine and tyrosine using liver and kidney brei and the Warburg manometric technique. With liver brei they found four atoms of oxygen were taken up per mole of tyrosine present. Phenylalanine was slowly oxidized under the same conditions. Kidney brei with phenylalanine gave an oxygen uptake of one atom per mole of amino acid, whereas tyrosine was not metabolized under these conditions.

The action of two different systems was shown by the fact cyanide inhibited the oxidation of tyrosine but not that of phenylalanine. Also of interest was the observation that no ammonia was produced in the metabolism of tyrosine thus showing no deamination. Phenylalanine, however, was oxidatively deaminated.

A later publication by Bernheim (130) presented studies of l- and dl-tyrosine with liver and kidney brei. Both the l- and d-isomers were metabolized by liver brei with an oxygen uptake corresponding to four atoms per mole of tyrosine present. Dilution of the brei did not effect the rate of metabolism of d-tyrosine but metabolism of the l-isomer gave a much slower rate under such conditions. With kidney brei d-tyrosine was metabolized with the same oxygen uptake as observed with liver brei. From dilution and inhibition experiments he obtained indications that the metabolism of l-tyrosine was stepwise, it appeared that under certain conditions the oxygen uptake always corresponded to one or to two atoms for each mole of substrate present instead of four.

Further and more satisfactory evidence for the stepwise degradation of l-tyrosine by liver brei was obtained by Felix, Zorn and Dirr-Kaltenbach (131). These investigators found the oxygen consumption of l-tyrosine to be dependent 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 showed a Millon's test still equivalent in intensity to the 2 mg. of l-tyrosine originally introduced as substrate. This seemed to indicate the primary oxidation product was still closely related to tyrosine and rupture of the benzinoid ring 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 corresponding to four atoms the Millon's test was negative. These authors also studied d-tyrosine, p-hydroxyphenylpyruvic acid, homogentisic acid and both of the isomers of phenylalanine. A compilation of their very interesting results is shown in table 3.

In 1941 Felix and Zorn (132) continued their report on studies of the metabolism of tyrosine with liver brei. On the basis of the identifiable end-products of tyrosine breakdown they set forth a new hypothesis. As no ammonia could be determined at the end of the reaction period they postulated the possibility an amino acid might be split off, most likely alanine. This they found to be the case when alanine determinations were carried out on control and experimental flasks. To explain and correlate results they set up a reaction scheme

Table 3

Summary of Results Obtained by Felix, Zorn and Dirr-Kaltenbach

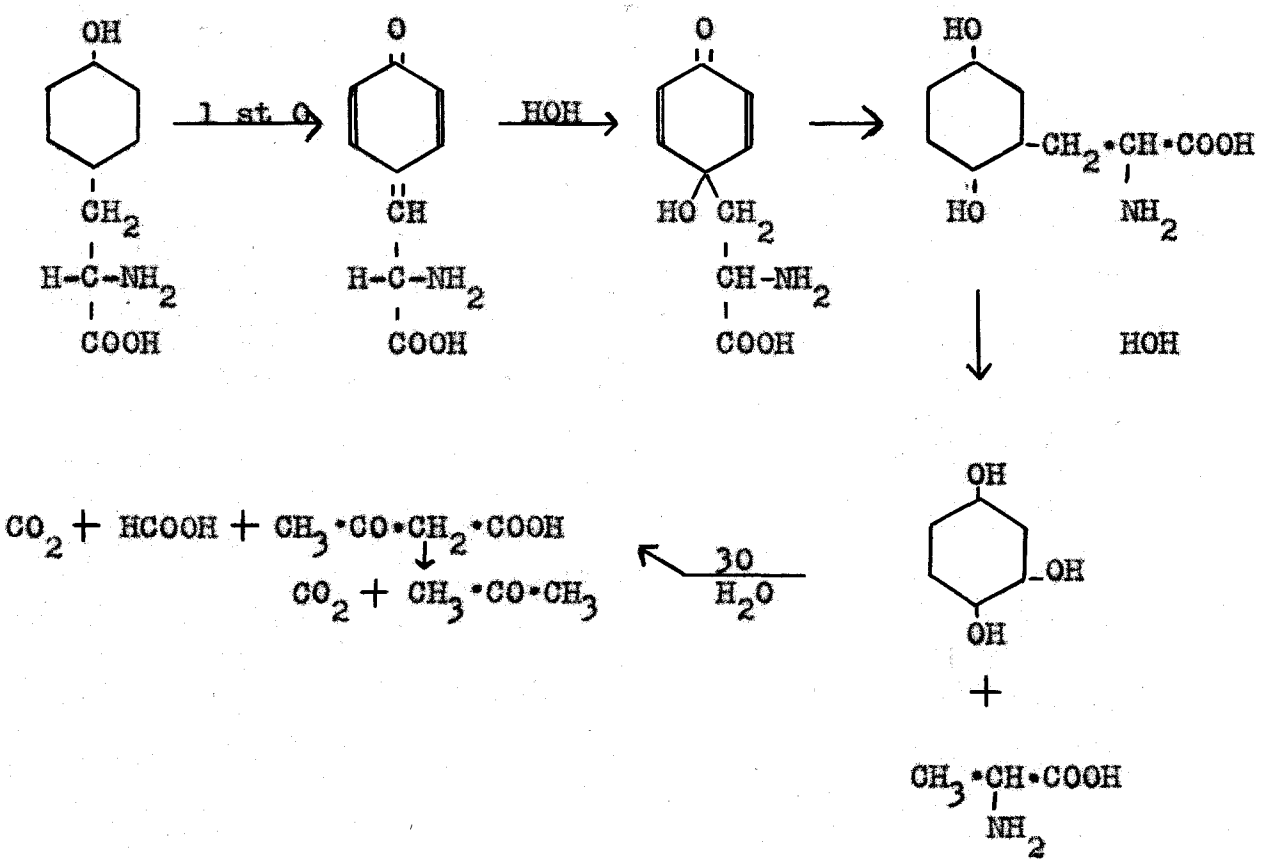
Compound	Oxygen Consumption[c]	Ammonia	Keto Acid	Acetone	Carbon Dioxide
l-tyrosine	(l)[a] 1 at pH 6.8	—	—	—	—
	(l) 2 at " 7.2	—	—	—	—
	(l) 4 at " 7.7	0	0	0.6-0.8	1.8
	(l) 1 at " 8.0	0	—	—	—
	(k)[b] 1 at " 7.8	0	—	—	—
dl-tyrosine	(l) 4 at " 7.6	0.5	0.5	0.6-0.7	—
	(k) 1 at " 7.6	0.5	0.5	—	—
p-hydroxyphenyl-pyruvic acid	(l) 3 at " 7.6	—	—	0.6-0.7	1
l-phenylalanine	(l) 1 at " 7.8	0	0	—	—
	(k) 1 at " 7.8	0	0	—	—
d-phenylalanine	(l) 1 at " 7.6	1	—	—	—
	(k) 1 at " 7.8	1	—	—	—
homogentisic acid	(l) 2 at " 7.6	—	—	0.7	—

a. Liver brei was used in all experiments with this designation.

b. Kidney brei was used in all experiments so designated.

c. Oxygen consumption is given in atoms per mole of substrate added. All other values in the table are in terms of moles of reactant produced per mole substrate.

which was in agreement with the end products identified and the oxygen uptake observed. As can be seen from a study of the formulas a positive Millon's test could be expected with all intermediates until the rupture of the benzene ring.



A new phase of the study of tyrosine and phenylalanine metabolism was entered with the disclosure by Sealock and Silberstein (133) that experimental alcaptonuria could be controlled through the feeding of vitamin C. This discovery was simultaneously and independently confirmed by the finding of Levine and coworkers (134) that a defect in the metabolism

of aromatic amino acids found in premature infants, and marked by excretion of intermediary metabolites of these substances, could be eliminated with vitamin C supplementation.

In a later publication, Sealock and Silberstein (135) showed that homogentisic acid and p-hydroxyphenylpyruvic acid excretion closely paralleled the state of vitamin C nutrition of the animal, and the effectiveness of vitamin C in preventing the excretion of intermediates arising from either tyrosine or phenylalanine metabolism was shown. Sealock, Perkinson and Basinski (136) further investigated the vitamin C problem using feeding experiments to test additional compounds to determine their role in the metabolic system involved. Although p-hydroxyphenylpyruvic acid was isolated from the urine of scorbutic animals ingesting phenylalanine, tyrosine or phenylpyruvic acid, in direct contrast it was found on feeding p-hydroxyphenylpyruvic acid that this substance's subsequent excretion was scarcely influenced by either a deficiency or adequacy of vitamin C. Additional studies of tyrosine analogues by Sealock and Basinski (137) have shown that the acetyl and methoxy derivatives of l-tyrosine, the d-isomers of tyrosine and phenylalanine along with the more complex analogue, benzyl cysteine, are all metabolized irrespective of the vitamin C intake of the experimental animal.

In vitro metabolism of tyrosine has been studied by Lan

and Sealock (138). Their experiments showed that the ability of slices of surviving liver tissue from guinea pigs to oxidize tyrosine was dependent upon a normal intake of ascorbic acid. A vitamin C-deficient liver exhibited almost no oxidation of tyrosine. Addition of vitamin C to scorbutic tissue, however, resulted in the return of the normal function with an oxygen uptake corresponding to four atoms per mole of tyrosine added.

Biochemical studies with ortho and meta tyrosine were initiated by Blum (69). The feeding of 5 gm. of o-tyrosine as the racemic mixture to a normal man resulted in the excretion of 1.25 gm. of o-hydroxyphenylacetic acid. In the belief his extraction procedure might have been responsible for the low recovery, Blum fed 10 gm. of the amino acid, he recovered but 2.4 gm. of the substituted acetic acid, therefore it appeared a part of the material was being metabolized. A trace of p-hydroxyphenylpyruvic acid was suspected on the basis of a positive ferric chloride color test. In experiments with an individual having alcaptonuria no increase in homogentisic acid excretion could be detected. The evidence cast doubt on any mechanism for homogentisic acid formation which considered rearrangement of the alanine side-chain the primary step before introduction of the second hydroxyl group.

With meta-tyrosine similar results were obtained. When 5 gm. of this amino acid was fed, but 1.2 gm. of m-hydroxy-

phenylacetic acid could be isolated. Thus it again appeared that a portion of the amino acid was being metabolized. An alcaptonuric showed no increase in homogentisic acid excretion when this compound was included in his diet. These observations were duplicated in general by Flatow (139), however, in addition to o-hydroxyphenylacetic acid he obtained the glucuronate of the enol of 3-ketocoumarin from the urine. The coumarin would be expected as resulting from ring-closure of o-hydroxyphenylpyruvic acid, the intermediate resulting from oxidative deamination of o-tyrosine. The proportion of material fed to metabolite isolated in the urine, however, remained near the Blum value; which allowed one to postulate that while the l-isomers of ortho and meta tyrosine were metabolized, the d-isomers were converted to the intermediates isolable from the urine.

The only metabolic experiments with di- or polyhydroxyphenylalanine which came within the scope of our discussion were those of Sealock and Lan (140). These investigators carried out in vitro experiments using 3,4-dihydroxyphenylalanine and guinea pig liver and kidney slices. In contrast with l-tyrosine, l-3,4-dihydroxyphenylalanine was readily metabolized by normal guinea pig kidney slices but not by kidney slices from a scorbutic animal. The addition of ascorbic acid to the reaction flask, however, brought about the normal increased oxygen uptake due to the presence of the

amino acid.

With liver slices the oxygen consumption was markedly below that observed in the kidney with 3,4-dihydroxyphenylalanine. Also, with this tissue, the oxygen uptake was apparently independent of the vitamin C intake of the animal. From this it appeared that a system existed in the kidney, distinct from the tyrosine metabolizing system of the liver, and dependent upon an adequate supply of ascorbic acid for normal activity in the metabolism of 3,4-dihydroxyphenylalanine. This discovery served to focus increased interest on the polyhydroxyphenylalanines in the belief that investigation of them would yield information as to the exact limits of variation possible in the arrangement of hydroxyl groups on the benzene nucleus before the molecule became dependent on the kidney metabolic system instead of the liver system, and before the molecule lost all dependency on vitamin C for its metabolism.

III. EXPERIMENTAL

Having considered the methods for synthesis, resolution and proof of configuration of aromatic amino acids, along with the metabolic handling of those which have been so investigated, our experimental observations and conclusions therefrom will be discussed, together with the experimental methods used in all cases. Brevity has been in a number of cases sacrificed in favor of minute detail to enable those without extensive synthetic organic chemical experience to duplicate the results or apply the information in similar investigations.

As a starting point in our discussion we will consider the information obtained in the synthesis, resolution, and metabolic investigation of meta-tyrosine.

A. The Synthesis of dl-meta-Tyrosine and Derivatives

Several methods have been described in the historical section for the synthesis of dl-meta-tyrosine. Most of these methods offered low yields, while starting material were not readily available for others, as has been pointed out. There also entered into consideration the fact that the amino acid would be wanted in the form of an N-acyl derivative for resolution purposes. As there were possibilities for the direct synthesis of N-acetyl-dl-meta-tyrosine through the catalytic

reduction of the corresponding α-acetamido-m-hydroxycinnamic acid, this approach was first investigated. The general method we had in mind was successfully used by Herbst and Shemin (53) for the preparation of N-acetylphenylalanine. The substituted cinnamic acid, and the desired azlactone could not be found in the literature. The equations for the proposed synthesis, together with formulas for the products actually obtained, are shown on the flow sheet on page 54. The starting material was the commercially available meta-nitrobenzaldehyde.

m-Hydroxybenzaldehyde. The method used was a modification of the procedure in "Organic Syntheses" (141) as the original method was not entirely satisfactory. To a solution of 450 gm. (2 moles) of C.P. stannous chloride in 600 ml. of concentrated hydrochloric acid contained in a 4-l. beaker provided with an efficient mechanical stirrer, was added 100 gm. (0.66 mole) of meta-nitrobenzaldehyde in one portion. The stirrer was started and as the reaction began the temperature rose spontaneously to 100°. After the temperature had dropped down below 50° an ice-salt bath was placed around the beaker. The stannichloride of m-aminobenzaldehyde was allowed to crystallize with slow stirring for three hours. The thick paste was filtered off with suction using a Büchner funnel provided with an asbestos filter mat. The salt was pulled as free from mother liquor as possible with the water-pump. The

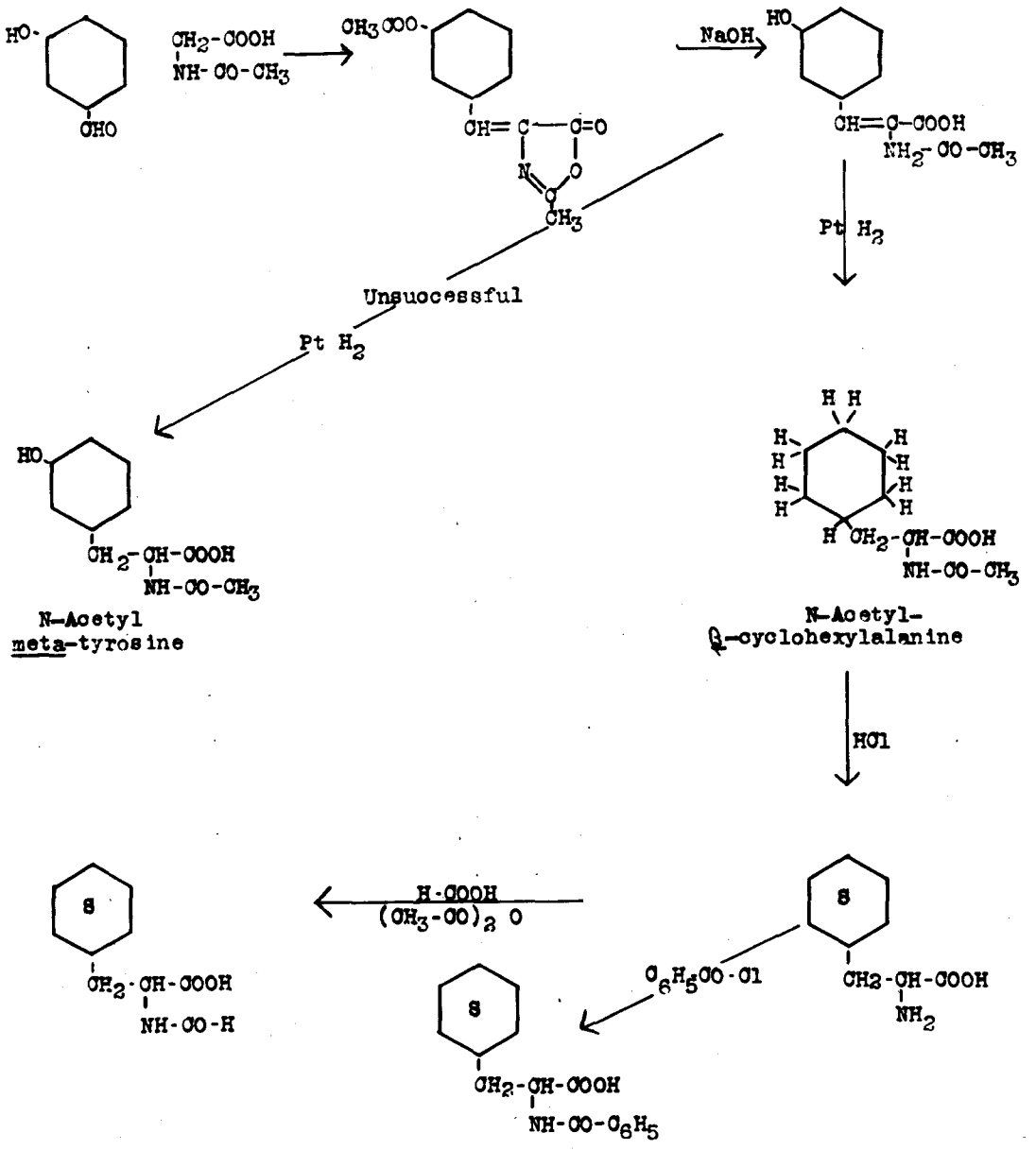


Fig. 1 Products Obtained in the Study of the Catalytic Reduction Method for meta-Tyrosine Synthesis

material was then suspended in 600 ml. of concentrated hydrochloric acid in a 3 l. beaker placed in an ice-salt bath. A solution of 55 gm. (0.8 mole) of sodium nitrite in 150 ml. of water was added below the surface of the liquid during a period of ninety minutes. After the addition, which was carried out at 4-5°, the temperature of the mixture was lowered to -5° and the slurry stirred for two hours at that temperature.

The precipitate remaining after the diazotization was filtered off using an asbestos mat as before. The damp complex was added with care to 1.7 l. of water boiling vigorously in a 4-l. beaker. Capryl alcohol was added when severe foaming appeared imminent. Water lost by evaporation was replaced. After the addition was completed, 5 gm. of Norite was added and the mixture filtered hot through a mat of Filter Cel and asbestos. The filtrate was cooled for twelve hours and yielded 50 to 60 gm. of crystals which melted at 102-104° (literature value 106-107°). The yield in a series of runs varied from 63 to 81% of theoretical.

As the azlactone produced through a condensation of m-hydroxybenzaldehyde with acetylglycine was not reported in the chemical literature, the general Erlenmeyer procedure was made use of.

2-methyl-4-(3'-acetoxybenzal)-5-oxazolone- In a liter Erlenmeyer flask were mixed 61 gm. (0.5 mole) of meta-hydroxy-

benzaldehyde, 58.6 gm. (0.5 mole) of acetylglycine, 41 gm. of anhydrous sodium acetate (0.5 mole) and 143.5 ml. (1.5 mole) of acetic anhydride. The mixture was shaken well, and then placed in a boiling water-bath for six hours. At the end of the reaction period the flask was cooled to room temperature and 300 ml. of water gradually worked into the semisolid mass. The diluted mixture was cooled 12 hours and the product separated as a light yellow solid, which was filtered with suction and pressed down well in the funnel. The cake was stirred up with several 100 ml. portions of cold water. After each washing the water was removed with suction. The air-dried product melted at 116-118°. Recrystallization from ethyl acetate through careful addition of Skellysolve B gave a light canary-yellow crystalline powder which melted at 118-120°. The yield in four trials varied from 82 to 92 gm. or 67 to 75% of the theoretical yield of 125 gm.

Anal. Calculated for $C_{13}H_{11}O_4N$

N, 5.71%

Found N, 5.73, 5.68, 5.68

α-Acetamido-m-hydroxycinnamic acid. In a 250 ml. flask was placed 24.5 gm. (0.1 mole) of 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone. A solution of 12 gm. (0.33 mole) of sodium hydroxide in 200 ml. of water was added, and the mixture warmed to 60° with vigorous stirring. After all the solid was in solution, 10 gm. of Norite was added and the mixture

stirred up well. The hot solution was filtered, and the Norite washed with several 10 ml. portions of boiling water. The filtrate was neutralized while warm. On cooling crystals with a slight yellow cast separated. These were filtered off and recrystallized from hot water using Norite. Pure white crystals melting at 140-142° were obtained. The yield was 18 gm. (80%). From the weight lost on drying and the analytical data it was established that the product was a monohydrate.

Anal. Calculated for $C_{11}H_{11}O_4N \cdot H_2O$

N, 5.86%; H_2O, 7.53%

Found N, 5.97, 5.97; H_2O, 7.83[2]

Catalytic reduction of α-acetamido-m-Hydroxycinnamic acid. A solution of 11.1 gm. (0.05 mole) of α-acetamido-m-hydroxycinnamic acid in 150 ml. of glacial acetic acid was placed in a hydrogenation bottle and attached to a Burgess-Parr type of hydrogenation apparatus. After the addition of 0.25 gm. of platinum oxide catalyst[1] and removal of the air from the flask with vacuum and subsequent sweeping with hydrogen, the pressure was raised to a gage reading of 40 lbs. After two hours and fifteen minutes, 13.5 lbs. less pressure was recorded on the gage, which corresponded to an absorption of 0.084 mole of hydrogen. The absorption rate at the time the reaction was stopped was a small fraction of the rate over

1. The catalyst was commercial Adam's catalyst purchased from the American Platinum Works, Newark, N.J.
2. Analyses by micro-Kjeldahl method.

the first two hours, which was nearly constant.

The catalyst was filtered off and washed with a little acetic acid. The filtrate was diluted with an equal volume of water and concentrated under reduced pressure. To remove the last traces of acetic acid, the residue was twice dissolved in 50 ml. portions of water and reconcentrated to dryness *in vacuo*. The residue was dissolved in 75 ml. of boiling water, treated with Norite, filtered and cooled. The first crop of crystals melted at 160-164°, but recrystallization raised the value to 174-175°. It was found that the recrystallized material did not give a Millon's reaction. This indicated that the reduction had in some manner altered the structure of the molecule so that a phenolic hydroxyl group was no longer present. Herbst and Shemin (53) stated that in their synthesis of phenylalanine through the reduction of the corresponding cinnamic acid, freshly prepared platinum oxide catalyst often reduced the ring. They determined conditions so that a synthetic procedure was developed for the preparation of β-cyclohexylalanine from α-acetamidocinnamic acid; through catalytic reduction using platinum oxide and subsequent hydrolysis of the N-acetyl-hexahydrophenylalanine thus formed (142). However, our belief that possible reduction to hexahydro-*meta*-tyrosine had taken place was not supported by analyses of our product for nitrogen, or analyses of hydrolysis products and derivatives for the same element.

The analytical data shown below shows that the nitrogen content was high, in comparison with the calculated value, for N-acetylhexahydro-meta-tyrosine.

Anal. Calculated for $C_{11}H_{19}O_4N$

N, 6.11%

Found N, 6.45, 6.43

Several investigators have studied the reduction of the benzene ring in tyrosine itself. Waser and Brauchli (143) and Karrer and Kehl (144) both observed that catalytic reduction using platinum oxide catalyst not only reduced the ring but also removed the hydroxyl group. It was established that only under carefully controlled conditions was the ring reduced without removal of the hydroxyl group. From this evidence it was considered possible that the product of our catalytic reduction of α-acetamido-m-hydroxycinnamic acid was β-cyclohexylalanine as the N-acetyl derivative. Comparison of the properties of the substance we had isolated with the N-acetyl-β-cyclohexylalanine prepared by Herbst and Shemin showed very close agreement. Our compound melted at 174-175°[1] uncorrected, while theirs melted at 178° corrected. The analytical results also were in good agreement. However, to

Anal. Calculated for $C_{11}H_{19}O_3N$

N, 6.57%

Found N, 6.45, 6.43

1. All melting points are uncorrected.

establish with certainty the nature of the material a portion of the acetyl derivative was hydrolyzed to β-cyclohexylalanine which was analysed.

β-Cyclohexylalanine. A solution of 5 gm. of the reduction product in 100 ml. of 1 N hydrochloric acid was refluxed for twelve hours. The excess acid was removed through concentration of the solution under reduced pressure. The residue was dissolved in 10 ml. of water and neutralized with ammonium hydroxide. A heavy precipitate separated, and 25 ml. of absolute ethyl alcohol was added to complete the precipitation. After cooling for twelve hours, the product was filtered with suction. The white light powder remaining was washed with cold water, alcohol, and ether. When dried the product melted at 229-230°. The melting point for the compound was not given by Herbst and Shemin.

Anal. Calculated for $C_9H_{17}O_2N$

N, 8.18%

Found N, 8.09, 8.17

N-Formylcyclohexylalanine. Following the method of Clark as applied by du Vigneaud and Meyer (147) a solution of 4 gm. (0.02 mole) of β-cyclohexylalanine in 43 ml. of 88% formic acid was heated to 45°, and 15 ml. of acetic anhydride added dropwise in thirty minutes. The temperature rose to 65° during the addition. After standing at room temperature for several hours, the liquid was diluted with an equal vol-

ume of water and concentrated under reduced pressure, followed by several reconcentrations with water to remove all formic acid. The oil remaining was dissolved in water and on cooling 3.2 gm. (80%) of crystals melting at 135-136° was obtained.

Anal. Calculated for $C_{10}H_{17}O_3N$

N, 7.03%

Found N, 7.13, 7.07

N-Benzoylhexahydrophenylalanine. In 3 ml. of water and 2 ml. of 2 N sodium hydroxide was dissolved 200 mg. of β-cyclohexylalanine. Approximately 200 mg. of benzoyl chloride was added with shaking. After warming the reaction mixture gently on the steam-bath for ten minutes, the solution was filtered and acidified with dilute hydrochloric acid. The white precipitate which separated was recrystallized from methyl alcohol-water mixture. After two recrystallizations the melting point was 184-185°. Herbst and Shemin gave 182-183.5° for the melting point of their N-benzoylcyclohexylalanine.

In view of the melting points and analyses of derivatives we felt confident in our belief that the reduction of N-acetyl-m-hydroxycinnamic acid yielded N-acetylcyclohexylalanine.

It thus appeared that considerable research, using a variety of catalysts, solvents and experimental conditions might be necessary before a good yield of N-acetyl-m-tyrosine could be obtained by a reduction procedure. Simultaneous investi-

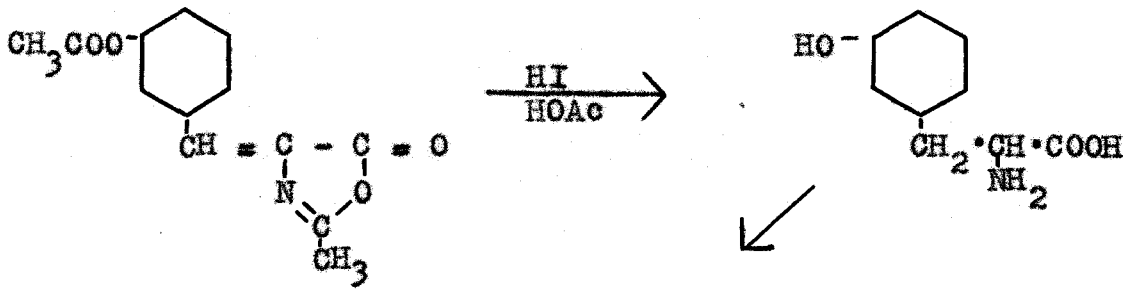
gations of other methods for the synthesis of m-tyrosine and its derivatives gave promise at this time, thus no further study of this procedure was considered justified.

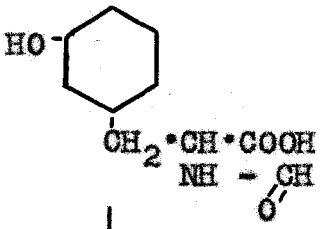
Alternative syntheses for the preparation of m-tyrosine and its derivatives were considered as soon as it became evident that large quantities of material would be difficult to handle by the catalytic reduction procedure. Of the approaches remaining, it appeared worthwhile to investigate the action of hydriodic acid on the 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone used as the starting material in the first approach explored. The series of reactions finally developed for the synthesis and resolution of meta-tyrosine involved the reductive hydrolysis of 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone with hydriodic acid and red phosphorus in acetic acid solution. The free dl amino acid was converted to the formyl derivative which was resolved as the brucine salt. Ethanol recrystallization yielded one diasterioisomeric brucine salt while the other isomer, obtained from the ethyl alcohol mother liquors, was purified through recrystallization from water. The isomeric N-formyl-meta-tyrosines were isolated after alkali decomposition of the brucine salts. Acid hydrolysis of the formyl derivatives then yielded dextro- and levo-rotatory meta-tyrosine. These reactions are indicated step by step in the accompanying chart.

dl-meta-Tyrosine. The procedure used was a modification

The Synthesis and Resolution of

meta-tyrosine





↓ Brucine

Brucine Salt

Alcohol Insoluble Salt		Alcohol Soluble Salt
↓ Five recrystallizations ethanol	Mother liquors →	↓ Three recrystallizations water
Brucine Salt		Brucine Salt
$(\alpha)_D^{26} = -37.6^\circ$		$(\alpha)_D^{26} = +0.73^\circ$
↓ NaOH		↓ NaOH
N-Formyl-m-tyrosine		N-Formyl-m-tyrosine
↓ HCl		↓ HCl
meta-tyrosine		meta-tyrosine
$(\alpha)_D^{26} = +8.8$ (0.2N HCl)		$(\alpha)_D^{26} = -8.8^\circ$ (0.2N HCl)

of that applied by Lamb and Robson (57) in the synthesis of tyrosine and phenylalanine.

A solution of 49 gm. (0.2 mole) of 2-methyl-4-(3'-acetoxy benzal)-5-oxazolone in 250 ml. of glacial acetic acid was refluxed for two and a half hours with 250 ml. of hydriodic acid (sp. g. 1.70) and 20 gm. of red phosphorus in an all-glass apparatus. The solution was filtered from the phosphorus using a sintered-glass funnel. The filtrate was concentrated under reduced pressure and reconcentrated with water to remove excess acid. The distillate was saved for re-use after redistillation. The residue, when free of hydriodic acid, was dissolved in 200 ml. of water and extracted twice with 50 ml. portions of ether to remove a small amount of colored impurity. The water layer was again concentrated to dryness and the crystalline residue dissolved in 25 ml. of boiling water. The solution was treated with Norite and filtered. The hot filtrate was carefully neutralized with ammonium hydroxide until basic to congo red indicator yet acid to litmus. About 100 ml. of absolute ethyl alcohol was added to the mixture which was then cooled for at least twenty-four hours. The white precipitate was filtered with suction and washed on the funnel with several 10-ml. portions of ice-water. The residue was then washed with three 25-ml. portions of ethyl alcohol followed by three 25-ml. portions of ether.

After standing a few hours in air the product was dry and

ether free. The yield was from 21 to 24 gm. or about 60 to 70% of the theoretical yield of 33.6 gm. The material melted at 275° with decomposition. Purification, through solution of the amino acid in alkali, decolorization with Norite, and reprecipitation with acid, yielded plate crystals which melted at 283°, when the bath was preheated to 250° before introduction of the sample. Blum (69) gave 280-281° for the melting point of the compound. Our product gave a strong Millon's test in the cold and a positive ninhydrin reaction.

Anal. Calculated for $C_9H_{11}NO_3$

N, 7.73%

Found N, 7.59, 7.59

Although this procedure was used in the preparation of two hundred grams of meta-tyrosine its behavior was found on a few occasions to be erratic. This was believed related to the concentration of hydriodic acid used. Good results were always obtained when hydriodic acid of specific gravity 1.70 was used. A number of runs using material of 1.50 specific gravity gave good results. Recovered hydriodic acid from previous runs was unsatisfactory unless fractionated to a sp. g. of 1.50 or better. The method was not satisfactory in larger runs as the yields decreased. The reaction gave large amounts of an ether soluble red oil when the hydriodic acid was weak. Attempts to crystallize this by-product were not successful. In order to ascertain if better yields could be

obtained through modification of the experimental details a number of small scale runs were carried out.

dl-meta-Tyrosine. (procedure b) A mixture of 4.5 gm. (0.02 mole) of α-acetamido-m-hydroxycinnamic acid was refluxed with 40 ml. of a mixture of equal parts hydriodic acid (sp. g. 1.50) and glacial acetic acid and 2 gm. of red phosphorus for seventy-five minutes. Evaporation of the reaction mixture under vacuum and extraction with ether was carried out as in the previous procedure. The final residue was dissolved in water and neutralized with ammonium hydroxide, after which several volumes of absolute alcohol was added to complete the separation of the amino acid. The yield was 1 gm. (27%) of product which melted at 280° with decomposition.

As the yield in this reaction was lower than that observed with the oxazolone, the use of the cinnamic acid offered no advantage. Also, the conversion of the azlactone to the cinnamic acid was not without loss.

2-Phenyl-4-(3'-acetoxybenzal)-5-oxazolone. The procedure of Erlenmeyer and Wittenberg (145) was used to synthesize this azlactone. Our purpose in the preparation of this substance was to compare the yield of m-tyrosine obtainable on hydriodic acid hydrolysis with the yield obtained with the 2-methyl-oxazolone. Work with the o-tyrosine had shown that the 2-phenyl-oxazolone gave a better yield of the o-amino acid than we were able to obtain of meta-tyrosine by any procedure.

A mixture of 48 gm. (0.4 mole) of m-hydroxybenzaldehyde, 72 gm. (0.4 mole) of hippuric acid, 114 ml. (1.2 moles) of acetic anhydride and 33 gm. (0.4 mole) of sodium acetate was heated in a boiling water-bath for six hours. After three hours, crystals were evident in the reaction flask. At the end of the reaction period the flask contents were solid. When cooled to room temperature, the reaction mixture was stirred up with 100 ml. of ethyl alcohol which was added slowly with cooling in 10 ml. portions. The mixture was then cooled for twelve hours and filtered with suction. The product was washed with alcohol and water and then air dried. The material melted at 148-150°. Erlenmeyer and Wittenberg gave a melting point of 145° for their product but did not give their purification procedure.

dl-meta-Tyrosine (c). Reductive hydrolysis of 50 gm. of 2-phenyl-4-(3'-acetoxybenzal)-5-oxazolone with 250 ml. of hydriodic acid (sp. g. 1.50) and 250 ml. of acetic acid in the presence of 20 gm. of red phosphorus was carried out according to the procedure used with the 2-methyl azlactone. A yield of 12 gm. (51%) of dl-m-tyrosine, identical in properties with material obtained in earlier reactions, was isolated.

As the various modifications of the first method for the synthesis of meta-tyrosine all gave lower yields, and in some cases a greater number of steps was involved, none were used for preparative purposes. However, it was considered of in-

terest to prepare meta-tyrosine by the one method which it appeared might equal the new procedure in yield and in simplicity of manipulation. That procedure was the synthesis making use of 2,5-diketopiperazine as applied by Ueda (71) to the preparation of m-tyrosine. The successful application of the Ueda method to our needs, however, was dependent on the success of a synthetic method of obtaining the diketopiperazine starting material. The simplest method for the preparation of this intermediate appeared to be that of Sannié (146) published in 1942. The general method involved heating glycine with ethylene glycol with the subsequent splitting out of water to give glycine anhydride or diketopiperazine. The use of the earlier syntheses for this compound could not be considered in view of the labor and expense involved. To be of any value, however, the Sannié method had to be successful in moderately large scale preparations. Thus, a trial run was carried out using twenty times the quantities given in the literature.

2,5-Diketopiperazine according to the Sannié procedure. A mixture of 100 gm. (1.33 moles) of glycine and 600 ml. of ethylene glycol was refluxed until all of the solid material had gone into solution. This required one hour and as the solution darkened considerably in the course of the reaction, it was somewhat difficult to establish when solution was complete. The reaction mixture was cooled somewhat, and then

concentrated *in vacuo* using a mechanical pump with a dry-ice trap to remove any entrained vapor. The mixture was brought to dryness in three hours. As the mixture had a strong tendency to bump in the first stages of the distillation, a good ebulator tube and careful control of the temperature was essential. The dry residue was dissolved in 500 ml. of water, and 20 gm. of decolorizing charcoal added. After digesting this mixture at the boiling point for several minutes, it was filtered through a warm Büchner funnel and then cooled. The first fraction of crystals weighed 33.5 gm. and melted at 308-310° (copper block melting point apparatus used). The mother liquors were concentrated to 150 ml. and again treated with charcoal followed by filtration. The yield was 22 gm. The total yield was 55.5 gm. of light brown crystals. Pure white material was isolated after a second recrystallization which yielded 48 gm. (64% yield) of product. The melting point was not altered by recrystallization. Sannié obtained a 67% yield with a small scale run.

3,6-Di-(3'-acetoxybenzal)-2,5-diketopiperazine. In the preparation of this intermediate the procedure of Dickenson and Marshall (72) was used as they claimed a 90% yield while Ueda (71) obtained 76%.

In a round-bottomed flask was placed 48 gm. (0.4 mole) of *m*-hydroxybenzaldehyde, 22 gm. (0.2 mole) of glycine anhydride, 60 gm. (0.74 mole) of sodium acetate and 90 ml. (0.95

mole) of acetic anhydride. The reactants were stirred up well and heated seven hours at 135-140°. The solid mass remaining was digested with water and the lumps broken up. The mixture was then filtered, the solid stirred up with 50 ml. of alcohol and then refiltered. The solid remaining was recrystallized from glacial acetic acid. The light yellow product melted at 267-269° in agreement with the literature value, while the yield was 31 gm. or 40% of theory. A similar run with half these quantities gave approximately the same yield. Although Dickenson and Marshall claimed their method gave a 90% yield they did not say if that was calculated on the basis of recrystallized product or crude material. It seems possible that this reaction procedure may lend itself only to synthesis on a small scale.

meta-Tyrosine from 3,6-di-(3'-acetoxybenzal)-2,5-diketopiperazine. Using an all-glass apparatus, 20 gm. of the diketopiperazine derivative was refluxed with 100 ml. of concentrated hydriodic acid (sp. g. 1.70) and 10 gm. of red phosphorus for twelve hours. The acid was then removed under reduced pressure and the excess removed through reconcentration with water. The residue was dissolved in 25 ml. of boiling water and the solution neutralized with ammonium hydroxide. After addition of two volumes of alcohol, the mixture was cooled for 48 hours. The amino acid was then filtered and washed with several portions of cold water, alcohol, and fin-

ally ether. The product weighed 17 gm. and had a light brown color. When purified through solution in alkali and reprecipitation with acid the amino acid melted at 280° with decomposition. The yield of purified material was 14.4 gm. or 79.5%.

In our hands the azlactone hydrolysis method for the preparation of m-tyrosine gave about a 15% better yield than the diketopiperazine procedure. In addition, the synthesis of diketopiperazine added quite a bit in the way of labor to the method. The acetylglycine needed in the azlactone method was purchased reasonably or prepared on a large scale by a very simple procedure. Thus, no advantage could be seen in use of the diketopiperazine method.

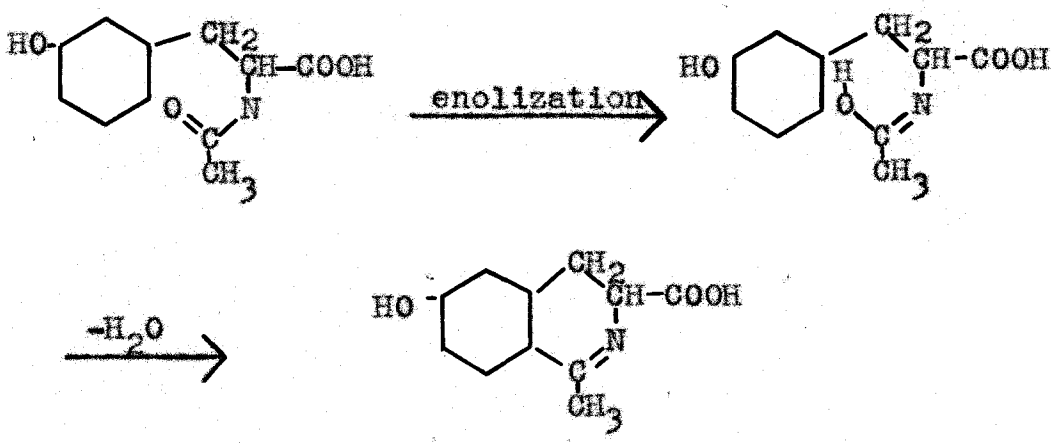
B. N-Formyl and N-Acetyl-meta-Tyrosine

With a satisfactory preparation method for dl-meta-tyrosine it remained to be determined what N-acyl derivative would be best in the resolution procedure. The N-benzoyl, formyl, and acetyl derivatives were used by various investigators with tyrosine. As the N-acetyl derivative as used by Sealock (102) gave excellent results with tyrosine it was decided to investigate the use of this derivative in the resolution of m-tyrosine. The acetylation procedure was that used by du Vigneaud and Meyer (147) for acetylation of tyrosine.

Attempted preparation of N-acetyl-m-tyrosine. A solution

of 15 gm. of purified m-tyrosine in 42.5 ml. of 2 N sodium hydroxide and 25 ml. of water was cooled in an ice-bath. In forty-five minutes 200 ml. of 2 N sodium hydroxide and 20 ml. of acetic anhydride were added in ten equal portions. The mixture was shaken well after each addition with care to keep the temperature down through immersion in the ice-bath. After the base and acetic anhydride had been introduced, the reaction was allowed to stand at room-temperature for twelve hours. After filtration, the solution was acidified with 83.5 ml. of 6 N sulfuric acid introduced carefully from a burette. The solution was concentrated *in vacuo* and the residue extracted with 400 ml. of acetone in five portions. The acetone was removed under reduced pressure and the residue reconcentrated with water to remove last traces of solvent which might interfere with crystallization. The slightly colored residue was dissolved in 30 ml. of boiling water, decolorizing charcoal added and the mixture allowed to digest at the boiling point for several minutes. The colorless filtrate deposited a minute amount of amorphous material which was filtered off. After three weeks in the ice-box no evidence of crystallization could be observed. Portions of the solution were concentrated and allowed to stand in the cold, while other portions were diluted somewhat in the hope crystallization would be induced. After six months no crystalline material was evident.

It was considered possible that the reaction might have given a different product than that expected. Most probable of the side reactions was ring closure to give an isoquinoline derivative as the following equation shows. It was appar-



ent that the ortho position to the alanine side chain would be highly activated, and this type of ring closure has been reported in the literature (148). This postulate was shown incorrect as hydrolysis of a portion of the acetylation reaction product gave an excellent return of m-tyrosine and nothing in the nature of an isoquinoline derivative could be isolated.

Hydrolysis of the oil obtained on attempted acetylation of m-tyrosine. A three gm. portion of the oil remaining after concentration of a portion of the uncrystallizable acetylation reaction product was refluxed for six hours with 25 ml. of 1 N hydrochloric acid. The mixture was diluted with an equal volume of water and concentrated to dryness. After re-

moval of excess acid through reconcentration with water, the residue was taken up in 5 ml. of water and neutralized with ammonium hydroxide. The white precipitate obtained melted at 282° with decomposition and gave a positive Millon's and ninhydrin reaction. In all respects it was identical with the m-tyrosine used as the starting material in the acetylation reaction.

As simultaneous experiments in regard to the preparation of N-formyl-dl-m-tyrosine showed this compound could be quite readily obtained in reasonable yield, and also appeared promising in preliminary resolution studies, other methods for the possible synthesis of N-acetyl-m-tyrosine were not investigated. The N-formyl-m-tyrosine was prepared by the method of Clarke as used by du Vigneaud and Meyer (147) for the formylation of tyrosine.

Synthesis and resolution of N-formyl-dl-m-tyrosine. A solution of 35.5 gm. (0.2 mole) of m-tyrosine in 348 ml. of 88% formic acid was prepared by warming the two substances to 45°. Over a ninety minute period 116 ml. (1.2 moles) of acetic anhydride was added in small portions with vigorous shaking. The temperature rose to 65° during the addition. After standing at room temperature for twelve hours the reaction mixture was filtered, diluted with an equal volume of water, and concentrated under reduced pressure. The residue was freed of formic acid through re-

concentration *in vacuo* with water. The residue, dissolved in 95 ml. of boiling water, was decolorized with Norite and filtered. The cooled solution deposited plate crystals after twelve hours. The yield was 31.5 gm. (77%). A further yield of 5 gm. was obtained through concentration of the mother liquors for a total yield of 89%. The product melted at 136-138°, was very soluble in hot water and moderately soluble in cold.

Anal. Calculated for $C_{10}H_{11}O_4N$

N, 6.69%

Found N, 6.67, 6.66

Preliminary experiments with 0.001 mole portions of brucine and N-formyl-*m*-tyrosine showed that separation of isomers resulted on recrystallization from alcohol. Thus larger scale experiments were tried which resulted in the isolation of the isomeric *meta*-tyrosines.

A mixture of 37 gm. (0.176 mole) of N-formyl-*dl*-*meta*-tyrosine and 69.3 gm. (0.176 mole) of anhydrous brucine was dissolved in 1,900 ml. of 95% ethyl alcohol by heating to the boiling point. The solution was filtered and allowed to cool slowly to room temperature. The material was then seeded with crystals obtained in the small scale experiments and within twenty-four hours the flask was filled with a mass of fine crystals. These were scraped down off of the sides and finally filtered off after four days. The mother liquors were

removed as completely as possible with suction and the solid then air-dried. The recrystallizations were carried out in the same manner, without seeding however. Samples from each recrystallization were dried to constant weight in the drying pistol, dissolved in water and readings taken with the polarimeter using a two decimeter tube. To give an observed reading which could be determined with minimum error, solutions of from one to one and a half percent concentration were used. The data obtained from two resolutions are shown in the following tables.

It was apparent that four recrystallizations served to bring the observed rotation to a constant value of $-37.6^{\circ} \pm .2$. For the first three recrystallizations the yield of brucine salt obtained was greater than the theory yield, which indicated the material was still quite contaminated with the more soluble isomer. The yield of alcohol insoluble isomer was, in both resolutions illustrated, about 69%. In the first resolution it appeared that maximum rotation was achieved when the yield was close to 80%. This was not observed in the later trials of the method.

The filtrates from all of the alcohol recrystallizations were saved in order to obtain from them the other diasterioisomer. These filtrates were concentrated under reduced pressure and reconcentrated with water to remove all traces of alcohol. The light brown syrup remaining was dissolved in

Table 4

Rotations and Yields of Brucine Salt Fractions Obtained from Alcohol in the Resolution of m-Tyrosine

Fraction	Rotation	Concentration	Yield	
		Run 1		
	o	%	gm.	%
1	-22.4	1.1	81.7	153
2	-31.0	.58	74.8	140
3	-33.8	1.37	55.0	103
4	-37.3	1.50	43.5	82
5	-37.6	.82	38.0	71
6	-37.4	1.40	37.0	69
		Run 2		
1	[1]	[1]	89	169
2	[1]	[1]	69	131
3	-33.2	0.31	46	86
4	-35.0	0.17	43	81
5	-37.6	0.39	36	68

1. As it was apparent that at least three recrystallizations were required before resolution could be expected, readings were not taken on the first two fractions of the second run.

three volumes of water, approximately 90 ml., and, after treatment with decolorizing charcoal, cooled in the ice-box. After two weeks of scratching and cooling crystals were observed. After another week these were filtered off and air-dried. The air-dried crystals lost weight in the drying pistol corresponding to four moles of water of crystallization.

Rotations were taken as previously described on each fraction. Three crystallizations from water were sufficient to bring the rotation to the calculated value. This calculated value was determined from the fact that an equimolecular mixture of brucine and formyl-dl-meta-tyrosine gave a rotation of-18.5°. As this value represented the rotation of a mixture of equal amounts of the isomeric brucine salts it represented the zero point of the resolution. As the alcohol insoluble isomer showed a maximum rotation of -37.6°, which was an increase in negative rotation of 19.1°, the pure water insoluble isomer should theoretically show a rotation 19.1° more positive than the zero value of -18.5°. From this it was readily seen that the theoretical rotation for the water insoluble isomer was +0.6°. As our experimental value after three recrystallizations was +0.73° the resolution was considered complete. The data obtained are shown in table 5.

The yields given for the water insoluble isomer are lower than observed with the alcohol insoluble isomer. The moderate solubility of the water soluble isomer in even cold water was

Table 5

Rotations and Yields of Brucine Salt Fractions Obtained From Water in the Resolution of m-Tyrosine

Fraction	Rotation	Concentration	Yield	
	°	%	gm.	%
1	-1.1	1.34	37	63.3
2	0.48	1.04	32	54
3	0.73	1.36	27	46

without doubt responsible.

Hydrolysis of the alcohol insoluble brucine salt to yield optically active N-formyl-meta-tyrosine. A solution of 37 gm. of the brucine salt of rotation -37.6° in 740 ml. of hot water was cooled to 40° and 35 ml. of 2 N sodium hydroxide added with vigorous stirring. The solution was then just alkaline to phenolphthalein. A heavy precipitate of brucine soon separated and precipitation was completed through twelve hours cooling in the ice-box. The brucine was filtered off and washed with ten 50-ml. portions of water with care to stir up the brucine mat well with each portion of wash water. After extraction of dissolved brucine with 300 ml. of chloroform in six portions, the combined filtrates were neutralized with 11.7 ml. of 6 N sulfuric acid. The brucine-free solution was then concentrated under reduced pressure. The residue was extracted with seven 100-ml. portions of moist acetone and the acetone

extracts in turn were concentrated to dryness under reduced pressure. Last traces of acetone were removed through several reconcentrations with 10-ml. portions of water. The final residue was dissolved in 20 ml. of water and cooled slowly with scratching to induce crystallization. After a few hours crystals formed. The product was filtered off after twelve hours and 7.4 gm. (40% yield) of beautiful plate crystals obtained. These melted at 146-148°. Considerable material remained in the mother liquors which were hydrolized with consiederable of the crystalline product to the free amino acid.

Anal. Calculated for $C_{10}H_{11}O_4N$

N, 6.69%

Found N, 6.51, 6.56

Rotation With 0.218 gm. of compound in 25 ml. of redistilled water the rotation observed was $\alpha = -0.78^{\circ}$ which calculated for a specific rotation of $(\alpha)_D^{27} = -44.7^{\circ}$.

Hydrolysis of N-formyl-meta-tyrosine from alcohol insoluble brucine salt to meta-tyrosine. The filtrate from the formyl-meta-tyrosine, along with 6 gm. of the crystalline material, was refluxed for four hours with 150 ml. of 10% hydrochloric acid. The excess acid was then removed through concentration under reduced pressure. The residue was dissolved in 20 ml. of water and neutralized with concentrated ammonium hydroxide until basic to congo red indicator yet acid to litmus. Crystallization was evident at once but the mixture was

cooled 24 hours in the ice-box to complete the separation of the product. The beautiful plate crystals obtained after filtration and careful washing with ice-cold water, alcohol, and ether melted at 275-276° with decomposition. The yield of pure amino acid was 8.9 gm. (56%). However, the total yield of this isomer isolated as the free amino acid or retained as the formyl derivative was 10.1 gm. (63%).

A series of rotations was taken with this amino acid using various amounts of acid and alkali. These will be discussed later as they were concerned with the proof of configuration of the isomer. The material was found to be dextrorotatory.

Hydrolysis of the water insoluble brucine salt to yield the corresponding N-formyl-meta-tyrosine. The same procedure as used in the case of the other isomer was applied in the hydrolysis of 27 gm. of the brucine salt with a specific rotation $(\alpha)_D^{27} = +0.73°$. The yield of crystals melting at 148-149° was 3.8 gm. obtained as the first crop. From the mother liquors 2 gm. more of pure compound was obtained for a total yield of 5.8 gm. (36.5%) on the basis of dl-formyl-m-tyrosine starting material.

Anal. Calculated for $C_{10}H_{11}O_4N$

N, 6.67%

Found N, 6.57, 6.78

Rotation A solution of 0.183 gm. of material in 25 ml. of water was used. The observed rotation using a two decimeter tube was $\alpha = +0.70^{\circ}$ which was calculated for a specific rotation of $+45.7^{\circ}$.

Hydrolysis of N-formyl-meta-tyrosine from water insoluble brucine salt to meta-tyrosine. The procedure used in the hydrolysis of the isomeric formyl derivative was applied with 2.8 gm. of the dextrorotatory compound. A quantitative yield of 2.4 gm. of m-tyrosine possessing a levo configuration was obtained. The product melted at 277° with decomposition. A series of rotations was taken with this levorotatory compound using different concentrations of acid and base. These are shown in table 7.

Anal. Calculated for $C_9H_{11}O_3N$

N, 7.73%

Found N, 7.51, 7.53

Thus, on hydrolysis of the isomeric brucine salts N-formyl derivatives of meta-tyrosine were obtained which rotated polarized light in the same direction as the brucine salt from which they were obtained. On hydrolysis of the formyl group, however, inversion of rotation was observed so that the dextrorotatory derivative gave a levorotatory amino acid and vice versa. Although this behavior paralleled the behavior of the acetyl derivative of naturally occurring tyrosine, analogy alone could not be considered evidence to es-

tablish our levo meta-tyrosine as the natural isomer. The establishment of configuration was thus our next step.

D. The Proof of Configuration of the Isomers of meta-Tyrosine

As has been discussed in the historical section of the thesis, the method of Lutz and Jirgensons (110,111) offered a convenient procedure for the establishment of optical configuration. To apply the method, rotations were taken of both isomers using definite molecular ratios of acid and base. These rotations are tabulated on page 84.

The data of the table can also be seen graphically on page 86. It can be readily seen that the dextrorotatory isomer had a decreasing positive rotation as acidity was increased. As the Lutz and Jirgenson rule designated the isomer with increasing positive or decreasing negative rotation as having the natural or levo configuration it was obvious that this particular isomer of meta-tyrosine possessed the unnatural or dextro configuration, and should be written as d(+)-meta-tyrosine.

The data on the levorotatory isomer of meta-tyrosine shown in table 7, and the graphical representation on page 86, show clearly that the rotation became more positive or less negative, as the ratio of acid to amino acid was increased. Thus, this isomer was shown to have the levo or natural con-

Table 6

Specific Rotations of Dextrorotatory m-Tyrosine in Acid and Basic Solution

Concentration (molarity)	Moles acid / Moles amino acid	Moles base / Moles amino acid	Rotation
molarity			
.05	0.0	0	+30.9°
.05	0.25	0	+25.2
.05	0.50	0	+20.9
.05	1.00	0	+14.9
.05	4.00	0	+ 8.8
.05	0.0	1	+19.2
.05	0.0	4	+13.2

Table 7

Specific Rotations of Levorotatory m-Tyrosine in Acid and Basic Solution

Concentration (molarity)	Moles acid / Moles amino acid	Moles base / Moles Amino acid	Rotation
molarity			
.05	0	0	-29.8°
.05	1	0	-15.4°
.05	4	0	- 8.8°
.05	0	0.5	-24.3°
.05	0	3.0	-13.8°

figuration, and should be designated as l(-)-meta-tyrosine.

Confirmation of the information obtained through the procedure of Lutz and Jirgenson was obtained through the application of an entirely biochemical procedure. As the amino acid isomers were prepared for enzymatic investigations, it was considered fitting to confirm configuration by an enzymatic method. The work of Krebs (149) established the specificity of the d-amino acid oxidase of the kidney for the deamination of amino acids of the unnatural configuration. Lipmann and coworkers (150) used the enzyme as a reagent for the detection of unnatural amino acids. Later Sealock (151) simplified the earlier procedures through the use of a colorimetric method which determined the amount of pyruvic acid formed in the enzyme reaction.

The use of the d-amino acid oxidase and the analytical method of Sealock offered an excellent method for proof of configuration. Application of the method would involve incubation of the isomers with the enzyme with subsequent analysis to determine the amount of keto acid formed in each case. The isomer giving the theoretical amount of keto acid would thus be established as having the unnatural or dextro configuration. At the same time the purity of the isomers would be established. With application of this technique in mind we obtained a preparation of the d-amino acid oxidase by the following procedure.

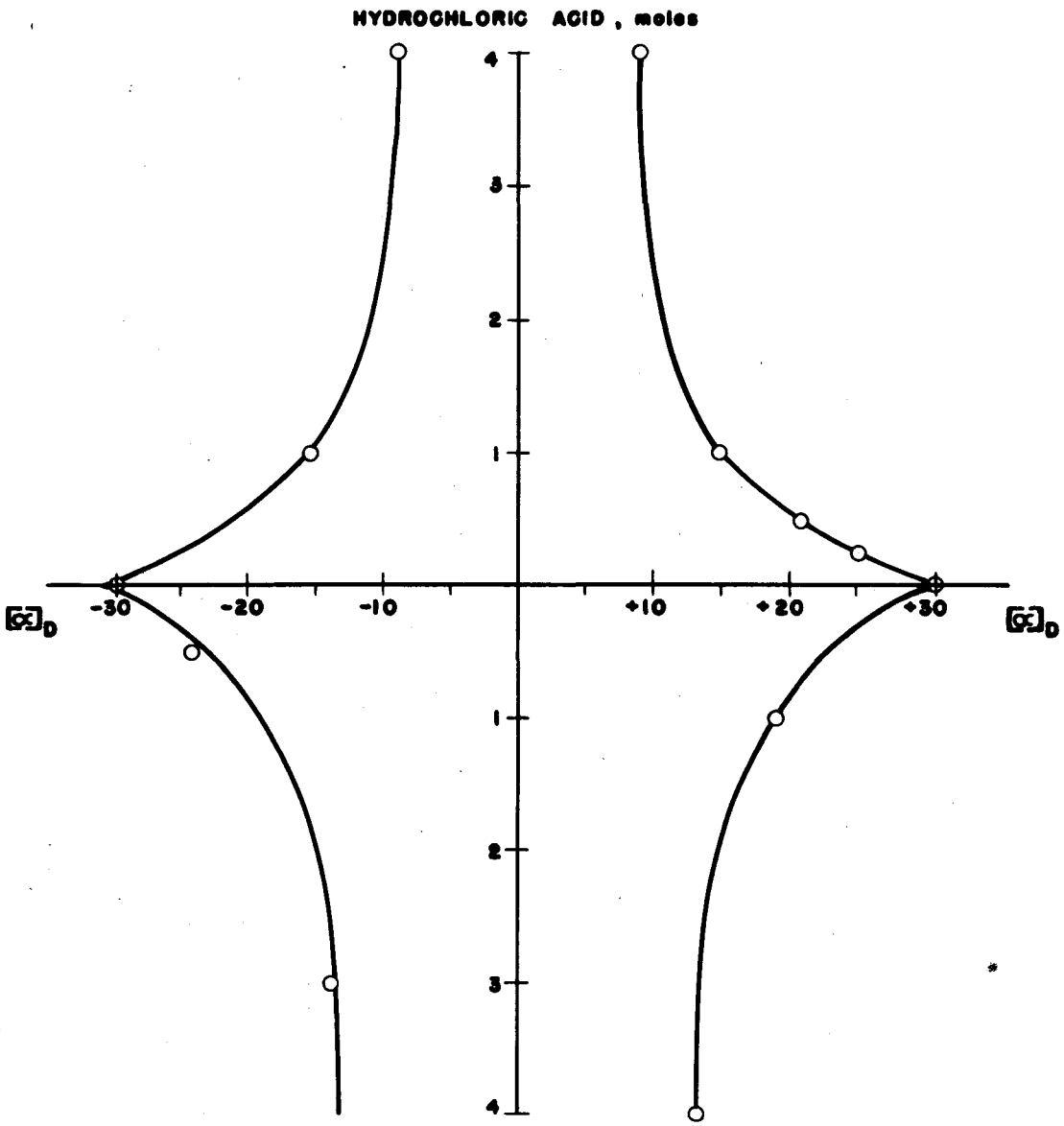


FIG. 2 ROTATIONS OF d AND l-META-TYROSINE

THE ACID AND ALKALI ARE REPRESENTED AS MOLES PER MOLE OF AMINO ACID

Extraction of the d-amino acid oxidase from kidney tissue. The active enzyme extract was most readily obtainable from desiccated kidney tissue. Desiccation was accomplished through the procedure of Neglein and Brömel (152) which involved homogenization of ground kidney cortex with acetone. After suspension of the filtered material in a second quantity of acetone, the desiccated tissue was refiltered and carefully freed from acetone. The final drying over phosphorus pentoxide in a vacuum desiccator gave a light fine powder which could be stored, when tightly stoppered, in the cold.

The desiccated powder thus obtained was converted to a highly purified enzyme preparation by following the Neglein and Brömel purification through step 1. This was considered necessary as the desiccated tissue still retained sufficient acetone to give a high control value with the highly sensitive 2,4-dinitrophenylhydrazine reagent. Seven gm. of dried kidney tissue, after being carried through the extraction and purification procedure, gave 60 ml. of enzyme preparation in 0.0166 M pyrophosphate buffer.

Application of the d-amino acid oxidase to the isomers of meta-tyrosine. In 125 ml. flasks were placed 1 ml. of a solution containing 0.1, 0.2 and 1.0 mg. of meta-l-tyrosine. A similar series of flasks were set up with identical quantities of d-meta-tyrosine. To each flask was added 4 ml. of the enzyme preparation and the contents shaken well. The

samples were then incubated at 38° along with control flasks which contained no added amino acid. The first two flasks and corresponding controls were removed after two hours, while the last flask was given a four hour reaction time. The flask contents were washed into centrifuge tubes, which contained 1 ml. of 5% metaphosphoric acid, with sufficient water to give a total volume of 10 ml. The mixtures were stirred up well, allowed to stand thirty minutes, and then centrifuged. A 1 ml. portion of each supernatent liquid was pipetted into a test-tube and 1 ml. of freshly filtered 0.008 molar 2,4-dinitro-phenylhydrazine reagent added. Blank tubes were prepared using 1 ml. of 0.1 N hydrochloric acid with 1 ml. of the reagent. After thirty minutes, 5 ml. of distilled water was added to each tube and 5 ml. of sodium hydroxide. Very vigorous shaking was necessary during the addition of base. After ten minutes, the samples were read with the Klett-Summerson photo-electric colorimeter using filter number 52.

The instrument was set at zero using the reagent blank, and the correct keto acid value obtained by subtracting the corresponding control flask value from each reading. The amount of keto acid formed was calculated in terms of p-hydroxy-phenylpyruvic acid, as in a standardization experiment one scale division was found to correspond to 0.00205 micromoles of that keto acid. The error in using that keto acid as a standard, however, was considered very slight. Thus, in table

Table 8

d-Amino Acid Oxidase Reaction with meta-Tyrosine Isomers

Amount Amino Acid	Scale Reading	Corrected Scale Reading	Keto Acid Found	Theory Found
moles			moles	%
control	8	—	—	—
0.553 d	34	26	0.533	96.4
0.553 l	7	0	0	0
control	6	—	—	—
1.106 d	64	58	1.19	107.0
1.106 l	8	2	0.04	3.6
control	13	—	—	—
5.53 d	299	286	5.86	106.0
5.53 l	13	0	0	0

8 the colorimeter readings, corrected readings, amount of keto acid formed, and the percent yield are shown.

The evidence obtained from the method of Lutz and Jirgensen in regard to configuration of the isomers obtained from the alcohol soluble and alcohol insoluble brucine salts was supported perfectly by this biochemical procedure. In all cases the isomer we designated as having the unnatural configuration, from the evidence presented earlier, gave close to 100% of the theoretical amount of keto acid, according to

this procedure. The other isomer, which theoretically should not be attacked by the specific d-amino acid oxidase, was found to yield absolutely no keto acid in two cases and 3% of the theoretically possible quantity in the other. The 3% found was well within the limits of experimental error of the method. Not only did this method give additional proof of the configuration of the isomers of meta-tyrosine but it also gave evidence that the resolution had yielded the isomers in a very high state of purity. With these facts established, the biochemical investigation of d and l-meta-tyrosine could be undertaken with confidence that the pure isomers were being studied.

E. Manometric Study of the Metabolism of d- and l-meta-Tyrosine

With pure d- and l-meta-tyrosine available through the resolution procedure, preliminary metabolic studies with the substances were undertaken. The method used for the investigation involved the application of the Warburg manometric technique. In this procedure the compounds were incubated with liver brei and the oxygen uptake over a three hour period determined. The difference between the oxygen absorbed in control flasks, without added tyrosine or tyrosine isomer, and the uptake in flasks containing the amino acid substrate was considered to be due to oxidation of the amino acid by an

enzyme system of the liver. As our interest in the metabolism of tyrosine isomers was involved mainly in the possibility of obtaining information through comparison of behavior of the isomers with tyrosine itself, our studies were carried out using tyrosine as a substrate in parallel experiments.

The animals used, mainly guinea pigs, were stunned by a blow on the back of the head and killed by bleeding from the severed jugular veins. The liver was then removed, weighed and cut into fine pieces. These were ground with acid washed sand with small amounts of 0.2 M phosphate buffer. The cell-free mixture was extracted with 2 ml. portions of buffer, centrifuged, and the liquid decanted until a brei of the desired concentration was obtained. This was usually in the order of 1 ml. of brei for each gm. of liver used.

The brei was adjusted to a pH of 7.4 and pipetted into the main chamber of Warburg vessels, which contained potassium hydroxide on filter paper in the center well to absorb the carbon dioxide produced. In the side arm was placed the substrate amino acid dissolved in phosphate buffer. The control flasks contained 1 ml. of the brei and 1 ml. of the buffer so that all flasks, after mixing, contained 2 ml. of material. The substrate in the side arms was run into the main chamber of the Warburg vessel by tilting the manometer and flask after an equilibration period. The manometer systems were then closed and shaking begun. Readings were taken

at 30, 60, 120 and 180 minutes.

After the incubation period, the reaction flasks were disconnected and the contents pipetted into 5% metaphosphoric acid to immediately stop enzyme action and precipitate the protein. The transfer was carried out quantitatively through careful rinsing of the side-arms and main compartments with three 2-ml. portions of distilled water. The solutions were diluted to 10 ml., stirred up well to break up all lumps, allowed to stand thirty minutes, and then centrifuged. The supernatant liquids were decanted into clean test tubes and saved for analyses which will be discussed in a later section. The results of a typical run are shown in table 9. The sim-

Table 9

The Oxygen Consumption of l-Tyrosine, l-meta-Tyrosine and d-meta-Tyrosine with Normal Liver Brei

Time	Control Flasks	l Tyrosine	l-meta Tyrosine	d-meta Tyrosine
minutes				
30	91.3[1]	109	157	88.6
60	130	164	208	126.0
120	182	245	256	176.0
180	219	302	290	212.0

1. All readings are in microliters of oxygen absorbed. These were obtained through multiplication of the manometer readings by appropriate flask constants.

ilarities and differences between l-tyrosine and l-meta-tyro-

sine can be quite well seen in the graphical representation of these results. These data are amplified in table 10 which summarizes the results of a series of experiments with guinea pig and rat liver brei.

From table 9 it can be seen that the rate of oxygen uptake was much faster in the flasks containing l-meta-tyrosine than in those containing tyrosine itself for the first hour. During the second and third hours, however, the oxygen uptake of the l-m-tyrosine flasks was just about equal to the basal value of the control flasks. In some cases the uptake over the last two hours with l-m-tyrosine was actually a little less than the basal. In general, the three hour values for oxygen uptake for l-tyrosine and l-meta-tyrosine were comparable and a parallel existed. The interesting rate differential which was observed can be very well shown graphically as in figure 4.

In contrast to the behavior of the l-meta-tyrosine the d-meta-tyrosine was metabolized in only one of three experiments with guinea pig liver brei. In that single case in which oxygen uptake was observed the amount was slight. As d-tyrosine itself behaved in a similar manner in parallel experiments it was assumed that our brei lacked the enzyme system responsible for the oxidation of d-amino acids in the liver.

A point of great interest in the study of the isomers

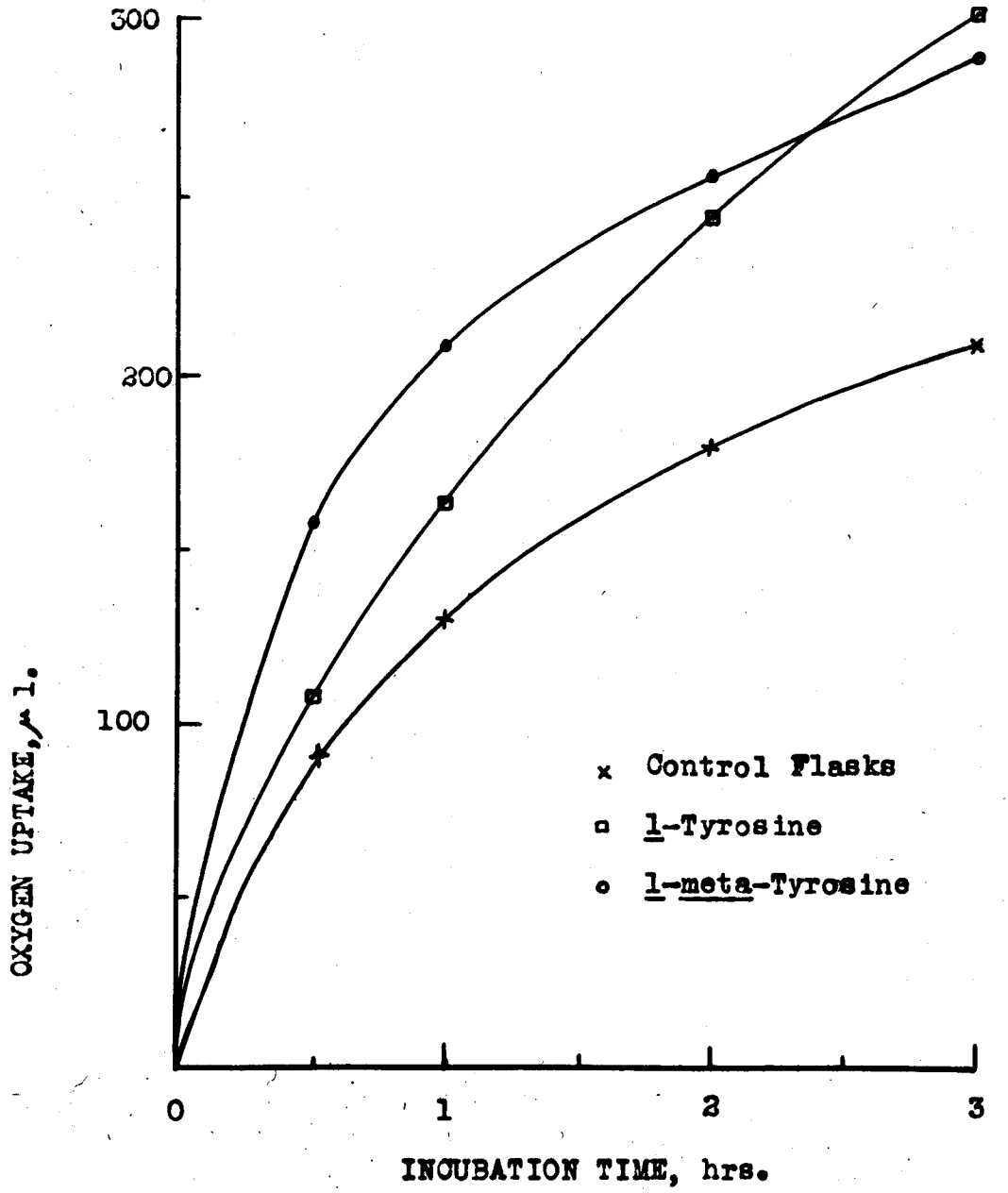


Fig. 3 The Oxygen Uptake of Normal Guinea Pig Liver Brei with l-Tyrosine and l-meta-Tyrosine.

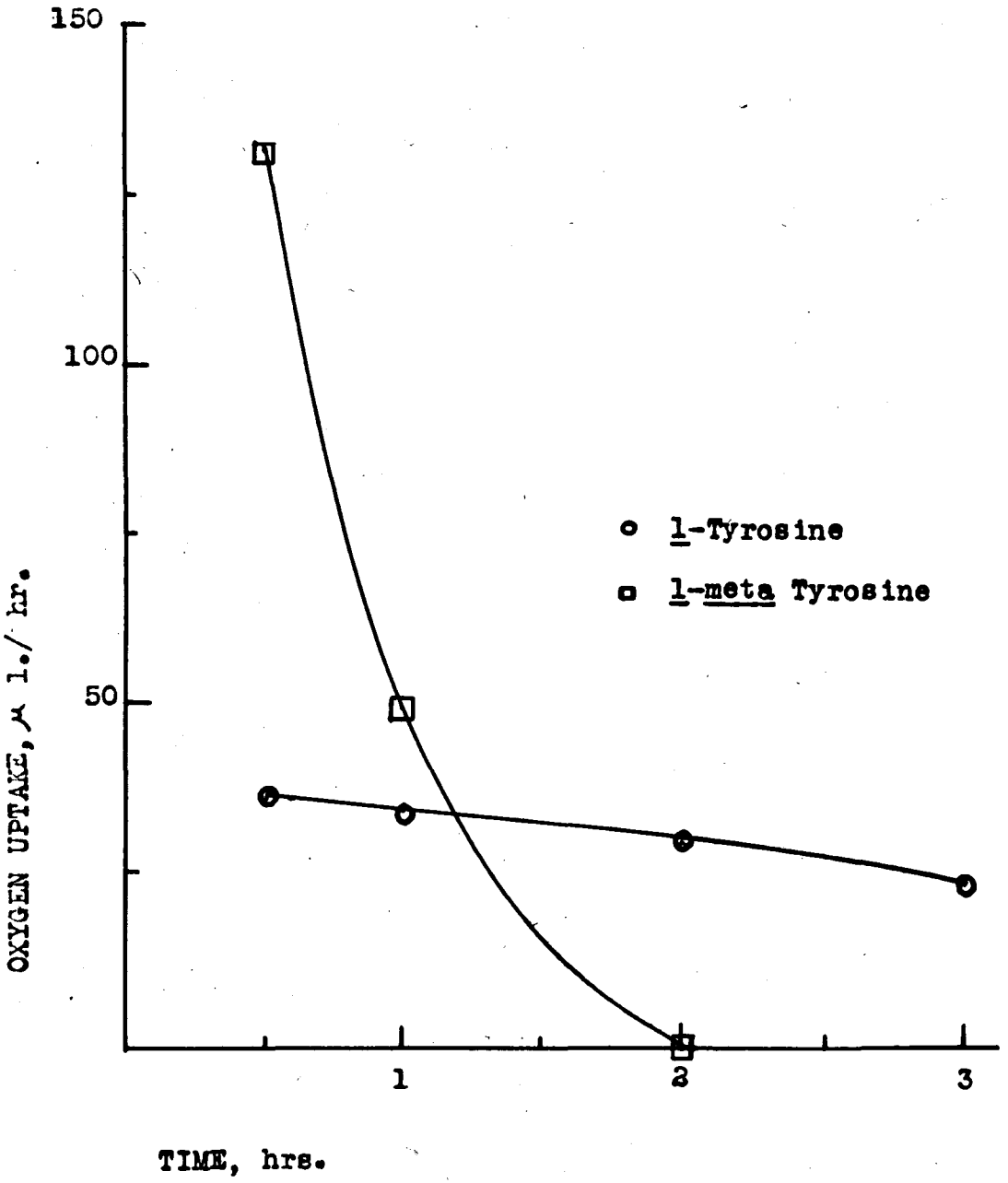


Fig. 4 The Rates of Oxygen Uptake Observed with l-Tyrosine and l-meta-Tyrosine with Normal Guinea Pig Liver Brei.

was the establishment of the atoms of oxygen taken up per mole of substrate added. As stated previously, this data has been summarized in table 10. It is evident from the table that a parallel existed between the O/T ratio observed for l-tyrosine and that found for l-meta-tyrosine, however, the values for the latter compound were consistently lower.

Because in these experiments the time was not sufficient for complete oxidation of the substrates, with the subsequent absorption of four atoms of oxygen per mole of l-tyrosine, analyses of the flask contents at the end of the incubation period was carried out to determine the amount of amino acid consumed. Two methods of analysis were used; the Folin-Ciocalteu (153) procedure and that of Theis and Benedict (154). The analytical data were interpreted in the following manner. The tyrosine value of the incubated tissue of the control flasks was determined through analysis of the deproteinized flask contents. This value was then subtracted from the colorimeter readings for each of the reaction flasks. Standards were prepared using tissue together with added tyrosine or tyrosine isomer. These standards were treated with metaphosphoric acid and diluted in the same manner as the reaction flask contents. The colorimeter value for the standard was corrected for tissue tyrosine through subtraction of the tyrosine value of an incubated tissue blank. The corrected tyrosine value of the reaction flasks divided by the corrected

Table 10

Oxygen Uptake Per Mole of Amino Acid Substrate

Guinea pig No.	gm. liver / mg. amino acid	Tyrosine O/T	m-Tyrosine O/T l-isomer	m-Tyrosine O/T d-isomer
		Normal Animals		
550	0.5	1.34	1.14	—[1]
552	1.33	1.16	0.785	—
553	1.33	1.35	1.05	0.324
555	1.0	1.61	1.27	
555	2.0	2.15	1.62	
558 560	1.0	5.92	1.22	
558 560	2.0	5.20	1.59	
561 562	1.0	2.26	1.12	
561 562	2.0	2.10	1.26	
557	1.0	1.62	1.01	
572	0.69	1.19	1.23	
Rat	1.334	2.19	0.712	0.679
		Scorbutic Animal		
551	0.5	0.436	0.283	

1. With d-meta-tyrosine the final reading was below the value of the control flasks.

standard value gave the fraction of tyrosine or tyrosine isomer which was not metabolized. From this the number of micromoles of amino acid metabolized could be determined. A sample calculation is shown below.

Incubated tissue, tyrosine value	38
l-Tyrosine reaction flask value	133
l-Tyrosine plus tissue, standard	294
Unincubated tissue, tyrosine value	132

133-38 = 95 Corrected tyrosine reaction flask value

294-132 = 162 Corrected tyrosine standard

$$\frac{95}{162} \times \underset{(5.52)}{\text{micromoles tyrosine}} \times \underset{(10)}{\text{dilution factor}} = \text{Tyrosine not Metabolized}$$

The micromoles of tyrosine not metabolized subtracted from 5.52 thus gave the number of micromoles metabolized and a corrected O/T ratio could be determined. The same general type of computation was carried out with the Theis-Benedict procedure. The O/T ratios are shown in table 11.

As the table shows, the corrected O/T ratios were in most cases high. The expected O/T ratio, from the work of Felix and Zorn (131,132) and the Bernheims (129) would be four. The average value with the Folin-Ciocalteu procedure was 5.02 while the Theis-Benedict method gave an average of about 10.33 atoms of oxygen absorbed per mole of tyrosine isomer metabolized.

Table 11

Comparison of O/T Ratios of l-meta-Tyrosine

Guinea pig no.	O/T Uncorr.	O/T Folin-Ciocalteu	O/T Theis-Benedict
552	1.14	6.77[2]	23.4[2]
552	.785	2.14	5.45
553	1.05	4.79	4.21
555	1.27	4.99	4.21
555	1.62	4.68	13.5
558 560	1.22	6.71	13.30
558 560	1.59	7.53	5.33
561 562	1.12	5.41	16.2
561 562	1.26	5.08	5.36
557	1.01	5.18	13.80
572	1.23	6.30	20.2
551[1]	.283	2.14	5.45
Rat	.712	3.96	3.89

1. Scorbutic animal.

2. All values are averages of two flasks.

As an explanation for the high averages the most logical supposition appeared to be that the oxidation of m-tyrosine by the liver brei produced a substance which gave high values

with both analytical procedures. Possible oxidation products to consider included the various dihydroxyphenylalanines. Thus, the flask contents in one experiment, after deproteinization and dilution according to the procedure used for the tyrosine analyses, were analysed for 3,4-dihydroxyphenylalanine by the Arnow procedure (154) and for 2,5-dihydroxyphenylalanine by the Brigg's method for hydroquinone determination (156). The analyses were carried out with 1 ml. portions of the diluted deproteinized flask contents and were in both cases negative. Blank determinations carried out using some of the centrifugates together with added 3,4-dihydroxyphenylalanine and hydroquinone gave the full value for the added substances which indicated the reagents used in deproteinization did not interfere with the analyses.

A major point of difference in metabolic behavior between l-tyrosine and l-meta-tyrosine, which has not been brought out in the tables, concerns the fact that at the end of one hour the oxygen uptake due to metabolism of m-tyrosine had stopped while l-tyrosine metabolism was still taking place at the end of three hours. This was a general finding through most of the runs. Thus the agreement in O/T ratios found in these experiments may not be so close when an O/T ratio of four for tyrosine is observed. On the other hand, the similarity observed in the metabolism of l-tyrosine and l-meta-tyrosine by normal guinea pig liver brei was also observed in

an experiment where scorbutic brei was used. The run with a scorbutic animal gave an O/T ratio approximately 23% of the average ratio observed with normal animals. From this result it appeared l-meta-tyrosine, like l-tyrosine, was dependent upon an adequate supply of vitamin C for its metabolism by the enzyme system of the liver.

The run with rat liver was carried out in the hope a higher O/T ratio than that observed with guinea pig brei might be observed. It has been shown that rat liver has a more active metabolic system for tyrosine than the guinea pig. The rat experiment, however, gave a lower O/T than the average of the guinea pig runs. Application of the Theis-Benedict and Folin-Ciocalteu analytical methods for tyrosine gave corrected O/T ratios which were very close to four.

From the experiments discussed, it was apparent that l-meta-tyrosine was metabolized by normal and scorbutic liver brei in a manner which paralleled the behavior of l-tyrosine under similar conditions. Although the obvious conclusions which could be reached have been presented, the field of theoretical speculation these screening experiments have opened up will be explored in another section of the thesis.

F. Investigation of Dakin's 2-Methyl-4-(2'-acetoxybenzal)-5-oxazolone

Concurrent with studies on the synthesis of the isomers

of meta-tyrosine, methods for the preparation and resolution of ortho-tyrosine were investigated. In general, the objections to methods used by earlier investigators for the preparation of m-tyrosine held equally well for the ortho isomer. Thus, it was our goal to determine how the amino acid might be prepared in reasonable yield, on a moderate scale, and with a minimum amount of manipulation.

The direct preparation of N-acetyl-o-tyrosine seemed possible through the conversion of 2-methyl-4-(2'-acetoxybenzal)-5-oxazolone to the corresponding cinnamic acid followed by catalytic reduction. The preparation of the desired azlactone had been reported by Dakin (157). The usual type of Erlenmeyer reaction, using acetylglycine, however, in place of the more commonly used hippuric acid, was employed.

2-Methyl-4-(2'-acetoxybenzal)-5-oxazolone attempted synthesis. In a round-bottomed flask were mixed 122 gm. (1.0 mole) of salicylaldehyde, 117 gm. (1.0 mole) of acetyl glycine, 82 gm. (1.0 mole) of fused sodium acetate, and 306 gm. (3.0 moles) of acetic anhydride. After three hours on a boiling water-bath, needle crystals began to separate. The flask was cooled, and 100 ml. of cold water worked into the mixture. The mass of long fine needles was filtered after twelve hours, washed well with water, and air dried. The yellow-orange needles melted at 203-205° which agreed with Dakin's observation. The yield was 65 gm.

In seeking to convert this supposed azlactone to the corresponding cinnamic acid through treatment with alkali and careful neutralization in the cold, white needle crystals identical, except for color, in appearance with the starting material were obtained. These also melted at 203-205° and were obtained by Dakin on treatment of the same so-called azlactone with alkali. He considered the substance to be 3-acetamidocoumarin and the analyses and properties were in agreement. From simple observation it appeared to us that the so-called azlactone and the coumarin were identical. This was supported by a mixed-melting point, which showed that the original product of the condensation reaction mixed with the product obtained after solution in alkali melted at 203-205°.

The color of the original condensation product was found to be readily removed through washing with alcohol. In addition it was found that the original condensation product analysed correctly for the composition of 3-acetamidocoumarin.

Anal. Calculated for $C_{11}H_9O_3N$

N, 6.89%

Found N, 6.86, 6.80

Use of O-acetylsalicylaldehyde in attempt to prepare 2-methyl-4-(2'-acetoxybenzal)-5-oxazolone. It appeared possible that azlactone formation rather than coumarin synthesis might take place in reasonable yield if the starting material were

O-acetylsalicylaldehyde. Thus, this substance was condensed with acetyl glycine. The acetylated aldehyde was prepared according to the procedure of Malkin and Nierenstein (158). However, a yield of approximately 50% of acetamidocoumarin was obtained. The mother liquors did not yield any azlactone. Thus, the O-acetyl group was lost in the course of the reaction and its protective influence was no longer available.

Attempts to convert 3-acetamidocoumarin to N-acetyl-o-tyrosine. As the coumarin ring readily opened with dilute base to give the sodium salt of α-acetamido-o-hydroxycinnamic acid, experiments were carried out with the hope of reducing the double bond under alkaline conditions. Several different methods were tried.

Catalytic method- In 150 ml. of 5% sodium hydroxide was dissolved 20.3 gm. (0.1 mole) of 3-acetamidocoumarin. After the addition of 1 gm. of palladium-barium sulphate catalyst prepared according to Schmidt (159), the mixture was shaken with hydrogen in a Burgess-Parr reduction apparatus. The original gage pressure was 40 lbs.; after one hour no change was observed. Adding another gram of catalyst and heating the mixture to 60° did not effect reduction at 40 lbs. pressure.

Similar reduction attempts using a solution of the coumarin in sodium ethoxide were carried out using the palladium-barium sulphate catalyst and also platinum oxide. No evidence of reduction was obtained. A final trial seeking to reduce

the coumarin itself in acetic acid solution using palladium-barium sulphate catalyst gave no evidence of reduction.

Sodium amalgam method- An 18 gm. portion (0.088 mole) of coumarin derivative was dissolved in 200 ml. of 5% sodium hydroxide. Over a two hour period, 100 gm. of 3% sodium amalgam was added, which corresponded to about 0.15 mole of sodium. The color of the solution faded during the reaction period, but the oil isolated after acidification, extraction with butanol, and concentration did not crystallize. In view of the difficulty observed in later experiments in the crystallization of N-acetyl-m-tyrosine from water, even when quite pure, it was not surprising we did not obtain the desired product. A reduction carried out using sodium in butyl alcohol with a suspension of acetamidocoumarin was not successful.

Synthesis of trans-3-acetamido-o-hydroxycinnamic acid. A number of methods have been used for the conversion of coumarin derivatives to trans-cinnamic acids. However, one of the most recent methods was that of Seshadri and Roa (160) who prepared trans-o-hydroxycinnamic acid from coumarin in good yield through shaking coumarin in basic solution with a suspension of mercuric oxide. We adapted this method to the preparation of the desired substituted cinnamic acid.

Ten gm. (0.05 mole) of 3-acetamidocoumarin was dissolved in 100 ml. of 10% sodium hydroxide. To this red solution 1

gm. of mercuric oxide was added, and the mixture shaken vigorously for one-half hour. The mixture was filtered from the inorganic material and acidified. After standing several hours in the ice-box, a yield of 8 gm. (72%) of red tinted crystals was obtained. Recrystallization from water was not possible, as at 100° in water solution the product was converted to the coumarin, which separated as slender white needles. Thus, the material was purified through dissolving in 1 N sodium hydroxide, stirring up well with Norite at 85°, and reprecipitating with acid while cooling in an ice-bath. The product then melted at 165-167°, and gave a correct analysis for the nitrogen content of the expected substituted cinnamic acid.

Anal. Calculated for $C_{11}H_{11}O_4N$

N, 6.33%

Found N, 6.31, 6.24

2-Methyl-4-(2'-acetoxybenzal)-5-oxazolone. To establish with certainty that Dakin did not have an azlactone as the product of the condensation of acetyl glycine with salicylaldehyde it was considered pertinent to synthesize the oxazolone. The properties of the authentic material could then be compared with those given for Dakin's "azlactone", which our results indicate was probably a coumarin derivative. As direct condensations did not give the azlactone, the molecule was prepared through the action of acetic anhydride and sodium

acetate on trans-α-acetamido-o-hydroxycinnamic acid. The product of the reaction was a yellow crystalline compound which analysed correctly as the azlactone and melted at 133-135°, in contrast to the melting point of 203° given for the molecule by Dakin.

A mixture of 5 gm. (0.025 mole) of trans-α-acetamido-o-hydroxycinnamic acid, 15 ml. of acetic anhydride, and 1 gm. of sodium acetate was refluxed for one and one-half hours. The reaction mixture was cooled, diluted with an equal volume of cold water, and placed in the ice-box. The yield of crude product was quantitative, 6.1 gm. Recrystallization of the material from dilute alcohol gave yellow plate crystals which melted at 133-135°. The material was insoluble in dilute alkali and gave a negative Millon's test in the cold.

Anal. Calculated for $C_{13}H_{11}O_4N$

N, 5.71%

Found N, 5.65, 5.57

Catalytic reduction of trans-α-acetamido-o-hydroxycinnamic acid. Although prepared by a different series of reactions than originally planned, the desired intermediate for hydrogenation to N-acetyl-o-tyrosine was obtained.

A reduction bottle was partly filled with a solution of 10 gm. (0.05 mole) of the cinnamic acid in 150 ml. of glacial acetic acid. After the addition of 0.25 gm. of platinum oxide catalyst, the mixture was shaken for two hours starting with

a gage pressure of 35 lbs. The pressure dropped 12 lbs., which indicated an absorption of 0.07 mole of hydrogen. The catalyst was filtered off, and an equal volume of water added to the filtrate. The mixture was concentrated *in vacuo* and the oil which remained recrystallized from 20 ml. of water. Granular crystals which melted at 83-85° were isolated in 25% yield (3 gm.). The product gave a strong Millon's test in the cold, which indicated hydrogenation of the nucleus could not have taken place. The product was found to be hydrated when a weighed sample was dried in the drying pistol. The nitrogen determined by analyses agreed well with the theoretical value for N-acetyl-*o*-tyrosine.

Anal. Calculated for $C_{11}H_{13}O_4N \cdot H_2O$
N, 5.80%; H_2O, 7.47%
Found N, 5.82, 5.82; H_2O, 7.23

G. The Synthesis of *ortho*-Tyrosine and Resolution Studies

Outlined in equation form in figure 5 is the method made use of in section F to prepare N-acetyl-*o*-tyrosine. As we were not satisfied with the yields obtained, particularly in the reduction step, other methods were simultaneously investigated. Subsequent developments showed *o*-tyrosine could be prepared in excellent yield through a modified Erlenmeyer synthesis. This method can also be seen in the following figure. To avoid the difficulty of coumarin formation in the

azlactone synthesis, the methyl ether of salicylaldehyde was made use of according to the procedure of Bergel, Haworth, Morrison, and Rinderknecht (161).

2-Phenyl-4-(2'-methoxybenzal)-5-oxazolone. A mixture of 68 gm. (0.5 mole) of o-methoxybenzaldehyde, 98.5 gm. (0.5 mole) of hippuric acid, 41 gm. (0.5 mole) of sodium acetate and 153 gm. (1.5 moles) of acetic anhydride was shaken until homogeneous. The material was then heated four hours in a boiling water-bath. The flask, now filled with a dense mass of yellow crystals, was cooled and 200 ml. of 95% alcohol added in one hour in small portions. The ice-cold mixture was filtered after twelve hours, washed with several portions of alcohol and water, and then air dried. The yield was 96 gm. or 68.5% of theory. As the product melted at 167° in agreement with the value given by Bergel and coworkers it was used without further purification.

dl-ortho-Tyrosine. A solution of 50 gm. (0.18 mole) of 2-phenyl-4-(2'-methoxybenzal)-5-oxazolone in 250 ml. of glacial acetic acid was mixed with 250 ml. of hydriodic acid (sp. g. 1.50) and 10 gm. of red phosphorus. The mixture was refluxed in an all-glass apparatus for six hours, using an oil-bath at 150-165°. The reaction mixture was filtered hot through a sintered-glass funnel, and the phosphorus residue washed with two 20-ml. portions of glacial acetic acid. The filtrate was concentrated under reduced pressure, and the

mixture of benzoic acid and amino acid salt taken up in 250 ml. of water and 100 ml. of ether. The ether layer was separated, and the water layer extracted twice more with 100 ml. portions of ether. The solution was then again taken to dryness under reduced pressure, and the residue freed from excess hydriodic acid through reconcentration with several portions of water under vacuum. The crystalline salt was dissolved in 50 ml. of boiling water, and the solution carefully neutralized with ammonium hydroxide. A heavy precipitate separated at once, and 100 ml. of ethyl alcohol was added to complete the separation.

After twenty-four hours, the amino acid was filtered with suction and washed with three 25-ml. portions of ice-water followed by two 50-ml. portions of alcohol. The product was dried through a final washing with ether and allowed to stand in air until all odor of solvent vanished. Some lots melted at this stage at the Blum value of 252° with decomposition. However, the yield at this point was quite often very close to theoretical, in which case the melting point was quite a bit lower. This indicated some contamination. Purification of 35 gm. of crude product through solution in 100 ml. of 10% sodium hydroxide, addition of 5 gm. of Norite, and filtration gave, after careful acidification, 22 gm. of pure amino acid. This was a yield of 68.7%. Concentration of the filtrate gave another gram of pure material for a total yield of 72%.

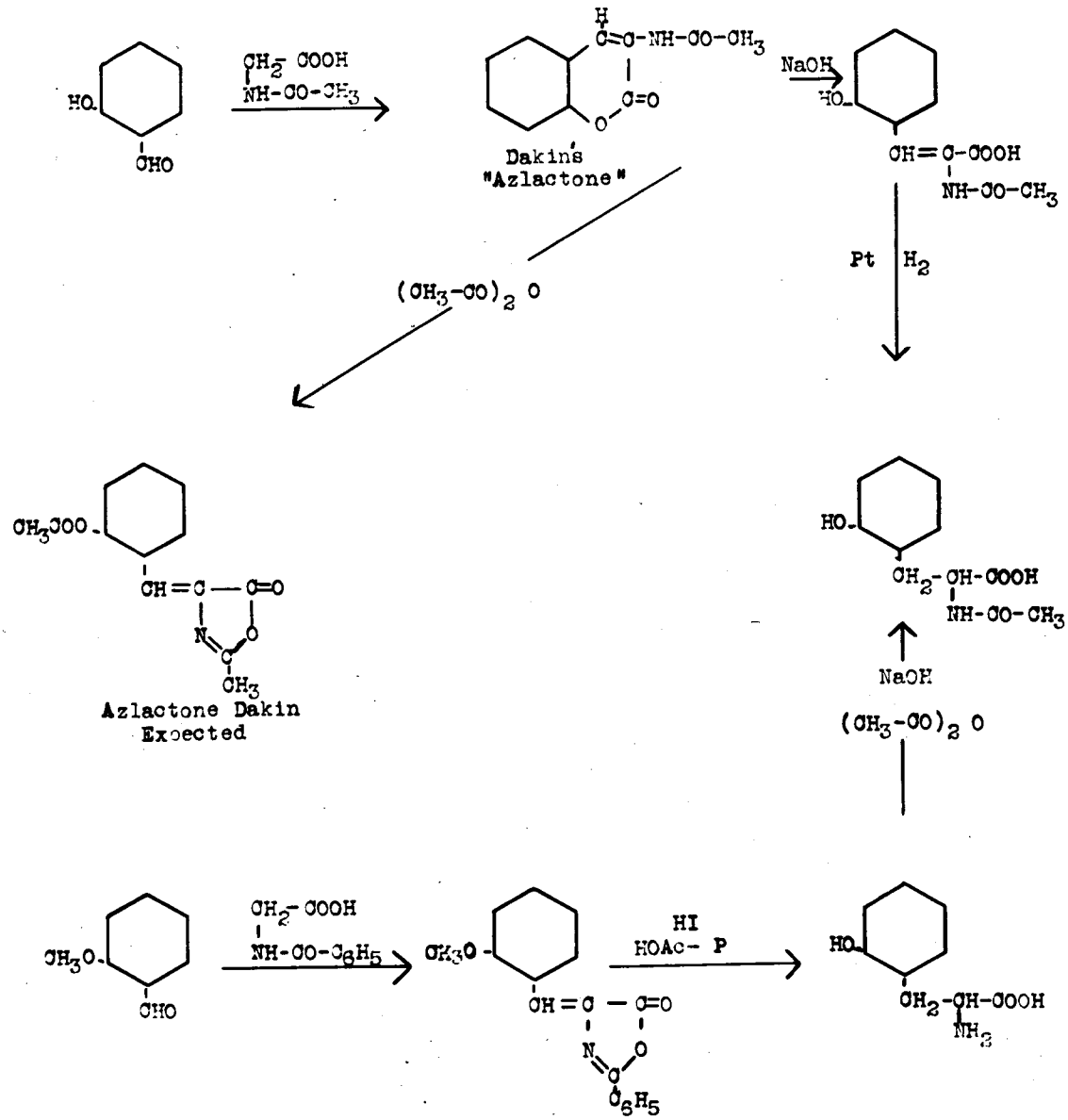


Fig. 5 The Synthesis of ortho-Tyrosine and Proof of Structure of Dakin's "Azlactone"

Yields of this order were consistently observed in a series of runs.

Anal. Calculated for $C_9H_{11}O_3N$

N, 7.73%

Found N, 7.85, 7.87

Reaction of 3-acetamidocoumarin with hydriodic acid and red phosphorus. The literature did not give information in regard to what might be expected from the reaction of an acetamidocoumarin with hydriodic acid. It appeared possible that reduction of the double bond might take place, in addition to the hydrolysis of the acetyl group, to give the inner anhydride of o-tyrosine. If successful, the reaction would allow one to prepare the amino acid starting with the more readily available salicylaldehyde instead of the methyl ether.

Using an all-glass apparatus, 10 gm. (0.05 mole) of 3-acetamidocoumarin and 5 gm. of red phosphorus were refluxed for two hours with 60 ml. of hydriodic acid (sp. g. 1.50). The mixture was filtered, and some organic material in the red phosphorus extracted with hot ethyl alcohol. When the alcohol evaporated the oil remaining crystallized at once. The material crystallized from water as long slender white needles which melted at 150-152°. The yield was 4 gm. The product was soluble in base but insoluble in acid. The elemental test for nitrogen was negative.

A product identical in all respects with the product

thus obtained was prepared by Erlenmeyer and Staldin (162). They prepared a compound, 3-ketocoumarin, through the alkaline hydrolysis of 3-benzoylamidocoumarin. The compound melted at 152°, produced a green color with ferric chloride as did the product from our reaction and formed a phenylhydrazone which melted at 173-174° as did the phenylhydrazine derivative of the product of our hydrolysis.

Phenylhydrazone of 3-ketocoumarin- in 10 ml. of water was suspended 1.4 gm. of 3-ketocoumarin. To this was added 1 gm. of phenylhydrazine hydrochloride and 3 gm. of sodium carbonate. This mixture was heated 20 minutes on a boiling water-bath. Acidification with acetic acid yielded yellow-brown crystals which melted at 173-174°. Erlenmeyer and Stadlin obtained the same melting-point.

N-Formyl-dl-ortho-tyrosine. Using the same procedure as given for the formylation of m-tyrosine, 18 gm. (0.1 mole) of o-tyrosine in 174 ml. of formic acid was reacted with 58 ml. of acetic anhydride at 45-65°. Concentration and recrystallization following the previously outlined method gave 12 gm. of crystals as a first crop. The product melted at 135-137°. The mother liquors yielded 5 gm. more of product for a total return of 81%.

Anal. Calculated for $C_{10}H_{11}O_4N$

N, 6.69%

Found N, 6.68, 6.68

Attempted resolution of N-formyl-o-tyrosine. Solutions containing 0.209 gm. (0.001 mole) of formyl-o-tyrosine and 0.394 gm. (0.001 mole) of brucine were prepared in various solvents using a slight excess over the minimum needed to bring all material into solution at the boiling point. The solutions were then cooled in an ice-salt bath. The crystals which formed were filtered and dried to constant weight in a Fischer drier. Rotations were then taken on the various fractions and these are recorded in table 12.

It was quite evident that the usual solvents employed in the resolution of an aromatic amino acid as the brucine salt were of no value in this application. It appeared that a racemic compound or mixture separated from both alcohol and water as the rotations of the crystals were identical, within limits of experimental error, with the rotation of the equilibrium mixture. In the cases where some separation of isomers seemed to occur the yields of material were very small, thus resolution using those solvents did not seem feasible.

N-Acetyl-dl-ortho-tyrosine. As the formyl derivative failed to offer promise as a derivative suitable for resolution, the N-acetyl derivative was prepared using the same procedure as in our earlier attempt to acetylate m-tyrosine. Forty gm. (0.22 mole) of o-tyrosine in 117 ml. of 2 N sodium hydroxide was acetylated through reaction with 55.2 ml. (0.58 mole) of acetic anhydride and 552 ml. of 2 N sodium hydroxide.

Table 12

Rotations of Brucine Salts of N-Formyl-o-Tyrosine

Solvent	Recrystallizations	Rotation
	Equilibrium mixture[a]	-17°
95% ethanol	1	-17
95% ethanol	2	-17.4
ethyl acetate[b]	1	-18
abs. alcohol	1	-18
butyl alcohol[c]	1	-20
95% ethanol[d]	1	-16.1
water[e]	1	-16.9

a. The equilibrium mixture was a solution of equimolecular amounts of brucine and formyl-o-tyrosine.

b. A large volume of solvent was needed and the yield was very low.

c. The yield was less than 10%.

d. This was a recrystallization of the ethyl acetate soluble material obtained on removal of the solvent under reduced pressure.

e. The material crystallized from water after a month in the ice-box.

The reaction mixture, after acidification with 240 ml. of 6 N sulfuric acid, was concentrated to dryness; the residue extracted with acetone, and the oil remaining after evaporation of the acetone under reduced pressure recrystallized from 100

ml. of water. The yield was 42.8 gm. of crystals (88%) which melted at 85-86° and were identical in all respects with the N-acetyl-o-tyrosine monohydrate prepared earlier through the catalytic reduction of trans-α-acetamido-o-hydroxycinnamic acid. The crystals prepared by the two methods melted undepressed when mixed.

Resolution of N-acetyl-dl-ortho-tyrosine as the brucine salt. In preliminary investigations using 0.001 molar portions of acetyl-o-tyrosine and brucine the only solvent from which crystalline material could be isolated was 95% alcohol. Absolute ethyl alcohol, methyl alcohol, and water failed to give crystals after weeks in the cold. The rotation of the fraction obtained from alcohol was $(\alpha)_D^{26} = -22.1^\circ$. As a mixture of brucine and acetyl-o-tyrosine in solution in equimolecular proportions gave a rotation of $(\alpha)_D^{26} = -17.3^\circ$, it appeared some separation of isomers was taking place. On a larger scale, crystallization and recrystallization of the brucine salt was repeated until a constant maximum value was reached.

A solution of 10 gm. (0.041 mole) of N-acetyl-o-tyrosine and 16.3 gm. (0.041 mole) of brucine in 104 ml. of 95% ethyl alcohol was allowed to cool to room temperature slowly and then was placed in the ice-box. The crystallization took place on the sides of the flask and these were scraped off with a stirring-rod daily. After a week, the crystals were

filtered with suction and air-dried. A portion of each crystallization was dried to constant weight and the rotation determined. Each subsequent recrystallization was carried out in a manner similar to the first using 3 to 4 volumes of 95% ethyl alcohol. The rotations of the fractions and the yields obtained are shown in the table. The rotations of the last three fractions were constant within limits of experimental error.

Table 13

Rotations of the Brucine Salt of Acetyl-o-Tyrosine on Recrystallization from Ethyl Alcohol

Crystallizations	Yield		Concentration	Rotation
	gm.	%	%	°
1[a]	13.3	100	0.924	-21.0
2	10.0	75	1.010	-23.8
3	7.5	56	1.218	-26.2
4	5.6	42	0.571	-26.8
5	___		0.559	-26.9
6	3.6	27	0.683	-27.1

a. All rotations were taken in water. The temperature varied from 25-27°.

A second resolution using 25 gm. of acetyl-o-tyrosine was carried out and after two recrystallizations the rotation

reached -27°. A third recrystallization yielded material with the same optical activity. The yield of brucine salt in this case was 23 gm. or 69% of the theoretical amount. The yield in the resolution reported in table 13 was 27.4%.

Preparation of dextrorotatory o-tyrosine from the alcohol insoluble brucine salt. Twenty-three gm. of the air-dried brucine salt was dissolved in 450 ml. of water and the solution warmed to 40°. From a burette was added 22 ml. of 2 N sodium hydroxide. At this point the material was basic to phenolphthalein indicator. After standing twelve hours in the ice-box, the mixture was filtered from precipitated brucine. The brucine was washed with eight 50-ml. portions of water with care to stir the precipitate well with each portion of solvent. The combined filtrates were neutralized with 7.33 ml. of 6 N sulfuric acid, after they had been extracted with five 25 ml. portions of chloroform. The water was removed under reduced pressure, and the N-acetyl-d-ortho-tyrosine extracted with five 100 ml. portions of moist acetone. The acetone was removed under reduced pressure, and the residue dissolved in 100 ml. of boiling 20% hydrochloric acid. The solution was refluxed for two hours, concentrated in vacuo, and reconcentrated with water to remove all excess of hydrochloric acid. The oil remaining was taken up in 15 ml. of boiling water and the resulting solution carefully neutralized at the boiling point with concentrated ammonium hydrox-

ide. Crystallization began at once at the neutral point and was completed through several days standing in the cold. The product was filtered off and washed with a small amount of ice-water, alcohol, and ether. Microscopically viewed, the crystals were beautifully formed plates. These melted at 239-240° with decomposition. The yield was 6.2 gm., which was 63% of the amount of isomer theoretically isolable. The rotation of the compound will be discussed in detail in a later section. However, an approximately one percent solution in water gave a specific rotation of +57.3°.

Anal. Calculated for $C_9H_{11}O_3N$

N, 7.73%

Found N, 7.69, 7.82

Investigation of the alcohol soluble brucine salt. The filtrates from the alcohol recrystallizations of the brucine salt were combined and concentrated to dryness under reduced pressure. The heavy oil which remained was tested with a number of solvents in attempts to induce crystallization. Water, methyl alcohol, ethyl alcohol, absolute ethyl alcohol, butyl alcohol, and ethyl acetate were tried under a variety of conditions. Of these only ethyl acetate deposited crystalline material, and from this solvent the crystallization was slow and the yield poor. Of 16.5 gm. of isomer theoretically isolable from the alcohol soluble fraction obtained from two resolutions, 3.0 gm. of material was obtained after

four recrystallizations. Even at this point the salt gave a rotation which showed some contamination by the other isomer. Table 14 shows the rotations observed on fractionation of the material from ethyl acetate.

Table 14

Rotations of Brucine Salt Fractions Obtained From Ethyl Acetate

Recrystallization	Conc.	Rotation
	%	°
1	1.05	-14.8
2	1.17	-13.2
3	.70	- 9.8
4	1.04	- 9.36
Calculated[1] goal	—	- 7.5

1. This was calculated from the maximum rotation observed with the ethyl alcohol fraction and the observation that a mixture of equal molecular amounts of brucine and the N-acetyl-dl-o-tyrosine gave a specific rotation of $(\alpha)_D^{26} = -17.30°$.

It was found difficult to get all the salt into solution at each recrystallization. Even with 2,000 ml. of solvent for 10 gm. of salt a residue remained which refused to dissolve in a fresh portion of boiling solvent. Also, it was necessary to concentrate the 2 l. of solvent to 300 ml. before a reasonable amount of material separated.

To compare the material from the ethyl acetate fractionation with that from ethyl alcohol, a portion with the lowest rotation was split to give the N-acetyl derivative and finally hydrolyzed to the amino acid.

Approximately 2.5 gm. of the brucine salt with a specific rotation of -9.36° was dissolved in 25 ml. of water at 40°. This was made basic with 2 ml. of 2 N sodium hydroxide and cooled overnight. The mixture was filtered, and the precipitate washed with 250 ml. of water in five portions. The filtrate was freed from brucine through extraction with five 20-ml. portions of chloroform. The solution was concentrated to 100 ml., and, after addition of 10 ml. of concentrated hydrochloric acid, refluxed for 12 hours. The mixture was then evaporated <u>in vacuo</u> and the residue dissolved in 10 ml. of water. Neutralization yielded a precipitate of light brown plate crystals which melted at 236° with decomposition. The material, when dissolved in water, gave a specific rotation of —51.9°. This indicated that about 10% racemic compound was present, as the isomer from alcohol gave a rotation of +57.3°. Two recrystallizations of a portion of the material obtained were carried out through solution of the material in a moderate volume of water and concentration <u>in vacuo</u> until crystallization was evident. The material was then cooled and the plate crystals filtered off. A final yield of 60 mg. was used for determination of rotation and although a

rotation of -57° was observed the error of observation at that concentration could well be $\pm 3^{\circ}$.

The crystals analysed correctly as o-tyrosine.

Anal. Calculated for $C_9H_{11}O_3N$

N, 7.73%

Found N, 7.81, 7.84

Configuration of the o-tyrosine isomers. Information in regard to the configuration of the optically active isomers of o-tyrosine isolated was obtained through application of the Lutz and Jirgenson procedure with the dextrorotatory material. Using the same concentrations of amino acid as used with meta-tyrosine isomers the rotation was determined in water and with one and four moles of acid present for each mole of amino acid. The rotation in water was $(\alpha)_D^{26} = +57.3^{\circ}$, with one mole of acid the rotation was $+32^{\circ}$, with four moles the rotation dropped to $+23.1^{\circ}$. Unfortunately sufficient of the other isomer was not obtained to confirm these observations. However, the information obtained showed that the isomer obtained from alcohol gave a decreasing positive rotation as the amount of acid added was increased. Thus, that isomer possessed the unnatural or dextro configuration.

This indicated that the isomer obtained in sufficient quantity for biochemical investigation was the d- isomer. As it appeared that our methods would not yield enough of the l-isomer for metabolic testing the d-isomer was saved for

later application in parallel experiments.

Use of cinchonine in o-tyrosine resolution. As the brucine salt could not be considered satisfactory in the resolution of o-tyrosine as the formyl or acetyl derivative, the possible use of cinchonine was investigated. A series of solutions was prepared using 0.294 gm. of cinchonine and 0.209 gm. of formyl o-tyrosine. The mixtures, which thus contained 0.001 mole of each constituent, were then dissolved in various solvents. Water, ethyl and methyl alcohol were investigated. The only solvent which gave any evidence of crystallization after several weeks in the cold was water. The amount of solid material, however, was only a few milligrams and the method was not considered worth further study.

Cinchonine was also studied with acetyl-o-tyrosine with approximately the same result. Water only gave a faint precipitate of material and the amount was too little to warrent investigation.

Action of the d-amino acid oxidase with o-tyrosine. As reasonable amounts of both the d and l isomers could not be prepared by the resolution methods studied, the possibility of obtaining pure l-o-tyrosine through the action of the d-amino acid oxidase on racemic o-tyrosine was investigated.

Two and four-tenths gm. of desiccated kidney tissue was stirred up with 12 ml. of 0.066 molar pyrophosphate buffer. The homogeneous suspension was diluted to 64 ml. with buffer

(pH 8.3) and stirred thirty minutes at 35-40°. The thick slurry was centrifuged and the supernatent liquid filtered through cotton into an Erlenmeyer flask of liter capacity. To the enzyme preparation was added 1.81 (0.01 mole) of o-tyrosine in 50 ml. of water adjusted to a pH of 8.3 with dilute sodium hydroxide. This mixture was aerated with pure oxygen for six hours while incubated at 35-40°. The progress of the reaction was followed through analysis of the reaction mixture for keto acid by the previously discussed 2,4-dinitrophenylhydrazine procedure. Some increase in value was noted, but it corresponded to only a 2 or 3% conversion of the d-amino acid present. Deproteinization of the reaction mixture and concentration of the filtrate gave crystals of amino acid with a rotation of $(\alpha)_D^{26} = -0.66°$.

A second run using 12 gm. of tissue with the same amount of dl-o-tyrosine was carried on as before for six hours. Analyses for keto acid indicated approximately 3% of the amino acid present had been acted upon by the enzyme. Thus, a second enzyme preparation was carried out using 6 gm. of desiccated tissue. This was added to the original reaction mixture and incubation continued for eight hours. Keto acid analyses indicated no further reaction was taking place. In spite of these negative indications the reaction mixture was deproteinized and concentrated. On adjustment to the neutral point no amino acid separated although sodium phosphate crys-

tals slowly formed in the cold. Several fractions of inorganic crystals were filtered off but no amino acid was obtained.

The method used by us was satisfactorily applied by Behrens (163) for the preparation of l-alanine from the racemic mixture. Our experiments appeared to indicate that o-tyrosine had a very slow rate of reaction with the enzyme. The reaction of the oxidase with aromatic amino acids may be too slow in most cases to be applicable for preparative purposes.

H. Approaches to the Preparation of 2,5-Dihydroxyphenylalanine

In the historical section we noted the fact that although this amino acid had been prepared by three groups of investigators contradictory properties were recorded. In particular, a melting point of 203° was given by Hirai (87) and by Freedman (88) who both prepared the amino acid through the diketopiperazine method. Schaaf and Labouchère (89) used the original Erlenmeyer method, which involved four steps, to obtain an amino acid which melted at 242°.

We have also called attention to why this amino acid has extensive biochemical interest, and, in particular, its relationship to the problem of tyrosine metabolism. In spite of the interest, however, 2,5-dihydroxyphenylalanine has not been studied metabolically. The difficulties involved in the synthesis of the molecule offer the only explanation for the

small amount of biochemical work done with the substance.

Both ortho-and meta-tyrosine were readily obtained in our experiments through the hydriodic acid reductive hydrolysis of suitable azlactones. Thus we set out to determine the applicability of this procedure to the preparation of 2,5-dihydroxyphenylalanine. The reactions used are diagrammed on the following page.

Preparation of 2,5-dimethoxybenzaldehyde. As this aldehyde did not appear readily available through literature methods, one of the more recently developed synthetic procedures for synthesis of phenolic aldehydes was investigated. Wood and Best (164) found N-methyl-formanilide reacted with phenols of the naphthalene series to yield substituted naphthaldehydes. We tried the Wood and Best method with hydroquinone dimethyl ether.

Using an all-glass apparatus a mixture of 13.8 gm. (0.1 mole) of hydroquinone dimethyl ether was reacted with 16 gm. (0.1 mole) of N-methyl-formanilide and 18 gm. (0.1 mole) of phosphorus oxychloride. This mixture was heated six hours in a boiling water-bath and then poured slowly into 100 ml. of 20% hydrochloric acid. After the mixture had stood one hour, it was steam distilled. Unreacted hydroquinone dimethyl ether distilled over rapidly with the first 200 ml. of water. When a ml. of distillate yielded only needle crystals and no plates on cooling in an ice-bath for a minute, the receiver

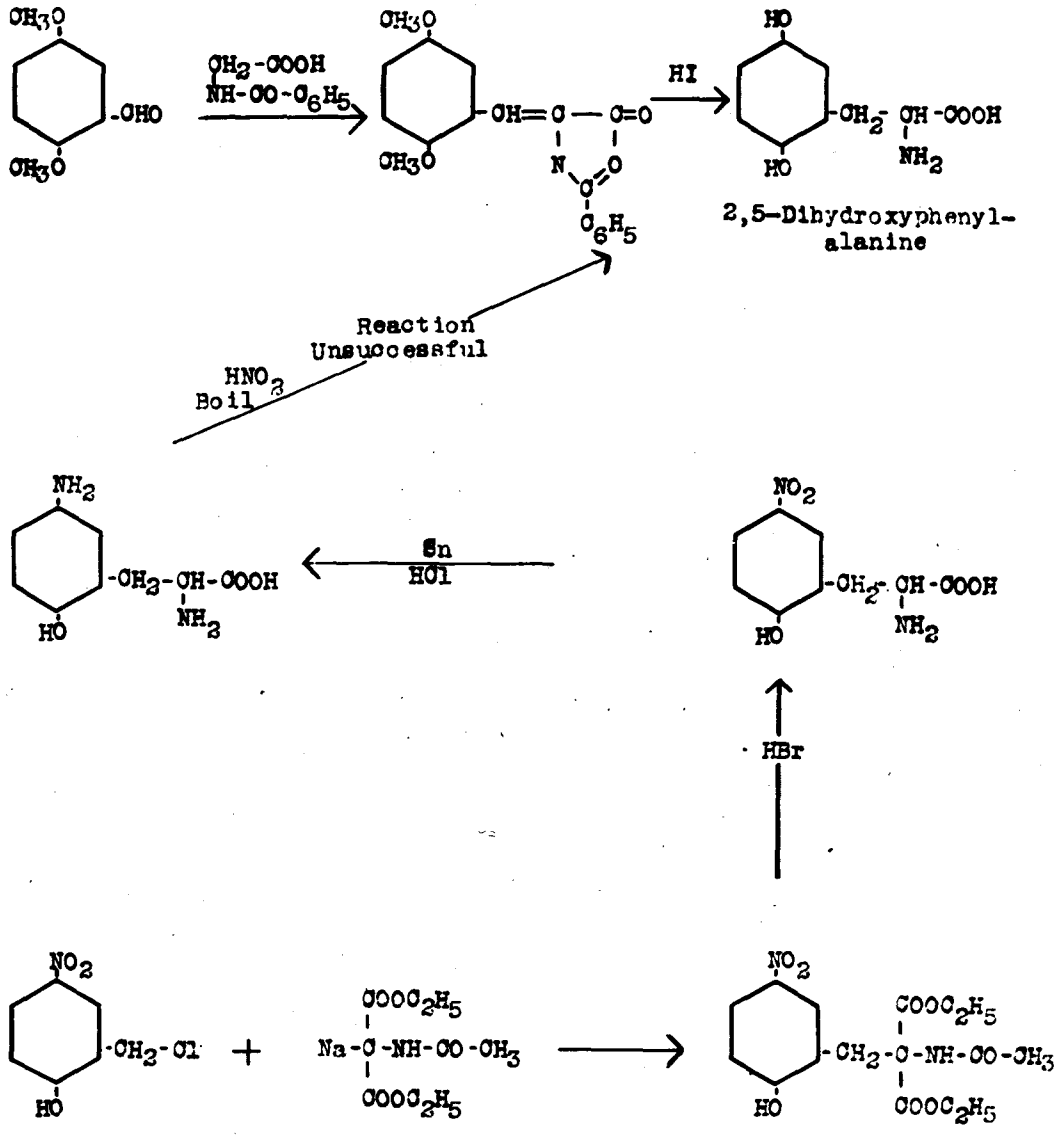


Fig. 6 Synthesis of 2,5-Dihydroxyphenylalanine by the Erlenmeyer Method and Attempted Synthesis Using Ethyl Acetamidomalonate

was changed. Two liters of distillate gave 2 gm. of long needle crystals, while 1.5 gm. was obtained through ether extraction of the filtrate. The yield was thus 21%. The crystals melted at 51-52°. The crystals melted undepressed with an authentic sample prepared by the modified Gattermann procedure which we will next discuss.

As this procedure offered only enough product for some preliminary studies we found the method of Gulland and Virden (165), although tedious and laborious, was more satisfactory for the quantities needed.

Preparation of 2,5-dimethoxybenzaldehyde by the modified Gattermann procedure. The Gattermann reaction in its original form involved the use of anhydrous hydrogen cyanide. Adams and Levine (166) and Adams and Montgomery (167) simplified the method through substitution of zinc cyanide for the hydrogen cyanide. Gulland and Virden, in turn, applied the Adams and Montgomery method to the synthesis of 2,5-dimethoxybenzaldehyde.

In a three-necked flask equipped with mechanical stirrer, reflux condenser, and a gas inlet tube were placed 50 gm. (0.36 mole) of hydroquinone dimethyl ether, 95 gm. (0.81 mole) of zinc cyanide and 350 ml. of sodium-dried benzene. This mixture was stirred mechanically while a rapid stream of dry hydrogen chloride was passed into the mixture. After twenty-four hours 150 gm. (1.1 moles) of anhydrous aluminum

chloride was added with vigorous stirring. The stream of hydrogen chloride was continued for another twelve hours, while the temperature of the reaction mixture was maintained at 50°. The benzene layer was decanted, and the heavy dark oil poured into a beaker filled with cracked ice. After standing over-night, the mixture was refluxed three hours in a good hood. The hot solution was then steam distilled and 8 l. of distillate collected. After cooling in an ice-salt bath the product was filtered off and the filtrates extracted with ether. A yield of 23.5 gm. (39%) was obtained. A run with an increased amount of aluminum chloride gave a lower yield.

2-Phenyl-4-(2'5'-dimethoxybenzal)-5-oxazolone. This intermediate was prepared according to the experimental details given by Gulland and Virden (165).

To 14.5 gm. (0.1 mole) of 2,5-dimethoxybenzaldehyde in a round-bottomed flask were added 23 gm. (0.13 mole) of hippuric acid, 38 ml. (0.4 mole) of acetic anhydride and 8.2 gm. (0.1 mole) of anhydrous sodium acetate. This mixture was shaken well and heated five hours in a boiling water-bath. About 35 ml. of ethyl alcohol was stirred into the cold reaction mixture. After twelve hours in the cold, the product was filtered off, washed with cold alcohol and water, and air-dried. The yield was 17.5 gm. (56%), and the product melted at 170-172° in agreement with the value given by Gul-

land and Virden.

Reaction of 2-phenyl-4-(2,5-dimethoxybenzal)-5-oxazolone with hydriodic acid. Ten gm. (0.032 mole) of the azlactone was refluxed for six hours with 50 ml. of hydriodic acid and 50 ml. of acetic acid along with 5 gm. of red phosphorus. The solution was filtered, concentrated, and neutralized as described in the synthesis of o-tyrosine and m-tyrosine. However, to prevent oxidation, several drops of water saturated with sulfur dioxide was added to the neutral mixture along with 25 ml. of ethyl alcohol. The yield of crude product was 3.4 gm. or 54%. Recrystallization of the material from 10 ml. of water with treatment with Norite yielded beautiful white prismatic crystals of ten sides which often grouped so as to form crosses. These melted at 242-243° which agreed with the value reported by Schaaf and Labouchère (89). The crystals gave a positive Millon's reaction and a positive ninhydrin test. A green color, which turned brown on standing, formed on addition of ferric chloride. In dilute base the product rapidly darkened, while Tollen's reagent was immediately reduced yielding a silver mirror in the cold. All the properties we observed agreed with those given for the molecule by Freedman (88) and Hirai (87). The only exception was the melting point. It appeared possible that the earlier workers had a somewhat impure preparation or else the compound might exist in polymorphic forms.

We found our product as obtained from water lost weight in the crying pistol corresponding to a little more than one mole of water of crystallization. The dried material also melted at 243°.

Anal. Calculated for $C_9H_{11}O_4N$

N, 7.10%

Found N, 6.97, 6.91

Preparation of the hydantoin of 2,5-dihydroxyphenylalanine. A suspension of 0.19 gm. (0.001 mole) of 2,5-dihydroxyphenylalanine in 5 ml. of water was heated to boiling and 0.4 gm. (0.05 mole) of potassium cyanate added in small portions after which the solution was boiled five minutes. The solution was then acidified with 3 ml. of 10% hydrochloric acid. After boiling the light brown solution for 15 minutes, small crystals could be seen in the solution. The reaction mixture was cooled and the fine crystalline precipitate recrystallized from 10 ml. of water with the use of Norite to remove a small amount of color. On cooling fine crystals separated which melted at 244-246°. These were dried in the drying pistol and analysed.

Anal. Calculated for $C_{10}H_{10}O_4N_2$

N, 12.60%

Found N, 12.48, 12.48

From the agreement of the analyses, and consideration of

the nature of the reactions used, we have concluded that our product from the azlactone hydrolysis was 2,5-dihydroxyphenylalanine.

I. Attempted Application of Ethyl Acetamidomalonate to the Synthesis of 2,5-Dihydroxyphenylalanine

Excellent yields have been reported in the literature for the preparation of aromatic amino acids through the use of ethyl acetamidomalonate. The applications of this substance were discussed in the historical section. The simplest application of this reagent for the preparation of 2,5-dihydroxyphenylalanine would involve a condensation between 2,5-dihydroxybenzylchloride and ethyl acetamidomalonate, followed by hydrolysis of the intermediate to the amino acid with hydrobromic acid.

As 2,5-dihydroxybenzyl chloride was not a known compound, and no reasonable synthesis for the molecule could be formulated, 2-hydroxy-5-nitrobenzyl chloride appeared to be the best starting material available. The substituted benzyl chloride needed was readily prepared through the chloromethylation of p-nitrophenol. The reaction series which follows is diagrammed in figure 6.

2-Hydroxy-5-nitrobenzyl chloride. This intermediate was readily prepared through the "Organic Syntheses" (168) procedure. We were able to use four times the quantities given

in their method without decrease in yield.

Condensation of 2-hydroxy-5-nitrobenzyl chloride with ethyl acetamidomalonate. A number of trial condensations were carried out which involved variations in the reaction conditions. By far the best over-all yields of amino acid were obtained with the following procedure. In this case the intermediate condensation product was hydrolyzed to the amino acid without purification.

In a three-necked flask, equipped with an addition funnel, reflux condenser, and mechanical stirrer was placed 250 ml. of absolute alcohol. The alcohol was dried according to the procedure of Lund and Bjerrum (169). To the alcohol was added 4.6 gm. (0.2 mole) of sodium. After the sodium had all gone into solution, 21.7 gm. (0.1 mole) of ethyl acetamidomalonate[1] was introduced. This compound dissolved on stirring. To the clear solution was added 18.7 gm. (0.1 mole) of 2-hydroxy-5-nitrobenzyl chloride dissolved in 50 ml. of absolute alcohol. A light yellow precipitate at once formed and the temperature rose 5°. The reaction was stirred for three hours at room temperature, and then refluxed two hours longer. The cooled solution was neutralized with hydrochloric acid, and the alcohol removed under reduced pressure. The residue was stirred with 200 ml. of water and cooled twelve hours. The precipitate was filtered with suction and washed

1. Furnished by Merck and Co.

with water. The crude product weighed 40 gm.

The crude ethyl α-acetamido-2-hydroxy-5-nitrobenzylmalonate was refluxed with 120 ml. of 48% hydrobromic acid for seven and one-half hours. The solution was cooled and decanted from a small amount of heavy oil. The mixture was concentrated under reduced pressure, and the residue dissolved in 100 ml. of boiling water. This solution was neutralized with concentrated ammonium hydroxide, and a heavy precipitate of light yellow amino acid separated at once. The cooled mixture was filtered and the product washed in the funnel with water, alcohol, and ether. The yield was 12.4 gm. (54.4%). The 2-hydroxy-5-nitrophenylalanine obtained was purified through solution in acid and precipitation with base. The resulting compound melted at 252° with decomposition, gave a positive ninhydrin reaction, and dissolved readily in acid and base. The basic solution was deep yellow in color. Like other nitrohydroxyphenylalanines the compound gave a negative Millon's reaction.

Anal. Calculated for $C_9H_{10}O_5N_2$

N, 12.38%

Found N, 12.22, 12.18[1]

Preparation of 2-hydroxy-5-nitrophenylalanine through

1. All nitro compounds of this series were analysed by the micro-Dumas method. All previous analyses were by the micro-Kjeldahl method.

the nitration of o-tyrosine. It was found that nitration of o-tyrosine gave the same product as isolated from the above series of reactions. This established that the acetamidomalonic ester synthesis had proceeded as expected, as nitration of o-tyrosine would give the 5-nitro derivative. The powerful para directive influence of the hydroxyl group would control the position of substitution.

Two gm. (0.011 mole) of dl-o-tyrosine was suspended in 7 ml. of water, to which was gradually added 5 ml. of concentrated nitric acid (sp. g. 1.5). The temperature was maintained below 25° through occasional immersion of the mixture in an ice-salt bath. Light brown crystals separated after one hour. The mixture was cooled several hours in the ice-salt bath and filtered through a sintered-glass funnel. The salt was dissolved in 10 ml. of water and neutralized with dilute ammonium hydroxide. The precipitate which separated was identical in appearance with that obtained from the previous synthesis. The material weighed 2 gm. Thus, the yield was 80%. The product was washed well with water, alcohol, and ether. When dried the compound melted at 252° with decomposition. Also, a mixture of this compound with the racemic product of the acetamidomalonic ester reaction decomposed at 252°. Although mixed decomposition points are not in all cases as reliable as mixed melting point determinations, in this case, where no depression of decomposition

point was observed, the evidence indicates the two reaction products were identical.

Isolation of ethyl α-acetamido-(2-hydroxy-5-nitrobenzyl)-malonate. In a condensation carried out according to the same procedure as the previous acetamidomalonic ester reaction, 9.35 gm. (0.5 mole) of 2-hydroxy-5-nitrobenzyl chloride was condensed with 16.27 gm. (0.075 mole) of the malonic ester derivative. The somewhat oily product isolated was recrystallized from 95% ethyl alcohol. Seven gm. (41% yield) of plate crystals were obtained which melted at 197-198° with decomposition.

Anal. Calculated for $C_{16}H_{20}O_8N_2$

N, 7.60

Found N, 7.80, 7.72

Preparation of N-acetyl-2-hydroxy-5-nitrophenylalanine. A mixture of 5 gm. of ethyl α-acetamido-2-hydroxy-5-nitrobenzylmalonate and 30 ml. of 10% sodium hydroxide was refluxed for four hours. The solution was acidified with 60 ml. of 3 N hydrochloric acid, and the mixture refluxed for one hour longer. Evolution of carbon dioxide was evident during the second reflux period. After concentration of the reaction mixture to 25 ml., plate crystals separated. These were recrystallized from boiling water and prism clusters separated which melted at 193-195°. As the starting material melted at

198°, a mixed melting point was taken. The mixture melted at 180-185°, a depression of about 15°. Also, the product was found to be readily soluble in sodium bicarbonate, in which the starting material was insoluble. The yield was 2.60 gm. (70%).

Anal. Calculated for $C_{11}H_{12}O_6N_2$

N, 10.45%

Found N, 10.35, 10.38

Synthesis of N-formyl-2-hydroxy-5-nitrophenylalanine. Eleven gm. (0.05 mole) of 2-hydroxy-5-nitrophenylalanine was dissolved in 87 ml. of formic acid. The solution was warmed to 45° and 29 ml. of acetic anhydride added dropwise over a 30 minute period. The reaction mixture was allowed to stand for twelve hours and was then concentrated under reduced pressure. On reconcentration with water the product crystallized in the distilling flask. The product was dried and recrystallized from a mixture of ethyl acetate and petroleum ether. The yield of 10 gm. was 79% of theory. The microscopic plate crystals obtained melted at 166-167° with decomposition.

Anal. Calculated for $C_{10}H_{10}O_6N_2$

N, 11.02%

Found N, 11.11, 11.01

Attempted resolution of N-formyl-2-hydroxy-5-nitrophenyl-

alanine. As it was uncertain that 2,5-dihydroxyphenylalanine could be resolved satisfactorily because of its ready oxidation in solution, the possibility of resolution at this intermediate stage of synthesis was investigated. Solutions containing 0.254 gm. (0.001 mole) of the N-formyl phenylalanine derivative and 0.394 gm. (0.001 mole) of brucine were prepared using ethyl alcohol, absolute ethyl alcohol, and water. Approximately 40 ml. of alcohol was required for each gram of salt. Water, however, required but 15-20 ml. per gm. of salt to bring about solution. All solvents gave crystals on cooling, and, as the yields were less than 40% on the basis of starting material weight, some separation of isomers was expected. However, the rotations of the alcohol fractions were -16.2° and the water fraction -17.3°. As the rotation of a mixture of equimolecular quantities of brucine and N-formyl-2-hydroxy-5-nitrophenylalanine was -17° no separation of isomers appeared to be taking place.

Reduction of 2-hydroxy-5-nitrophenylalanine. This compound was reduced by the procedure used by Waser and Lewandowski (85) in the preparation of 3-amino-4-hydroxyphenylalanine.

Twelve gm. (0.054 mole) of 2-hydroxy-5-nitrophenylalanine was dissolved in a mixture of 48 ml. of concentrated hydrochloric acid and 42 ml. of water. The solution was heated to boiling and mossy-tin added through the top of the condenser

as rapidly as the vigor of the reaction permitted. About 24 gm. of tin was added, and after the mixture had refluxed for one hour several grams remained undissolved. The mixture was filtered through a sintered-glass funnel, and the reaction flask and funnel rinsed with several portions of dilute acid. The combined filtrates were concentrated under reduced pressure, and the excess acid removed as completely as possible through reconcentration with water. The thick, oily residue was dissolved in 200 ml. of boiling water and a rapid stream of hydrogen sulphide passed into the mixture to precipitate the tin. The mixture was filtered while hot from the tin sulphide, and the precipitate was washed well with boiling water. The product gave a beautiful purple color with ferric chloride solution, and this reaction was used as a test on the filtrate to determine when all of the product had been removed from the precipitate. The filtrate and washings were concentrated under reduced pressure, and reconcentrated as before. The residue was dissolved in 200 ml. of boiling water and hydrogen sulphide again passed into the mixture. This second precipitation of tin was found necessary in all runs. A third hydrogen sulphide treatment was found not necessary.

After the tin was completely removed, the solution was again evaporated <u>in vacuo</u> and the residue dissolved in 5 ml. of boiling water. The solution was neutralized with concentrated ammonium hydroxide, and a few drops of a saturated

solution of sulphur dioxide in water added to prevent discoloration. Crystallization started as soon as the neutral point was reached. The mixture was cooled in the ice-box for forty-eight hours and the product filtered. The plate crystals, which weighed 8.5 gm. (80% yield), had a light pink color which quite rapidly darkened in air. They were well washed on the funnel with alcohol and ether. The product decomposed without melting at 270°. When rapidly heated on a spatula tip, a liquid stage was evident followed by instantaneous carbonization. The material was recrystallized from water through the addition of an equal volume of alcohol. The crystals separated with a pink tinge. These also decomposed without melting at 270°. The 2-hydroxy-5-aminophenylalanine dissolved in alkali with rapid discoloration, gave a positive ninhydrin reaction, and a pale yellow Millon's test, which turned red-orange on warming.

Anal. Calculated for $C_9H_{12}O_3N_2$

N, 14.28%

Found N, 14.35, 14.40

Attempted preparation of 2,5-dihydroxyphenylalanine through the diazotization of 2-hydroxy-5-aminophenylalanine. This reaction was carried out according to the method used by Waser and Lewandowski (85) for the preparation of 3,4-dihydroxyphenylalanine.

A solution of 4 gm. (0.018 mole) of 2-hydroxy-5-amino-

phenylalanine in 32 ml. of 20% sulfuric acid was cooled to 0° and diazotized with 1.4 gm. (0.02 mole) of sodium nitrite dissolved in 40 ml. of water. After 30 minutes in the cold, the diazotized amine was added to a boiling solution of 40 gm. of copper sulphate in 40 gm. of water. After the addition, during which extensive gas evolution was evident, the mixture was cooled and filtered. The filtrate was diluted to 500 ml. and saturated with hydrogen sulphide. The heavy copper sulphide precipitate was filtered with suction, and washed with 200 ml. of boiling water in several portions. The copper-free filtrate was treated with a water suspension of barium hydroxide until no longer acid to congo red. Barium carbonate was then carefully added until the mixture was very close to the neutral point. Care was taken to avoid a localized excess of base in any part of the mixture through slow addition of the reagents with vigorous agitation. The precipitate was then filtered off and washed with several portions of boiling water.

The filtrate obtained from the above operations was evaporated to dryness <u>in vacuo</u> using a system which passed carbon dioxide through the ebulator in place of air. The residue was dissolved in 5 ml. of boiling water and the solution very carefully neutralized with a little concentrated ammonium hydroxide. After prolonged standing in the cold, a small amount of amorphous material separated from the solu-

tion. The material did not melt. The solution was tested with Millon's reagent but no color developed. A ferric chloride test was also negative.

A second attempt to prepare this compound was carried out using 4 gm. of material. The diazotization was carried out as before, and the solution tested through addition of a few drops to 5% alpha-naphthol in sodium hydroxide. The formation of a beautiful crimson color indicated a diazotized amine was present. The diazotized amine was then added to 50 ml. of boiling 20% sulfuric acid. After the addition, the solution was diluted to 500 ml., and the sulphate precipitated with barium hydroxide. After filtration of the mixture, and concentration to dryness, only a trace of uncrystallizable oil remained. This material did not give any characteristic amino acid reactions.

A final reaction attempt was carried out using the same amounts of materials. In this run the diazotized amine was heated rapidly to the boiling point, concentrated under reduced pressure, and the residue dissolved in 5 ml. of water. The solution was neutralized with ammonium hydroxide and cooled. The reaction mixture yielded only inorganic crystalline material. A strong phenolic odor was detected in the concentrated reaction mixture.

Attempted Duplication of the Hirai synthesis of 2,5-dihydroxyphenylalanine. It was considered possible that Hirai

(87) had obtained the same amino acid through his synthetic procedure as Schaaf and Labouchère (89), but had not purified it sufficiently so as to obtain the correct melting point. Thus, repetition of their work was attempted. As their experimental detail was followed closely it will not be repeated here. The condensation of 2,5-dimethoxybenzaldehyde with diketopiperazine proceeded without difficulty. The hydriodic acid hydrolysis, however, did not yield any identifiable product. After precipitation of the iodide ion with lead acetate, the solution was made basic with ammonium hydroxide to precipitate the lead salt of 2,5-dihydroxyphenylalanine. No precipitate separated, however, nor could any amino acid be isolated through other procedures.

Thus, of methods for the synthesis of 2,5-dihydroxyphenylalanine, the modification of Schaaf and Labouchère (89) method was the only one successful. Unfortunately the acetamidomalonic ester method failed at the last step of the five step procedure investigated. In view of the success of the modified Erlenmeyer method the only objection which could be put forth in regard to the synthesis concerned the availability of 2,5-dimethoxybenzaldehyde. If a simpler procedure existed for the preparation of this aldehyde, 2,5-dihydroxyphenylalanine could be considered as readily available through application of our procedure.

J. The Synthesis of 2,3-Dihydroxyphenylalanine

Two of the five possible dihydroxyphenylalanines have not been reported in the chemical literature. The two evidently uninvestigated were the 2,3-dihydroxyphenylalanine and the 2,6-dihydroxyphenylalanine. As 2,3-dimethoxybenzaldehyde was readily available commercially, it appeared possible that the corresponding phenylalanine might be prepared through the same modification of the Erlenmeyer reaction as used by us successfully in the preparation of ortho and meta-tyrosine, and 2,5-dihydroxyphenylalanine. Thus, the necessary azlactone was prepared through the method available in the literature, and the amino acid was obtained when the azlactone was reacted with hydriodic acid according to the procedure previously used. The most readily available derivative of the amino acid was prepared for preliminary resolution experiments. In this case that derivative was the N-formyl compound. Acetylation did not seem applicable as the amino acid darkened rapidly in the basic reaction media used in the modified Schotten-Baumann procedure.

2-Phenyl-4-(2'3'-dimethoxybenzal)-5-oxazolone. This azlactone was prepared according to the method of Salyeadranath and Swaminathian (170).

To a mixture of 66.4 gm. (0.4 mole) of 2,3-dimethoxybenzaldehyde, 72 gm. (0.4 mole) of hippuric acid and 32 gm. (0.4 mole) of sodium acetate was added 104 ml. (1.2 moles) of

acetic anhydride. The well-shaken mixture was heated for four hours on a boiling water-bath. The reaction mixture was cooled and 100 ml. of alcohol worked into the mixture. After twelve hours in the cold, the product was filtered with suction, and washed well with water and a small amount of alcohol. The melting point of 168-170° obtained for the product agreed with the value given by the Indian workers. The product was used without further purification.

2,3-Dihydroxyphenylalanine. Fifty gm. (0.16 mole) of the azlactone was refluxed with 250 ml. of hydriodic acid, (sp. g. 1.50) and 250 ml. of glacial acetic acid, and 15 gm. of red phosphorus for six hours. The reaction mixture was filtered, concentrated, and the product isolated in the identical manner as described for the preparation of o-tyrosine, and m-tyrosine. The amino acid isolated melted at 240° with decomposition. When purified through solution in acid and precipitation at the boiling point with base the amino acid melted at 242°. The yield of purified material was 25.3 gm. (80%).

The amino acid gave a strong Millon's reaction and a positive ninhydrin test. A red color was given with a small amount of amino acid and dilute ferric chloride solution. The solution in alkali rapidly darkened. The 2,3-dihydroxyphenylalanine was slightly soluble in cold water and moderately soluble in boiling water. Once dissolved in water,

however, the compound crystallized very slowly.

Anal. Calculated for $C_9H_{11}O_4N$

N, 7.10%

Found N, 7.00, 7.00

N-Formyl-2,3-dihydroxyphenylalanine. In 309 ml. of 88% formic acid was dissolved 30 gm. (0.15 mole) of 2,3-dihydroxyphenylalanine. The solution was heated to 45° and 104 ml. (1.1 moles) of acetic anhydride added in ten portions over an hour period. After standing at room temperature for twelve hours, the reaction mixture was diluted with an equal volume of water and concentrated, in vacuo, to dryness. The product crystallized during the concentration and was recrystallized from 125 ml. of boiling water. Light yellow plate crystals were obtained which melted at 165-167°. A second recrystallization from water which contained a little sulfur dioxide yielded lighter colored crystals of the same melting point. The yield was 28 gm. (85%).

Anal. Calculated for $C_{10}H_{11}O_5N$

N, 6.22%

Found N, 6.01, 6.01

Resolution experiments with N-formyl-2,3-dihydroxyphenylalanine. Preliminary experiments with solutions which contained 0.225 gm. (0.001 mole) of N-formyl-2,3-dihydroxyphenylalanine and 0.394 gm. (0.001 mole) of brucine showed that a

brucine salt crystallized readily from water, absolute ethyl alcohol, and methyl alcohol. Using water solutions of the crystals obtained for polarimetric readings, it appeared some separation of isomers was accomplished. When a moderate scale resolution was attempted, however, and rotations taken in alcohol because of the color of the samples in water solution, very little separation of isomers was detectable. These results are shown in table 15.

The values in the table can be best viewed in consideration of the rotation observed for a solution of N-formyl-dl-2,3-dihydroxyphenylalanine in alcohol with an equimolecular quantity of brucine. This value was found to be -14.6°. As the maximum increase in rotation observed was but 2° in a series of crystallizations in which there was a loss of material corresponding to 75%, in terms of one isomer, it appeared resolution through this method was not feasible.

It is evident that in this investigation all possible solvents under all possible conditions have not been studied. However, in view of the fact water and ethyl alcohol yielded crystalline salts which hardly differed a degree in rotation after two recrystallizations, it did not appear this amino acid would parallel those successfully resolved as the brucine salt of the formyl derivative.

Table 15

Attempt to Resolve 2,3-Dihydroxyphenylalanine

Solvent[1]	Number of Crystallizations	Rotation
		°
water	1	-15.6
water	2	-14.9
methanol[2]	1	-16.8
methanol	2	-16.6
ethanol (abs.)	1	-16.4

1. The solvent for the polarimeter readings was in all cases ethyl alcohol (95%). Concentrations of the solutions read were 0.5%.

2. The material recrystallized from methanol was the residue left on evaporation of the mother liquors from the first water recrystallization.

K. Attempted Synthesis of 2,4-Dihydroxyphenylalanine by the Modified Erlenmeyer Method

The amino acid 2,4-dihydroxyphenylalanine has a close structural relationship to tyrosine. For this reason the synthesis of the compound was investigated. The method of choice for attempted preparation of the molecule was the modified Erlenmeyer procedure which was applied successfully in the previous syntheses. However, in direct contrast with the results obtained in other applications of the method, none of

the desired amino acid could be obtained by the exact procedure previously used, or by any of a number of modifications tested. However, in preliminary investigations of other procedures by which the molecule might be obtained, it was found that the Hirai diketopiperazine method could be modified to yield the amino acid in approximately 32% over-all yield.

Synthesis of 2,4-dimethoxybenzaldehyde. Although a number of workers have used this compound in various syntheses, the experimental details for the preparation of it using dimethyl sulphate were not given in the chemical literature.

In a three-necked flask equipped with stirrer, condenser, and addition funnel was placed 35 gm. (0.254 mole) of 2,4-dihydroxybenzaldehyde, and 25 gm. (0.62 mole) of sodium hydroxide dissolved in 200 ml. of water. This mixture was heated to boiling and placed in a boiling water-bath. To the hot mixture 78 gm. (0.62 mole) of dimethyl sulphate was added dropwise with stirring. To the slightly acid mixture was added 10 gm. (0.25 mole) of sodium hydroxide followed by 31.5 gm. (0.25 mole) of dimethyl sulphate. Alternate additions in this manner were continued until 1.5 moles or 189 gm. of dimethyl sulphate had been introduced. The addition required three hours. When the addition was completed, the mixture was made strongly basic with 10 gm. of sodium hydroxide and heated for one hour longer. The mixture was cooled in the ice-box and the crystals filtered with suction after twelve

hours. The 41 gm. of crude crystals obtained were dried and recrystallized from ligroin. The yield of pure aldehyde obtained was 32 gm. or 78% of theory. The product melted at 68-69° in agreement with the value reported by Tiemann and Parrisius (171).

Preparation of 2-phenyl-4-(2'4'-dimethoxybenzal)-5-oxazolone. This compound was prepared according to the general procedure of Deulofeu (91).

Sixteen gm. (0.1 mole) of 2,4-dimethoxybenzaldehyde was condensed with 17.5 gm. (0.1 mole) of hippuric acid in the presence of 8 gm. (0.1 mole) of sodium acetate and 30.4 gm. (0.3 mole) of acetic anhydride. The reaction was brought about by heating the mixture for 4 hours in a boiling water-bath. The mixture was cooled to room temperature and 50 ml. of ethyl alcohol added to destroy the acetic anhydride. The product was filtered with suction, washed well with water, and air dried. The yield was 16.5 gm. (53%) of product which melted at 165-167° in agreement with the value given by Deulofeu.

Reaction of 2-phenyl-4-(2',4'-dimethoxybenzal)-5-oxazolone with hydriodic acid. A mixture of 16 gm. (0.05 mole) of the oxazolone with 75 ml. of hydriodic acid (sp. g. 1.50), 75 ml. of glacial acetic acid, and 10 gm. of red phosphorus was refluxed for a total of seven and one-half hours. After the first hour, a heavy red precipitate was evident in

the mixture. The mixture was cooled, the lumps disintegrated and heating continued. The precipitate did not decrease in quantity during the reaction period. The hot solution was filtered through a sintered-glass funnel and concentrated to dryness under reduced pressure. The residue consisted of a small amount of red oil which was dissolved in 1 ml. of water and neutralized. Several volumes of alcohol was added but even after prolonged standing in the cold no product separated.

The red precipitate, which was mixed with red phosphorus in the funnel, was suspended in water, and, being lighter than the phosphorus, was decanted in suspension. The material was quite porous and appeared amorphous. The product had no definite melting point but decomposed and caught fire when heated on a spatula tip. The material was not attacked by boiling 10% sodium hydroxide or boiling hydrochloric acid. Organic solvents which had no effect on the by-product included ether, alcohol, toluene, chloroform, carbon tetrachloride, and ethylene glycol. Hot acetic acid dissolved enough material to take on a slight red color. When cooled, however, crystals did not separate nor could they be obtained on evaporation of the solvent.

This reaction was repeated using hydriodic acid alone, hydriodic acid and acetic anhydride, and hydriodic acid diluted with water. In all cases the same amorphous product

was obtained as in the first reaction. These reactions were all carried out in a carbon dioxide atmosphere.

Preparation of α-benzoylamido-2,4-dimethoxycinnamic acid. The method of Deulofeu (91) was used to convert the azlactone used in the previous reactions to the corresponding cinnamic acid.

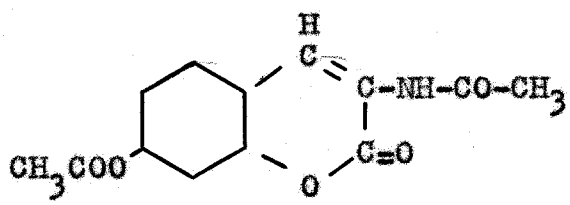
Five gm. (0.012 mole) of 2-phenyl-4-(2',4'-dimethoxybenzal)-5-oxazolone was suspended in 500 ml. of water in which was dissolved 5 gm. of sodium hydroxide. The mixture was stirred three hours in a boiling water-bath and all the material dissolved. The reaction mixture was decolorized with Norite, filtered, and acidified. A nearly quantitative yield of 5 gm. was obtained. The product melted at 214-215° which agreed with Deulofeu's value.

Reaction of α-benzamido-2,4-dimethoxycinnamic acid with hydriodic acid. Two gm. (0.006 mole) of the substituted cinnamic acid was refluxed with 10 ml. of concentrated hydriodic acid and 10 ml. of glacial acetic acid in the presence of 1 gm. of red phosphorus. After a few minutes, a heavy red precipitate separated and did not dissolve during a six hour reaction period. The red precipitate was the only product which could be isolated from the reaction mixture. The appearance and properties of the product were identical in all respects with the material isolated in the first reaction.

Condensation of 2,4-dihydroxybenzaldehyde with acetyl-

glycine. This reaction was investigated because of the possibility azlactone formation might predominate over coumarin formation. The subsequent reaction of the azlactone with hydriodic acid could either yield the desired amino acid or give more information in regard to the mechanism of formation of the peculiar material isolated from the previous reactions.

In 47.5 ml. (0.5 mole) of acetic anhydride was dissolved 13.8 gm. (0.1 mole) of 2,4-dihydroxybenzaldehyde. To this mixture was added 12 gm. (0.1 mole) of acetyl glycine and 8.2 gm. (0.1 mole) of sodium acetate. After one hour in a boiling water-bath, the reaction mixture began to crystallize. After two hours, the crystals were filtered off and the filtrate mixed with 100 ml. of cold water. The crystals which separated from the diluted filtrate were identical with those filtered from the original reaction mixture. The total yield was 7 gm. The product melted at 228-230°. As the product was pure white and had a high melting point it was suspected that a coumarin derivative was obtained in the reaction. The analytical results supported the belief the product was the coumarin shown in the equation below. The nitrogen content of the molecule was found to be 5.48%.



The calculated nitrogen content of 3-acetamido-7-acetoxycoumarin was 5.37%. In contrast, if the product were 2-methyl-4(2',4'-acetoxybenzal)-5-oxazolone the nitrogen analyses should have given a value near 4.61%. It was considered possible that an azlactone might have been synthesized without the hydroxyl groups being acetylated. As the Millon's test was negative with the product obtained this was shown not to be the case.

Anal. Calculated for $C_{10}H_{13}O_5N$

N, 5.37%

Found N, 5.48, 5.48

Reaction of 3-acetamido-7-acetoxycoumarin with hydriodic acid. Five gm. (0.02 mole) of 3-acetamido-7-acetoxycoumarin was heated for one hour with equal parts of a mixture of water and concentrated hydriodic acid. The reaction mixture was filtered hot, but some material crystallized in the funnel. The filtrate yielded granular crystals when cooled which were soluble in hot water. Neutralization of a water solution of the material with sodium hydroxide gave needle crystals which were soluble in acid and base. The material gave a positive Millon's test and melted at 238-240° with decomposition. The properties of the compound and the analytical data established the product to be 3-amino-7-hydroxycoumarin.

Anal. Calculated for $C_9H_7O_3N$

N, 7.91%

Found N, 7.70, 7.73

3,6-Di-(2',4'-dimethoxybenzal)-2,5-diketopiperazine. This compound was prepared according to the method of Hirai (90).

A mixture of 33.2 gm. (0.2 mole) of 2,4-dimethoxybenzaldehyde, 11.4 gm. (0.1 mole) of glycine anhydride, 44.6 gm. (0.44 mole) of acetic anhydride, and 28 gm. (0.34 mole) of sodium acetate was heated in an oil-bath six and one-half hours at 155-165°. After the mixture was cooled to room temperature, 70 ml. of ethyl alcohol was worked into the hard mass with a stirring-rod. The cooled mixture was filtered with suction, and the solid washed well with alcohol and water. The yield was 32 gm. (78%). The product melted at 284-286°. Hirai claimed a melting point of 286-287° for his product.

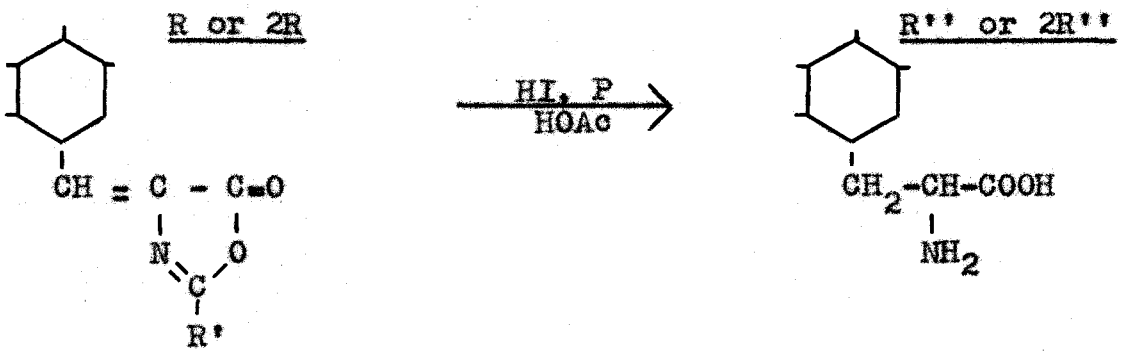
2,4-Dihydroxyphenylalanine. Four gm. (0.01 mole) of the diketopiperazine derivative was refluxed for eight hours in an all-glass apparatus with 60 ml. of constant-boiling hydriodic acid and 2 gm. of red phosphorus. The reaction mixture was filtered and concentrated to dryness under reduced pressure. The yellow crystalline residue which remained was freed from excess acid through reconcentration of the material with water *in vacuo*. The residue was dissolved in 5 ml. of water and neutralized with ammonium hydroxide. An equal volume of alcohol was added and the mixture was cooled in the

ice-box. After twenty-four hours a heavy precipitate of plate crystals separated. The product was filtered with suction, and washed with alcohol and ether. The crude amino acid melted at 215° with decomposition. The product was purified through dissolving it in water and reprecipitating with an equal volume of alcohol. The amino acid separated slowly and when isolated melted at 220°. Hirai claimed the compound melted at 223-224°. Our yield was 1.6 gm. or 41%. The properties of the product obtained agreed in all respects with those given by Hirai. The compound gave a green color with ferric chloride which turned red-brown on standing. Millon's reagent gave a red-orange color and the solution of the compound in base was pale green which turned brown on heating.

The first part of this synthesis followed the directions of Hirai. However, the amino acid was isolated through simple neutralization of the concentrated reaction mixture, in place of the lead acetate precipitation procedure of Hirai. The yield obtained compared very favorably with that reported in the literature.

IV. DISCUSSION

The successful preparation of ortho-tyrosine, meta-tyrosine, 2,5-dihydroxyphenylalanine, and 2,3-dihydroxyphenylalanine was accomplished by one general reaction procedure. The synthetic method which was successfully applied in these syntheses is shown in the general equation shown below.



R	CH_3O- or CH_3COO-
R'	CH_3- or C_6H_5-
R''	HO-

Several restrictions, however, must be added to the above general equation before it is in agreement with all of the experimental evidence. In view of our experiments and those of Lamb and Robson (57) all of the possible monophenolic phenylalanines can be prepared in good yield by this method. Of the dihydroxyphenylalanines the 2,3-, 3,4- and 2,5-isomers can be obtained in yields equal or better than those reported for any general method for the synthesis of aromatic amino

acids. For satisfactory results, however, a hydroxyl group ortho to the aldehyde group must be protected by a stable substituent. In our experience the group was satisfactorily blocked as the methyl ether. Simple acetylation of the hydroxyl group did not prevent coumarin formation.

The nature of the group R' did not appear too crucial in regard to the success or failure of the reaction. On the other hand, the synthesis of 2-phenyl oxazolones was found to be somewhat easier than the synthesis of corresponding 2-methyl derivatives. In the case of the 2-phenyl oxazolones the reaction mixtures could be worked up through simple addition of ethyl alcohol at the end of the reaction period. This served to precipitate the product in crystalline form as the acetic anhydride was gradually converted to ethyl acetate. In contrast, to obtain a good yield of 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone water had to be added to the reaction mixture which precipitated the product with much contaminating impurity.

In table 16 the yields of intermediates and amino acids obtained through the methods used in our experiments are shown. Remarkably, the yield of 2,3-dihydroxyphenylalanine was as good as that obtained from the less complex reactions which did not involve the hydrolysis of ether linkages. The 30% yield obtained of 2,5-dihydroxyphenylalanine is best viewed in light of the fact the best previous yield given in

Table 16

Yields of Products and Intermediates in the Modified Erlenmeyer Synthesis of Amino Acids

Substituted Benzaldehyde	Other Reactant	Yield Azlactone	Yield Amino Acid	Over-all Yield
		%	%	%
m-hydroxy	A. g.[1]	67-75	60-70	40-53
o-methoxy	H. a.[2]	68	69	47
2,5-dimethoxy	H. a.	56	54	30
2,3-dimethoxy	H. a.	62	80	50
2,4-dimethoxy	H. a.	53	0	—

1. Acetyl glycine was used in this preparation.

2. Hippuric acid was used in all syntheses so designated.

the literature was about 6%.

As yet unexplained is the peculiarity observed in the attempted synthesis of 2,4-dihydroxyphenylalanine from 2-phenyl-4-(2',4'-dimethoxybenzal)-5-oxazolone. The formation of an apparently amorphous polymeric product was not observed in any of the other syntheses. In this case it was the only product isolable from the reaction mixture. The same behavior was observed in the attempted conversion of α-benzoylamido-2,4-dimethoxycinnamic acid to the amino acid through reaction with hydriodic acid. In contrast, the 3,6-di-(2',4'-

dimethoxybenzal)-2,5-diketopiperazine was converted to the 2,4-dihydroxyphenylalanine without any formation of the by-product. This leads one to suspect that the rates of hydrolysis of the various groups involved, together with the rate of reduction of the double bond, caused the phenomenon. It seems possible that the benzoylamido linkage of the azlactone or substituted cinnamic acid may have hydrolized before the double bond was reduced. The formation of the by-product would then involve subsequent reactions of the substituted phenylpyruvic acid produced. On the other hand, the diketopiperazine derivative was so resistant to hydrolysis that the double bonds were reduced before the ring was opened. Thus, no by-product was produced.

The related α-benzoylamido-2,3,4-trimethoxyphenylalanine could not be reduced by Schaaf and Labouchere (89) to the corresponding phenylalanine derivative. Also, they found 2,3,4-trimethoxybenzalhydantoin could not be converted with hydriodic acid to the desired amino acid. Although by-products were obtained, they did not state if they resembled what we isolated from the 2,4-dimethoxy azlactone reaction.

Investigated previous to the application of the modified Erlenmeyer synthesis to the preparation of o-tyrosine and m-tyrosine was the possibility of application of a catalytic reduction procedure. In neither case was the method satisfactory. However, the tests made were not complete as

the equipment available could not be trusted to give a true value for the amount of hydrogen absorbed. This proved to be the crucial factor in the reduction of α-acetamido-m-hydroxy-cinnamic acid. Without accurate information as to the hydrogen absorbed some time was required before it was established that nuclear reduction was taking place in addition to saturation of the ethylenic double bond. However, in this case other factors appear to have been involved. According to Herbst and Shemin (142) platinum oxide catalyst with phenylalanine gives only nuclear reduction if kept moist and freshly prepared. Our catalyst was a commercial product which was shipped dry and thus would not be expected to catalyze benzenoid reduction. This leads one to suspect that the m-hydroxyl-group might have particularly labilized the ring toward reduction. With accurate control of hydrogenation conditions, however, this method may prove more readily applicable than our results indicate.

In the resolution of meta-tyrosine the N-formyl derivative proved satisfactory as one of the diasterioisomers which this compound formed with brucine proved to be insoluble in alcohol but soluble in water, while the other diasterioisomer showed the inverse behavior. The N-formyl derivatives of amino acids were found more readily prepared with less labor than the corresponding acetyl derivatives. Also, the N-formyl derivatives appeared more readily crystallizable. The N-

acetyl derivative of meta-tyrosine could not be crystallized in spite of intensive efforts.

As a result of our experience in the resolution of meta-tyrosine, we sought to apply the same method of resolution to other amino acids in view of the structural similarities evident. With N-formyl-o-tyrosine, N-formyl-2-hydroxy-5-nitrophenylalanine, and N-formyl-2,3-dihydroxyphenylalanine the brucine salts formed were entirely unsatisfactory for resolution purposes. Each of these compounds gave crystalline salts from alcohol and from water, but the difference in rotation shown by the two fractions was usually about one degree. In contrast, meta-tyrosine, when recrystallized as the brucine salt of the formyl derivative, gave fractions from water and from alcohol which on the first crystallization differed by almost 15° in rotation. Tyrosine itself cannot be completely resolved as the N-formyl derivative, and in our experience o-tyrosine as the formyl derivative gave only racemic compounds or mixtures with brucine. In contrast, meta-tyrosine, with a hydroxyl group in an intermediate position to those of tyrosine and o-tyrosine, was resolved successfully. From this it can be seen that it would be an error to attempt to deduce resolution methods from experimental data with related compounds. As it would be hard to visualize substances more closely related than the ortho and meta tyrosines and tyrosine itself, it appears trial and error is as

yet the best guide to the choice of a resolution method for an aromatic amino acid.

Although the formyl derivative of o-tyrosine could not be resolved as the brucine or cinchonine salt, the pure d-isomer was obtained in reasonable yield from the brucine salt of the acetyl derivative. Like acetyltyrosine and the acetyl derivative of 3,4-dihydroxyphenylalanine (84) the brucine salt which crystallized out of alcohol yielded the unnatural or d isomer. Similar solubility behavior has been shown by the formyl derivatives of meta-tyrosine and phenylalanine, whose alcohol insoluble brucine salts gave the unnatural isomer. The natural isomers, on the other hand, were obtained in the case of tyrosine, meta-tyrosine, phenylalanine, and 3,4-dihydroxyphenylalanine through recrystallization of the brucine salts of acetyl or formyl derivatives from water. This observation made water the logical choice for a recrystallization agent for the alcohol soluble material from the o-tyrosine resolution. In spite of many efforts a crystalline brucine salt of acetyl-o-tyrosine could not be obtained from water.

The literature offered little in the way of suggestions for alternative solvents for the recrystallization of the alcohol soluble isomers of aromatic amino acids as the brucine salt. Through trial and error, after a number of solvents were investigated, ethyl acetate was found to give crystalline

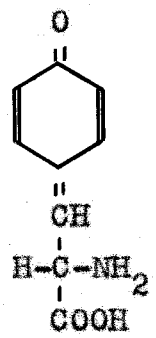
material. The yields were so small and the procedure so laborious that only a few hundred mg. of product could be isolated. The primary product isolated on decomposition of the brucine salt was contaminated with the d-isomer as the rotation was 6° lower than that observed for the other enantiomorph. Water recrystallization served to raise the rotation to the value of the other isomer, but so little material was available for the rotation the experimental error may have been moderately large. However, that observation makes it appear worthwhile to investigate the alcohol soluble brucine salt fraction further. Through direct decomposition of the brucine salt, followed by water recrystallization of the free amino acid obtained, the l-isomer may possibly be isolated. It would appear that water recrystallization might remove the racemic compound remaining to give the pure l-isomer. The alternative left in the resolution of o-tyrosine would be to investigate other of the alkaloid resolution reagents.

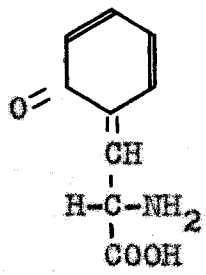
The proof of configuration of the meta-tyrosine isomers, and the isomer of ortho-tyrosine isolated, was readily accomplished through application of the Lutz and Jirgensons (110, 111) method. In both cases the decrease in rotation observed was in the order of 20° in the range of acidity studied. This left no doubt regarding which isomer possessed the natural and which the unnatural configuration. The first appli-

cation of the d-amino acid oxidase to the proof of configuration of a resolved amino acid proved very satisfactory. The fact that the isomer considered to have the natural configuration gave a keto acid value almost identical with the tissue blank showed that our l-isomer was not contaminated with the unnatural isomer. On the other hand, the 100% yields of keto acid obtained on incubation of the unnatural isomer with the enzyme showed that the material was free of levo configuration material. Thus, this method should find extensive future application in amino acid research as it not only offers a simple proof of configuration method but also gives evidence as to the purity of the isomers isolated and the success of the separation method.

The manometric investigation of the dextro and levo isomers of meta-tyrosine has opened up an extensive field for further investigation. Consideration of the metabolic schemes of Neubauer (116) and Felix and Zorn (132) as outlined in the historical section shows that both mechanisms postulate an intermediate quinoid compound. While a quinoid structure can be readily shown for tyrosine and o-tyrosine as illustrated in the formulas, a corresponding intermediate cannot be drawn for meta-tyrosine.

This might be considered evidence in favor of the view that the oxidation of meta-tyrosine observed in the Warburg experiments was due to the action of a metabolic system dif-





ferent from that which oxidizes naturally occurring tyrosine. On the other hand, the evidence might be considered to cast doubt on the Neubauer (116) and Felix and Zorn (132) mechanisms. This would definitely be the case if it could be established that the enzyme system acting on meta-tyrosine and that metabolizing tyrosine were the same. This might be established through inhibition experiments together with evidence showing the same or similar intermediates and final products were formed in each case. Proof that alanine is split off in the oxidation of meta-tyrosine, as well as in the case of tyrosine as shown by Felix and Zorn, would be strong evidence that the same system was acting in both cases.

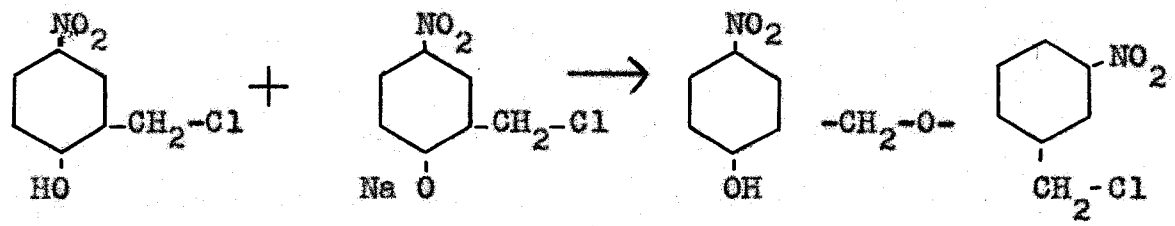
The consistent observation of an O/T ratio of approximately 1.2 for meta-tyrosine could be interpreted as indicating one atom of oxygen was being taken up by each molecule of substrate with a halt in oxidation at that point. If this point is established by further investigation the isolation of the first intermediate in the oxidative scheme may be accomplished using tissue brei on a larger scale with meta-

tyrosine. The O/T ratio of 1.2 observed in a number of runs on the basis of meta-tyrosine added was not in agreement with the O/T ratio calculated from analytical data. The extremely high values for O/T ratios obtained in some of these experiments indicated that the intermediate oxidation products must react with the reagents used as well as with the unoxidized tyrosine. The problem thus arises as to whether it is more logical to postulate that a small amount of the material was being completely oxidized or that all the substrate added was being oxidized one step. The rate of reaction observed, with the cessation of oxygen uptake after the first hour, makes the latter supposition quite probable. Another possibility, however, is the production of a substance during the first hour which was a powerful inhibitor of the enzyme system during the second and third hours.

The elucidation of the problems postulated on the basis of our preliminary experiments awaits further study with in vivo and in vitro methods. In addition, the study of the other isomers and analogues of tyrosine, which have been made available for resolution and metabolic studies through our fundamental investigation of synthetic methods, should give additional information and open more avenues of approach in the study of the problem of tyrosine metabolism.

A synthetic method which was found very satisfactory, as far as the fundamental reaction concerned, was the use of

acetamidomalonic ester. In a simple two step reaction, 2-hydroxy-5-nitrophenylalanine was prepared in about 55% yield. The condensation of 2-hydroxy-5-nitrobenzyl chloride with acetamidomalonic ester proceded in good yield in spite of the fact an obvious side reaction could occur. The substituted



benzyl chloride could readily condense with itself under the conditions of the condensation to give the substituted benzyl ether shown in the equation. As a small amount of oil innert to boiling hydrochloric acid was isolated from the reaction mixtures, this reaction may have taken place to some extent. The amount, in view of the yields of product obtained, must have been only a few percent of that possible.

It was not found possible to convert 2-hydroxy-5-amino-phenylalanine to 2,5-dihydroxyphenylalanine by any of a number of diazotization methods. The procedure of Waser and Lewandowski (85) which was satisfactory for the preparation of 2,4-dihydroxyphenylalanine was unsuccessful. The conversion of amino-phenols to diphenols has always been difficult through diazotization and hydrolysis methods. It may be yet possible to convert this intermediate to the desired amino

acid through the Bucherer (172) reaction. The Bucherer method, however, has found little application outside of the naphthaline series because of low yields. With this procedure the synthesis of 2,5-dihydroxyphenylalanine would offer an independent proof of structure for the molecule. However, because of the labor and time involved it does not appear applicable to large scale synthesis of the amino acid.

The resolution of N-formyl-2-hydroxy-5-nitrophenylalanine was investigated as this compound offered greater stability than the N-formyl derivative of 2,5-dihydroxyphenylalanine itself. As 2,5-dihydroxyphenylalanine rapidly becomes colored in basic solution, it appeared extensive loss of resolved amino acid might result when the brucine salts were split with alkali. This would not be the case with N-formyl-2-hydroxy-5-nitrophenylalanine as oxidation to a quinone could not readily take place. The conversion of the 2-hydroxy-5-nitrophenylalanine to 2,5-dihydroxyphenylalanine could then be accomplished after the resolution. Both the resolution and subsequent conversion were unsuccessful.

The synthesis by Dakin of a compound supposedly 2-methyl-4-(2'-acetoxybenzal)-5-oxazolone was shown by our studies to be an error. The compound actually obtained by Dakin was 3-acetamidocoumarin, although the correct analytical data was given by Dakin for the azlactone. This was established through mixed-melting point determinations which showed the original

condensation product melted undepressed with an authentic sample of the coumarin. Also, 2-methyl-4-(2'-acetoxybenzal)-5-oxazolone was synthesized through the action of acetic anhydride and sodium acetate on trans-α-acetamido-o-hydroxycinnamic acid. The compound obtained from this reaction melted at 133° which was in definite contrast to the value of 203-204° given by Dakin. This low melting point is well within the range of values commonly observed for 2-methyl oxazolones, while the 203° melting point is much higher than is common for these compounds.

The same tendency of o-hydroxy aldehydes to form coumarins was observed in our studies with 2,4-dihydroxybenzaldehyde. A product identified as 3-acetamido-7-acetoxycoumarin was readily isolated from the condensation of the aldehyde with acetylglycine and acetic anhydride. No evidence of azlactone formation was obtained. The reaction of hydriodic acid with the coumarin served to hydrolyze off the N-acetyl and O-acetyl groups to give 3-amino-7-hydroxycoumarin. This result was surprising as the hydriodic acid hydrolysis of 3-acetamidocoumarin gave 3-ketocoumarin. The synthesis of amino acids or their derivatives from coumarins was shown to be possible by our experiments in the preparation of N-acetyl-o-tyrosine by reduction. However, the yields involved in the synthesis of coumarins is usually lower than the yields of the corresponding azlactones obtained if the ortho hydroxyl

groups are protected. Also, the necessary conversion of the coumarin to a trans-cinnamic acid often involves difficulty in view of the readiness with which the reversal to coumarin structure takes place.

V. SUMMARY

1. A historical survey of the methods used in the synthesis of tyrosine, phenylalanine, and various tyrosine isomers and analogues has been made. In addition, the methods for the resolution and proof of configuration of these compounds have been reviewed, together with the metabolic studies *in vivo* and *in vitro* which have been carried out.

2. The synthesis of *meta*- and *ortho*-tyrosine derivatives through catalytic reduction of suitable cinnamic acid derivatives has been studied and the labile nature of the benzene ring noted, in the case of the *meta*-compound, toward nuclear hydrogenation.

3. The reaction of hydriodic acid with 2-methyl-4-(3'-acetoxybenzal)-5-oxazolone, 2-phenyl-4-(3'-acetoxybenzal)-5-oxazolone, and α-acetamido-*m*-hydroxycinnamic acid gave *meta*-tyrosine in each case. The *meta*-tyrosine was resolved to obtain the pure *d* and *l* isomers through the use of brucine with the N-formyl derivative. The configuration of the isomers isolated was determined by the method of Lutz and Jirgensons and confirmed by a biochemical procedure which made use of the specific *d*-amino acid oxidase of kidney tissue. The oxidation of the isomers of *meta*-tyrosine was studied using normal and scorbutic guinea pig liver brei and the Warburg manometric technique. The behavior of *l*-*meta*-tyrosine

paralleled the behavior of l-tyrosine in these experiments. Also d-meta-tyrosine and d-tyrosine paralled each other in preliminary screening tests.

4. The reaction of hydriodic acid with 2-phenyl-4-(2'-methoxybenzal)-5-oxazolone gave o-tyrosine in good yield. The amino acid could not be resolved as the brucine salt of the formyl derivative, or as the cinchonine salt of either the acetyl or formyl derivative. The N-acetyl derivative was resolved as the brucine salt, and the d- or unnatural isomer obtained. Small amounts of impure l isomer were obtained through recrystallization of the alcohol soluble isomer from ethyl acetate. The yields were minute from this solvent so the method was not feasible for even small scale resolution.

5. It was found possible to synthesize 2,5-dihydroxyphenylalanine from 2-phenyl-4-(2,5'-dimethoxybenzal)-5-oxazolone. This was accomplished through the same method as used with ortho- and meta-tyrosine in yields approximately six times better than previously reported in the literature. The compound was characterized through the hydantoin derivative. A series of reactions calculated to yield 2,5-dihydroxyphenylalanine through the application of acetamidomalonic ester failed as in the last step 2-hydroxy-5-aminophenylalanine could not be converted to the diphenol.

7. The isomeric 2,3-dihydroxyphenylalanine was readily obtained from the corresponding azlactone through reaction

with hydriodic acid. The N-formyl derivative of the amino acid could not be resolved as the brucine salt.

8. The 2,4-dihydroxyphenylalanine could not be prepared from the azlactone through reaction with hydriodic acid. An intractable red amorphous solid was obtained. The amino acid was successfully prepared through a modification of the original literature method using diketopiperazine.

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