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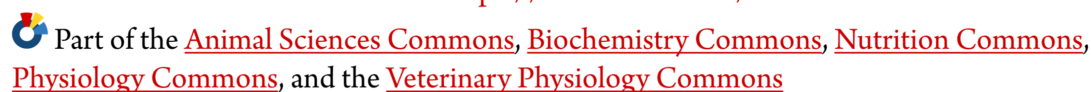
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DIHYDROXYPHENYLALANINE METABOLISM BY KIDNEY TISSUE

by

Robert Edward Clegg

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Physiological and Nutritional Chemistry

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I. INTRODUCTION

The metabolism and physiological behavior of the aromatic amino acids has been the subject of much study since their discovery and isolation from protein hydrolyzates. As each of the various amino acids was isolated and characterized, the relative importance of these compounds in nutrition was established, but the intermediary metabolism of the amino acids is still the object of intense study.

The metabolism of the aromatic amino acids related to phenylalanine was the subject of early investigations because of the relationship of these amino acids to the inherited disease, alcaptonuria. The rather recent demonstration of experimental alcaptonuria by feeding excessive amounts of phenylalanine has again stimulated new research in this field, and, in addition, the discovery of an experimental alcaptonuria in the scorbutic guinea pig has emphasized the importance of vitamin C in the metabolism of these amino acids.

During this same period, the role of dihydroxyphenylalanine in essential hypertension has been the object of many investigations and has led to the discovery of the ability of kidney tissue to metabolize this compound. For the most part, this work has centered in the decarboxylation and deamination of the alanine side chain and information concern-

ing the fate of the aromatic nucleus is practically non-existant. The combined efforts of organic chemists, bio-chemists and pharmacologists have demonstrated that the presence of the 3,4-dihydroxyphenyl group of dihydroxyphenylalanine enhances the effect of sympathomimetic amines. This interesting observation has made the metabolism of the amino acid a problem of primary importance. Furthermore, the recent discovery of the ability of vitamin C to correct the inability of surviving slices from kidneys of scorbutic guinea pigs to oxidize dihydroxyphenylalanine has also added to the interest in the fate of this amino acid in animal tissue.

However, to analyze the series of reactions concerned with the metabolism of a complex substance, the cellular organization must be disrupted so that the enzymes can be separated one from the other and the properties of these enzymes studied. Therefore, an investigation of the ability of cell-free kidney extracts to metabolize dihydroxyphenylalanine is essential for a better understanding of the complete metabolism of the aromatic portion of the amino acid by animal tissue and of the role of vitamin C in the ability of the animals to metabolize the amino acid, _l_-3,4-dihydroxyphenylalanine.

II. HISTORICAL

A. Dihydroxyphenylalanine

A study of compounds structurally similar to adrenalin led to the synthesis of d,l-3,4-dihydroxyphenylalanine by Funk (1) in 1911. By condensing 3,4-carbonyldihydroxybenzaldehyde and hippuric acid and subjecting the resulting oxazolone of α-benzoylamino-3,4-dihydroxycinnamic acid to treatment with 10 per cent sodium hydroxide, he prepared α-benzoylamino-3,4-dihydroxycinnamic acid. By reducing this compound with sodium amalgam and hydrolyzing with 20 per cent hydrochloric acid, Funk prepared the d,l-amino acid. The over-all yield was 8.3 per cent of the theoretical. The resolution was not attempted.

In 1913 Torquati (2,3) noted the presence of a nitrogenous substance in the germ of the seed and in the green pod of the common velvet bean, Vicia faba, but it was Guggenheim (4), using essentially the same extraction technique of Torquati, who finally identified this substance as dihydroxyphenylalanine by preparing protocatechuic acid, tribromodihydroxyphenylalanine and tribenzoyldihydroxyphenylalanine. Guggenheim demonstrated that the compound was levo rotary. Another method of synthesizing the amino acid from piperonyl

bromide and ethylphthalimidomalonate was reported by Stephen and Weizmann (5) in 1914. These authors did not mention their yield and calculation of the yield was not possible from the information given in their report. In 1920 Miller (6) extracted the amino acid from the velvet bean, and identified it by the preparation of the tribenzoyl derivative and protocatechuic acid. In rapid succession Waser and Lewandowski (7), Sugii (8), Hirai (9) and Harington and McCartney (10) synthesized the amino acid. Waser and Lewandowski (7) prepared the amino acid by nitrating tyrosine, reducing the nitrotyrosine to aminotyrosine, diazotizing the aminotyrosine and then preparing the amino acid by heating the diazotized aminotyrosine preparation. The yield for the first step was not given but the over-all yield for the other steps was 51 per cent. Sugii (8) synthesized the amino acid by condensing vanillin with hippuric acid to form the azlactone; then, by treating the azlactone with hot sodium hydroxide, he prepared p-hydroxy-m-methoxy-α-benzoylaminocinnamic acid which was subsequently reduced by sodium amalgam to p-hydroxy-m-methoxy-α-benzoylaminohydrocinnamic acid. The benzyl and methyl groups were removed by treatment with 3 per cent hydrochloric acid in a sealed tube at 150° C. for 8 hours. The amino acid was precipitated in crystalline form by the addition of ammonium hydroxide. No yield was given.

Hirai (9) condensed glycine anhydride and vanillin, and

refluxed the resulting compound, di-(3-acetoxy-4-methoxybenzol)glycine anhydride, with hydriodic acid and red phosphorous. Subsequent acidification and treatment with lead acetate and ammonium hydroxide led to the precipitation of the lead salt of dihydroxyphenylalanine. The lead was removed with hydrogen sulfide, the filtrate concentrated under carbon dioxide and the amino acid allowed to crystallize. The over-all yield was 51.3 per cent.

Harington and McCartney (10) prepared the azlactone from vanillin and hippuric acid, and then prepared ethyl-α-benzoyl-3-methoxy-4-hydroxycinnamate by treating the azlactone with concentrated sulfuric acid. By refluxing this compound with hydriodic acid, acetic anhydride and red phosphorous under hydrogen, they succeeded in preparing the amino acid with an over-all yield of 24.4 per cent.

In 1927 Lieben (11) suggested a colorimetric method for the determination of adrenaline and dihydroxyphenylalanine which was based upon a reaction with 1 per cent ferric chloride in the presence of 10 per cent sodium carbonate (red color) or 10 per cent sodium acetate (blue-violet color). The following year, 1928, Abderhalden and Rassner (12), in a study of the ultraviolet absorption of the α-amino acids, demonstrated a maximum extinction coefficient at approximately 3600 A. and a minimum at 4000 A. No values were determined at wave lengths less than 3300 A.

In 1931 Harington and Randall (13) synthesized the acetyl racemate of dihydroxyphenylalanine by reducing the azlactone, formed by condensing protocatechuic aldehyde and acetylglycine, with sodium amalgam. The acetyl racemate was then resolved with brucine. However, the crystalline brucine salt was not obtained until the acetyl groups had hydrolyzed from the compound. This hydrolysis occurred slowly while the solution was standing and was recognized by the odor of ethyl acetate. Both the _dextro_ rotary and the _levo_ rotary forms of the amino acid were prepared, and the _levo_ rotary isomer was found to be identical with a sample of the natural amino acid prepared by Guggenheim from _Vicia faba_ (4).

An analytical method for determining dihydroxyphenylalanine, based on a reaction of the 3,4-dihydroxyphenyl group, was developed by Arnow (14). The method consists of treating an acid solution of the amino acid with a nitrite-molybdate reagent and developing the nitroso derivative formed with alkali. This method is positive for catechol, epinephrine, and other catechol derivatives. Arnow (15) also studied the formation of dihydroxyphenylalanine from tyrosine by ultraviolet radiation and, shortly thereafter, published a kinetic study of the reaction (16). The conversion of tyrosine to dihydroxyphenylalanine under the influence of ultraviolet light was a first order reaction. The reaction would not proceed _in vacuo_ but in the presence of oxygen

the conversion proceeded readily. Sborov, Peters and Arnow (17), employing the analytical procedure mentioned above, failed in an attempt to demonstrate the presence of dihydroxyphenylalanine in the acid hydrolyzates of egg albumin, casein, edestin and fibrin, although they did realize good recoveries of added dihydroxyphenylalanine under the conditions of acid hydrolysis. To date the amino acid has not been isolated from protein hydrolyzates, and Schmidt (18) states that this may be due to its ease of oxidation. However, it is possible that dihydroxyphenylalanine is not present in animal tissue.

B. Biochemistry of Dihydroxyphenylalanine

Since the discovery by Stådeler (19) of volatile phenols in the urine of the horse, the cow and man, and the observation by Mühlmann (20) of a rise in blood pressure produced by the administration of catechol, many investigations have been initiated to determine the site and nature of phenol metabolism. Among the early investigators, Embden and Glaessner (21) and Elliot (22), by the use of the perfusion technique, reported that the detoxification of phenols, and related compounds, by conjugation occurs mainly in the liver. Barttelli (23), by limiting the oxygen supply of perfused livers, noted that the disappearance of the phenols was, to some extent, determined by the oxygen supply. During this same period Dakin's study (24) of the physiological activity

of substances indirectly related to adrenalin, such as chloro-acetylcatechol, methylamino-acetylcatechol and acetylcatechol, demonstrated the importance of the 3,4-dihydroxybenzene nucleus in the production of physiologically active substances of the adrenalin type. Catechol produced a marked increase in the blood pressure of the rabbit. However, no such effect on the blood pressure was observed when the hydrogen of one of the hydroxyl groups was replaced with a methyl radical.

In 1910 Barger and Dale (25) investigated the relationship of the chemical structure of amines to their sympathomimetic activity and found amines containing the 3,4-diphenolic group more active than the other aromatic amines. Of the amines studied, hydroxytyramine, which is decarboxylated 3,4-dihydroxyphenylalanine, had the greatest activity. During the same period Ewins and Laidlaw (26) observed the appearance of p-hydroxyphenylacetic acid when p-hydroxyphenylethylamine (tyramine) was administered to the intact animal and perfused through an isolated liver. This was the first indication of the deamination of aliphatic amines. Guggenheim (4), after his isolation of dihydroxyphenylalanine from the seeds of Vicia faba, investigated the physiological activity of the compound. When fed to rabbits, dihydroxyphenylalanine produced no essential variation in the blood pressure or respiration. The urine of the rabbits contained an ether soluble substance which produced a green color with ferric chloride and the mother liquor contained a compound which

reacted in a similar fashion. The amino acid had no effect on smooth muscle (uterus, diaphragm). When Guggenheim ingested 2.5 grams he became ill within 10 minutes. In 1927 Hirai and Gondo (27) determined the blood sugar levels at various intervals after the injection of the amino acid and noted the hyperglycemic effect of d,l-3,4-dihydroxyphenylalanine. He also observed the inability of the d,l-2,4- and the d,l-2,5- compounds to produce such an effect.

Although biological deamination of aliphatic amines had been discovered by Ewins and Laidlaw (26) in 1910, it was not until 1928 that Hare (28) demonstrated deamination of tyramine with a simultaneous uptake of oxygen in cell-free liver extracts in vitro. The deamination was proportional to the oxygen consumption and she claimed that the production of ammonia ceased when the excess oxygen consumption stopped. This was the first introduction to the enzyme which was to be known as amine oxidase. Using milk peroxidase, which in the presence of hydrogen peroxide oxidizes nitrite to nitrate, Hare demonstrated the production of hydrogen peroxide in the course of the reaction. Maximal activity of the enzyme was observed at pH 8.0, the enzyme was destroyed at pH 11.5 and, although the activity was greatly reduced, it was not destroyed at pH 4.4. The presence of 0.002 M hydrogen cyanide did not inhibit the reaction. Bernheim (29), in 1931, confirmed the discovery of Hare and, in addition, demonstrated the inability of 0.05 M pyrophosphate to interfere with the deamination of

tyramine. She isolated p-hydroxyphenylacetic acid, and an investigation of the activity of her enzyme preparations at various pH values relative to the age and concentration of her extracts produced conflicting results as to the amount of oxygen consumed in the reaction. When tyramine was the substrate, fresh liver extracts at pH 5.2 consumed 4 atoms of extra oxygen per mole of substrate, while at pH 8.0 only one atom was consumed. However, 36-hour samples of the extract at pH 8.0 and pH 5.2 consumed 2 atoms of oxygen. According to Bernheim the consumption corresponding to 2 atoms of oxygen is the most stable system, and aging the preparation results in the destruction of the other systems.

In 1932 the study of dihydroxyphenylalanine metabolism shifted from the use of tissue extracts to the use of the intact animal. Edmunds and Smith (30) reported that the recovery of dogs subjected to experimental adrenalin depletion by the administration physostigmine, which reduced the adrenalin content of the adrenal glands 60 to 65 per cent, was slightly accelerated by the administration of dihydroxyphenylalanine.

Shortly thereafter, Medes (31) described a case of tyrosinosis -- the only case to date -- in which l-3,4-dihydroxyphenylalanine was excreted under conditions of high tyrosine intake. This condition is due to a failure by the by the organism to oxidize the aromatic acid, tyrosine, completely and is characterized by the excretion of p-hydroxy-

phenylpyruvic acid. In her opinion, the individual manifesting the symptoms of tyrosinosis is able to oxidize homogentisic acid completely so that probably the mechanism for the conversion to the 2,5-dihydroxy derivative is lacking. However, the conversion of the administered tyrosine to the 3,4-dihydroxy derivative was considered very important, for it established the fact that the formation of the 3,4-dihydroxybenzyl group can occur in the animal body. According to Medes, the 3,4- compound must be oxidized with difficulty in the body because it appeared in large quantities in the urine while the 2,5- compound, homogentisic acid, was readily oxidized. Medes theorized that the 3,4- oxidation product may be used for some synthesis in the body, probably adrenaline, while the 2,5- compound is the normal catabolic breakdown of tyrosine. The possible role of dihydroxyphenylalanine in adrenaline formation was more clearly defined when Heard and Raper (32) noted the inability of the adrenal gland, perfused with a solution of N-methyl-3,4-dihydroxyphenylalanine, to synthesize adrenalin. They concluded that, if _l_-3,4-dihydroxyphenylalanine is used in the synthesis of adrenalin, the amino acid must be decarboxylated before methylation occurs.

The sympathomimetic action of compounds with and without the 3,4-diphenolic nucleus was also investigated during this period. Weinstein and Manning (33) noted that monomethylaminoethanol-3,4-quinone had no effect on the blood pressure of cats and suggested the necessity of the hydroxyl groups

for the sympathomimetic action of adrenalin-type compounds. An investigation of the physiological activity of catechol and catechol derivatives by Mulinos and Osborne (34) led to the conclusion that, as had been suggested by Barger and Dale (25), the 3,4-diphenolic nucleus was, in great part, responsible for the activity of epinephrine. In a later report (35) these authors described the sympathomimetic effect of catechol and catechol derivatives in both intact and pithed animals.

At this point, the use of cell-free enzyme preparations of the various tissues became popular in the study of systems intimately related to the metabolism of dihydroxyphenylalanine. Keilin and Hartree (36) noted that kidney slices or fresh kidney tissue, macerated with sand and suspended in a buffer at pH 7.8, could, in addition to oxidative deamination, promote oxidation of both optical isomers of dihydroxyphenylalanine by attacking the ring portion of the molecule. According to the authors, this ring oxidation, which was evidenced by the darkening of the extracts, was promoted by a polyphenol oxidase, which also oxidized catechol, pyrogallol and adrenalin but not monophenols. The reaction was inhibited by cyanide. In view of investigations to be discussed shortly the oxidation of both optical isomers may have been nonspecific, for Keilin and Hartree, conducting their incubation at pH 7.8, would have experienced considerable autooxidation of the substrates. They did not mention the use of controls in

these experiments.

In 1937 a series of papers which did much to characterize the amine oxidase appeared in the Biochemical Journal, and, as this enzyme will be shown to be very important in the metabolism of dihydroxyphenylalanine, these papers will be reviewed in some detail. Pugh and Quastel (37), using surviving slices of guinea-pig kidney and liver, demonstrated the deamination of butylamine and the ability of brain tissue, which does not deaminate l-amino acids, to deaminate aliphatic amines. This indicated that the amine oxidase discovered by Hare (28) was not identical with the d- or l-amino acid oxidases. By employing both liver slices and liver extracts the view that the amine oxidase was an aerobic oxidase was substantiated by Philpot (38) who also confirmed the formation of hydrogen peroxide by employing the principle of coupled oxidation in which the hydrogen peroxide oxidized ethyl alcohol to acetaldehyde. The principle of coupled oxidation is based on the formation of hydrogen peroxide which, in the presence of either catalase or peroxidase, will oxidize a suitable substrate. In this case the hydrogen peroxide formed in the amine oxidation was used to oxidize ethyl alcohol to acetaldehyde. This prevented the oxygen, formed by the action of catalase on the hydrogen peroxide, from returning to the atmosphere of the flask and resulted in almost doubling the excess oxygen uptake. Hydrogen

cyanide which is a good inhibitor of catalase inhibited the coupled oxidation by about 50 per cent. Blaschko, Richter and Schlossmann (39) studied the rate of deamination of sixty-six amines and, of all the amines assayed, the enzyme was most active toward hydroxytyramine. On the basis of tyramine as 100 the relative rates of oxidation of substrates important to this discussion are shown in Table I. Of the compounds

TABLE I

ACTIVITY OF AMINE OXIDASE (39)

3,4-Dihydroxyphenylethylamine	140
Tyramine	100
Butylamine	54

tested only those with an amino group on the end carbon of the hydrocarbon chain were deaminated. Compounds with the amino group on the second carbon atom -- isopropylamine, benzedrine, ephedrine, amino acids -- and short chain amines -- propylamine and below -- were not oxidized. Hydrogen cyanide and semicarbazide did not affect the course of the reaction but positive inhibition was obtained with octyl alcohol and thymol. An investigation of the distribution of the enzyme system indicated its presence in the liver, intestine, brain and kidney of the guinea pig. Other forms of life such as the pigeon, tortoise, frog and trout gave a positive reaction. Richter (40), using guinea-pig liver as the source of the

enzyme and the presence of 0.05 molar semicarbazide to protect the aldehydes from destruction by aldomutase, isolated the aldehydes formed from benzylamine, phenylethylamine, tyramine, and adrenaline with 2,4-dinitrophenylhydrazine. The reaction mixture was deproteinized by boiling at pH 5.3, acidified with hydrochloric acid and then treated with a saturated solution of 2,4-dinitrophenylhydrazine in 2N hydrochloric acid. By studying the oxidation of aliphatic amines in the presence of ethyl alcohol Pugh and Quastel (41) confirmed the formation of hydrogen peroxide. Dialysis against phosphate-Locke solution for twenty hours through cellophane had no apparent diminishing effect on the activity of the enzyme preparation, and, although arsenite and hydrogen cyanide did not effect the activity, in the presence of the hydrogen cyanide only one atom of oxygen was consumed for each mole of amine consumed and one mole of ammonia was liberated. It was shown that hydrogen cyanide reduces the side reactions occurring during the incubation,making possible the realization of the theoretical consumption of oxygen. The 1:1 oxygen to ammonia ration was confirmed by Kohn (42) who showed that, as the pH of the reaction mixture was reduced, tyramine oxidation was also reduced. However, the theoretical 1:1 ratio was still obtained at pH 6.3 even though the oxygen consumption was only 40 per cent of that observed at pH 7.7. The hydrogen cyanide concentration was increased to 0.01 M with

no apparent effect on the deamination except to reduce the value of the control and to inhibit extraneous oxidation. Neither hydroxylamine nor iodoacetic acid at a concentration of 0.01 M inhibited the reaction, but 0.01 M hydrazine hydrate or ethylurethane inhibited the deamination approximately 30 and 4 per cent, respectively. Dialysis did not decrease the activity and the formation of hydrogen peroxide was again indicated by the use of both peroxidase and coupled alcohol oxidation. Kohn was the first to demonstrate the relationship of the activity of the amine oxidase to the oxygen tension. The increase in rate as the atmosphere was changed from air to pure oxygen amounted to 200 to 250 per cent. Two lines of evidence favor the formation of the aldehyde when tyramine is oxidized: (1) the aldehyde formation can be detected manometrically by adding Schardinger enzyme which increases the oxygen consumption up to 75 per cent; (2) a 2,4-dinitrophenylhydrazone derivative was obtained from the reaction mixture. In 1938 Werle, et al (43) confirmed the optimum pH of 8.0 reported by Hare for the amine oxidase system.

The characterization of the amine oxidase was very important, for the system was soon shown to be essential in the metabolism of dihydroxyphenylalanine by kidney extracts. In 1938 Holtz (44) and Holtz, Heise and Lüdtke (45) indicated the formation of hydroxytyramine from the amino acid by demonstrating the pressor action of the amine formed, by the isolation of the tribenzoyl derivative and by manometrically

measuring the carbon dioxide evolved. Conversion of the dihydroxyphenylalanine to hydroxytyramine was found to be about 80 per cent of the theoretical. The importance of anaerobic conditions was emphasized by the action of the amine oxidase on the hydroxytyramine when the reaction was attempted in the presence of air. The hydroxytyramine was oxidized in the same manner as other aliphatic amines by the amine oxidase present in kidney extracts. A preparation of d-amino acid oxidase did not oxidize the l-amino acid or hydroxytyramine but gave the expected oxygen consumption with d,l-3,4-dihydroxyphenylalanine. Some degree of specificity was demonstrated for only one-half the activity was realized when d,l-3,4-dihydroxyphenylalanine was the substrate. The pH-activity relationship was not explored completely, but the decarboxylase was reported to be most active between pH 7.0 and 8.0 while at pH 5.0 the system was virtually inactive. The enzyme was sensitive to cyanide inhibition. The decarboxylase was shown to be present in the tissue extracts of the guinea pig, rabbit and swine. The activity of the amine oxidase present in the kidney extracts was indicated by the oxygen consumption when the reaction was carried out aerobically. By employing semicarbazide, to prevent the destruction of the aldehyde formed by the deamination, Holtz, Heise and Lüdtke isolated 3,4-dihydroxyphenylacetaldehyde and identified this compound by its 2,4-dinitrophenylhydrazone derivative. The

blood pressure response of cats subjected to injections of the extracts which had been incubated anaerobically was typical of the response caused by the injection of sympathomimetic amines. If the reaction was carried out aerobically no such rise in blood pressure resulted. Furthermore, if the aerobic reaction was carried out in the presence of vitamin C, subsequent injection produced an actual reduction in the blood pressure. The authors suggested, in view of this evidence, that the ascorbic acid protects the 3,4-dihydroxyphenylacetaldehyde from destruction and that the aldehyde produces the depressor effect. The addition of ascorbic acid to the kidney extracts prevented the extracts from becoming dark brown in the course of the reaction. This was probably due to the well-known antioxidant properties of vitamin C, for vitamin C has been used by other investigators (46) to prevent miscellaneous oxidations. Although the amine oxidase system was not subject to cyanide inhibition, it was sensitive to the presence of octyl alcohol.

Catechol oxidase enzyme systems were still receiving the attention of various investigators. In 1938 a wide variety of materials was examined for a phenol oxidase of the catechol oxidase type by Bhagvat and Richter (47). All vertebrate tissues tested, including guinea-pig tissue, were comparatively inactive. The small oxygen consumption in the case of the vertebrate tissues was ascribed, in part, to non-enzymatic

catalysis by traces of heavy metal and, in part, to the cytochrome-cytochrome oxidase system, which catalyzes the slow oxidation of catechol derivatives. On the basis of these results the authors questioned the presence of a phenol oxidase in animal tissue. The authors investigated the importance of small quantities of metals in the oxidation of the catechols, and the amount of copper, as copper sulfate, needed to promote oxidation of dihydroxyphenylalanine was found to be as little as 0.025 mg. Other metallic ions -- iron, nickel, cobalt and manganese -- were also found to increase the oxygen consumption with catechol, homocatechol and adrenaline.

In 1939 Holtz, et al, continuing the interesting series on the dihydroxyphenylalanine decarboxylase, found the enzyme in guinea-pig liver (48), in the liver and kidney of sheep, goat, pigs and hens but not in rats, mice and cattle (49) and in the mucous membrane of the small intestine of the guinea pig (50). Holtz (51) demonstrated that the activity in pig kidney was only 75 per cent, and the activity of the rabbit kidney was only 20 to 25 per cent that of the guinea-pig kidney tissue, and he also indicated that the rat kidney was practically devoid of this enzyme system. By the use of d,l- and l-dihydroxyphenylalanine the ability of the rat kidney extracts to oxidize half of the d,l- form of the amino acid and none of the l- form of the amino acid was noted. The

activity of the rat tissue was attributed to the very active d-amino acid oxidase of this animal. Blaschko (52) confirmed the discovery of the decarboxylase in liver and, by using a pure form of d-3,4-dihydroxyphenylalanine, demonstrated the inactivity of the extracts in the presence of this isomer. The inactivity of the enzyme system in the presence of d,l-N-methyl-3,4-dihydroxyphenylalanine, l-tyrosine, d,l-N-methyl-tyrosine, l-phenylalanine and l- and d-histidine was further evidence of the degree of specificity of the decarboxylase. During this period Bhagvat, Blaschko and Richter (53) found amine oxidase widely distributed and Blaschko (54), in 1940, proposed the general rule that β-aliphatic amines inhibit the amine oxidase system. This was attributed to competition between the refractory amine and the active substrate for the enzyme.

The reports of the formation of adrenaline by extracts of the adrenal gland by Vinet (55,56) in 1940 are of primary interest in this connection. By adapting the analytical technique developed by Meunier (57) to mixtures of dihydroxy-phenylalanine, hydroxytyramine and adrenaline, Vinet obtained evidence which led her to report the formation of adrenaline from dihydroxyphenylalanine and hydroxytyramine by extracts of the adrenal gland. Tyramine under similar conditions was not converted to adrenaline. In the same year Hasiguti (58) reported no change in the adrenal glands after injections of

dihydroxyphenylalanine or liver extracts, but noted a marked increase of the vacuoles and of the chromaffin substances after the injection of both, and believed that this indicated the formation of adrenaline from the dihydroxyphenylalanine in the presence of a substance from the liver.

The reports of the effect of the enzymatic decomposition products of dihydroxyphenylalanine on both experimental and normal hypertension was of considerable interest during this period. Bing (59), and Bing and Zucker (60), perfusing cat kidney with a solution of l-3,4-dihydroxyphenylalanine and testing the activity of the resulting perfusate by its effect on the blood pressure rise of cats, reported that the ischemic kidney released a pressor substance having the properties of 3,4-dihydroxyphenylethylamine. The perfused liver and gut of the cat under analogous conditions did not produce pressor substances. Bing, Zucker and Perkins (61), while studying the comparative destruction of angiotonin, hydroxytyramine and tyramine by renal extracts, noted the inactivation of hydroxytyramine by kidney tissue in the presence of octyl alcohol while tyramine was not affected. They suggested the existence of an aerobic oxidase, not sensitive to octyl alcohol inhibition, which might be inactivating the hydroxytyramine, but submitted no data on oxygen consumption to support their suggestion. They did demonstrate the stability of hydroxytyramine under anaerobic conditions. These experi-

ments were carried out in 500 ml. flasks at 40° C. The activity of the products of these incubations was estimated by injecting the products intravenously into anesthetized cats and measuring the blood pressure response. Relating oxygen consumption to the inactivation of hydroxytyramine was impossible under such experimental conditions and oxidation of the hydroxytyramine in the preparation of their extracts for injection may have caused the inactivation of the hydroxytyramine. Bing and Zucker (62) demonstrated the *in vivo* effect of dihydroxyphenylalanine in animals in which partially ischemic kidneys were produced by applying the Goldblatt clamp on the renal artery. Control animals did not produce any blood pressure rise when injected with the amino acid.

Oster and Sorkin (63) obtained a marked rise in the blood pressure of cats with experimental hypertension when dihydroxyphenylalanine was injected intravenously but noted no such effect in cats with acute renal ischemia or in normal cats. Intravenous injection of the amino acid into humans with essential hypertension produced a similar pressor response, however, a much less marked pressor effect was obtained in humans with normal blood pressure. The possibility of a defective renal deamination due to kidney ischemia was suggested as being responsible for the pressor effect of the *l*-3,4-dihydroxyphenylalanine in the case of essential human hypertension.

Schroeder (64) noted only a transient hypertension in rats after dihydroxyphenylalanine administration, and Greengard, Roback and Ivy (65) reported that dihydroxyphenylalanine stimulated pancreatic secretion.

The relative importance of the dihydroxyphenylalanine decarboxylase was strengthened by Blaschko (66). He obtained 1 mole of carbon dioxide per mole of amine formed and noted that about one-half of the carbon dioxide formed during the reaction was retained by the enzyme solution, but that this was released when sulfuric acid was added at the completion of the experiment. Dialysis against distilled water for forty hours at 1° C. did not decrease the activity of the enzyme preparation. The attempt by Blaschko to inhibit the action of the decarboxylase is summarized in Table II.

TABLE II

INHIBITION OF DIHYDROXYPHENYLALANINE DECARBOXYLASE (66)

Inhibitor	Inhibition per cent
Cyanide (0.001 M)	72.5
Hydroxytyramine (0.02 M)	71.0
Suramine (0.0001 M)	57.0
Trypan blue (0.0001 M)	33.0
Sodium sulfide (0.02 M)	21.0
Cyanide (0.0001 M)	00.0
Sodium azide (0.002 M)	00.0
Carbon Monoxide	00.0

The 71 per cent inhibition by hydroxytyramine was very interesting for the ability of the product of the reaction to inhibit the decarboxylase was thus demonstrated. The cyanide inhibition was also shown to be reversible. Twelve amino acids were tested but carbon dioxide was evolved only when l-3,4-dihydroxyphenylalanine was the substrate (Table III).

During this period Holtz and Credner (67) and Holtz, Credner and Koepp (68) reported the presence of a hydroxytyramine-like substance in the urine of the animals fed l-3,4-dihydroxyphenylalanine, and furthermore, demonstrated the possibility of a conjugated form of hydroxytyramine in the urine of these animals. When the l-amino acid was administered orally the hydroxytyramine was excreted slowly and over an extended period (four hours) but when injected intravenously the maximum excretion occurred in the first thirty minutes and was completed in about an hour; only 12 per cent of the dihydroxyphenylalanine was recovered as hydroxytyramine when taken orally, but 36 per cent was recovered when injected intravenously. The hydroxytyramine of the hydrolyzed and unhydrolyzed urine samples, when separated from the urine by adsorption on aluminum hydroxide and recovered by elution, presented the same picture. As much as 50 per cent of amine was recovered in the conjugated form. A large volume of normal urine was concentrated *in vacuo* to prevent the destruction of easily oxidizable substances, and from the blood pressure response obtained by

TABLE III

SPECIFICITY OF THE DECARBOXYLASE (66)

Substrate	Pig	Guinea-pig		Rhesus	
	Kidney	Liver	Kidney	Liver	Kidney
l(-)-dopa	+	+	+	+	+
d(+)-dopa	-	-			
d,l-N-methyldopa	-	-			-
l(-)-tyrosine	-			-	-
d,l-N-methyltyrosine (synthet. surinamine)		-			
d,l-phenylalanine			-		
l(-)-tryptophan		-			
l(-)-histidine	-	-	-	-	-
d(+)-histidine	-				
d,l-alanine			-		
d,l-proline			-		
d,l-hydroxyproline			-		
d,l-serine			-		
l(-)-leucine			-		

- signifies formation of carbon dioxide
- signifies no formation of carbon dioxide
Blank signifies no test made

injecting hydrolyzed and unhydrolyzed samples of this concentrate, the authors suspected the presence of small quantities of a hydroxytyramine-like substance in normal urine. Holtz and Credner (69), determining hydroxytyramine in the urine by its effect on the blood pressure, found no reason to assume that _d_-3,4-dihydroxyphenylalanine was metabolized in the guinea pig and the rabbit, for when equivalent amounts of the _l_- and _d,l_- form of the amino acid was ingested the activity of the urine from the guinea pig fed the _l_- form was twice as great as the activity of the urine from the animal fed the _d,l_- form. However, the rat apparently can change the _d_- form to the _l_- form for the urine of the rat gave the same blood pressure response when both the _l_- and the _d_- form of the acid was administered. The authors again suggest that probably the abundance of the _d_-amino acid oxidase in rat tissue was responsible for the conversion of the _d_- form of the amino acid to the _l_- form and then decarboxylation to the hydroxytyramine was possible. In 1942 Holtz, Credner and Strübung (70), in a continuation of their work on the amino acid decarboxylases, reported a slight decarboxylase activity in rat kidney and liver and found guinea-pig liver to be one-third as active as guinea-pig kidney. The pancreas also exhibited decarboxylase activity. Curves of the carbon dioxide evolved showed that the reaction was almost completed in the first thirty minutes

of the reaction. Beyer (71) pursuing his studies on sympathomimetic amines confirmed the report of Blaschko (54) that isopropyl derivatives will inhibit the activity of the amine oxidase and that the terminal carbon-nitrogen bond was necessary for this reaction to proceed.

The presence of a catechol oxidase in animal tissue was reported by two groups of investigators in 1942. Cadden and Dill (72) prepared a cell-free extract of pig kidney which was active toward catechol and hydroquinone when incubated in a phosphate-acetate buffer at pH 7.0 - 7.2, but which would not oxidize tyrosine, dihydroxyphenylalanine and _p_-cresol. Although the oxygen consumption was measured, no data which would show the relationship between the color appearing during the incubation and the oxygen consumption was reported. The preparation was reported to increase the rate of substrate oxidation with increase in pH. Only partial inactivation was noted when the preparation was heated in a water bath for one hour at 80 - 100° C. and, as no information was offered relative to the per cent inactivation and as to whether the dry powder or a buffered extract was heated, it is very difficult to draw any conclusions from this paper. The fact that the heat treatment did not totally inactivate the preparation is surprising and, if the preparation had considerable activity after such heat treatment, a nonspecific oxidation or metallic ion catalysis might be expected. The only data actually reported were concerned with the oxidation of

a catechol-hydroquinone mixture and the relative rates of the activity of the enzyme with catechol and hydroquinone, when used separately, were not stated. Hogeboom and Adams (73) were able to obtain extractable enzymes from a mouse tumor which catalyzed the oxidation of both tyrosine and dihydroxyphenylalanine to melanin. The total consumption of oxygen after completion of the reaction approximated four atoms of oxygen per molecule of l-3,4-dihydroxyphenylalanine. This is similar to the reaction of the tyrosinase from such sources as mushrooms and potatoes, and, although this is a report of an oxidase in abnormal tissue, it is one of the few instances of a catechol-type oxidase in animal tissue. The activity of the enzyme at pH 6.0 is such that four atoms of oxygen are consumed per mole of substrate. This fact divorces this enzyme from the system under discussion.

In 1943 considerable emphasis was placed on the study of the amine oxidase. Raska (74) found the oxygen consumption of both slices and extracts of the ischemic kidney in the presence of l-3,4-dihydroxyphenylalanine to be less than that of the normal kidney. In Raska's opinion the equilibrium between the decarboxylating system and the amine oxidase system in the ischemic kidney is altered in such a way that the former is in pathological excess, i. e., the anaerobic degradation process may exceed the aerobic oxidation process. His examinations demonstrate that in ischemic kidney the

respiration of the kidney slices and extracts were markedly reduced even when neither necrosis nor atrophy could be found. This decrease in oxidative ability was paralleled by a decrease in the amount of ammonia produced during the incubation. However, no ammonia values were presented for the _l_-3,4-dihydroxyphenylalanine experiments, and from the variety of the amines used as substrates, one would conclude that Raska was dealing with the amine oxidase system. The report makes no effort to correlate these results with the disappearance of the diphenolic nucleus of _l_-3,4-dihydroxyphenylalanine, since the latter was not measured.

To study the substrate specificity and the kinetics of the oxidation of certain amines, Alles and Heegaard (75) prepared a purified amine oxidase. Previously, Hare (28) and Werle (43) had reported an optimum pH of 8.0 for the amine oxidase with a rapid decrease at both higher and lower pH values, but Alles and Heegaard obtained a variation of the pH of maximum activity depending on the substrate used. For example: with butylamine, amylamine, hexylamine, heptylamine and octylamine as substrates the points of maximum activity were pH 8.1, 7.5, 7.1, 6.9 and 7.1, respectively. Results obtained with a series of phenylalkylamines and reproduced here from a graphical representation show even wider variation. The optimum pH of phenmethylamine was 8.6, phenethylamine - 6.1, phenpropylamine - 7.8, and phenbutylamine and phenamylamine - 6.8. The change in the

optimum of the pH-activity curve of an enzyme with a change in substrate, when the substrate is ionizable, is discussed by Baldwin (76). As is well known from numerous studies, the optimum pH, when the substrate is ionizable, is the resultant of the variation of the enzyme activity and the variation of the ionization of the substrate with pH. Alles and Heegaard suggest the existence of more than one amine oxidase in their enzyme preparation but the explanation of Baldwin seems more logical for Alles and Heegaard have noted that the pK_b values for the amines mentioned above vary considerably. The list of substrates studied contained aliphatic primary amines, N-methyl derivatives of aliphatic amines, phenylaliphatic amines and ring-substituted phenylaliphatic amines. Among the last mentioned substrates were the phenolic derivatives which are of special interest to this discussion. Using phenethylamine as a base of 100, the maximum oxidation rates, relative to phenylethylamine at pH 7.0 and 30° C., were tyramine 80 and hydroxytyramine 68. These figures do not agree exactly with those previously mentioned in the report of Blaschko, Richter and Schlossmann (39) but the experimental conditions were so different in each case that comparison is almost impossible. Blaschko, et al were using crude extracts and Alles and Heegaard were using a partially purified preparation. Heegaard and Alles (77) studied aliphatic amines and phenylaliphatic amines as inhibitors of the amine oxidase system and concluded that

the secondary and tertiary carbinamines are inhibitors and, in addition, are not oxidized.

In 1944 Gonoid and Pelau (78) reported that the kidney extracts of the guinea pig and the rabbit did not decarboxylate l-tyrosine but did decarboxylate l- and d,l-dihydroxyphenylalanine equally well in air or nitrogen. This is in complete disagreement with the reports of Holtz, et al (44,70) and Blaschko (66) who found the decarboxylase to be very specific with regard to configuration.

In 1944 Holtz, Credner and Strübung (79) demonstrated the hyperglycemic effect resulting from the injection of hydroxytyramine and dihydroxyphenylalanine and attributed this effect to the hydroxytyramine produced by the decarboxylase. In the case of the hydroxytyramine the blood sugar exhibited an early rise and fall, but in the case of the dihydroxyphenylalanine the curve was not as high and was maintained for as long as three hours. The effect of the l- and d,l- amino acids was also illustrated and, gram for gram, the d,l- form was only half as active as the l- form in producing the hyperglycemic effect. Holtz and Credner (80), reporting further on the metabolism of the l- and d,l- amino acids first mentioned by these authors in 1941 (69), gave figures of the ratio of the hydroxytyramine produced by the administration of l- and d,l- amino acids as 1/0.45, 1/0.77, 1/0.33 and 1/0.66, which again indicates that the d- form is not metabolized in the guinea pig.

In 1945 a study of the amine oxidase system was reported. Blaschko and Duthie (81) demonstrated that amidines -- mono- and diamidine, mono- and diguanidine, diisothiourea -- inhibited the enzyme, and very shortly thereafter (82) showed that, in the very short chain diamines (up to C_8), the second amino group interfered with the affinity of the substrate for the amine oxidase but, when long chain diamines (C_{14}) were employed, the interference disappeared. The authors suggested that the amino groups of the long chains were far enough apart for the enzyme to act on one amino group without interference from the other.

After 1945 reports on the amine oxidase and the catechol conjugation system have not appeared. Instead, research interest shifted to the study of the decarboxylase. After a report by Page and Reed (83) on the hypertension effect of dihydroxyphenylalanine and related compounds in the rat, Page (84) failed in an attempt to prepare an acetone dessicated decarboxylase from guinea-pig kidney. Drying in the cold by a lyophilic process reduced the activity by at least 50 per cent and on two occasions completely abolished the activity. A stable preparation of variable activity was prepared by drying a buffered saline extract of the tissue in a cellophane sac at room temperature and storing the powder *in vacuo*. Page did not give the details of this preparation. Dialysis of the extracts for twenty-four hours at 5° to 7° C. did not

reduce the activity of the preparations. In the presence of excess l-3,4-dihydroxyphenylalanine the yield of hydroxytyramine was directly proportional to the amount of substrate present. Using a biological assay (pressor effect), comparison of tissue extracts from various sources demonstrated that rabbit and monkey kidneys were about one-tenth as active as guinea-pig kidney but three times as active as human kidney.

The work on the bacterial decarboxylases led to the demonstration by Green, Leloir and Nocita (85), in 1945, that phosphorylated pyridoxal was as active as bacterial codecarboxylase in regenerating the activity of mammalian 3,4-dihydroxyphenylalanine decarboxylase. Shortly after Gunsalus and Bellamy (86) showed that Streptococcus fecalis required pyridoxine in order to exhibit maximum decarboxylation of tyrosine, the vitamin was shown to be important in the decarboxylation of arginine (87,88), ornithine (87), glutamic acid (88), tyrosine (87,89,90), lysine (91), and l-3,4-dihydroxyphenylalanine (92). Gale and Epps (91) succeeded in extracting a codecarboxylase from bacteria and demonstrated its wide distribution in animals and bacteria. Umbreit, Bellamy and Gunsalus (93) discovered that pyridoxal, which had been subjected to both chemical and enzymatic phosphorylation, would replace the codecarboxylase of Gale and Epps, and this set the stage for Green, Leloir and Nocito (85) to demonstrate the necessity of the phosphorylated pyridoxal in l-3,4-dihydroxyphenylalanine decarboxylase.

These authors prepared a purified decarboxylase from pig liver, which was not active unless supplemented with a coenzyme found in boiled liver and heart. The components of the liver soluble in 0.8 per cent sodium chloride were fractionated with ammonium sulfate and then dialyzed against dilute ammonia (0.002 M) at 0° C. for four days. The preparation was virtually inactive but the presence of the codecarboxylase of Gale and Epps (94) and, in addition, reducing substances such as cysteine or glutathione regenerated the activity of the system. Green's group also found that the pyridoxal-1-phosphate of Umbreit, et al (93) was as active as codecarboxylase in regenerating the activity of their enzyme preparation and thus demonstrated the need of the system for the phosphorylated product.

In 1945 Schapira (95) noted the high decarboxylating activity of the guinea-pig tissue and also reported the inactivity of adrenal tissue.

Another report of the discovery of a catechol-type oxidase in animal tissue strengthened the previous reports of Cadden and Dill (72) and Hogeboom and Adams (73). Hermann and Boss (96) prepared an extract from ciliary body which did not accelerate the oxidation of hydroquinone or catechol in the absence of cytochrome-C, but did enhance the oxygen consumption with l-3,4-dihydroxyphenylalanine as a substrate. The preparations most active with respect to l-3,4-dihydroxy-

phenylalanine oxidation showed the lowest cytochrome oxidase activity. They suggest the possibility of a "dopa oxidase" which could possibly be separated from the cytochrome system and which would also oxidize compounds containing the 3,4-diphenolic nucleus. This work supports the view of Hogeboom and Adams (73) but again the absence of experiments which measured the disappearance of the substrates makes interpretation difficult.

In 1946 Beyer (97) reviewing the subject of pressor amines summarizes as follows:

> "If the nucleus is unsubstituted by hydroxyl groups, derivatives of phenylethylamine, which have the amino group on the terminal carbon atom, are deaminated in the body and are not excreted as such. Where the amino group is not on the terminal carbon atom the compound is refractory to deamination . . . and are excreted for the most part as such. p-Hydroxy derivatives of β-phenylethylamine, regardless of the position of the amino group on the side chain, are inactivated in the body but are conjugated, at least in part, in so loose a manner as to be indistinguishable from the free forms of the compound after excretion. Compounds having a catechol nucleus are excreted predominantly in the conjugated form when administered orally . . . . On hydrolysis of the urine the free form on the sympathomimetic amine is hydrolyzed from its conjugate."

In this review Beyer notes that when β-3,4-dihydroxyphenylethylamine was administered orally the original compound, as shown by chemical and biological analysis, could be recovered from the urine after acid hydrolysis.

In 1946 the effect of desoxypyridoxine on the decarboxylase was noted. Desoxypyridoxine has been shown to be an effective inhibitor of pyridoxine (98). Recently, Beiler

and Gustav (99) have shown that desoxypyridoxine does not inhibit tyrosine decarboxylase, but that phosphorylated desoxypyridoxine will replace pyridoxal phosphate in the tyrosine decarboxylase system. As certain other decarboxylases have been shown to be dependent upon the presence of the pyridoxal phosphate for their maximum activity, the above inhibition study and the previously mentioned discovery by Green, et al (85) would suggest that the l-3,4-dihydroxyphenylalanine decarboxylase system might also be inhibited by the phosphorylated desoxypyridoxine. However, in a later paper Martin and Beiler (100) found that desoxypyridoxine phosphate did not inhibit the decarboxylation but that two folic acid displacing agents -- 7-methylfolic acid and the aspartic acid analog of folic acid -- were efective as inhibitors of the decarboxylation of the amino acid.

C. Ascorbic Acid and Aromatic Amino Acid Metabolism

The relationship of vitamin C to the metabolism of the aromatic amino acids is a rather recent development, but the history of the abnormal condition which eventually led to the recognition of this relationship goes back to the middle of the nineteenth century. As the purpose of this discussion is not a detailed review of this phase of the metabolism of the aromatic amino acids, this early history will be treated

briefly. A more detailed discussion of this early period may be found in the review by Dakin (101).

Thirty years after the original announcement on alcaptonuria by Bödeker (102), Walkow and Baumann (103), in 1891, isolated homogentisic acid from the urine and established the dependence of the appearance of this metabolite on the ingestion of proteins and tyrosine. The fact that tyrosine was almost completely converted to homogentisic acid led to the conclusion, with the support of additional evidence, that tyrosine is the substance from which homogentisic acid is formed.

During the following years considerable discussion revolved around the question of whether or not homogentisic acid was the product of normal or abnormal metabolism. Neubauer (104), as the result of studies with an alcaptonuric patient, was able to demonstrate the role of tyrosine, phenylalanine, phenylpyruvic acid and p-hydroxyphenylpyruvic acid in the formation of homogentisic acid, and postulated a scheme of intermediary metabolism for these amino acids based on the supposition that homogentisic acid was a normal intermediate. In his opinion the peculiarity of the alcaptonuric consequently resides in his inability to carry the oxidation beyond the homogentisic acid stage.

On the other hand Dakin (105) concluded that the production of homogentisic acid was an abnormal metabolism. He

based his conclusions on feeding experiments conducted by Abderhalden (106) in which phenylpyruvic and p-hydroxyphenylpyruvic acids were excreted after feeding large amounts of phenylalanine, and on his own feeding of phenylalanine derivatives which because of their constitution were incapable of forming p-quinone structures. According to Dakin the only path of normal oxidation lies through p-hydroxyphenylpyruvic acid, in which ring opening occurs. This debate has continued through the years and a definite decision is still to be realized.

The first indication of the importance of vitamin C in the metabolism of the aromatic amino acids was the report by Szent-Györgyi (107) of the removal of the pigmentation appearing in Addison's disease by the administration of vitamin C. Previously Bruno Block, in 1917, (108) had indicated the importance of l-3,4-dihydroxyphenylalanine in melanin formation in animal tissue and Raper, in 1928, (109) had successfully related the amino acids, tyrosine and dihydroxyphenylalanine,to the formation of melanin in plant tissue. The discovery of Szentz-Györgyi gave added impetus to the study of the metabolism of these amino acids in animal tissue. Interposed between the announcement of Szentz-Györgyi and future reports of the importance of vitamin C in the metabolism of the aromatic amino acids was a demonstration by Papageorge and Lewis (110) of the excretion of homogentisic acid after prolonged and excessive feeding of phenylalanine.

On the basis of this evidence the authors regarded homogentisic acid as an intermediate in normal metabolism. Butts, et al (111) soon confirmed this report but were unable to find homogentisic acid after tyrosine feeding.

In 1939 Sealock, Ziegler and Driver (112) noted the increased requirement of the guinea pig for vitamin C when the diet was supplemented with tyrosine and dihydroxyphenylalanine. In the same year Sealock and Silberstein (113,114) identified homogentisic acid and other tyrosine metabolites in the urine of vitamin C deficient guinea pigs, and were able to cause removal of these metabolites by the administration of vitamin C. Simultaneously Levine, Marples and Gordon (115) reported that the urine of premature infants receiving cow's milk as a source of protein contained α-hydroxy and α-keto acids derived from tyrosine. Furthermore, they were able to demonstrate the disappearance of these acids from the urine when vitamin C was administered (116, 117). The possible relationship of vitamin C to the alcaptonuric individual was tested by Monsonyi (118), Diaz, Mendoza and Rodriquez (119) and Sealock, Gladstone and Steele (120) but, even though the last mentioned investigators administered large doses of vitamin C, the vitamin was found to be ineffective in correcting the condition. However, this fact did not detract from the importance of the experimental alcaptonuria for as Sealock, et al concluded:

"When one recalls that the majority of metabolic reactions are chain reactions proceeding under the influence of many different factors and enzyme systems the above difference is not surprising. In the guinea pig the missing factor is ascorbic acid while in the alkaptonuric patient it is not the vitamin but some other factor as yet unknown."

The work of Sealock and his associates has done much to clarify the relationship of vitamin C to the metabolism of tyrosine and tyrosine derivatives. The role of these compounds in the production of tyrosine metabolites was explored further (121). The feeding of extra l-phenylalanine and phenylpyruvic acid to scorbutic guinea pigs resulted in the appearance of tyrosine metabolites which could be eliminated by the administration of vitamin C, whereas the metabolism of p-hydroxyphenylpyruvic acid was only slightly effected by the ingestion of this vitamin. The possible role of the dicarboxylic acids in mobilizing the tissue storage of vitamin C has been noted (122). Single doses of glutamic acid resulted in the transient disappearance of the metabolites from the urine of scorbutic guinea pigs fed extra l-tyrosine but repeated administration of the dicarboxylic acid had no permanent effect. Previously Hawley, et al (123) had demonstrated the importance of acid-base balance in vitamin C storage and in the light of these results, Sealock suggests:

" . . . that the acidifying agent in our own experiments mobilizes tissue reserves of ascorbic acid not otherwise called upon, and this newly mobilized vitamin then exerts its usual action in tyrosine metabolism."

The structural specificity of tyrosine in relation to the metabolic action of ascorbic acid was demonstrated (124) by the fact that the urinary excretion of keto acids and the "tyrosyl" value for d-tyrosine, d-phenylalanine, N-acetyl-l-phenylalanine, N-acetyl-l-tyrosine, diacetyl-l-tyrosine, l-p-methoxyphenylalanine, d,l-phenylaminobutyric acid, and l-S-benzylcysteine was in no way related to the state of vitamin C nutrition.

After the effect of vitamin C on the metabolism of tyrosine was firmly established, the in vitro metabolism of the amino acid was investigated. This effort resulted in the demonstration by Lan and Sealock (125) of the in vitro oxidation of l-tyrosine by the liver slices of normal guinea pigs and the inability of the same tissue from C-deficient animals to exhibit a similar effect. The same effect was noted in the kidney slices although the importance of the kidney appears to be definitely less than that of the liver.

The feeding experiments of Sealock, Ziegler and Driver (112) had indicated an increased need for vitamin C in the albino guinea pig even though these animals are incapable of forming melanin, and in view of the above demonstrations of the importance of the vitamin in the in vitro oxidation of tyrosine, the behavior of dihydroxyphenylalanine was examined by the use of surviving liver and kidney slices. In the case of dihydroxyphenylalanine (126) kidney slices of the normal guinea pig were able to readily oxidize the amino acid but

kidney slices from the scorbutic guinea-pig did not show this ability. Either the administration of vitamin C in quantities sufficient to cure the scurvy or the addition of ascorbic acid to the scorbutic slices resulted in a return of the ability of the tissue to oxidize the substrate. In the case of dihydroxyphenylalanine, however, the ability of the liver slices to oxidize the amino acid was not as great as the kidney slices and it is at this point that a very distinct difference is evident. In the case of tyrosine oxidation the liver is the most active while the kidney is the most active tissue when dihydroxyphenylalanine is the substrate.

Previous reports of the presence of tyrosine metabolites as the result of dihydroxyphenylalanine feeding would lead to the supposition that dihydroxyphenylalanine might be a link in the scheme concerned with the metabolism of tyrosine. The relative inability of the liver slices to metabolize dihydroxyphenylalanine discourages this idea and this difference is a strong argument for the existence of two different systems. In the light of recent work on the dihydroxyphenylalanine decarboxylase and amine oxidase the activity of the kidney slices is not surprising, but the effect of vitamin C on this activity is very interesting and invites further work on the possible inter-relationships of the systems involved.

In 1947 Sealock, Goodland and White (127), by the use of homogenates and breis from normal and scorbutic guinea pigs, were able to demonstrate a reduced ability of the preparation from the scorbutic animals to metabolize tyrosine. The success of Sealock, et al in the investigation of the metabolism of tyrosine suggests a possible parallel in the ability of kidney extracts to metabolize dihydroxyphenylalanine. If such a parallel exists the use of kidney extracts will furnish a means of studying the enzymes involved, and could eventually lead to the chemical characterization of the enzyme systems responsible for the metabolic series of events in dihydroxyphenylalanine metabolism and to the identification of the intermediary products.

III. EXPERIMENTAL

In a successful investigation of a series of metabolic events, the various enzymes responsible for the complex reactions must be separated one from the other. This separation cannot be accomplished by the use of tissue slices where the cellular structure is intact. However, disruption of the cellular organization will usually effect such a separation. Some enzymes are not readily soluble, but a remarkable number of enzyme systems have been successfully investigated by the use of extracts prepared by rupturing the cell in the presence of a suitable buffer. Although the spacial relationships have been disturbed, these extracts contain many of the soluble components of the original material and, if the interrelationships of the components are the same in the cell-free extracts as in the intact cell, it may be possible to characterize the enzymes involved and to identify the intermediary metabolic reaction products. Therefore, in order to analyze the enzyme systems involved in dihydroxyphenylalanine metabolism and to investigate the role of vitamin C in this process, cell-free extracts, prepared from guinea-pig kidney, were employed.

A. Procedure

The results of Warburg constant-volume respirometer experiments, in which the oxygen consumption, amino acid disappearance and, in some instances, the carbon dioxide evolution and ammonia formation were measured, form the basis on which the activity of the kidney extracts was determined. With the exception of a few experiments to determine the ability of the extracts of rat kidney to metabolize dihydroxyphenylalanine, guinea-pig kidney extracts were employed in this investigation.

The guinea pigs were maintained on Purina Rabbit Chow, Checkers, (complete ration) plus an adequate supply of mixed green food. This diet was varied when scorbutic guinea pigs were required, but this variation will be described in connection with the appropriate experiments.

The extracts were prepared as follows: The non-fasted animals were stunned by a blow on the occiput and bled by severing the jugular veins. The kidneys were then removed, cleaned, weighed, and placed in an ice cooled, micro Waring blendor jar with enough cold 0.1 M phosphate buffer to cover the blendor knives and thus prevent excessive splashing. Homogenization was continued, with occasional cooling in an ice bath, until the preparation was essentially homogeneous. Approximately fifteen minutes were required. The mixture was then centrifuged at 2500 rpm for five minutes. The super-

natant was removed, the residue re-extracted with phosphate buffer and again centrifuged. The combined supernatants were then diluted to the required volume according to the following equation:

$$\text{Final volume} = \frac{\text{Weight of Kidney}}{\text{Final Concentration in gm. per ml.}}$$

After the extract was adjusted to the correct volume, the pH was adjusted with 1 N sodium hydroxide or 0.5 N hydrochloric acid, care being taken to stir the extract well during the addition to prevent local excesses of acid or base.

One ml. of this extract was then placed in the reaction compartment of the vessel. Either the substrate dissolved in 1.0 or 0.5 ml. of phosphate buffer, or an equivalent amount of buffer, was introduced into the side arm. Twenty per cent potassium hydroxide (0.3 ml.) and a filter paper roll (Whatman No. 40) were introduced into the center well to absorb the carbon dioxide evolved during the reaction. The contents of the flasks, after equilibration to the temperature of the bath, were incubated for three hours at 37.5° C. with constant shaking. The oxygen consumed was measured at the end of 30, 60, 120 and 180 minutes. After the last manometer reading, the contents of each vessel were quantitatively transferred into 1 ml. of 10 per cent metaphosphoric acid contained in a graduated 15 ml. centrifuge tube, and the volume adjusted to 10 ml. with distilled water. This mixture was well stirred, allowed to stand for one-half hour and the protein precipitate

removed by centrifuging for five minutes at 2000 rpm. The clear supernatant was transferred to a test tube and stored in the refrigerator until analyzed.

The analysis of the unreacted substrate in the incubated extracts was entirely dependent upon the use of proper tissue controls to correct for possible interference by substances present in the tissue extracts. This was accomplished by incubating the extracts with and without the substrate, and the interference then eliminated by substracting the diphenolic value of the tissue control from that of the tissue experimental.

In the same manner a correction was made for tissue interference in the "zero-time" analyses. The "zero-time" metaphosphate filtrates were prepared in the same fashion as the incubated tissue filtrates, except that the tissue extract and substrate were added to the 10 per cent metaphosphate without subjecting them to an incubation period. These standards, against which the substrate disappearance was measured, were prepared as follows: One ml. of the substrate, or 1 ml. of buffer, and 1 ml. of the tissue extract were added to 1 ml. of 10 per cent (1.25 N) metaphosphoric acid solution contained in a graduated 15 ml. centrifuge tube. This mixture was subjected to the same treatment as the incubated samples.

A metaphosphoric acid filtrate, in which phosphate buffer was substituted for the tissue extract, was prepared for each analysis to make certain that the tissue extracts

did not adversely influence the method of analysis.

The Arnow method for the analysis of dihydroxyphenylalanine (14) was used to determine the unreacted 3,4-dihydroxyphenyl group of the amino acid. In an acid solution the catechol portion of the molecule will react with the nitrite of the nitrite-molybdate reagent to form a nitroso compound which, on the addition of alkali, turns red. The reagents, as described by Arnow, are 0.5 N hydrochloric acid, 1 N sodium hydroxide and the nitrite-molybdate reagent, which is prepared by dissolving 10 gm. of sodium nitrite and 10 gm. of sodium molybdate in 100 ml. of distilled water.

The Arnow method was originally studied with water solutions of dihydroxyphenylalanine and, therefore, an investigation of its applicability to the present problem was initiated. The change in the intensity of the color with time was determined, for, if possible, the color values should be read when the intensity of the color is not changing. As is illustrated in Figure 1, the intensity of the color decreased for thirty minutes but remained constant thereafter. For this reason the solutions were allowed to stand for thirty minutes before the color value was read. An investigation of the proportionality obtained with the Arnow method at various wave lengths of light was initiated so that the most appropriate light filter could be employed. The color values of solutions containing from 0.0 to 0.1 mg. of dihydroxyphenylalanine were determined at various wave

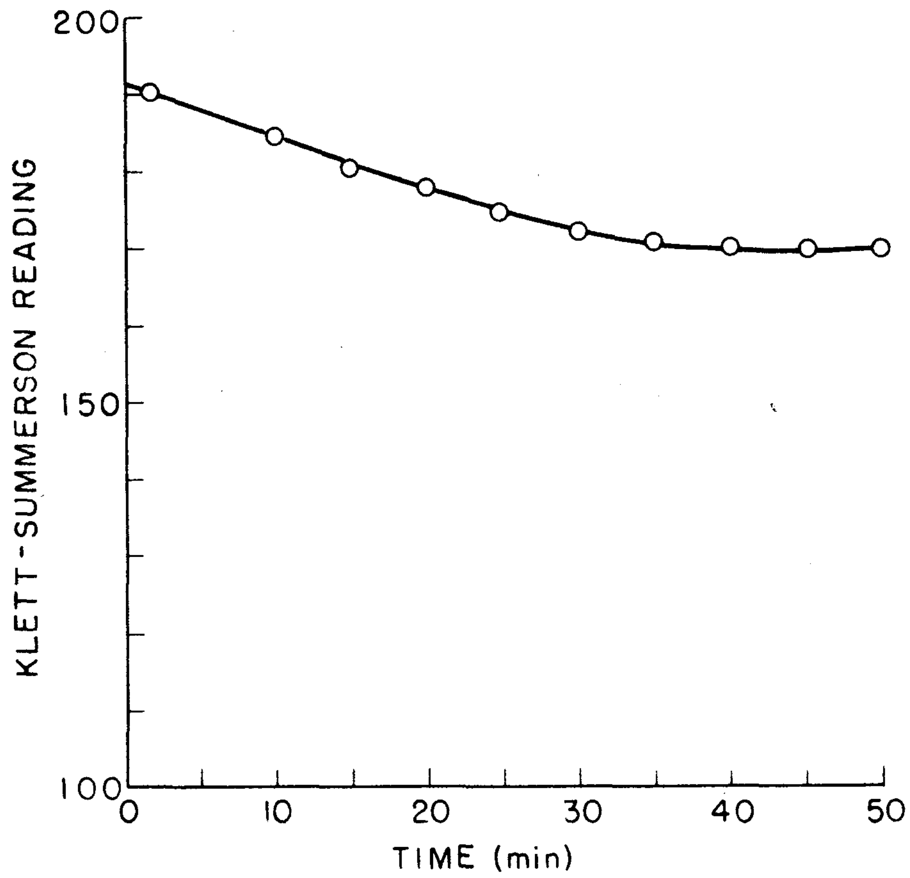

FIGURE I. STABILITY OF COLOR VALUE WITH TIME, ARNOW METHOD

lengths by means of the Coleman, Model 11, Universal Spectrophotometer, and a direct proportionality between the color value and the milligrams of amino acids was evident at 420 millimicrons. The data obtained when these same concentrations of dihydroxyphenylalanine were compared in a Klett-Summerson photoelectric colorimeter, using filter No. 420, are summarized in Figure 2, and illustrate the direct proportionality over a limited range realized with this light filter. The reagent blank gave a constant, but very low reading, and it should be emphasized that the straight line relationship in Figure 2 has been corrected for this reagent blank.

The analysis was dependent upon good recovery of the added substrate. Dihydroxyphenylalanine was added to unincubated kidney extracts, the resulting solution deproteinized with 10 per cent metaphosphoric acid and then treated in the same manner as formerly prescribed for the incubation extracts. The results of nine such experiments are summarized in Table IV, and an average recovery of 100.5 $\pm$ 2.2 per cent was realized.

As a result of these preliminary studies, the method employed was as follows: To 1 ml. of standard, or unknown, in 0.125 N metaphosphoric acid in a test tube, 3 ml. of 0.125 N metaphosphoric acid, 2 ml. of 0.5 N hydrochloric acid, 2 ml. of nitrite-molybdate reagent and 2 ml. of 1 N

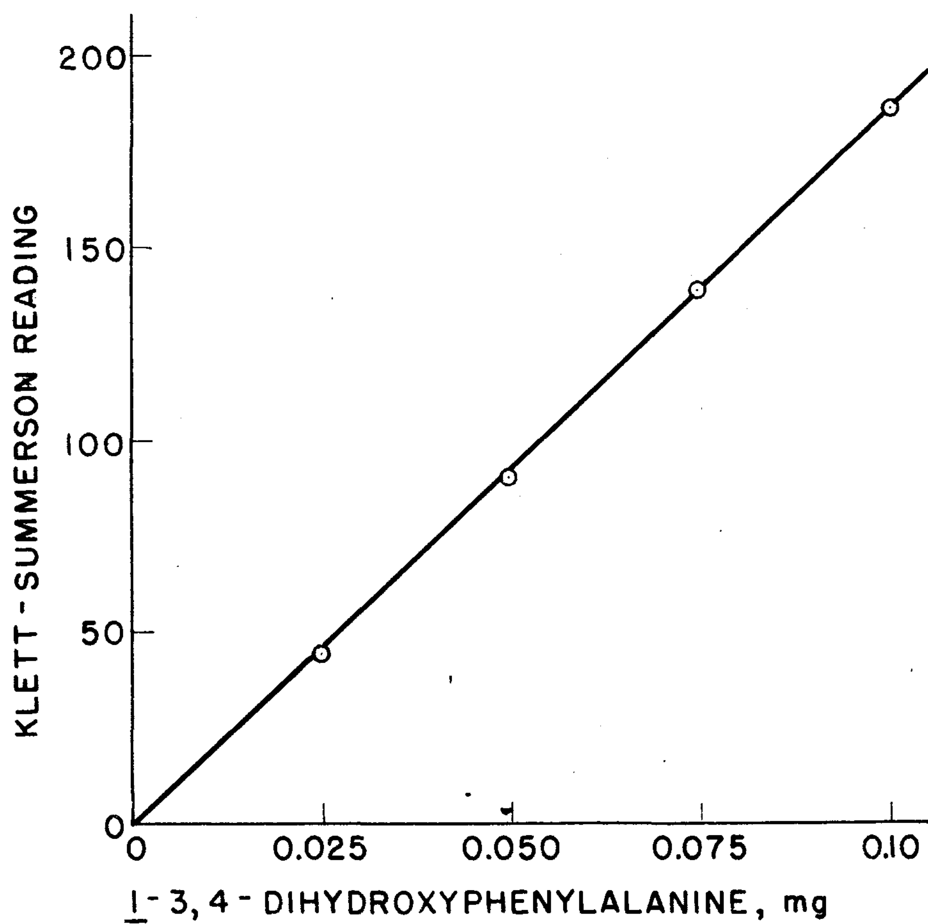

FIGURE 2. PROPORTIONALITY OF THE ARNOW METHOD

TABLE IV

RECOVERY OF DIHYDROXYPHENYLALANINE, ARNOW METHOD

Sample	Tissue	Dihydroxyphenylalanine			
		Added to tissue	Total found	Recovered	
	μmoles	μmoles	μmoles	μmoles	per cent
1	0.09	5.08	5.42	5.33	105.0
2	0.12	5.08	5.17	5.06	99.5
3	0.13	5.08	5.49	5.36	105.1
4	0.09	5.08	5.17	5.08	100.1
5	0.09	5.08	5.23	5.14	101.1
6	0.14	5.08	5.03	4.89	96.3
7	0.08	5.08	5.17	5.09	100.1
8	0.15	5.08	5.23	5.08	100.0
9	0.07	4.44	4.31	4.24	95.6
				Average	100.3 ± 2.2

sodium hydroxide were added in succession. The resulting solution was allowed to stand for thirty minutes and then the color value read in a Klett-Summerson photoelectric colorimeter using blue filter No. 420, the instrument being adjusted to zero with the reagent blank. The following equation was used to calculate the micromoles of dihydroxyphenylalanine (diphenolic value) present.

$$\text{Diphenolic value} = \frac{\text{Unknown}}{\text{Standard}} \times \frac{\text{Micromoles}}{\text{Standard}} \times \frac{\text{Total volume}}{\text{Unknown aliquot}}$$

The amount of substrate disappearance and the oxygen consumption were two quantities which could be related in the form of a ratio. In the early stages of this investigation the possible role of oxygen in the disappearance of the diphenolic portion of the amino acid was considered, and the ratio of the oxygen consumed to the substrate initially present, and to the substrate disappearance was calculated in an attempt to relate these quantities. These ratios were defined as follows:

O/D_p -- the ratio of the microatoms of oxygen consumed during the incubation period to the micromoles of substrate <u>present</u> at the start of the incubation.

O/D_c -- the calculated ratio of the microatoms of oxygen consumed during the incubation period to the micromoles of diphenolic <u>disappearance</u>.

The O/D_c makes possible the determination of the atoms of oxygen involved in the metabolism of one mole of substrate.

By comparing the O/D_p with O/D_e the degree of completion of the reaction could also be ascertained, for as long as the reaction was incomplete the O/D_p would be less than the O/D_e. However, when the reaction was complete the ratios should be equal.

The procedure, as outlined in this section was on occasion modified in an attempt to obtain additional information which would shed special light on the systems involved in the metabolism of dihydroxyphenylalanine. These variations will be described in connection with the appropriate experiments.

B. Vitamin C and Dihydroxyphenylalanine Metabolism

Sealock and his associates have shown that the scorbutic guinea pig cannot oxidize dihydroxyphenylalanine to the same degree as the normal guinea pig. Furthermore, kidney slices from the normal animals readily oxidize the amino acid, whereas slices of the same organ from the scorbutic guinea pig do not exhibit this property to the same degree. The possible role of enzyme systems in this metabolic process has been suggested. However, in order to analyze this question, cell-free preparations must be used. The first step in this analysis is an attempt to demonstrate a difference in the cell-free extracts comparable with the results observed in the case of kidney slices.

The guinea pigs were maintained on a vitamin C free

basal diet of ground and aerated Purina Rabbit Chow (complete ration). Approximately 0.9 gm. of Squibbs Brewers' Yeast was sprinkled on top of the feed every third day, and all animals received 1 ml. of cod liver oil per os weekly. Half of the animals received, in addition, 20 mg. of crystalline ascorbic acid each day. The animals on the C-deficient diet were used when gross evidence of scurvy was present and, to eliminate age variations, were matched against animals maintained on the supplemented diet for approximately the same length of time. In Table V the initial and final weight of the guinea pigs are recorded to show that, in addition to the usual signs of scurvy -- tender joints, "scurvy position", "face-ache position" and both subcutaneous and intramuscular hemorrhages --, the weight of the animals on the vitamin C deficient diet was lower than the initial starting weight of the animals, while the guinea pigs on the supplemented diet had gained considerable weight over the same period.

The growth of guinea pig No. 620 was used to demonstrate that ascorbic acid was the only factor missing from the diet. When 13 mg. of ascorbic acid was administered on the twenty-fourth day, the growth response was immediate and pronounced. The animal responded to further depletion and was finally used on the forty-seventh day.

The ability of extracts of the normal and scorbutic guinea pig kidneys to metabolize l-3,4-dihydroxyphenylalanine was compared, and the results of these experiments are shown

TABLE V

SUMMARY OF WEIGHT CHANGES
OF NORMAL AND SCORBUTIC GUINEA PIGS

Guinea pig No.	Initial weight	Final weight	Days on diet
	gm.	gm.	
Normal			
611	204	382	40
612	306	394	17
613	294	388	23
614	310	450	31
615	312	418	29
Scorbutic			
616	262	216	23
617	302	238	20
618	330	298	26
619	326	186	27
620	333	314	47

in Table VI. The average excess oxygen consumption for the normal kidney extracts was 73.1 microliters as compared with an average of 71.3 microliters for the scorbutic extracts, and the average per cent substrate disappearance was 84.5 per cent for the normal and 81.8 per cent for the scorbutic kidney extracts. The excess oxygen consumption and substrate disappearance, for the three-hour incubation period, were essentially the same for the normal and scorbutic kidney extracts. However, a slight difference in the rate of the oxygen consumption was evident. The average excess oxygen consumption of the normal and scorbutic kidney extracts are illustrated in graphical form in Figure 3. Although the oxygen consumption of the normal and scorbutic extracts were almost the same at the end of the three-hour period, the oxygen consumption of the normal extracts was greater in the early stages of the reaction.

While the comparison of the extracts of normal and scorbutic guinea-pig kidneys was in progress, the effect of added vitamin C on the metabolism of dihydroxyphenylalanine by kidney extracts from the scorbutic guinea pigs was investigated. Vitamin C -- 0.01, 0.05 and 0.1 mg., respectively -- was added to 1 ml. of the kidney extracts of scorbutic guinea pigs, and the activity of these supplemented extracts compared with the activity of the scorbutic kidney extracts. The results are tabulated in Table VII. The oxygen consumption

TABLE VI

DIHYDROXYPHENYLALANINE OXIDATION BY KIDNEY EXTRACTS OF NORMAL AND SCORBUTIC GUINEA PIGS

Guinea pig No.	Oxygen consumption Basal	Oxygen consumption Excess	O/D_p	O/D_o	Cpd. disap.
	μl.	μl.			per cent
Normal					
611	32.0	80.2	1.41	1.61	87.4
612	33.8	73.4	1.29	1.50	87.5
613	32.2	69.3	1.22	-----*	-----*
614	27.2	75.0	1.32	1.58	84.4
615	30.3	67.6	1.19	1.55	76.8
		73.1	1.28	1.56	84.5
Scorbutic					
616	27.1	76.9	1.35	1.70	79.3
617	32.0	68.8	1.21	1.47	83.3
618	29.4	64.9	1.14	1.42	80.3
619	24.2	70.8	1.26	1.56	79.7
620	30.5	76.3	1.34	1.61	82.8
		71.3	1.22	1.56	81.8

* These values are not available because an accident prevented analysis of the sample.

The tissue extract concentration was 10 per cent. The incubations were carried out for three hours at pH 7.4 and at 37.5° C.

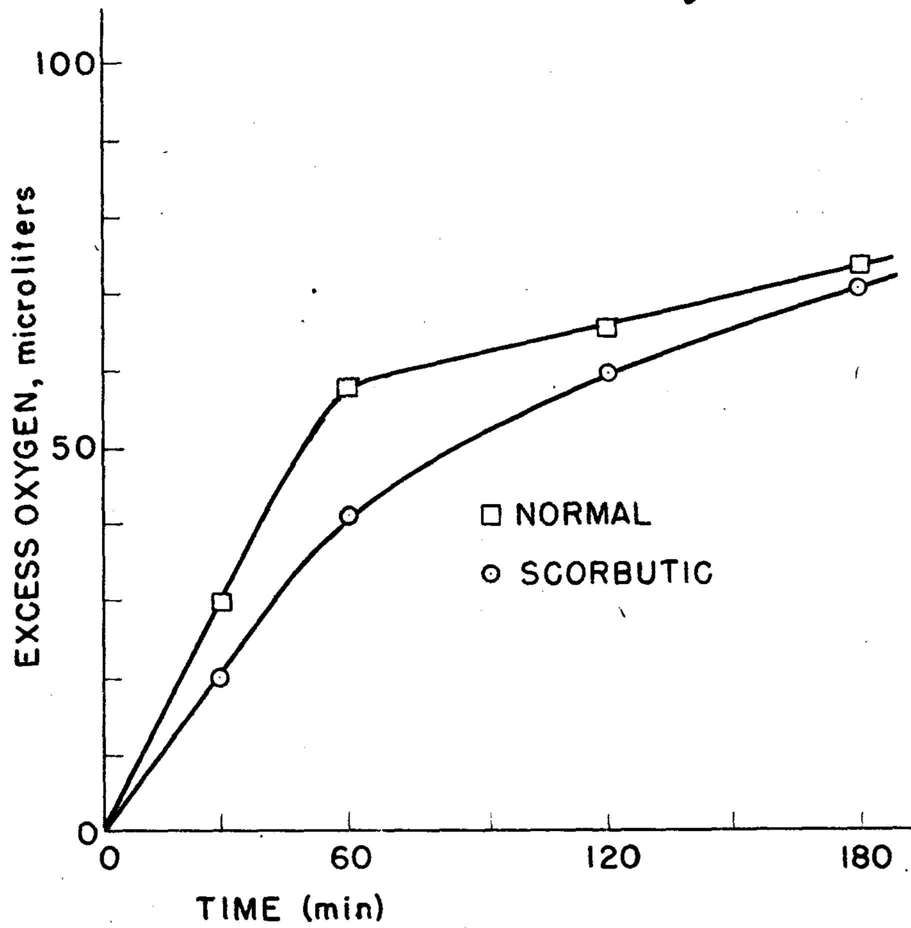

FIGURE 3. EXCESS OXYGEN OF NORMAL AND SCORBUTIC GUINEA-PIG EXTRACTS

TABLE VII

DIHYDROXYPHENYLALANINE OXIDATION BY KIDNEY EXTRACTS FROM SCORBUTIC GUINEA PIGS WITH ADDED ASCORBIC ACID

Guinea pig No.	Oxygen consumption Basal μl.	Oxygen consumption Excess μl.	O/D_p	O/Dc	Cpd. disap. per cent	Ascorbic acid mg.
Without ascorbic acid						
616	27.1	76.9	1.35	1.70	79.4	
618	28.8	64.7	1.14	1.41	80.2	
619	23.8	70.8	1.25	1.55	79.8	
		70.8	1.24	1.55	79.8	
With ascorbic acid						
616	37.2	81.2	1.42	1.79	79.4	0.1
618	29.2	63.6	1.12	1.39	80.4	0.01
619	32.8	61.5	1.08	1.41	76.5	0.05
		68.7	1.21	1.54	78.8	

The three-hour incubations were carried out at pH 7.4 and at 37.5° C. Concentration of the tissue extract was 10 per cent.

and per cent substrate disappearance were almost identical, which demonstrates that the added vitamin C did not effect the ability of the scorbutic kidney extracts to metabolize the amino acid.

Although the guinea pigs used in the previous tests showed gross symptoms of scurvy just prior to use, another investigation was designed to test the effect of vitamin C depletion on the activity of the kidney extracts. The increased ascorbic acid requirement of the guinea pig when tyrosine is included in the diet has been demonstrated by Sealock, Ziegler and Driver (112). Furthermore, Lan and Sealock (125) showed that the oxygen consumption of liver slices from scorbutic guinea pigs is lower than the oxygen consumption of the slices from normal animals. Later, Sealock, White and Goodland (127) noted this same effect in liver breis.

The effect of tyrosine feeding on guinea pigs deprived of vitamin C is very striking. The animal begins to excrete tyrosine metabolites very quickly and the effect of the tyrosine is to accelerate the depletion of the vitamin C stores of the guinea pig. The result is a vitamin C deficient animal much sooner than could be expected by feeding the C-free diet alone. The state of vitamin C depletion of these animals could then be investigated by determining the activity of the liver extracts of these animals in the presence of <u>l</u>-tyrosine. In a scorbutic guinea pig the ability of liver

extracts to metabolize tyrosine is reduced and, if the vitamin C stores of the guinea pigs fed tyrosine are sufficiently lowered, the same effect should be experienced in the liver extracts of these animals. In addition, the ability of the kidney extracts of these animals to metabolize dihydroxyphenylalanine could also be determined in order to observe the effect of the tyrosine feeding on dihydroxyphenylalanine metabolism by kidney extracts. If the ability of the liver extracts to metabolize tyrosine was lower than that observed in normal animals and the ability of the kidney extracts was not affected, the observations of the previous experiments would be confirmed. On the other hand, a more complete vitamin C deficiency may result from the tyrosine feeding, and the kidney extract of such guinea pigs may exhibit a reduced ability to metabolize dihydroxyphenylalanine.

The guinea pigs were maintained on the vitamin C free basal diet, supplemented with 10 per cent l-tyrosine, for eight and ten days, respectively. At the end of this period the animals had lost considerable weight and were beginning to refuse food. The guinea pigs were then sacrificed, and the activity of kidney extracts and liver extracts compared with the activity of similar extracts from normal animals using l-3,4-dihydroxyphenylalanine with the kidney extracts and l-tyrosine with the liver extracts. The metabolism of tyrosine by the liver extracts from the scorbutic

animals was greatly reduced[1]. However, the results of the kidney experiments, as summarized in Table VIII, demonstrate that the ability of kidney extracts of 2.5, 5.0 and 10 per cent was identical with that of kidney extracts, of similar concentration, from normal guinea pigs.

The attempt to demonstrate a difference in the ability of extracts from normal and scorbutic guinea-pig kidney to metabolize dihydroxyphenylalanine was not successful. However, this should not be interpreted to mean that such a difference does not exist, for the decreased ability of kidney slices from scorbutic guinea pigs to metabolize dihydroxyphenylalanine has been demonstrated (126). Possibly the integrity of the cellular structure is necessary for such a relationship to hold. On the other hand, the amount of vitamin C present in the scorbutic kidney extract is probably much greater than that present in the tissue slices. Only a very small amount of tissue slice is necessary for a successful experiment, whereas a rather large amount of tissue must be homogenized to obtain an extract, and all the soluble vitamin C present in the tissue would be present in such an extract. This would upset the ratio of vitamin C to enzyme present in the original tissue and may counteract the effect of depleting the animal prior to use.

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[1] Sealock, R. R. and White, P. L., unpublished data.

TABLE VIII

DIHYDROXYPHENYLALANINE METABOLISM OF EXTRACTS FROM THE KIDNEYS OF GUINEA PIGS FED A BASAL RATION CONTAINING 10 PER CENT TYROSINE

Extract conc.	Oxygen consumption		O/D_p	O/D_c	Cpd. disap.
	Basal	Excess			
per cent	$\mu l.$	$\mu l.$			per cent
Guinea pigs fed 10 per cent tyrosine					
2.5[a]	12.8	24.6	0.43	1.20	36.0
5.0[a]	17.0	49.5	0.87	1.51	57.4
10.0[a]	31.5	60.8	1.07	1.45	74.0
10.0[b]	23.9	56.9	1.00	1.36	73.4
Normal guinea pig					
2.5	10.3	27.1	0.48	1.54	31.1
5.0	17.4	54.1	0.95	1.56	60.7
10.0	25.8	58.4	1.03	1.43	71.6

a Guinea pig was fed 10 per cent tyrosine for eight days.
b Guinea pig was fed 10 per cent tyrosine for ten days.

The incubations were carried out at pH 6.8 and at 37.5° C.

One outstanding fact was evident: The oxygen consumption did not agree with the diphenolic disappearance. Other investigators (45,48) had attributed the oxygen consumption of kidney extracts in the presence of dihydroxyphenylalanine to oxidative deamination of the hydroxytyramine formed in the decarboxylation of the amino acid. The results of this series indicate the consumption of approximately 1.5 atoms of oxygen for each molecule of substrate which disappeared in the reaction. If this oxygen was used to oxidize the diphenolic groups of the amino acid, very little could be attributed to amine oxidation. This posed the following question: If the oxygen consumption of the extracts was due to enzymatic deamination, as has been reported by other investigators, what mechanism was the cause of the diphenolic disappearance?

This question can only be answered by a careful analysis of the ability of the kidney extracts from normal guinea pigs to metabolize the amino acid, and such an analysis must precede any further attempt to study the role of vitamin C in the metabolism of dihydroxyphenylalanine by the kidney extracts.

C. General Behavior and Properties of the Kidney Extracts

The behavior of normal kidney extracts in the presence of the amino acid must be investigated before any attack on

the role of vitamin C in the metabolism of dihydroxyphenylalanine can be attempted with any reasonable assurance of success. This includes a study of the variation of activity with pH and with various concentrations of substrate and extract, as well as an investigation of the relationship between the oxygen consumed and the ammonia and carbon dioxide produced during the incubation period. The relationship between the rate of the oxygen consumed and the rate of the disappearance of the catechol value was also a very important consideration in determining the sequence of the reactions involved in the metabolism of the substrate. This investigation of the general behavior and properties of the kidney extracts was initiated in order to obtain information which would also indicate the enzymatic nature of the diphenolic disappearance.

1. Effect of pH variation

As a rule the catalytic power of an enzyme is exercised over a somewhat restricted range of pH, with a sharp decrease in activity at the higher and lower pH values. Other considerations may complicate this picture. For instance, in the case investigated, the more alkaline pH values were avoided because l-3,4-dihydroxyphenylalanine is subject to non-enzymatic oxidation in the more alkaline solutions. For this reason the highest pH used was 8.0.

The variation of the activity of the kidney extract with pH was investigated within the pH range, 6.6 - 8.0, and the results appear in Table IX. Although the oxygen consumption of the extracts incubated at pH 8.0 was approximately double that of the oxygen consumption of the extract incubated at pH 6.6 (89.9 microliters and 38.6 microliters, respectively), the percentage diphenolic disappearance was only increased from 63.6 per cent to 78.1 per cent -- an increase of about one-fourth. This discrepancy is in keeping with the theory, which will be developed throughout this discussion, that oxidation, enzymatically or otherwise, by the oxygen of the atmosphere in the flasks is not responsible for the disappearance of the greater part of the diphenolic portion of _l_-3,4-dihydroxyphenylalanine.

The color of the incubated samples varied from a very dark, almost black, shade at pH 8.0 to a very light brown at pH 6.6, which suggested that a side reaction, possibly autooxidation of the substrate in the more alkaline solution, was occurring. To test this hypothesis a series of experiments, employing boiled extracts, was carried out. The extracts were prepared in the same manner as the extracts used for a normal reaction but, in addition, they were placed in a boiling water bath for ten minutes, and then rehomogenized in a test tube homogenizer and the pH adjusted before use. The results of these experiments are summarized in Table X. At pH 6.6 the oxygen consumption was insignificant and the

TABLE IX

THE INFLUENCE OF pH ON THE METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE BY KIDNEY EXTRACTS

pH	Oxygen consumption		O/D_p	O/D_c	Compound disappearance	
	Basal	Excess				Average
	μl.	μl.			per cent	per cent
6.6	17.6	38.6	0.68	1.07	63.6	63.6
6.8	16.5	44.7	0.79	1.14	68.7	
	27.1	51.8	0.91	1.34	68.0	
	21.2	57.6	1.01	1.49	67.6	68.1
7.0	23.4	66.6	1.17	1.52	76.7	
	26.7	69.1	1.21	1.42	85.3	
	22.8	53.2	0.93	1.23	76.0	79.3
7.2	23.9	68.3	1.20	1.53	78.0	
	26.9	67.4	1.20	1.39	85.3	81.6
7.4	23.7	72.0	1.26	1.56	80.7	
	25.9	73.3	1.28	1.44	89.2	
	34.2	61.5	1.08	1.43	75.3	81.7
7.6	27.2	66.6	1.17	1.46	80.3	
	26.4	71.6	1.28	1.40	89.7	85.0
7.8	25.7	66.7	1.17	1.60	75.1	
	24.6	76.4	1.34	1.63	80.6	77.8
8.0	24.4	86.0	1.51	1.98	76.2	
	22.5	93.8	1.65	2.01	80.3	78.1

The incubations were carried out at 37.5° C. The concentration of the substrate was 5.08 micromoles.

TABLE X

THE INFLUENCE OF pH ON THE EFFECT OF BOILED KIDNEY EXTRACTS ON l-3,4-DIHYDROXYPHENYLALANINE

pH	Oxygen consumption		Compound disappearance	
	Basal	Excess		
	μl.	μl.	μmoles	per cent
6.6	8.5	0.3	0.23	4.5
6.8	17.9*	6.3	0.03	1.0
7.0	9.0	11.6	0.36	7.1
7.2	8.9	20.3	0.75	16.2
7.4	9.3	25.5	1.19	23.4
7.6	11.1	39.8	1.70	33.4

* This basal was high but the excess oxygen was in line with the general decrease of activity as the pH was lowered.

Incubations were carried out at 37.5° C. The extracts were heated in a boiling water bath for ten minutes and then homogenized in a test tube homogenizer before used. The boiled extract was equivalent to a 10 per cent extract.

diphenolic disappearance was very slight (4.5 per cent). However, as the pH was increased both the oxygen consumption and the diphenolic disappearance increased, until, at pH 7.6, 39.8 microliters of oxygen were consumed and 33.4 per cent of the diphenolic nucleus, as measured by the Arnow test, had disappeared. Furthermore, an investigation of the oxygen consumption of phosphate buffer solutions of the substrate under similar conditions gave similar results. Therefore, autooxidation in the unheated extracts at the more alkaline pH values was very significant, and if the diphenolic disappearance due to this extraneous oxidation is subtracted from the normal oxidation, mentioned above, the resulting pH-activity curve exhibits a maximum at approximately pH 7.0 (Table XI and Figure 4).

This significant amount of autooxidation at the more alkaline pH values resulted in reducing the pH of most of the following incubations from 7.4 to 6.8, where the amount of autooxidation was not a major consideration.

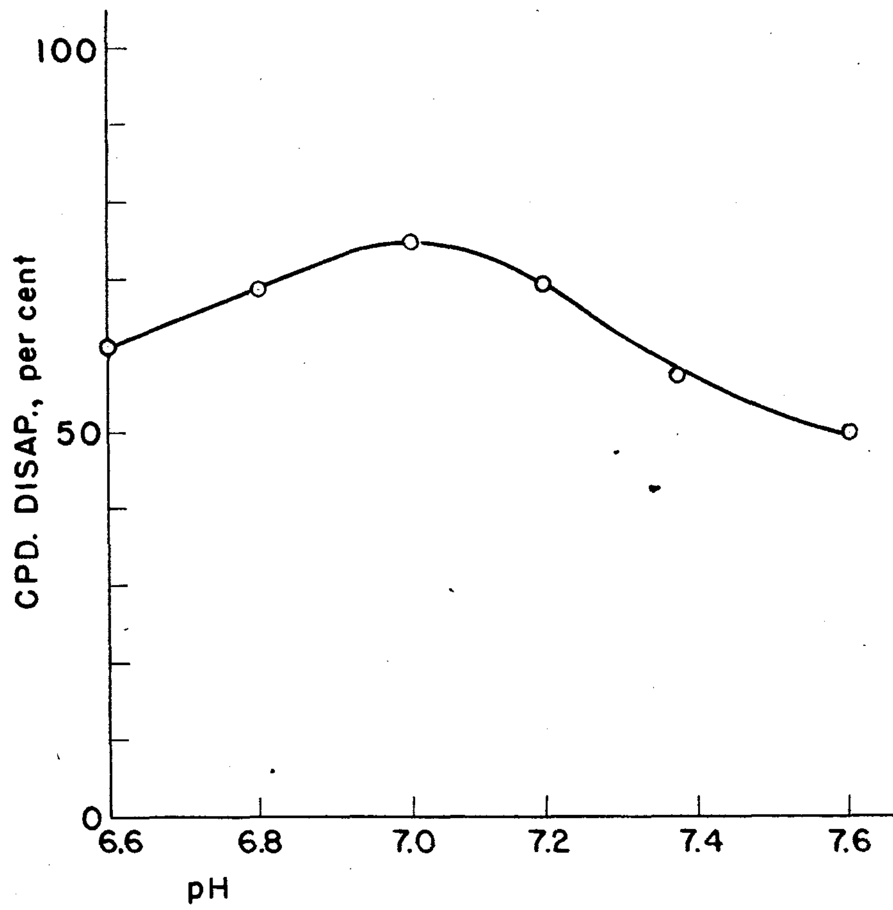


FIGURE 4. pH-ACTIVITY RELATIONSHIP

TABLE XI

THE DIPHENOLIC DISAPPEARANCE
OF THE NORMAL REACTION CORRECTED FOR AUTOOXIDATION

pH	Cpd. disap.		Diff.	Cpd. disap.
	Boiled extract μmoles	Normal extract μmoles	μmoles	per cent
6.6	0.23	3.32	3.09	60.8
6.8	0.03	3.38	3.35	65.8
7.0	0.36	4.11	3.75	73.7
7.2	0.75	4.25	3.50	68.9
7.4	1.19	4.10	2.91	57.2
7.6	1.70	4.21	2.51	49.4

2. Effect of varying substrate concentration

The rate of an enzymatic process depends upon the concentration of the enzyme and of the substrate. In most cases if the quantity of the enzyme is fixed the reaction velocity will increase until a limiting value is reached. In order to discover if the systems involved in the metabolism of the dihydroxyphenylalanine by the kidney extracts exhibited this property, the amount of substrate present at the beginning of the reaction was varied while the concentration of the tissue extracts was maintained at 0.2 gm. per ml. (which represents a working concentration of 10 per cent).

The substrate concentration was varied from 0.0006 M

to 0.0288 M and the oxygen consumption and diphenolic disappearance realized at the conclusion of the usual three-hour Warburg run are summarized in Table XII. Both the rate of the oxygen consumption and the diphenolic disappearance were influenced by the initial concentration of the substrate. As the concentration of the substrate was increased from 0.0006 M to 0.0288 M the velocity of the reaction increased until the substrate concentration reached 0.0116 M. At higher concentrations of the substrate the velocity of the oxygen consumption leveled off, and the velocity of the diphenolic disappearance reached a maximum. This fact is better illustrated in Figure 5, where the velocities, calculated as microliters of oxygen consumed per 10 minutes, based on the oxygen consumption at the end of the first thirty minutes, are graphically illustrated. With the lower concentrations of the substrate (0.0006, 0.0012, 0.0024 and 0.0058 M), the velocity of the oxygen consumption increases markedly, but at the higher concentrations the velocity was constant, leveling off at about 9.0 microliters of oxygen per 10 minutes.

In Figure 6 the diphenolic disappearance for the three-hour period is graphically illustrated, and as the initial concentration of the substrate increases the micromoles compound disappearance for the three-hour incubation period leveled off in much the same manner as the oxygen consumption. From an initial substrate quantity of zero micromoles to one

TABLE XII

SUBSTRATE CONCENTRATION AND
l-3,4-DIHYDROXYPHENYLALANINE METABOLISM

Substrate conc.	Oxygen consumption			Compound disappearance	
	Basal	Excess			
molar	µl.	µl.	µatoms	µmoles	per cent
0.0006	30.6	14.7	1.31	0.94	74.0
0.0012	29.0	27.6	2.47	1.81	71.2
0.0024	28.8	50.9	4.53	3.44	67.6
0.0058	31.9	80.6	7.20	5.59	55.1
0.0116	30.7	118.5	10.60	6.76	33.3
0.0192	21.4	132.8	11.80	5.83	14.4
0.0240	21.4	140.6	13.00	6.40	12.5
0.0288	21.4	140.2	12.50	5.50	9.0

The concentration of the kidney extract was 0.2 gm. per ml. The above are calculated from the results of a three-hour incubation at pH 6.8 and 37.5° C.

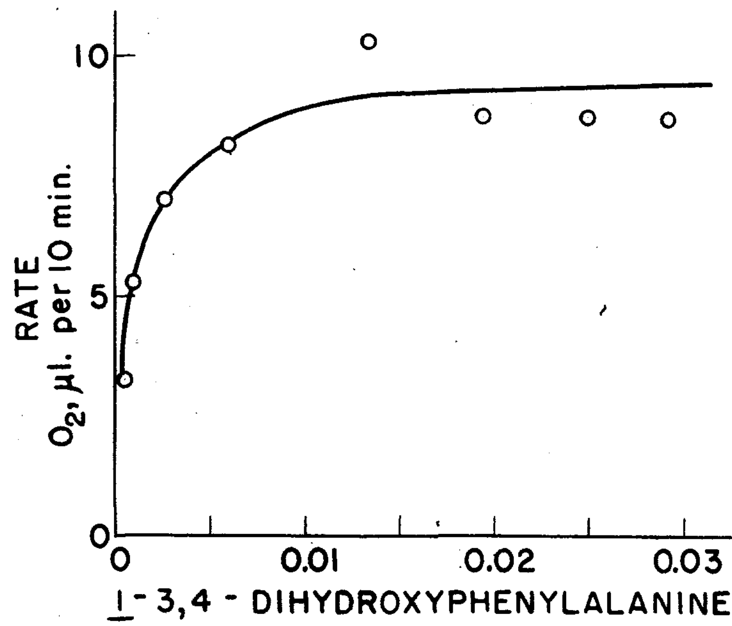


FIGURE 5. VARIATION OF REACTION VELOCITY WITH SUBSTRATE CONCENTRATION

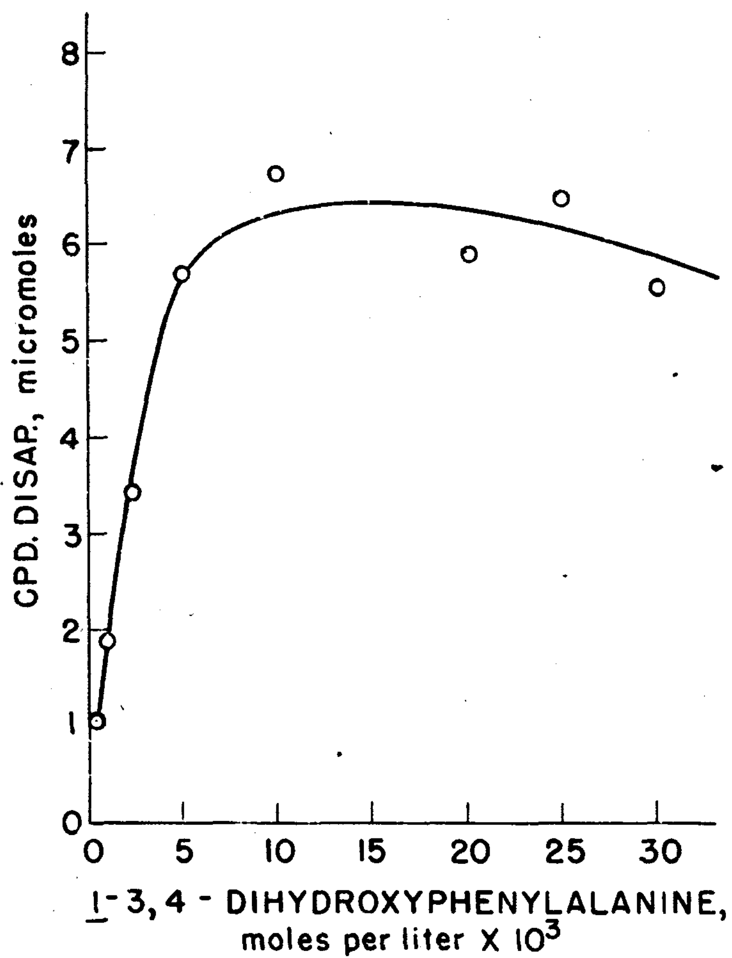


FIGURE 6. VARIATION OF COMPOUND DISAPPEARANCE WITH SUBSTRATE CONCENTRATION

of 10 micromoles, the micromoles of diphenolic disappearance increased very sharply to about 5.6 micromoles for the three-hour period, but a further increase in the substrate concentration did not increase this value appreciably. This is in agreement with the modern theory of enzyme action which suggests that an unstable intermediate compound is formed, which then dissociates into two compounds or decomposes with the formation of the free enzyme and the products of the reaction. If excess substrate is available to saturate the enzyme in question, the velocity of the reaction will be constant until the concentration of the substrate is reduced to the point where the concentration of the enzyme is not the limiting factor. At this point the amount of substrate is the limiting factor, and as this concentration is reduced, the velocity of the reaction for a given period will decrease.

This trend is also reflected in the O/D_p values. O/D_p is the ratio of the oxygen consumed to the amount of substrate present at the start of the reaction. As the reaction goes toward completion, the O/D_p values should approach the O/D_c values, which are the ratio of oxygen consumed to the actual substrate disappearance as measured by the Arnow test. In Table XIII the O/D_p and O/D_c values for this series are recorded. In those experiments in which the concentration of substrate employed was very low, the agreement between O/D_p and O/D_c was fairly close. In the high substrate con-

TABLE XIII

VARIATION OF THE O/D_p AND O/D_e VALUES OF SUBSTRATE CONCENTRATION SERIES

Substrate conc. molar	O/D_p	O/D_e
0.0006	1.03	1.39
0.0012	0.97	1.37
0.0024	0.89	1.32
0.0058	0.71	1.29
0.0116	0.52	1.57
0.0192	0.29	2.02
0.0240	0.26	2.03
0.0288	0.21	2.27

centrations the ratio, O/D_p, reach very low values, while the O/D_o values are not appreciably affected, indicating that the reaction had involved only a small portion of the substrate. At the very high substrate concentrations the O/D_o is higher than that previously experienced. However, this is understandable if extraneous oxidation is considered. With the excessive amount of substrate present the autooxidation of a very small percentage of the total would amount to considerable oxygen uptake.

3. Effect of varying extract concentration

Other factors being equal in an enzyme catalyzed reaction, the limiting velocity attained in the presence of an excess of the substrate should be proportional to the concentration of the enzyme. This has been demonstrated by many investigators and is well illustrated in the discussion of enzyme action by Baldwin (76). Therefore, if the activity of the kidney extracts is of an enzymatic nature, any variation in activity should be proportional to the concentration of the kidney extracts.

The concentration of the extracts of guinea-pig kidney was varied from 0.05 gm. per ml. to 0.6 gm. per ml. which represents a working concentration of 2.5 to 30.0 per cent. The activity of these extracts, as shown by the oxygen consumption and the diphenolic disappearance was measured at pH 6.8, using as the substrate 5.08 micromoles of l-3,4-dihydroxyphenyl-

alanine. The results of this series are summarized in Table XIV. As the concentration of the tissue extracts is increased, the excess oxygen consumption and the percentage diphenolic disappearance also increased. The excess oxygen consumption varies from 10.3 microliters for a tissue concentration of 2.5 per cent to 61.0 microliters for a tissue concentration of 30 per cent; for the same tissue concentrations the diphenolic disappearance was 31.1 per cent and 97.4 per cent, respectively.

The values in Table XIV are three-hour values, and an examination of the oxygen-consumption curves for four concentrations of the series (Figure 7) shows that, in the case of the high tissue concentration, the reaction was completed in the first hour. The reaction at the lowest tissue concentration was still proceeding. An examination of the velocities calculated from the three-hour values would, therefore, not show any appreciable difference in the ability of the extracts to metabolize the amino acid, for at that time the oxygen consumption of the 10 per cent tissue concentration had almost equalled the oxygen consumption of the high tissue concentration (30 per cent). The oxygen consumption of the low tissue concentration (2.5 per cent) was less than half of that of the 30 per cent extract. However, if the velocities are compared in the early stages of the reaction, where the reaction in all cases was vigorous, a comparison of the activity of the various concentrations was possible. The same tissue extract was used to prepare these three tissue

TABLE XIV

EFFECT OF INCREASING THE CONCENTRATION OF THE TISSUE EXTRACT ON THE METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE

Extract conc.	Oxygen consumption		O/D_p	O/D_e	Cpd. disap.
	Basal	Excess			
per cent	µl.	µl.			per cent
2.5	12.8	24.6	0.43	1.20	36.0
2.5	10.3	27.0	0.48	1.54	31.1
5.0	17.0	49.5	0.87	1.51	52.4
5.0	17.4	54.1	0.95	1.56	60.8
10.0	21.2	57.6	1.01	1.49	67.7
10.0	27.1	51.8	0.91	1.34	68.0
10.0	31.5	60.8	1.07	1.45	74.0
10.0	25.8	58.4	1.03	1.43	71.6
10.0	27.3	48.0	0.84	1.26	66.8
20.0	47.1	55.6	0.98	1.15	84.7
25.0	54.0	65.1	1.13	1.39	82.1
25.0	59.9	51.7	0.91	1.14	79.0
25.0	61.9	70.6	1.24	1.37	90.2
30.0	83.8	61.0	1.07	1.10	97.4

This series was carried out at pH 6.8 and 37.5° C. The substrate concentration was 0.0025 M (5.08 micromoles). All values are calculated from the results of a three-hour incubation. The enzyme concentration is based on the grams of fresh kidney used to prepare 1 ml. of kidney extract; for example, a working concentration of 0.1 gm. per ml. equals a 10 per cent extract.

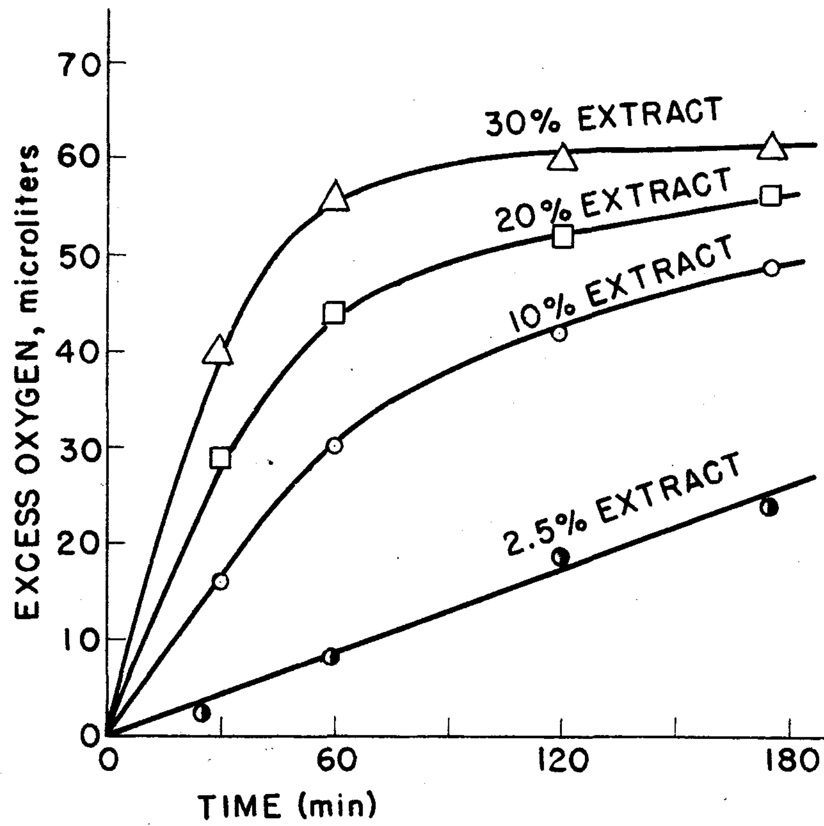


FIGURE 7. EXCESS OXYGEN CONSUMPTION

concentrations (10, 20 and 30 per cent) to avoid variations in the preparation of the extracts. The excess oxygen consumption values obtained at the 30 minute interval were used to compare the activities of the tissue extracts under consideration. The rates of the oxygen consumption at the 30 minute interval for the above mentioned three tissue concentrations were calculated. As shown in Figure 8, the reaction velocity increase is approximately doubled when the extract concentration is increased from 10 to 20 per cent and is almost tripled when the extract concentration is increased from 10 to 30 per cent. The reaction velocity increase is approximately directly proportional to the increase in the concentration of the tissue extract. This is in accord with the law of mass action which states that the velocity of a reaction is proportional to the concentration of reacting substances, and, other things being equal, this is one of the properties of an enzyme system.

4. Oxygen consumption and carbon dioxide production

The fate of the excess oxygen consumed during the incubation period is of primary importance. This oxygen could account for the production of carbon dioxide, for the oxidative deamination of the amino acid or for the oxidation of some other portion of the dihydroxyphenylalanine molecule. In any event, the actual role of this oxygen in the metabolism of the amino acid must be clarified if the elements in the extracts responsible for the disappearance of the dihydroxy-

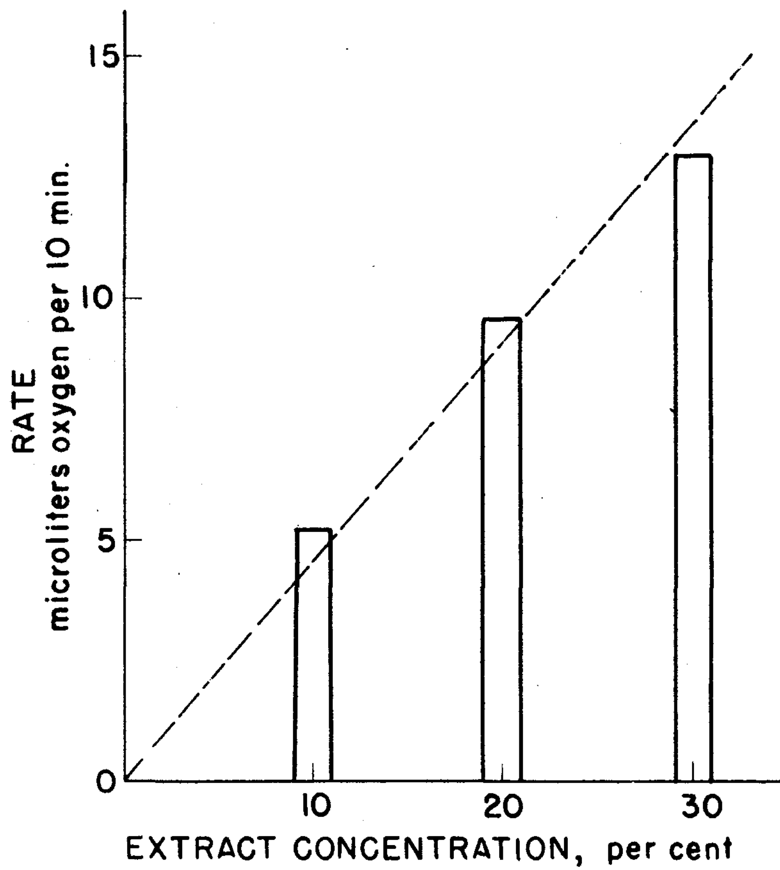


FIGURE 8. EFFECT OF EXTRACT CONCENTRATION ON RATE OF OXYGEN CONSUMPTION

phenylalanine are to be characterized.

In the first experiment, the amount of carbon dioxide evolved in a normal reaction with two concentrations of extract, 10 and 25 per cent, was determined. The extract, either 0.2 or 0.5 gm. per ml., was pipetted into one compartment of the Murlin-Marsh vessel and the substrate pipetted into the corresponding side arm. One-half ml. of potassium hydroxide (approximately 3.5 N) and 1 ml. of sulfuric acid (5 N) were pipetted into the other compartment and side arm, respectively. The contents of the flasks were incubated at 37.5° C. for three hours. At the end of the incubation period the sulfuric acid was dumped from the side arm, mixed with the flask contents, and then the flasks equilibrated at 37.5° C. until a constant manometer reading was obtained. The results of these experiments are shown in Table XV. There was a decided disagreement between the oxygen consumption and the carbon dioxide evolution during the incubation period. Using the 10 per cent extract experiment as an example, we find that 4.43 microatoms of oxygen were consumed and 3.06 micromoles of carbon dioxide evolved. If the oxygen was used for the production of the carbon dioxide, 6.14 microatoms of oxygen would be needed to obtain an oxygen to carbon dioxide balance. An examination of the experiment with the more concentrated extract resulted in essentially the same conclusion. Either some of the carbon dioxide produced was

not the result of oxidation or possibly all of the carbon dioxide was not the result of oxidation. The oxygen may have been used in some other process.

TABLE XV

CARBON DIOXIDE EVOLUTION AND THE OXYGEN CONSUMPTION DURING A THREE-HOUR INCUBATION PERIOD

Extract conc.	Oxygen consumption			Cpd. disap.	Carbon dioxide	
	Basal	Excess				
per cent	μl.	μl.	μatoms	per cent	μl.	μmoles
10.0*	30.6	49.7	4.43	60.7	68.6	3.06
25.0**	54.0	65.1	5.81	82.0	79.0	3.52

* For the 10 per cent extract concentration the O/D_c was 1.43 and the CO_2/D_c was 1.00.
** For the 25 per cent extract concentration the O/D_c was 1.38 and the CO_2/D_c was 0.85

Incubation at pH 6.8 and 37.5° C. using the Murlin-Marsh vessels. The carbon dioxide values were obtained by dumping sulfuric acid from the side arm at the three-hour interval and the flasks equilibrated at 37.5° C. until a constant reading was obtained.

By determining the rate of carbon dioxide evolution during an anaerobic incubation and comparing this with the rate of the oxygen consumed during the same period, a separation of these reactions was possible. The same extract was used in both experiments so that variations in the preparation would be eliminated. The results of this experiment

are illustrated in Table XVI and Figure 9. Carbon dioxide was evolved during the anaerobic experiment. This fact completely divorces the carbon dioxide evolution from the oxygen consumption. The amount of carbon dioxide produced during the anaerobic incubation was not as great as that obtained with the Murlin-Marsh vessels, but this is understandable, for it has been shown by Blaschko (66) that hydroxytyramine, which is the product of the decarboxylation of the amino acid, inhibits the decarboxylation. Under anaerobic conditions no oxygen was present to enable amine oxidase to remove the hydroxytyramine as it was formed. In the normal reaction in the Murlin-Marsh vessels, the amine oxidase, in the presence of air, was able to deaminate the hydroxytyramine formed, and a higher carbon dioxide evolution was realized.

An examination of the early part of the time curve of the anaerobic and aerobic reactions (Figure 9) shows that the carbon dioxide production during the first thirty minutes of the incubation was over twice that of the oxygen consumption; 49.4 microliters of carbon dioxide was evolved and 22.2 microliters of oxygen consumed. The relationship between the carbon dioxide evolved and the oxygen consumption will be clearly defined in the following discussion in which the oxygen consumption is shown to be the result of amine oxidation by the enzyme, amine oxidase.

TABLE XVI

RATE OF CARBON DIOXIDE EVOLUTION
AND OXYGEN CONSUMPTION

Expt.	Extract conc.	Gas exchange*					Cpd. disap.
		Basal	30 min.	60 min.	120 min.	180 min.	180 min.
	per cent	μl.	μl.	μl.	μl.	μl.	per cent
aerobic	10.0	27.9	22.2	33.7	52.2	59.0	70.0
anaerobic	10.0	6.9	49.4	51.2	52.6	54.2	7.1

* Figures for the aerobic experiment are oxygen and the figures for the anaerobic experiment are carbon dioxide.

The anaerobic experiment was carried out in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. Before the substrate was added, the anaerobic flasks were equilibrated in the bath at 37.5° C. until all absorbed gases were evolved, as indicated by no change in the manometer reading over a five-minute period. The incubations were carried out at pH 6.8 and 37.5° C. Substrate = 5.08 micromoles dihydroxyphenylalanine.

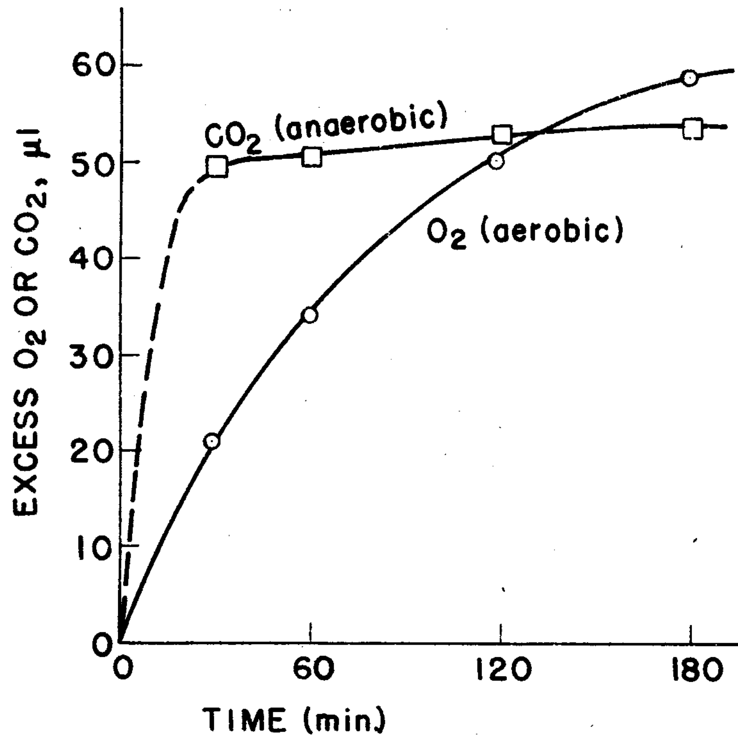


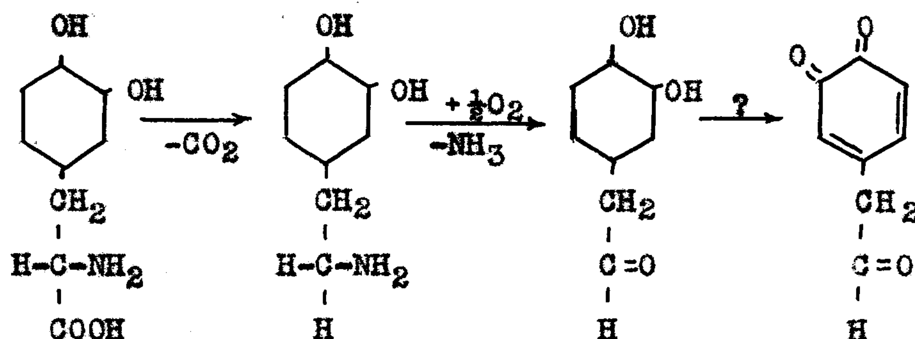
FIGURE 9. CARBON DIOXIDE EVOLUTION AND OXYGEN CONSUMPTION

5. Oxygen consumption and ammonia production

With the demonstration of the production of carbon dioxide by the kidney extracts when l-3,4-dihydroxyphenylalanine was the substrate came the realization that the extracts contained the dihydroxyphenylalanine decarboxylase discovered by Holtz (44), and that, in all probability, the decarboxylation was followed by the oxidation of the hydroxytyramine by amine oxidase. However, as no previous work correlated the oxygen consumption with the disappearance of the catechol portion of the amino acid, it was important to investigate this relationship in order to determine whether or not the diphenolic disappearance was due to oxidation by the atmosphere of the flask.

Various investigators have studied the relationship between the oxygen consumed and the ammonia formed during deamination by the amine oxidase (41,42), and have concluded that the ratio of atoms of oxygen consumed to moles of ammonia formed is one. In the light of the previous results of the present study, it is important to know if the oxygen consumed during the reaction will account for both the formation of ammonia and the diphenolic disappearance. The incubations were carried out in the usual manner. In addition, the ammonia content of 5 ml. of the metaphosphoric acid filtrate was determined. The procedure was to distill the ammonia from the filtrate by means of the Parnas borate method (128)

and to determine the nitrogen by Nesselerization, using the Klett-Summerson Photoelectric Colorimeter and Filter No. 54. The results of these experiments are summarized in Table XVII. If we assume the following reaction:



and also assume that one atom of oxygen is used to form the quinone from the diphenolic nucleus of the amino acid, the atoms of oxygen needed would be equal to the moles of ammonia formed plus the moles of dihydroxyphenylalanine which disappeared. However, a decided discrepancy between the theoretical, as calculated above, and the actual oxygen consumption was observed. For example, in the first experiment of Table XVII the oxygen theoretically needed as calculated from the ammonia formed and the diphenolic disappearance was 6.65 microliters. However, only 4.61 microliters of oxygen were consumed. The oxygen consumption was only 69.3 per cent of that theoretically required.

The average oxygen consumption of all the experiments was only 71.9 per cent of the theoretical. Blaschko, et al

TABLE XVII

OXYGEN CONSUMPTION, DIHYDROXY DISAPPEARANCE AND AMMONIA FORMATION

No. of Expts.	Ammonia formation	Cpd. disap.	Oxygen consumption		
			Calculated	Found	Per cent of calculated
	μatoms	μmoles	μl.	μl.	
2	2.64	4.01	6.65	4.61	69.3
2	3.21	4.58	7.79	6.36	81.4
1	3.00	3.85	6.85	5.13	74.9
1	3.00	2.26	5.26	3.21	61.0*
1	2.00	3.87	5.81	4.91	83.7
1	2.00	0.70	2.70	1.82	67.5**
4	7.42	7.46	15.05	9.81	65.2
					71.9

* In the presence of 0.015 M ascorbic acid.
** In the presence of 0.00046 M hydrogen cyanide.

The three-hour incubations were carried out at pH 6.8 and at 37.5° C. Substrate was 5.08 micromoles except in the last experiment using four flasks, in which 10.16 micromoles of substrate were used.

(39) have demonstrated that the oxygen to ammonia ratio of one is only obtained in amine oxidase reactions when cyanide is added to prevent side reactions. It has been demonstrated, in the previous experiments of this series that at pH 6.8 the O/D_0 ratio very seldom exceeds 1.5 -- for the most part the values range between 1.2 and 1.4. If the previous assumption that one atom of oxygen is used to oxidize the diphenolic group is valid, the remaining oxygen consumed is not sufficient to account for the ammonia formed during the incubation. This is the first positive evidence that the oxygen consumed is not involved in the diphenolic disappearance.

6. Comparison of l-3,4-dihydroxyphenylalanine and hydroxytyramine as substrates

At this stage of the investigation the action of l-3,4-dihydroxyphenylalanine decarboxylase and amine oxidase was fairly well established. However, the relationship between these two enzymes could be very conclusively illustrated by comparing the activity of the extracts in the presence of the amino acid and hydroxytyramine. Hydroxytyramine is decarboxylated dihydroxyphenylalanine, and the use of this compound as the substrate would effectively eliminate the activity of the decarboxylase. However, as has been previously demonstrated, the decarboxylase is more active than the amine oxidase in the kidney extracts, and it is conceivable

that the oxygen uptake in the presence of both substrates would be essentially the same. In addition, if the diphenolic disappearance was in any way associated with the action of the decarboxylase, the use of hydroxytyramine should demonstrate this fact.

The hydroxytyramine was prepared by utilizing the enzymatic method of Holtz, Heise and Lüdtke (45) and Bing (59) to obtain a crude preparation and then adapting the extraction and crystallization process employed by Johnson and Daschavsky (129) in the preparation of tyramine to purify the compound. This procedure involved decarboxylation of l-3,4-dihydroxyphenylalanine to hydroxytyramine, anaerobically, by an extract of guinea-pig kidney. The extract was then deproteinized with trichloroacetic acid, and the filtrate evaporated to dryness under an atmosphere of carbon dioxide. The residue was extracted with hot, absolute ethanol, the ethanol solution concentrated and the hydroxytyramine hydrochloride precipitated in crystalline form by bubbling hydrogen chloride through the solution.

The kidneys from three guinea pigs (approximately 12 gm.) were homogenized, and the fragments removed from the homogenate in the same manner as described for the preparation of the kidney extracts. The extract was then diluted to 250 ml. by the addition of distilled water and 0.2 M phosphate buffer to a final buffer concentration of 0.05 M, and adjusted to pH 7.4. The extract was transferred to a 500 ml. erlenmeyer

flask, 125 mg. of l-3,4-dihydroxyphenylalanine added, 3 ml. of toluene introduced, and nitrogen bubbled through the solution for five minutes. The flask was then stoppered and incubated at 37.5° C. for eight hours. For the first few hours the contents of the flask were shaken every half hour to insure adequate mixing. At the completion of the eight-hour incubation period, 7 ml. of 50 per cent trichloroacetic acid were added, with shaking, and the contents filtered with suction. The resulting filtrate was extracted three times with 100 ml. of ether to remove the trichloroacetic and then evaporated to dryness under carbon dioxide, *in vacuo*. The temperature of the oil bath was never allowed to exceed 50° C. The residue was extracted with four, 5 ml. portions of hot absolute ethanol and the alcohol extract was then subjected to a stream of hydrogen chloride for fifteen minutes. The resulting precipitate was discarded, for it did not respond to the Arnow test for the 3,4-diphenolic group. The alcoholic solution was placed in a cold water bath and subjected to further hydrogen chloride treatment. The volume was then reduced to approximately 4 ml. by placing the test tube containing the extract into a hot water bath (70° C.) and subjecting the solution to an air stream. This concentration was very rapid and resulted in practically no darkening of the solution. The concentrate was then cooled, and a precipitate appeared almost immediately. The solution was stored in a refrigerator for two to three hours, and then the product filtered from the

concentrate. Recrystallization was accomplished by dissolving the precipitate in the minimum amount of hot ethanol, saturating the solution with hydrogen chloride and cooling. The product was filtered from the solution, washed with a few drops of cold ethanol and dried with ether. Forty-five milligrams of hydroxytyramine-hydrochloride was obtained in the first crystallization. The melting point of this substance was 137-39° C. compared to 140-41° C. reported in the literature.

Equimolar concentrations of both substrates, dihydroxyphenylalanine and hydroxytyramine, were employed in matched experiments -- experiments in which both substrates were incubated with identical extracts to eliminate variations due to differences in preparation -- and the results are summarized in Table XVIII. The oxygen consumptions are compared in Figure 10. With the possible exception of a slightly faster rate in the case of the hydroxytyramine the oxygen consumption is very similar for both substrates, while the diphenolic disappearance is only a few per cent higher when hydroxytyramine is the substrate. As far as can be determined from these results the reactions are identical. Therefore, in the following sections of this study it was possible to use either dihydroxyphenylalanine or hydroxytyramine to investigate the diphenolic disappearance. This was very valuable when enzyme inhibitors were employed for by

TABLE XVIII

METABOLISM OF HYDROXYTYRAMINE AND l-3,4-DI-HYDROXYPHENYLALANINE BY EXTRACTS OF GUINEA-PIG KIDNEYS

Substrate*	Oxygen consumption Basal	Oxygen consumption Excess	O/D_p	O/D_c	Cpd. disap.
	µl.	µl.			per cent
Experiment 1					
Dihydroxyphenyl-alanine	21.2	57.6	1.01	1.49	68.7
Hydroxytyramine	21.2	54.3	0.96	1.23	73.7
Experiment 2					
Dihydroxyphenyl-alanine	27.1	51.8	0.91	1.34	68.0
Hydroxytyramine	27.1	54.4	0.95	1.27	75.3

* Substrate ≏ 5.08 micromoles

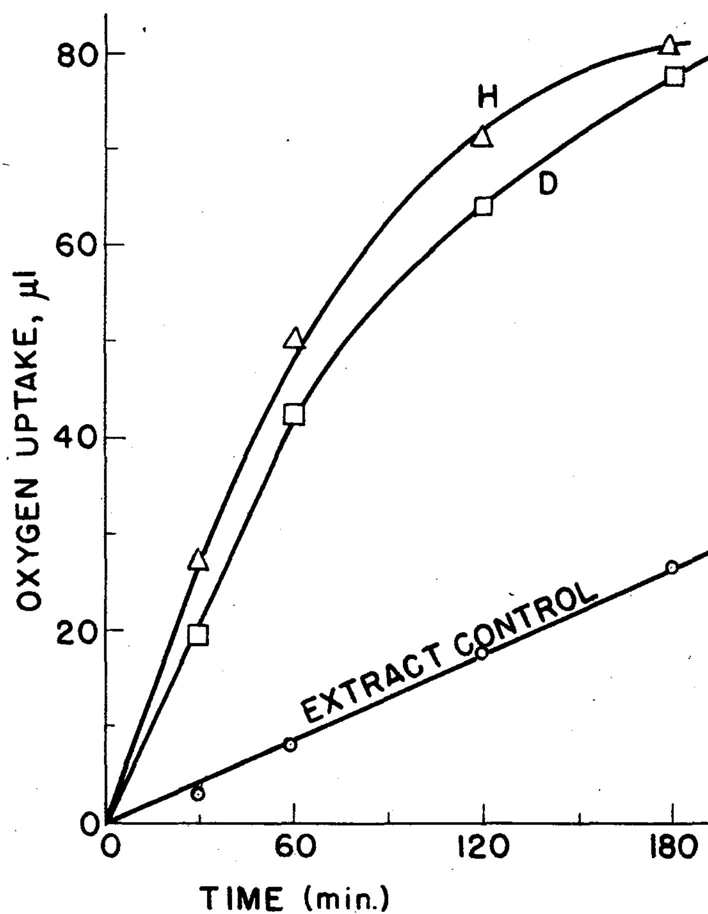


FIGURE 10. METABOLISM OF HYDROXYTYRAMINE DIHYDROXYPHENYLALANINE

H, HYDROXYTYRAMINE
D, DIHYDROXYPHENYLALANINE

the use of hydroxytyramine it was possible to eliminate the action of the decarboxylase.

7. Rate of oxygen consumption and diphenolic disappearance

The previous experiments have demonstrated that the amino acid is first decarboxylated and that then the amine formed is oxidized to form ammonia. Holtz, et al (45) have shown that one of the other products is 3,4-dihydroxyphenylacetaldehyde. The results of this investigation have also shown that the dihydroxy groups of the amino acid disappear during the incubation period. However, the relationship between the rate of the oxygen consumption and the rate of the disappearance of the dihydroxy portion of the amino acid has not been fully explored. The analyses at three hours fail to disclose any information relative to the respective rates in the early part of the reaction. Therefore, an investigation of the rates in the early stages of the reaction was necessary to obtain information which would demonstrate the relationship between the oxygen consumption and the diphenolic disappearance.

A rate experiment, in which a control and an experimental flask were removed from the bath at the 15, 30, 45, 60, 120 and 180 minute intervals, gave the first indication of a difference in the rate of oxygen consumption and the rate of disappearance of the amino acid as shown by analysis.

Table XIX summarizes the results of this experiment and Figure 11 illustrates how widely separated the rates are in the early stages of the reaction. The rate for the oxygen consumption for the first fifteen minutes was 4.69 microatoms per hour and fell rather steeply to a low of 0.95 microatoms per hour for the three-hour period. The rate for the disappearance of the diphenolic nucleus increases very quickly from 1.40 micromoles per hour during the first fifteen minutes to a high of 2.54 micromoles per hour at the forty-five minute interval and then fell to 1.15 micromoles per hour for the three-hour period. This lag period noted in the early stages of diphenolic disappearance is evidence which demonstrates that the oxygen consumption precedes the disappearance of the diphenolic group. The product of the deamination is then subjected to some other reaction which is responsible for the diphenolic disappearance. The rate of diphenolic disappearance was not directly proportional to the oxygen consumption.

An indication that oxidation by the atmosphere of the flask was not the cause of the diphenolic disappearance has been suggested by the discrepancy in O/D_c, the agreement between the ammonia formation and oxygen consumption and the demonstration in this experiment of a difference in the rates of oxygen consumption and diphenolic disappearance. This suggestion will be supported by experiments to be discussed shortly.

TABLE XIX

RATE OF OXYGEN CONSUMPTION AND DISAPPEARANCE OF THE DIHYDROXYPHENYLALANINE

Time	Oxygen consumption		Cpd. disap.	
min.	μatoms	μatoms per hour*	μmoles	μmoles per hour**
15	1.16	4.69	0.35	1.40
30	1.96	3.92	1.11	2.22
45	2.69	3.58	1.91	2.54
60	3.47	3.47	2.29	2.29
120	4.17	2.08	3.17	1.51
180	5.02	1.67	3.46	1.15

* Rate of oxygen consumption = atoms $O_2 \times \frac{60}{\text{incubation time}}$

** Rate of Cpd. disap. = moles Cpd. disap. $\times \frac{60}{\text{incubation time}}$

Extracts were incubated at pH 6.8 and 37.5° C. Extract concentration was 10 per cent.

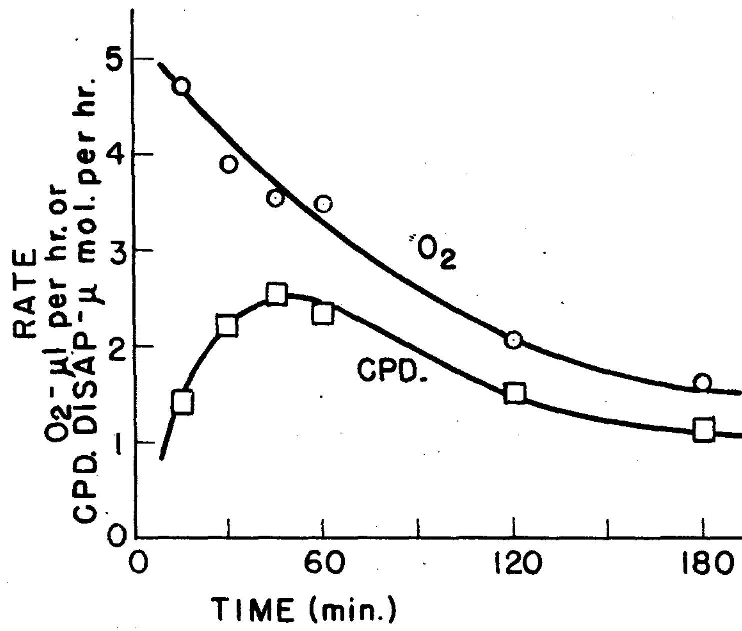


FIGURE 11. RATE OF OXYGEN CONSUMPTION AND SUBSTRATE DISAPPEARANCE

8. <u>Activity of guinea pig liver and rat kidney extracts</u>

The presence of l-3,4-dihydroxyphenylalanine decarboxylase and amine oxidase has been demonstrated in the kidney extracts, and a slight digression was made at this point to examine the ability of other tissues to cause the disappearance of the dihydroxy groups of the amino acid.

Lan and Sealock had observed the increased ability of kidney slices of the guinea pig to consume oxygen in the presence of dihydroxyphenylalanine but found the response of liver slices under the same conditions to be not nearly as marked. As no analysis for the dihydroxy disappearance was attempted, it was not possible to determine the limiting factor in the liver slices.

The availability of hydroxytyramine made possible a more complete examination of the ability of liver extracts to metabolize the amino acid than was possible in the above mentioned liver-slice experiments. The reaction with the amino acid would indicate the presence of the decarboxylase, while the reaction with hydroxytyramine would give some idea of the amount of amine oxidase present.

Equimolar amounts of dihydroxyphenylalanine and hydroxytyramine were incubated with extracts of guinea-pig liver in the same manner as in the case of the kidney extract experiments. The data obtained are shown in Table XX and Figure 12. With hydroxytyramine as a substrate the reaction

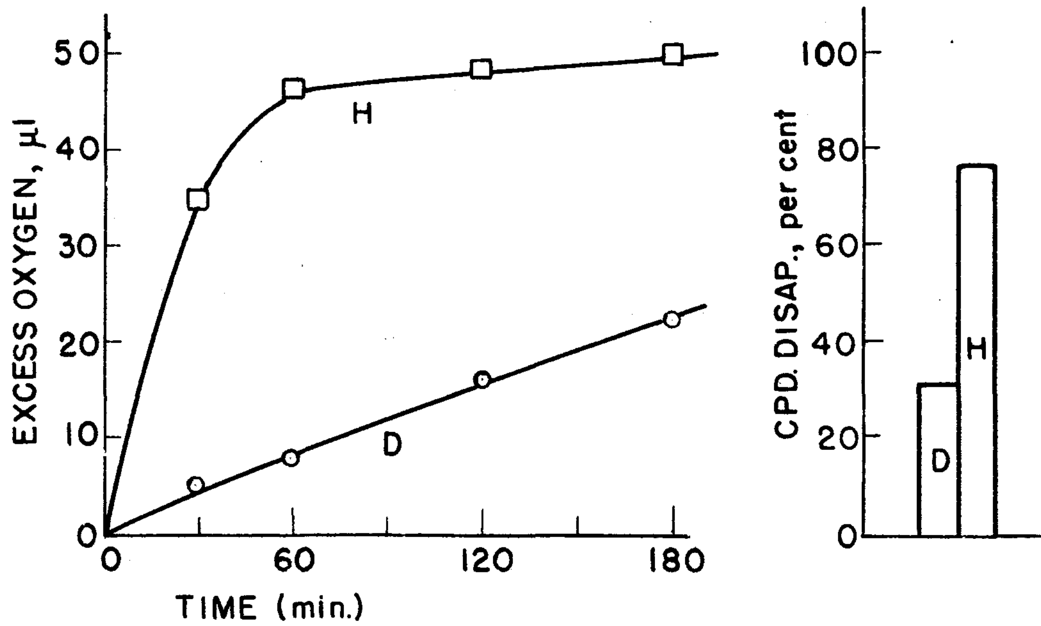


FIGURE 12. ACTIVITY OF LIVER EXTRACTS

D, l 3,4 - DIHYDROXYPHENYLALANINE
H, HYDROXYTYRAMINE

TABLE XX

ACTIVITY OF EXTRACTS OF GUINEA-PIG LIVER
WITH DIHYDROXYPHENYLALANINE AND HYDROXYTYRAMINE

Substrate*	Oxygen consumption		O/D_p	O/D_c	Compound disappearance	
	Basal µl.	Excess µl.			µmoles	per cent
l-3,4-Dihydroxy-phenylalanine	54.6	21.8	0.38	1.19	1.63	32.0
Hydroxytyramine	54.6	49.9	0.88	1.18	3.79	78.1

* Substrate = 5.08 micromoles.

The three-hour incubations were carried out at pH 6.8 and at 37.5° C. Concentration of the tissue extract was 10 per cent.

was very similar to the action of kidney extracts, however, when the amino acid was the substrate, the activity of the preparation was very low. According to the Arnow test, 78 per cent of the hydroxytyramine had disappeared while only 32 per cent of the amino acid had disappeared. This trend was also reflected in the oxygen consumption. With hydroxytyramine, 49.9 microliters of oxygen were consumed while, in the case of the amino acid, the oxygen consumption was only 21.8 micromoles. Therefore, it is apparent that the decarboxylase is the limiting enzyme in the liver extracts for the amino acid must be decarboxylated to hydroxytyramine before the amine oxidase can oxidize the amine. The low activity observed by Lan and Sealock (126) could have been caused by the low activity of

of the decarboxylase in the liver slices, however, no information is available relative to the activity of liver slices in the presence of hydroxytyramine and the solution of this problem must be left for future investigation. After decarboxylation has occurred, the deamination and diphenolic disappearance apparently proceed as in the kidney extract, but the total reaction is reduced by the limiting action of the decarboxylase.

A few exploratory experiments with rat kidney were carried out to ascertain if the same system was operative in another animal. These experiments include an enzyme concentration series and a pH-activity series. The extracts were prepared in the same manner as the guinea-pig extracts and the activity tested in the same manner as the guinea-pig kidney extracts (Table XXI). In general, the same trend was present here as has been shown in the guinea-pig series. As the concentration of the extracts was increased, the per cent diphenolic disappearance increased until at the high tissue concentration of 25 per cent, 95.6 per cent of the diphenolic value disappeared. In the same manner, the O/D_p increases and the O/D_c was remarkably constant. This probably indicates that the same reaction was taking place at each tissue concentration; the only difference being in amount. The O/D_c of 2.0 in this series was higher than the O/D_c of approximately 1.5 experienced with guinea-pig kidney at the same pH

TABLE XXI

ACTIVITY OF EXTRACTS OF RAT KIDNEY

Extract conc.	Oxygen consumption		O/D_p	O/D_o	Cpd. disap.
	Basal	Excess			
per cent	μl.	μl.			per cent
0.32	15.8	11.9	0.21	1.83	11.4
0.63	14.8	13.9	0.24	2.54	9.6
1.25	14.6	17.2	0.33	2.08	16.1
2.50	18.0	36.7	0.64	2.06	29.4
5.00	30.5	61.5	1.07	2.01	53.5
10.00	44.4	86.0	1.50	2.10	71.8
25.00	113.0	116.0	2.04	2.13	95.6

The three-hour incubation was carried out at pH 7.4 and at 37.5° C. The substrate concentration was 5.08 micromoles l-3,4-dihydroxyphenylalanine.

and it may be that another system was present in the rat tissue.

The short study of the pH-activity relationship exhibited by rat kidney extracts, summarized in Table XXII, demonstrates

TABLE XXII

THE INFLUENCE OF pH ON THE ACTIVITY OF RAT KIDNEY EXTRACTS

pH	Oxygen consumption		O/D_p	O/D_c	Cpd. disap.
	Basal µl.	Excess µl.			per cent
6.50	40.3	5.3	0.09	0.63	14.5
6.95	39.1	43.8	0.79	1.69	46.1
7.40	44.4	86.0	1.50	2.10	71.8

The incubations were carried out in 0.1 M phosphate buffer at 37.5° C. Concentration of extracts was 10 per cent.

a much sharper fall in activity than was experienced in the guinea-pig kidney extracts. At pH 6.5 the lowest pH recorded, the activity was very low when compared with the activity of the guinea-pig kidney extract of pH 6.6. Only 14.7 per cent dihydroxy disappearance was noted with the rat kidney extracts, at pH 6.5, whereas 63.6 per cent dihydroxy disappearance was noted with the guinea-pig kidney at pH 6.6. Even at pH 6.95 the activity, as indicated by oxygen consumption and diphenolic disappearance, was not as high as experienced with the guinea-

pig kidney at pH 7.0. Only 46.1 per cent dihydroxy disappearance was found in the rat kidney extract at pH 6.95, while at pH 7.0, the guinea-pig kidney exhibited 79.3 per cent dihydroxy disappearance. As the pH-activity curve and the O/D_o of the extracts prepared from the rat kidney were not in agreement with the guinea-pig kidney extracts, the attempt to use rat kidney extracts in this investigation was abandoned. The ability of the rat kidney extracts to metabolize dihydroxyphenylalanine must be further explored before an intelligent comparison of the extracts from the rat and guinea pig can be made.

D. Inhibition

Through application of enzyme inhibitors of known chemical reactivities valuable and informative knowledge of complex enzyme systems has been obtained. By the addition of substances which would stop one or more of the reactions in the metabolism of a substrate, various schemes have been derived to account for the disappearance of the substrate in the living organism. In view of the success that has resulted from this procedure, an investigation of the activity of the kidney extracts in the presence of a series of such compounds was initiated.

The inhibition of purified enzyme preparations usually requires a very small quantity of the inhibitor, but when crude

tissue extracts are employed more inhibitor is necessary to correct for losses due to reactions with other substances present in the reaction mixture. For this reason, a series of experiments were initiated to find the lowest inhibitor concentration which could be used to scan a number of compounds with a reasonable degree of success. Preliminary experiments demonstrated the inhibitory powers of sodium diethyldithiocarbamate, sodium azide, hydroxylamine and 8-hydroxyquinoline, and, therefore, these compounds were used for this investigation. Concentrations between 0.001 M and 0.01 M were employed. The results are summarized in Table XXIII, but for the purpose of this discussion the graphical presentation in Figure 13 is more useful. As the concentration of the inhibitor was increased, the per cent inhibition of both the oxygen consumption and the diphenolic disappearance increased. At the very low concentrations the inhibition was not very significant, but at 0.005 M the inhibition was great enough to be readily observed in all cases. Therefore, 0.005 M solutions of the various inhibitors were employed to investigate the effect of other substances on the metabolism of dihydroxyphenylalanine by the kidney extracts.

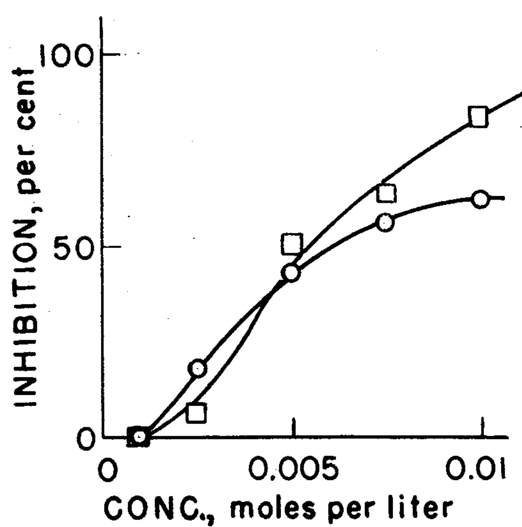
In most cases this concentration was realized by adding 0.1 ml. of a buffered solution of the substance to the tissue extract in the reaction chamber of the vessel. Due to the solubility difficulties, in some cases, other volumes were

TABLE XXIII

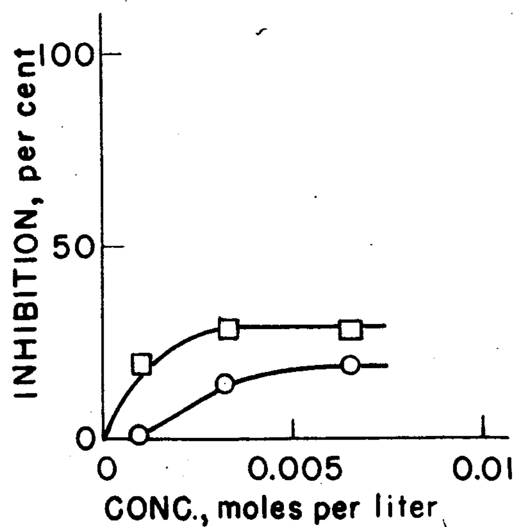
PER CENT INHIBITION BY SODIUM DIETHYLDITHIOCARBAMATE, SODIUM AZIDE, 8-HYDROXYQUINOLINE AND HYDROXYLAMINE

Inhibitor	Concentration of Inhibitor, moles per liter x 10^3						
	1.0	2.5	3.3	5.0	6.6	7.5	10.0
A. Based on l-3,4-dihydroxyphenylalanine analysis							
Sodium diethyl-dithiocarbamate	0.0	18.0		42.5		55.0	61.8
Sodium azide	2.2		13.1		18.7		
Hydroxylamine	19.2		70.0		86.3		81.3
8-Hydroxy-quinoline	5.7	11.5		17.7		39.3	61.3
B. Based on oxygen consumption							
Sodium diethyl-dithiocarbamate	00.0	6.3		50.3		63.0	84.5
Sodium azide	19.2		28.6		28.2		
Hydroxylamine	7.3		83.6		92.0		79.6
8-Hydroxy-quinoline	0.0	14.8		33.3		40.0	74.1

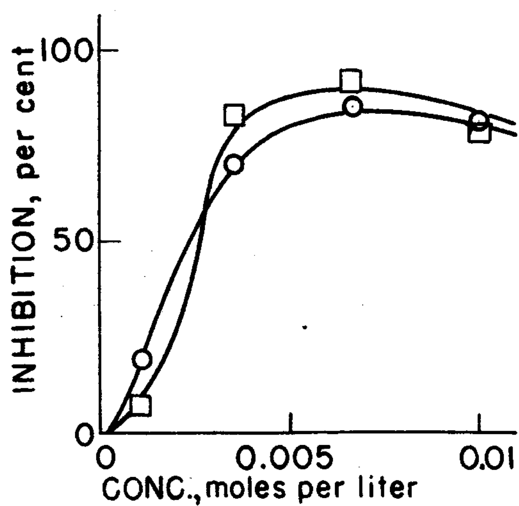
The three-hour incubations were carried out at pH 6.8 and at 37.5° C. Concentration of the kidney extracts was 10 per cent. Substrate was 5.08 micromoles of dihydroxyphenylalanine.



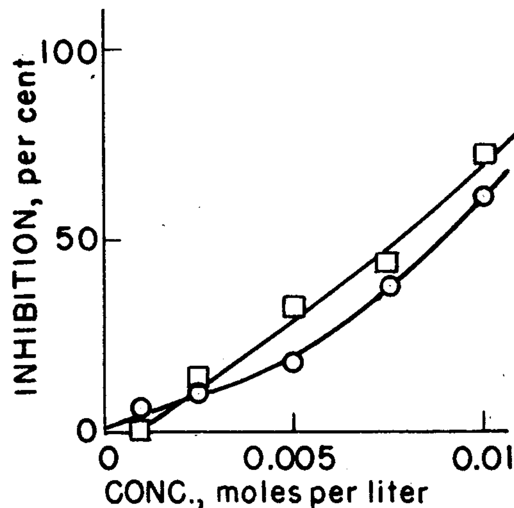
SODIUM DIETHYLDITHIOCARBAMATE



SODIUM AZIDE



HYDROXYLAMINE



8 - HYDROXYQUINOLINE

FIGURE 13. INHIBITION

□ CPD. DISAP

O OXYGEN CONSUMPTION

used, but in every case the final concentration was as recorded. Included among the substances employed as inhibitors were compounds which previously have been reported to inhibit either the decarboxylase or the amine oxidase, and, in addition, certain other compounds which, as far as could be discovered, had not been used on these systems.

In the first series l-3,4-dihydroxyphenylalanine was employed as the substrate. Sodium fluoride (0.005 M), sulfapyridine[1] and catechol (0.005 M) had no effect on the activity of the kidney extracts in the presence of the amino acid. The other substances employed -- sodium azide, sodium diethyldithiocarbamate, hydroxylamine, semicarbazide, cysteine, mercuric chloride and zinc sulfate -- inhibited the excess oxygen consumption and the diphenolic disappearance to approximately the same extent (Table XXIV). This could be interpreted as inhibition of the decarboxylase or as inhibition of the oxygen consumption and, in addition, as inhibition of diphenolic disappearance. The substances could inhibit one or all of the systems involved in the metabolism of dihydroxyphenylalanine. However, since the decarboxylation must precede the action of the amine oxidase, the decision as to whether the substance inhibits either the decarboxylation or the amine oxidation cannot be made from the results of an aerobic experiment employing dihydroxyphenylalanine as the substrate. This

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[1] An attempt was made to use 1 mg. per ml. but it was impossible to completely dissolve the inhibitor.

TABLE XXIV

INHIBITION OF THE METABOLISM
OF l-3,4-DIHYDROXYPHENYLALANINE

Inhibitor	Inhibitor conc.	pH	Inhibition	
			Oxygen consumption	Cpd. disap.
	molar		per cent	per cent
Sodium azide	0.001	7.4	19.0	13.8
Sodium azide	0.010	6.8	31.9	32.2
Sodium diethyldithiocarbamate	0.010	6.8	66.0	65.0
Hydroxylamine	0.005	6.8	89.2	84.6
Hydroxylamine	0.010	6.8	91.0	84.4
Semicarbazide	0.050	6.8	80.7	81.2
Cysteine	0.010	6.8	59.4	50.1
Mercuric chloride	0.005	7.4	79.9	69.0
Zinc sulfate	0.005	7.4	16.5	4.0
Potassium cyanide	0.00046*	6.8	53.7	72.0

* Concentration maintained by use of potassium cyanide-potassium hydroxide center-well additions (130).

The three-hour incubations were carried out at 37.5° C. Substrate was 5.08 micromoles dihydroxyphenylalanine.

is well illustrated by cyanide inhibition. Inhibition by cyanide, which has been reported as an inhibitor of the decarboxylase but not of the amine oxidase, was demonstrated by the greatly reduced oxygen consumption. This is understandable, for the amino acid must be decarboxylated before deamination by amine oxidase can occur.

To avoid inhibition of the decarboxylase, hydroxytyramine was used as the substrate in the next series. As this compound is decarboxylated dihydroxyphenylalanine, the first reaction of the metabolic series was eliminated. In this case, the inhibitors act either on the amine oxidase system or on any possible system involved in the diphenolic disappearance.

Sodium pyrophosphate, malonic acid, iodoacetic acid, thiourea and succinic acid, at a concentration of 0.005 M, had no observable effect on either the oxygen consumption or on the diphenolic disappearance. The remaining substances can be divided into three groups: (1) those substances which inhibit the oxygen consumption and diphenolic disappearance to about the same extent, (2) those which inhibit the oxygen consumption more than the diphenolic disappearance and (3) those which inhibit the diphenolic disappearance to a much greater extent than the oxygen consumption.

Table XXV summarizes the inhibition produced by the compounds in group 1 -- α,α-dipyridyl, sodium selenite, caprylic alcohol, potassium ethylxanthate, cysteine, sodium azide and

and hydroxylamine -- which inhibit the oxygen consumption and diphenolic disappearance to the same extent, and which may, by inhibiting the amine oxidase reaction, reflect this inhibition in the diphenolic disappearance.

TABLE XXV

INHIBITION OF THE METABOLISM OF HYDROXYTYRAMINE

Inhibitor	Inhibitor conc. molar	pH	Inhibition	
			Oxygen consumption per cent	Cpd. disap. per cent
α,α'-Dipyridyl	0.005	6.8	18.4	28.3
Sodium selenite	0.005	6.8	29.2	30.7
Caprylic alcohol	*	6.8	81.6	87.0
Potassium ethylxanthate	0.005	6.8	48.0	46.1
Cysteine	0.010	6.8	13.6	16.2
Sodium azide	0.010	6.8	49.3	38.4
Hydroxylamine	0.005	6.8	54.7	51.7

* In this case, 0.2 ml. of the caprylic alcohol was added directly to the contents of the flask.

The compounds of the second group (Table XXVI) -- phenylmercuric chloride, sodium diethyldithiocarbamate and p-hydroxybenzaldehyde -- which inhibit the oxygen consumption to a greater extent than the diphenolic disappearance may

TABLE XXVI

INHIBITION OF THE METABOLISM OF HYDROXYTYRAMINE

Inhibitor	Inhibitor conc. molar	pH	Inhibition: Oxygen consumption per cent	Inhibition: Cpd. disap. per cent
Phenylmercuric chloride	0.001	6.8	58.3	30.6
Sodium diethyldithiocarbamate	0.008	6.8	71.1	54.8
p-Hydroxybenzaldehyde	0.050	6.8	100.0	68.2
"	0.050	6.8	100.0	55.8
"	0.025	6.8	92.1	66.9
"	0.005	6.8	66.1	54.6

exhibit the increased oxygen inhibition by the prevention of various side reactions which, in all probability, account for some of the oxygen consumption. However one outstanding exception should be noted. In the case of p-hydroxybenzaldehyde, the oxygen consumption was inhibited 100 per cent, but the disappearance of the diphenolic value was only inhibited 55 to 65 per cent. As the concentration of the p-hydroxybenzaldehyde is decreased from 0.05 to 0.005 M, the inhibition of the oxygen consumption is reduced from 100 to 66.1 per cent, but inhibition of diphenolic disappearance (analysis) does not decrease a similar amount. At the high concentration,

possibly the p-hydroxybenzaldehyde forms a Schiff's base type of compound with the amine and prevents enzymatic oxidation. However, the inhibition of the diphenolic disappearance did not decrease when the concentration of the p-hydroxybenzaldehyde was reduced and this fact is indicative of the enzymatic nature of the dihydroxy disappearance. On the other hand, no oxygen consumption is evident at the high concentration of p-hydroxybenzaldehyde. Therefore, it is reasonable to assume that oxidation was also not the cause of the diphenolic disappearance at the lower concentration of the inhibitor.

Of the third group -- potassium cyanide, semicarbazide and ethyl-N-phenylcarbamate --, which do not inhibit the oxygen consumption to the same extent as the diphenolic disappearance, cyanide has been used by previous investigators to eliminate the various side reactions in the deamination by amine oxidase (41,42), while semicarbazide has been used to isolate the product of such deaminations (40,45). However, the action of these reagents has not previously been correlated with the effect of the tissue extracts on the dihydroxy portion of the molecule. The results of these experiments are shown in Table XXVII. The inhibition of the oxygen consumption is low, 12.0 to 32.3 per cent, whereas the inhibition of the diphenolic disappearance (60 to 70 per cent) is very high. This observed reduction in oxygen consumption is, in all probability, due to elimination of miscellaneous oxidations

by metallic ions and the cytochrome system, for the oxygen consumption is not great enough to account for the very high inhibition of the diphenolic disappearance.

TABLE XXVII

INHIBITION OF THE METABOLISM
OF HYDROXYTYRAMINE

Inhibitor	Inhibitor conc. molar	pH	Inhibition Oxygen consumption per cent	Cpd. disap. per cent
Ethyl-N-phenyl-carbamate	0.005	6.8	19.6	43.8
Semicarbazide	0.050	6.8	32.3	70.8
"	0.005	6.8	12.0	60.2
Potassium cyanide	0.00046*	6.8	27.7	69.6

* Concentration maintained by use of potassium cyanide-potassium hydroxide center-well addition (130).

The effect of the inhibitors on both substrates -- l-3,4-dihydroxyphenylalanine and hydroxytyramine -- has been summarized in Table XVIII in order to better illustrate the relationships between the reactions involved in the metabolism of these substrates. These are matched experiments inasmuch as the same extract was used to test the activity of the inhibitor in the presence of both substrates. This fact would eliminate differences in the results which might be caused by

TABLE XXVIII

RELATIVE EFFECT OF INHIBITORS ON THE METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE AND HYDROXYTYRAMINE

Inhibitor	Substrate	Inhibition	
		Oxygen consumption per cent	Cpd. disap. per cent
Sodium diethyl-dithiocarbamate (0.008 M)	Dihydroxy-phenylalanine	91.6	63.2
	Hydroxytyramine	71.1	54.8
Sodium azide (0.01 M)	Dihydroxy-phenylalanine	31.9	32.2
	Hydroxytyramine	49.3	38.4
Hydroxylamine (0.005 M)	Dihydroxy-phenylalanine	89.2	84.6
	Hydroxytyramine	54.7	51.7
Cysteine (0.01 M)	Dihydroxy-phenylalanine	59.4	50.1
	Hydroxytyramine	13.6	26.2
Cyanide (0.00046 M)	Dihydroxy-phenylalanine	53.7	72.0
	Hydroxytyramine	27.7	69.6
Semicarbazide (0.05 M)	Dihydroxy-phenylalanine	80.7	81.2
	Hydroxytyramine	32.3	70.8

inequalities in the preparation of the extracts.

The agreement between the inhibition of the oxygen consumption and diphenolic disappearance points to the probable series of reactions involved in the metabolism of the substrate, l-3,4-dihydroxyphenylalanine. Agreement between the inhibition of oxygen consumption and dihydroxy disappearance, in all but the cyanide and semicarbazide experiments, demonstrates that dihydroxy disappearance is probably dependent upon the decarboxylation and the deamination, because it does not occur to any appreciable extent until these reactions have taken place. On the other hand, cyanide and semicarbazide inhibit the diphenolic disappearance to a much greater extent than the oxygen consumption when hydroxytyramine was employed as the substrate. Therefore, deamination can occur without dihydroxy disappearance.

A further analysis of the dihydroxyphenylalanine and hydroxytyramine experiments are illustrated in Table XXIX and Figure 14. The inhibition of the oxygen consumption, even in the early stages of the reaction, was very low in the case of the hydroxytyramine. At the three-hour interval the inhibition of the oxygen consumption was 27.7 per cent and the inhibition of the diphenolic disappearance was 69.6 per cent. When the amino acid was the substrate, the inhibition of both the oxygen consumption and diphenolic disappearance was high; 72.0 per cent for the diphenolic disappearance and 53.3 per cent for the oxygen consumption. When hydroxy-

TABLE XXIX

EFFECT OF CYANIDE ON THE METABOLISM OF *l*-3,4-DIHYDROXYPHENYLALANINE AND 3,4-DIHYDROXYPHENYLETHYLAMINE

Substrate	Inhibition per cent*				
	Based on oxygen consumption				Cpd. disap.
	30 min.	60 min.	120 min.	180 min.	180 min.
l-3,4-dihydroxy-phenylalanine	83.6	65.6	63.0	53.3	72.0
3,4-dihydroxy-phenylethylamine	11.8	20.8	17.8	27.7	69.6

* Inhibition per cent = $100 - \frac{(\text{inhibited})}{(\text{normal})} \times 100$

The concentration of the cyanide ion in the reaction mixture was 0.00046 M, with a center-well addition of potassium cyanide-potassium hydroxide to maintain this concentration.

The incubations were carried out at pH 6.8 and at 37.5^{o} C. The concentration of tissue was 10 per cent. Substrate concentration was 5.08 micromoles.

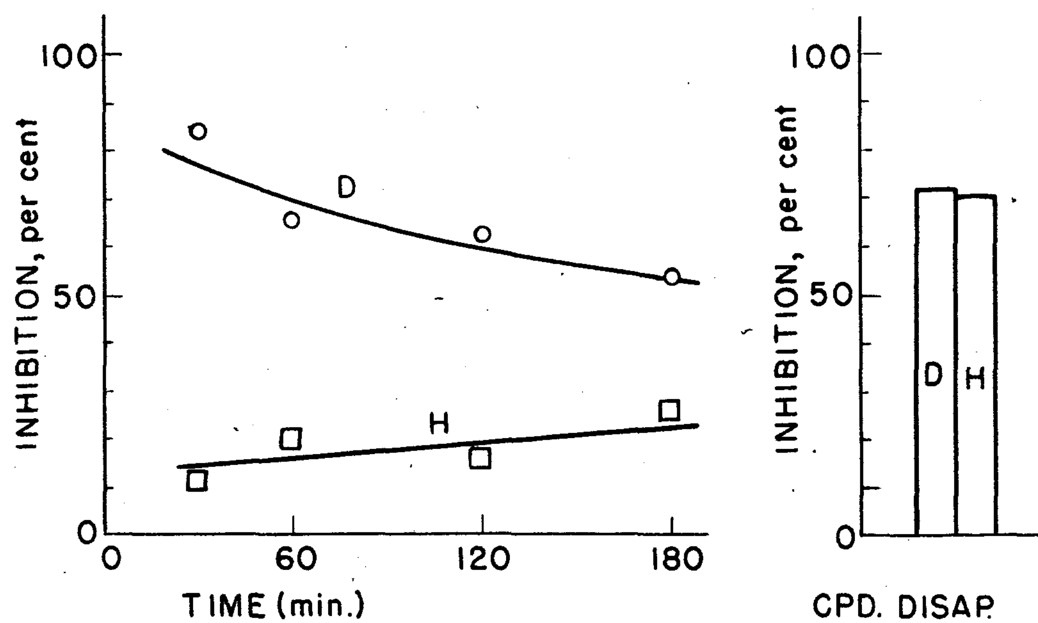


FIGURE 14. CYANIDE INHIBITION

D, DIHYDROXYPHENYLALANINE

H, HYDROXYTYRAMINE

tyramine was the substrate, although the inhibition of the oxygen consumption during the reaction was low, the diphenolic disappearance was of the same order as when the amino acid was used. The inhibition of the reaction with l-3,4-dihydroxyphenylalanine as the substrate was in keeping with the fact that cyanide inhibits the action of the decarboxylase. The great difference between the inhibition of the diphenolic disappearance and of the oxygen consumption when hydroxytyramine was the substrate again demonstrates that oxidation by the atmosphere of the flask was not the cause of the dihydroxyphenylalanine disappearance.

The subject of cyanide inhibition was explored more completely in an attempt to discover a relationship which would separate the decarboxylase and amine oxidase from the diphenolic disappearance. Using 0.005 and 0.01 M cyanide concentrations and l-3,4-dihydroxyphenylalanine as the substrate, a reversible inhibition was demonstrated (Table XXX). When cyanide is introduced into the reaction mixture without potassium cyanide in the center well, the cyanide will distill from the reaction mixture into the potassium hydroxide in the center well and in this way the cyanide concentration of the extract will be lowered. The inhibition of the oxygen consumption at the end of the experiment (180 minutes) was only half the inhibition at the thirty minute interval. For example, when the initial cyanide concentration was 0.005 M

TABLE XXX

REVERSIBLE INHIBITION BY CYANIDE

Initial cyanide conc.	Inhibition per cent				
	Based on oxygen consumption				Cpd. disap.
molar	30 min.	60 min.	120 min.	180 min.	180 min.
0.005	65	60	53	34	30.5
0.01*	46	43	32	23	

* This brei was stored in 0.01 M cyanide overnight before use and some of the cyanide would be lost under these conditions. The cyanide concentration at the beginning of the incubation is, therefore, questionable.

Three-hour incubations were carried out at pH 7.4 and at 37.5° C. Concentration of extracts was 10 per cent; substrate was 5.08 micromoles.

the inhibition of the oxygen consumption at thirty minutes was 65 per cent, compared with 34 per cent at the three-hour interval. Therefore, reversible cyanide inhibition was demonstrated in the case of the decarboxylase.

On the other hand, if the cyanide concentration of the reaction mixture is maintained, the per cent inhibition should be the same throughout the experiment. This was accomplished by using a potassium hydroxide-potassium cyanide solution in the center well instead of the potassium hydroxide. The concentration of potassium cyanide in the potassium hydroxide was such that the center well solution was saturated with

cyanide, and therefore, no cyanide would be lost from the reaction mixture. The report of Robbie (130) was consulted for the correct potassium hydroxide-potassium cyanide ratio. The inhibition obtained by the use of this technique was fairly constant throughout the reaction. At the sixty minute interval, the inhibition varied from 48 to 65.6 per cent while at the end of three hours it varied from 53.0 to 63.1 per cent. Table XXXI summarizes the information obtained in the above experiments.

TABLE XXXI

INHIBITION IN THE PRESENCE OF A CONSTANT CYANIDE CONCENTRATION

Initial cyanide conc.	Inhibition per cent				
	Based on oxygen consumption				Cpd. disap.
molar	30 min.	60 min.	120 min.	180 min.	180 min.
0.00046	38.0	48.0	54.0	53.0	71.2
0.00046	83.8	65.6	63.0	53.3	72.0
0.00046	58.3	61.8	64.4	63.1	81.9

Three hour incubations were carried out at pH 6.8 and at 37.5° C. Concentration of extracts was 10 per cent. Substrate was 5.08 micromoles of dihydroxyphenylalanine.

The difference between these experiments is best shown in Figure 15 in which the data of Table XXX and Table XXXI are graphically illustrated. In the experiments with cyanide

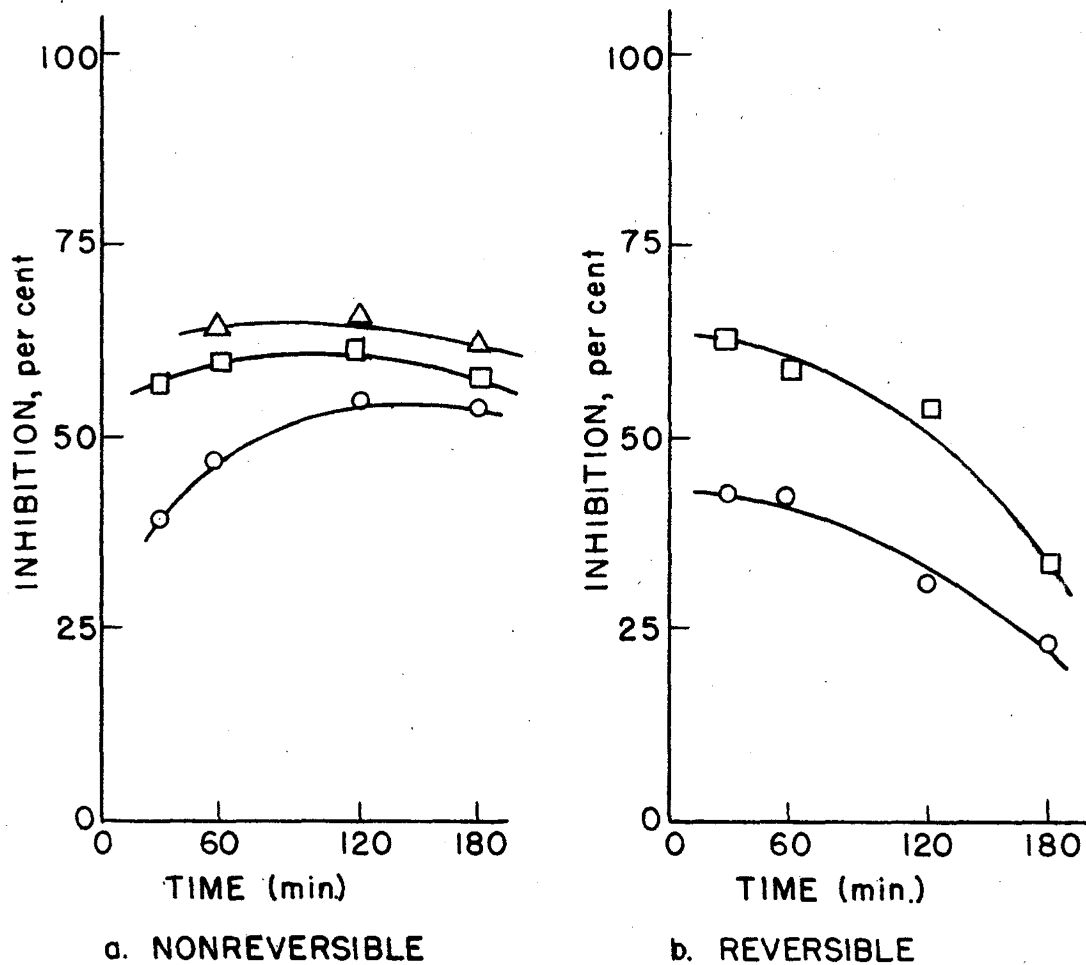


FIGURE 15. CYANIDE INHIBITION

in the center well, the curve is fairly flat during the last two hours of the incubation. In the other experiments, the curve fell off rather steeply near the end of the experiment. The center well cyanide maintained the concentration in the reaction mixture and, by so doing, maintained the inhibition of the decarboxylase.

The previous experiments, employing cyanide in the center well and hydroxytyramine as the substrate, demonstrated a very high inhibition of the diphenolic disappearance but, at the same time, exhibited a comparatively low inhibition of the oxygen consumption (Table XXIX). If the cyanide inhibition was reversible, as has been shown with the decarboxylase, this probably could be demonstrated by adding cyanide to the extracts and allowing this cyanide to distill into the potassium hydroxide in the center well. This would allow a fairly high concentration of cyanide to be present during the early part of the reaction, but the distillation of cyanide into the potassium hydroxide would gradually reduce this concentration and so exhibit reversible inhibition. Three initial concentrations of cyanide were employed -- 0.0008, 0.005 and 0.01 M -- and the inhibition of the oxygen consumption and the inhibition of the diphenolic disappearance at the 30 minute and 180 minute intervals are summarized in Table XXXII.

TABLE XXXII

CYANIDE INHIBITION
WITH HYDROXYTYRAMINE AS THE SUBSTRATE

Cyanide	Inhibition per cent			
	30 minutes		180 minutes	
	Oxygen consumption	Cpd. disap.	Oxygen consumption	Cpd. disap.
molar				
0.0008	21.6	11.8	25.4	8.4
0.005	26.0	30.8	23.2	43.7
0.01	14.3	47.8	27.0	41.2

Substrate was 5.08 micromoles hydroxytyramine.

At the low cyanide concentration (0.0008 M) the inhibition of the oxygen consumption was slightly higher than the diphenolic disappearance at both the 30 and 180 minute intervals. It is evident that at the low concentration the cyanide does not appreciably affect the diphenolic disappearance. Although the higher cyanide concentrations (0.005 and 0.01 M) did not increase the inhibition of the oxygen consumption, the inhibition of the diphenolic disappearance was increased to 43.7 and 41.2 per cent. Therefore, increasing the cyanide concentration affected only the diphenolic disappearance. The inhibition at the 180 minute interval was equal to the inhibition at the 30 minute interval, therefore, reversible inhibition was not demonstrated.

At this point of the discussion, the inhibition obtained

by semicarbazide should be re-emphasized. Even when the concentration with semicarbazide was reduced from 0.05 to 0.005 M, the inhibition of the diphenolic disappearance was not appreciably lowered. In both cases, the concentrations employed were more than twice the concentration of the substrate, and it is conceivable that the aldehyde formed by the deamination is prohibited from further reaction by reacting with the cyanide or the semicarbazide to form a cyanohydrin or a semicarbazone, respectively, and that in these combinations the diphenolic groups are immune to further reaction. However, the inhibition at a concentration of 0.005 M can not be explained on this basis, for it is very possible that some of the cyanide and semicarbazide was inactivated by reacting with the extraneous protein in the extracts. If a more highly purified enzyme preparation had been available, perhaps a much lower inhibitor concentration would have produced the same effect.

Derivatives of isopropyl amine have been shown to act as competitive inhibitors in the amine oxidase reaction. These amines have the amino group on the β-carbon atom and, although they compete with the substrate for the enzyme, they are not oxidized by the amine oxidase. The over-all effect is to reduce the concentration of the substrate in contact with the enzyme. This is evidenced by a reduced oxygen consumption. Alles and Heegaard (77) have used β-amino octane

for this purpose with excellent results and, as this compound was readily available, an experiment was designed to determine whether or not deamination must occur before the disappearance of the diphenolic groups. The β-amino octane was used to inhibit the amine oxidase with both l-3,4-dihydroxyphenylalanine and hydroxytyramine as substrates. The hydroxytyramine experiment was inserted to eliminate the possibility of the β-amine inhibiting the decarboxylase, and, by so doing, lead to a misinterpretation of the data obtained, for previous results have indicated that if the decarboxylase was blocked amine oxidation would not occur. When the amine oxidase is inhibited with β-amino octane, the reaction responsible for the disappearance of the diphenolic portion of the amino acid is also inhibited to about the same extent (Table XXXIII). At a β-amino octane concentration of 0.004 M the inhibition of both the oxygen consumption and diphenolic disappearance, when the amino acid was the substrate, was between 59.5 and 68.3 per cent. When hydroxytyramine was the substrate, the inhibition of the oxygen consumption was comparable with the above while the inhibition of the diphenolic disappearance was 49.8 per cent. Therefore, the deamination probably occurs before the diphenolic groups will be affected to any extent.

TABLE XXXIII

INHIBITION OF THE METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE AND HYDROXYTYRAMINE BY β-AMINO-OCTANE

Inhibitor	Oxygen consumption		Cpd. disap.	Inhibition	
				Oxygen consumption	Cpd. disap.
molar	Basal μl.	Excess μl.	μmoles	per cent	per cent
			Experiment 1[a]		
0.000	32.2	43.5	3.67		
0.004	30.9	17.6	1.25	59.5	65.9
0.016	30.4	8.8	0.83	79.8	77.4
			Experiment 2[a]		
0.000	21.2	57.6	3.44		
0.004	23.3	19.3	1.09	66.5	68.3
			Experiment 3[b]		
0.000	21.2	54.3	3.85		
0.004	23.3	17.6	1.92	67.6	49.8

a Substrate was l-3,4-dihydroxyphenylalanine (5.08 micromoles).
b Substrate was hydroxytyramine (5.08 micromoles).

Incubations were carried out at pH 6.8 and at 37.5° C., with an extract concentration of 10 per cent.

E. Effect of Dialysis on the Activity of the Kidney Extract

With but one exception in previous investigations the dialysis of tissue extracts did not reduce the activity either of the decarboxylase or of the amine oxidase. The one exception was the more recent note by Green (85) which states that a four-day dialysis against a dilute ammonia solution inactivated the decarboxylase. However, the system involved in the disappearance of the diphenolic nucleus was the object of this investigation and a study of the effect of dialysis on this system was initiated. In the first series, kidney extracts contained in a cellophane sac were dialyzed at refrigerator temperatures against distilled water for 3, 6, 12 and 48 hours and at various pH's. The results, as summarized in Table XXXIV, show that, except at pH 4.0 where the activity was completely lost in both the stored and dialyzed extracts, the dialysis did not cause any reduction in the oxygen consumption or diphenolic disappearance. On the contrary the activity increased after dialysis. Apparently the dialysis removed at least one substance which inhibited both the oxygen consumption and the diphenolic disappearance. To test this hypothesis the dialyzate was added to both stored and dialyzed brei. The processing of the dialyzate involved concentration, *in vacuo*, under a carbon dioxide atmosphere and at a temperature not exceeding 50° C. The dialyzate was

TABLE XXXIV

EFFECT OF DIALYSIS AGAINST DISTILLED WATER ON THE ACTIVITY OF THE KIDNEY EXTRACTS

Conditions of dialysis		Activity of the extracts			
pH	Time	Excess oxygen consumption		Compound disappearance	
		Stored	Dialyzed	Stored	Dialyzed
	hrs.	μl.	per cent of stored	μmoles	per cent of stored
7.4	48	18.4	149.0	1.00	149.0
7.4	12	72.6	101.0	3.82	108.0
7.4	6	68.4	93.8	3.63	103.0
7.4	6	58.0	85.9	3.60	75.0*
7.4	6	48.8	124.0	2.92	111.0
6.0	12	52.0	109.0	2.95	118.0
5.0	3	12.8	134.0	1.08	85.0
4.0	3	09.0	000.0	0.00	000.0

* In this incubation the dialyzed extract was stored in a more dilute form than the stored brei. This may have accounted for the reduction of activity.

The incubations were carried out at 37.5° C. and at pH 7.4.

reduced to about 2 ml. and then diluted with a few milliliters of distilled water until 0.5 ml. was equivalent to 1 ml. of the original extract. After the pH had been adjusted to 6.8, 0.5 ml. of this concentrate was added to 1 ml. of the normal and dialyzed extracts. As is shown in Table XXXV the dialyzate actually inhibited both the oxygen consumption and diphenolic disappearance. The oxygen consumption of the stored extract plus the dialyzate was 52.3 to 81.9 per cent of that of the stored extract while the diphenolic disappearance was from 72.8 to 95.6 per cent of the stored extract. The dialyzed extract did not exhibit such a marked difference but the activity of the dialyzed plus the dialyzate was less than that of the dialyzed brei.

Other investigators have removed copper from enzyme systems of the polyphenol oxidase type by dialyzing their preparations against cyanide. Since both cyanide and sodium diethyldithiocarbamate reduced the activity of the extracts, the preparations were dialyzed against solutions of these reagents in an attempt to reduce the activity of the extracts. If a dialyzable metal group, such as copper, was a prosthetic group, it should be removed by this process. Concentrations of 0.01 M and 0.001 M were employed and the results of these experiments are summarized in Table XXXVI. Dialysis against a water solution of both reagents resulted in an increase in activity as measured by the oxygen consumption and diphenolic disappearance. These results are comparable with the results

TABLE XXXV

EFFECT OF DIALYZATE ON THE ACTIVITY OF DIALYZED AND STORED KIDNEY EXTRACTS

Experiment	Activity of Extract			
	Excess oxygen consumption		Compound disappearance	
	Stored	Dialyzed	Stored	Dialyzed
	μl.	per cent of stored	μmoles	per cent of stored
Stored extract plus dialyzate				
1	68.4	81.9	3.63	88.2
2	48.8	79.5	2.92	95.6
3	72.6	63.8	3.82	89.8
4	74.1	52.3	2.49	72.8
Dialyzed extract plus dialyzate				
5	64.3	87.8	3.75	90.8
6	60.4	86.7	3.25	96.0
7	73.8	88.1	4.14	92.2

The incubations were carried out at pH 7.4 and at 37.5° C. The dialyzate had practically no effect on the oxidation of the substrate in boiled extracts or in phosphate buffer under similar conditions.

TABLE XXXVI

EFFECT OF DIALYSIS AGAINST CYANIDE AND SODIUM DIETHYL-DITHIOCARBAMATE ON THE ACTIVITY OF KIDNEY EXTRACTS

Conditions of Dialysis			Activity of Extract			
pH	Time	Reagent	Excess oxygen consumption		Compound disappearance	
			Stored	Dialyzed	Stored	Dialyzed
	hrs.*		μl.	per cent of stored	μmoles	per cent of stored
7.4	0-12**	Potassium cyanide	57.6	109.0		
7.4	3-9	Potassium cyanide (0.01 M)	52.0	123.0	2.95	124.0
7.4	4-2	"	35.8	157.8	2.85	181.2
6.8	4-4	Sodium diethyl-dithiocarbamate (0.001 M)	38.4	139.0	2.47	126.0
6.8	4-4	Sodium diethyl-dithiocarbamate (0.01 M)	38.4	164.0	2.47	144.0

* First number represents the number of hours dialyzed against reagent and the second number represents the number of hours dialyzed against distilled water.

** Extract containing 0.01 M potassium cyanide was dialyzed 12 hours against distilled water.

obtained by dialysis against distilled water. Dialysis against distilled water, cyanide and sodium diethyldithiocarbamate did not reduce the activity of the system.

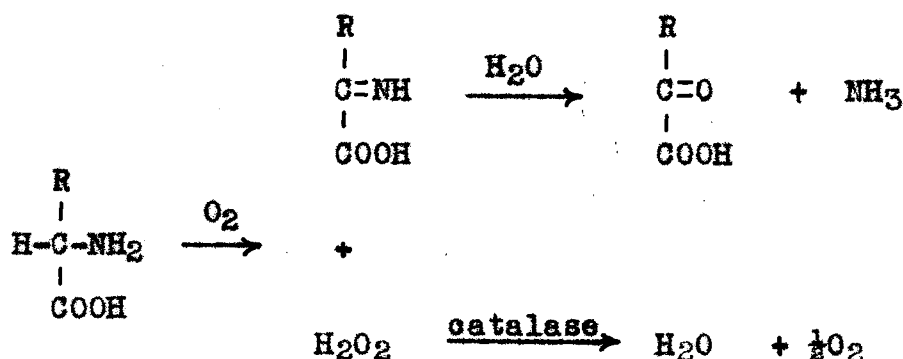
F. Effect of Hydrogen Peroxide on the Substrate Disappearance

The lack of agreement between the oxygen consumption, ammonia formation and diphenolic disappearance together with the results of the inhibitor experiments suggested a pathway other than oxidation by atmospheric oxygen as the method by which the 3,4-dihydroxybenzene group was disappearing from the extracts during the incubation period. Various investigators have demonstrated the production of hydrogen peroxide when an aliphatic amine is deaminated by amine oxidase. In order to further test the possibility of oxidation by oxygen as the cause of the disappearance of the diphenolic nucleus, oxidation of the amino acid by the hydrogen peroxide formed was investigated. Two approaches, one employing _d_-amino acid oxidase and a suitable substrate, and another employing competing substrates, were designed to investigate the possibility of a hydrogen peroxide effect.

1. Effect of d-amino acid oxidase

_d_-Amino acid oxidase in the presence of a suitable substrate -- in this case, _d,l_-methionine -- will produce hydro-

gen peroxide according to the following over-all reaction (131):



$$R\text{-}CH(NH_2)\text{-}COOH \xrightarrow{O_2} R\text{-}C(=NH)\text{-}COOH + H_2O_2$$

$$R\text{-}C(=NH)\text{-}COOH \xrightarrow{H_2O} R\text{-}C(=O)\text{-}COOH + NH_3$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2$$

The hydrogen peroxide is then decomposed by the catalase present and the oxygen, which is normally returned to the atmosphere of the flask, may act on any easily oxidized material present. If hydrogen peroxide is responsible for the disappearance of the diphenolic nucleus, it should react with added l-3,4-dihydroxyphenylalanine and by so doing increase the oxygen consumption of the system and reduce the amount of unreacted amino acid as shown by the Arnow test.

The d-amino acid oxidase extract was prepared from acetone-desiccated lamb kidney tissue furnished by Speeter (132):

> "The active enzyme extract was most readily obtainable from desiccated kidney tissue. Desiccation was accomplished through the procedure of Neglein and Bromel [(133)] 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."

For the purpose of this experiment 10 gm. of the above acetone desiccated enzyme preparation were ground in a mortar with the gradual addition of 400 ml. of 0.015 M pyrophosphate buffer (pH 8.3). The resulting extract was stored at 38° C. for forty-five minutes. The extract was then cooled, centrifuged and the supernatant poured through gauze. The extract was adjusted to pH 6.8 and used immediately.

Three experiments were designed to test the effect of the hydrogen peroxide formed in the _d_-amino acid oxidase reaction on _l_-3,4-dihydroxyphenylalanine.

The first demonstrated the effect of the _d_-amino acid oxidase on _l_-3,4-dihydroxyphenylalanine; this experiment was a control experiment in that it measured any extraneous oxidations occurring during the reaction. The second illustrated the effect of the enzyme on _d,l_-methionine. The _d_-amino acid was a normal substrate and this was a normal reaction for this enzyme. In this case, the hydrogen peroxide would be decomposed by the catalase present and the oxygen not used for extraneous oxidation returned to the atmosphere of the flask. The third experiment demonstrated the effect of hydrogen peroxide formed in the deamination of the _d,l_-methionine on dihydroxyphenylalanine. In this experiment equimolar amounts of both _d,l_-methionine and dihydroxyphenylalanine were added to the _d_-amino acid oxidase preparation.

The results of these experiments are summarized in Table XXXVII. The addition of d-amino acid oxidase had no

TABLE XXXVII

EFFECT OF d-AMINO ACID OXIDASE ON THE STABILITY OF l-3,4-DIHYDROXYPHENYLALANINE

Substrate	Oxygen consumption			Ammonia formation	Cpd. disap.
	Basal	Excess			
	μl.	μl.	μatoms	μatoms	μmoles
l-3,4-Dihydroxy-phenylalanine	10.8	0.0	0.00	0.00	0.22
d,l-methionine	10.8	22.6	2.01	1.85	0.00
l-3,4-dihydroxy-phenylalanine and d,l-methionine	10.8	22.2	1.98	1.85	0.23

Substrate concentrations were 5.08 micromoles l-3,4-dihydroxyphenylalanine and 5.08 micromoles d,l-methionine. Incubations were carried out in pyrophosphate buffer at pH 6.8 and at 37.5° C. Concentration of d-amino oxidase preparation was 0.025 gm. per ml.

effect on the l-3,4-dihydroxyphenylalanine either in the presence of d,l-methionine or when the d,l-methionine was absent. A very slight amount (0.22 micromoles) of l-3,4-dihydroxyphenylalanine -- no more than would be expected in a boiled extract -- disappeared when the combined amino acids were incubated with the d-amino acid oxidase. However, a comparable amount disappeared when only the l-3,4-dihydroxyphenylalanine was present. The oxygen consumption was the

same with and without the l-3,4-dihydroxyphenylalanine and the ammonia produced was in fair agreement with the micro-atoms of oxygen consumed. The presence of catalase in the d-amino acid preparation was indicated by the agreement between the oxygen consumption and ammonia formed, for if catalase had been absent, the oxygen consumption would have been approximately doubled.

Therefore, hydrogen peroxide, either by itself or in the presence of catalase, was not responsible for the disappearance of the diphenolic radical of the l-3,4-dihydroxyphenylalanine.

2. The effect of competing substrates

In order to test the hydrogen peroxide effect further, competing substrates -- substrates capable of producing hydrogen peroxide in excess of that formed by the action of the amine oxidase on l-3,4-dihydroxyphenylalanine or hydroxytyramine -- were employed.

Blaschko, et al (82) and Alles and Heegaard (75) have demonstrated the activity of amine oxidase on various amines and, from the many compounds cited, n-butyl amine and tyramine were selected for use in the following experiments to study the effect of hydrogen peroxide on the metabolism of l-3,4-dihydroxyphenylalanine by extracts of guinea-pig kidney. An examination of the results obtained by adding the above compounds to a normal reaction, in which l-3,4-dihydroxyphenyl-

alanine was present, led to the conclusion that the hydrogen peroxide formed does not act either on the amino acid or on the decomposition products of the amino acid formed in the reaction. The results are summarized in Table XXXVIII. In the presence of n-butylamine the diphenolic disappearance was inhibited; 14 per cent when 13.7 micromoles of amine were present, and 18 per cent when 27.4 micromoles of the amine were present. The oxygen consumption was also reduced from 59.6 to 42.9 microliters with 13.7 micromoles of amine and from 59.6 to 39.7 microliters when 27.4 micromoles of amine were present. As n-butylamine was a competing substrate, a reduction in the diphenolic disappearance would be expected for the activity of n-butylamine as a substrate is very low.

In order that a large quantity of hydrogen peroxide would be formed, tyramine, which is of the same order of activity as hydroxytyramine, was used. Again a decrease in the substrate disappearance was noted in spite of the fact that the oxygen consumption was almost three times as great during the three-hour incubation period. In this case, the hydrogen peroxide formed by the action of the amine oxidase appeared to have either no positive effect on the diphenolic radical of the amino acid or no appreciable effect on the product formed from the amino acid by the decarboxylase or the amine oxidase.

In the presence of cyanide the decarboxylase is inhibited

TABLE XXXVIII

EFFECT OF COMPETING SUBSTRATES ON THE METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE BY KIDNEY EXTRACTS

Competing substrate	Excess oxygen consumption	Cpd. disap.	Inhibition
	μl.	μmoles	per cent
	Experiment 1		
None	59.6	3.70	
n-Butylamine (13.7 moles)	42.9	3.18	14.0
n-Butylamine (27.4 moles)	39.7	3.04	18.0
	Experiment 2		
None	55.3	3.87	
Tyramine (10.16 moles)	114.5	3.30	14.8

The per cent inhibition calculated from the analysis can be considered correct because the substances used as competing substrates will not respond to the Arnow test.

Incubations were carried out at pH 6.8 and at 37.5° C.
Concentration of extracts was 10 per cent.
Substrate was 5.08 micromoles dihydroxyphenylalanine.

but the activity of the amine oxidase is not affected. This suggested another experiment which would indicate whether or not hydrogen peroxide had any effect on the amino acid. The reaction was carried out in the presence of butylamine or tyramine and correlated with identical reactions in the presence of cyanide. The per cent inhibition should be of the same order if the hydrogen peroxide is not effective in oxidizing the dihydroxyphenylalanine. Such a series of experiments are summarized in Table XXXIX. There is no appreciable difference in the per cent inhibition of the diphenolic disappearance even in the case when tyramine is present. The high oxygen consumption when tyramine was present is indicative of appreciable hydrogen peroxide formation; 89.3 microliters of oxygen were consumed in the presence of the cyanide inhibited reaction when tyramine was added. Therefore, the hydrogen peroxide formed as an action of the amine oxidase on the amine was not causing the disappearance of the dihydroxy group of the amino acid.

G. Effect of Vitamin C and Aniline on the Activity of the Kidney Extracts

The elimination of oxidation as the means by which the diphenolic value is reduced has been tested in the previous sections. These indications have been based, for the most part, on the fact that enough oxygen was not consumed to account for both the amine oxidation and the oxidation of

TABLE XXXIX

EFFECT OF THE HYDROGEN PEROXIDE
PRODUCED BY COMPETING SUBSTRATES

Potassium cyanide	Excess oxygen consumption*	Cpd. disap.	Inhibition
	μl.	μmoles	per cent
Substrate - l-3,4-dihydroxyphenylalanine, 5.08 micromoles			
-	55.3	3.87	
+	20.4	0.70	81.9
Substrate - l-3,4-dihydroxyphenylalanine, 5.08 micromoles - butylamine, 27.4 micromoles			
-	55.4	3.60	
+	18.9	0.87	75.1
Substrate - l-3,4-dihydroxyphenylalanine, 5.08 micromoles - tyramine, 10.16 micromoles			
-	155.6	3.30	
+	89.3	0.71	78.5

* Excess oxygen, in this case, was oxygen in excess of the normal control reaction for it was not possible, under the conditions used, to determine the per cent of reaction due to each substrate.

The incubations were carried out at pH 6.8 and at 37.5° C. Concentration of the extracts was 10 per cent. Potassium cyanide was 0.00046 M.

the ring portion of the amino acid. If oxidation of the ring did occur, probably the first step would be the formation of the *o*-quinone from the amino acid. Two methods were employed to test for the possible formation of the quinone: (1) the addition of vitamin C to reduce the quinone formed to the dihydroxy form and (2) the addition of aniline to react with the quinone and remove it from the reaction.

1. Effect of vitamin C

Ascorbic acid has been used by Green and Richter (134) and Holtz, et al (45) to prevent extraneous oxidation in oxidations of the phenol oxidase type. The ascorbic acid is a readily reduced substance and will keep the easily oxidized 3,4-dihydroxy compounds in the dihydroxy form.

If the quinone formed is constantly being reduced to the dihydroxy form this should maintain the dihydroxy compound at the expense of vitamin C. The oxygen consumption would remain the same or probably increase because of the large amount of dihydroxy compound available for the reaction. The total result should be the oxidation of the vitamin C and the maintenance of the 3,4-dihydroxyphenyl portion of the amino acid.

High concentrations of ascorbic acid (0.015 and 0.030 M) were employed to be sure adequate ascorbic acid would be present throughout the reaction. In spite of the high

concentration of ascorbic acid, 2.26 and 2.36 micromoles of the amino acid disappeared (Table XL) and the contents of the flasks were not discolored. Therefore, oxidation to the *o*-quinone was probably not a step in the diphenolic disappearance.

TABLE XL

ACTIVITY OF KIDNEY EXTRACTS IN THE PRESENCE OF HIGH CONCENTRATIONS OF ASCORBIC ACID

Ascorbic acid conc.	pH	Inhibition		
		Oxygen consumption		Cpd. disap.
		per cent	per cent	μmoles
0.015	6.8	37.3	38.7	2.36
0.030	6.8	51.3	41.3	2.26

2. Effect of Aniline

Richter (135) noted that the action of the catechol oxidase of potatoes was subject to an inhibition which he concluded was caused by the product of the reaction, the *o*-quinone. To test this hypothesis, he added aniline which would react with the *o*-quinone and so remove the product of the catechol oxidase reaction. The addition of 0.05 M aniline apparently removed the quinone, for the oxygen consumption was increased and the reaction proceeded on the

expected course. Richter also used o-phenylenediamine and obtained similar results. In view of Richter's success, aniline was used to test the possibility of quinone formation in the l-3,4-dihydroxyphenylalanine reaction under consideration. Aniline, at concentrations of 0.025 and 0.05 M, was employed. These concentrations were five and ten times the substrate concentration and therefore were in considerable excess. The result was an inhibition of the oxygen consumption at both concentrations (Table XLI): 11 per cent for the

TABLE XLI

METABOLISM OF l-3,4-DIHYDROXYPHENYLALANINE BY KIDNEY EXTRACTS IN THE PRESENCE OF HIGH CONCENTRATIONS OF ANILINE

Aniline conc.	pH	Inhibition Oxygen consumption
molar		per cent
0.025	6.8	11.0
0.050	6.8	46.1

0.025 M aniline and 46.1 per cent for the 0.05 M aniline. The most that can be said for these experiments is that, if Richter's hypothesis is correct, the oxygen consumption in this experiment was probably not due to the formation of the o-quinone for that would have resulted in an increased oxygen consumption. Unfortunately, this high concentration of

aniline interfered with the Arnow test and no appreciable difference was observed between the control and experimental metaphosphoric extracts. The same was true of the standard analyses. Therefore, the diphenolic values could not be obtained.

H. Conjugation and Diphenolic Disappearance

Conjugation has been demonstrated to be a means by which phenolic- and catecholic-type compounds are excreted by the intact animal. Recently, DeMeio, et al (136) and Bernheim and Bernheim (137) have demonstrated conjugation by employing the slice technique, but they were unable to demonstrate a similar reaction in tissue extracts. The evidence presented in the previous experiments of this study has indicated that oxidation was probably not important in the diphenolic disappearance and, as conjugation was a possibility, the metaphosphoric acid filtrates were tested for the presence of conjugates. Many investigators have demonstrated the presence of conjugates of diphenolic compounds by hydrolyzing such conjugates found in the urine of experimental animals, and such a hydrolysis has resulted in increasing the recovery of the diphenolic compounds by as much as 50 per cent.

In Experiments 1 and 2 of this series, 2 ml. of 0.5 M

hydrochloric acid were added to 1 ml. of the metaphosphoric acid filtrate and the resulting solution placed in a boiling water bath for one hour. The necessary control -- a similar amount of filtrate not subjected to heat treatment -- was also carried through the determination. At the end of the hour, 3 ml. of distilled water, 2 ml. of the Arnow Reagent and 2 ml. of 1.0 N sodium hydroxide were added. The resulting solutions were compared in the Klett-Summerson photoelectric colorimeter. Experiment 3 of the series was carried through a similar heat treatment with a higher concentration of hydrochloric acid to make sure that the hydrochloric acid concentration was high enough to insure hydrolysis. One ml. of 5.0 N hydrochloric acid was added to 4 ml. of metaphosphoric acid filtrate and placed in a boiling water bath for thirty minutes. One ml. was then analyzed by the Arnow test. The results of these experiments, as summarized in Table XLII, demonstrate no appreciable difference between the Klett-Summerson readings of the hydrolyzed and unheated samples. If all the diphenolic value which had disappeared in the reaction had been recovered, the readings should have been between 160 and 165, and the very small increase encountered may be taken as fairly conclusive evidence that the diphenolic disappearance was not the result of conjugation.

TABLE XLII

ATTEMPT TO DEMONSTRATE PRESENCE OF A CONJUGATE BY EMPLOYING ACID HYDROLYSIS

Sample	Analytical control		Hydrolyzed	
	Klett-Summerson readings	Difference	Klett-Summerson readings	Difference
		Experiment 1*		
Control	48	58	52	62
Experimental	106		114	
		Experiment 2*		
Control	47	51	48	54
Experimental	98		102	
		Experiment 3		
Control	2	56	3	60
Experimental	58		63	

* The controls and experimentals in these experiments are high because they are not corrected for a reagent blank. Experiment 3 has been corrected for the reagent blank.

I. Fractionation

The isolation and purification of the enzymes involved in metabolic processes is one of the primary aims in any investigation of a metabolic process. If successful, the isolated system can be studied with no interfering side reactions and, in many cases, stable and very active preparations have been prepared. Usually these investigations have followed the classic line of ammonium sulfate fractionation and isoelectric separations. Therefore, fractionation experiments were undertaken in this investigation in an attempt to prepare a concentrated enzyme preparation and to accomplish this the extracts were subjected to ammonium sulfate fractionation and isoelectric precipitation.

In the first series, the fractions insoluble in 50 per cent and 100 per cent ammonium sulfate were separated and then the fraction insoluble in 50 per cent ammonium sulfate was further fractionated by isoelectric precipitation. The actual procedure was as follows: One volume of saturated ammonium sulfate was added to one volume of kidney extracts (0.4 gm. per ml.), and the resulting solution allowed to stand one and one-half hour in the refrigerator. At the end of this period the resulting solution was filtered with suction until the cake was almost dry. Three hours were necessary for this filtration. The moist cake was dissolved in 15 ml. of distilled water and dialyzed 27 hours against 0.01

M phosphate buffer (pH 6.8) to free the extract of ammonium ions. The volume of this dialyzed extract was diluted with 0.1 M phosphate buffer to the volume of the original kidney extract and the resulting solution adjusted to pH 6.8. This solution was designated as fraction I.

To the filtrate from the above precipitation enough ammonium sulfate was added to saturate the solution and the mixture was allowed to stand for three-quarters of an hour in the refrigerator. At the end of this time the solution was filtered with suction. The moist cake was dissolved in 12 ml. of distilled water and dialyzed for 26 hours against 0.01 M phosphate buffer to free the extract of ammonium ions. At the end of this time the preparation was diluted to the original volume of the extract with 0.1 M phosphate buffer and adjusted to pH 6.8. This was designated as fraction II.

The activity of fractions I and II was tested in the usual manner and all the activity was concentrated in fraction I. Fraction I caused 48.0 per cent of the diphenolic groups to disappear. In an attempt to further concentrate the activity, the pH of fraction I was adjusted to pH 5.5 and centrifuged immediately. The clear liquid layer was decanted, made up to the required volume with 0.1 M phosphate buffer and adjusted to pH 6.8. This fraction was designated as fraction I-B. The precipitate was dissolved in 0.1 M phosphate buffer and made up to the same volume as fraction

I-B. The pH was adjusted to 6.8 and this fraction was designated as I-A. A test of the activity of these fractions demonstrated that fraction I-A was very slightly active (7.8 per cent diphenolic disappearance) whereas fraction I-B was totally inactive.

The pH of 5.5 used in the isoelectric separation of Fraction I may have been low enough to cause appreciable destruction of the enzyme systems. Therefore, a determination of the effect of the exposure of the kidney extracts to hydrogen-ion concentrations far removed from the conditions of incubation (pH 6.8) was undertaken. At room temperature, extracts were adjusted to pH 6.0 and pH 5.0, allowed to remain at these pH's for five minutes and then adjusted to pH 6.8 for incubation. The activity of the extract, exposed to pH 6.0, was not appreciable affected but the activity of the extract, adjusted to pH 5.0, was reduced to less than one-half that of the untreated extract (Table XLIII). Another such treatment of a longer duration, in which the extracts were adjusted to pH 5.0 and pH 4.0 and allowed to remain under these conditions for three hours in the refrigerator, demonstrated an even greater loss of activity. The extract adjusted to pH 5.0 was only about one-fourth as active while the extract exposed to pH 4.0 exhibited a complete loss of activity. It was evident that considerable inactivation of the extract was experienced at pH 5.0 but

TABLE XLIII

EFFECT OF EXPOSURE TO VARIOUS HYDROGEN ION CONCENTRATIONS ON THE ACTIVITY OF THE KIDNEY EXTRACTS

pH	Extract conc.	Oxygen consumption		O/D_p	O/D_c	Cpd. disap.
		Basal	Excess			
	gm./ml.	µl.	µl.			per cent
			Experiment 1*			
6.8	0.17	26.4	58.0	1.02	1.33	76.3
6.0	0.17	28.9	56.7	0.99	1.50	66.1
5.0	0.17	23.6	15.8	0.28	0.89	31.3
			Experiment 2**			
5.0	0.125	7.4	12.8	0.22	1.08	21.3
4.0	0.125	6.1	1.8	0.00	0.00	00.0

* The extracts were adjusted to pH 5.0 and pH 6.0, allowed to remain at these pH's for five minutes and then adjusted to pH 6.8 for analysis.

** The extracts were stored in the refrigerator for three hours at pH 5.0 and pH 4.0, then adjusted to pH 6.8 for analysis.

that at pH 6.0 the activity was only slightly diminished.

Another series of ammonium sulfate and isoelectric separations were attempted in which the time necessary to complete the operation was reduced by eliminating the time necessary to test the activity of fraction I. The pH of the isoelectric separation was also raised from pH 5.5 to pH 6.0 in order to avoid the loss of activity noted above. The actual procedure was as follows: One volume of saturated ammonium sulfate was added to one volume of a kidney extract (0.4 gm. per ml.). This mixture was allowed to stand in the refrigerator for two hours and then filtered with suction. Two hours were necessary to filter this solution. The residue was dissolved in 10 ml. of distilled water and dialyzed 29 hours against 0.01 M phosphate buffer until free of ammonium ions. The dialyzed portion was then adjusted to pH 6.0 and centrifuged. The supernatant was diluted to the volume of the original extract and the pH adjusted to 6.8 (fraction I-BI). The precipitate was dissolved in 0.1 M phosphate buffer and diluted to the volume of the original extract and the pH adjusted to 6.8 (fraction I-AI). The activity of these fractions were tested immediately. Fraction I-AI metabolized only 30 per cent and fraction I-BI metabolized 6.7 per cent of the amino acid.

This activity was still considerably lower than the 65 to 75 per cent diphenolic disappearance experienced with the

fresh extracts, therefore, an attempt was made to reduce the time necessary to complete the filtration of the fraction insoluble in 50 per cent ammonium sulfate. If this time could be reduced, the extract would be subjected to less exposure during the filtration period. To accomplish this, the extract was filtered through a filter pack to remove fragments not separated from the extract by centrifuging the homogenate prepared in the Waring blendor. However, the use of the filter pack followed by ammonium sulfate fractionation resulted in a very low activity in fraction I. Fraction I caused the disappearance of only 17.7 per cent of the diphenolic value. The extracts when filtered through a filter pack were relatively clear. This removal of the activity by the filter pack is in keeping with the work of Kohn who stated that the activity of the amine oxidase system is associated with very turbid solutions. As the particles taken from the solution by the filter pack were distributed throughout a considerable portion of the pack, it was not possible to measure the activity of these fragments.

A considerable loss of activity was experienced in the ammonia sulfate and isoelectric separations. This discouraged further attempts at this time to prepare a tissue concentrate which had the ability to metabolize the amino acid.

J. Nature of the End Product

Ultraviolet absorption spectra have been used rather frequently in biological studies to illustrate the disappearance of a substrate or to determine the nature of the end products of the reaction. Recently, this method has been employed by Mason (138) in a study of the tyrosinase reaction, and by Fishberg (139) in an investigation resulting in the identification of p-benzoquinoneacetic acid in the urine of vitamin C deficient patients. Therefore, to obtain further data which might confirm the dihydroxy disappearance in this investigation a study of the ultraviolet absorption of the metaphosphoric extracts was undertaken. For this purpose a Beckman spectrophotometer was used. The slit-width limits, within which the measurements were made, varied from a low of 0.48 mm. for a wave length of 350 millimicrons to 2.00 mm. for the lower wave lengths. For the purpose of comparison the optical density, D, which is $\log_{10} I_o/I$ for whatever conditions of concentration and cell length employed, was used in plotting the data obtained.

As a basis of reference the ultraviolet absorption of 0.004 M catechol and 0.005 M dihydroxyphenylalanine were determined and as is demonstrated in Figure 16 the maximum value observed at approximately 280 millimicrons is due to the catechol portion of the dihydroxyphenylalanine.

The next step in this investigation was a comparison of

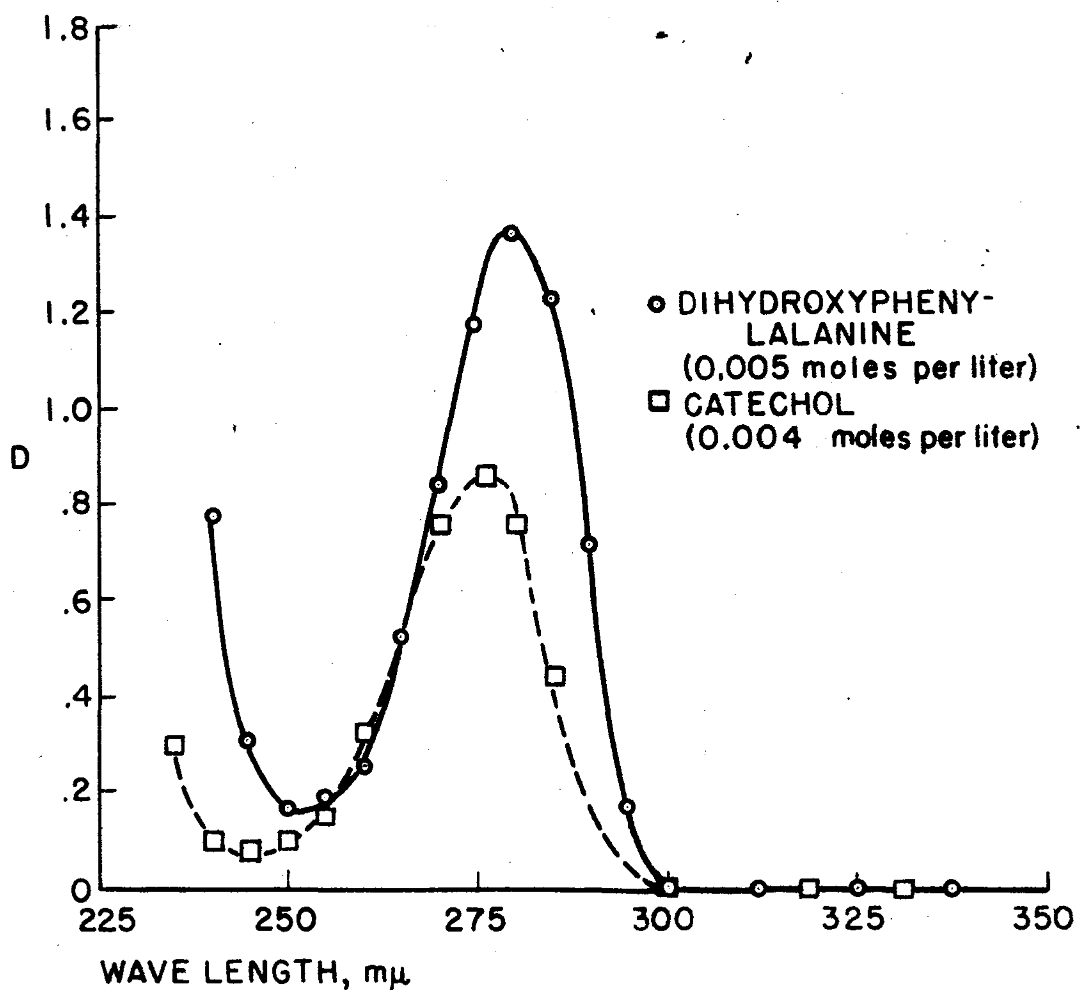


FIGURE 16. ULTRAVIOLET ABSORPTION SPECTRA OF CATECHOL AND DIHYDROXYPHENILALANINE IN 0.1 N HYDROCHLORIC ACID

the absorption of the metaphosphoric extracts of the incubated samples with the dihydroxyphenylalanine solution. The samples were prepared by diluting 1 ml. of the 0.125 N metaphosphoric acid filtrate to 10 ml. with 0.1 N hydrochloric acid. In Figure 17 the curve representing the incubated sample was corrected for tissue interference by subtracting the optical density of the control flask from that of the experimental flask. This eliminated the background of the tissue extract and only the products of dihydroxyphenylalanine metabolism and any unreacted substrate should be contained in this curve. The character of this curve is very different from the dihydroxyphenylalanine curve. The maximum at 280 millimicrons has been eliminated and a new maximum at 255 - 260 millimicrons is evident. Another maximum at 315 millimicrons has also appeared. The analysis of the diphenolic disappearance indicated that during the experiment, from which the above data were obtained, 67 per cent of the diphenolic value had disappeared from the extract.

An attempt was made to extract the substances responsible for the character of the curve of the metaphosphoric filtrate of the experimental flask. Butyl alcohol was chosen as the extracting agent. Dihydroxyphenylalanine is not soluble in butyl alcohol and unreacted dihydroxyphenylalanine would not be extracted. The extraction was accomplished by shaking 5 ml. of metaphosphoric extract with 5 ml. of butyl alcohol and then separating the layers by centrifugation.

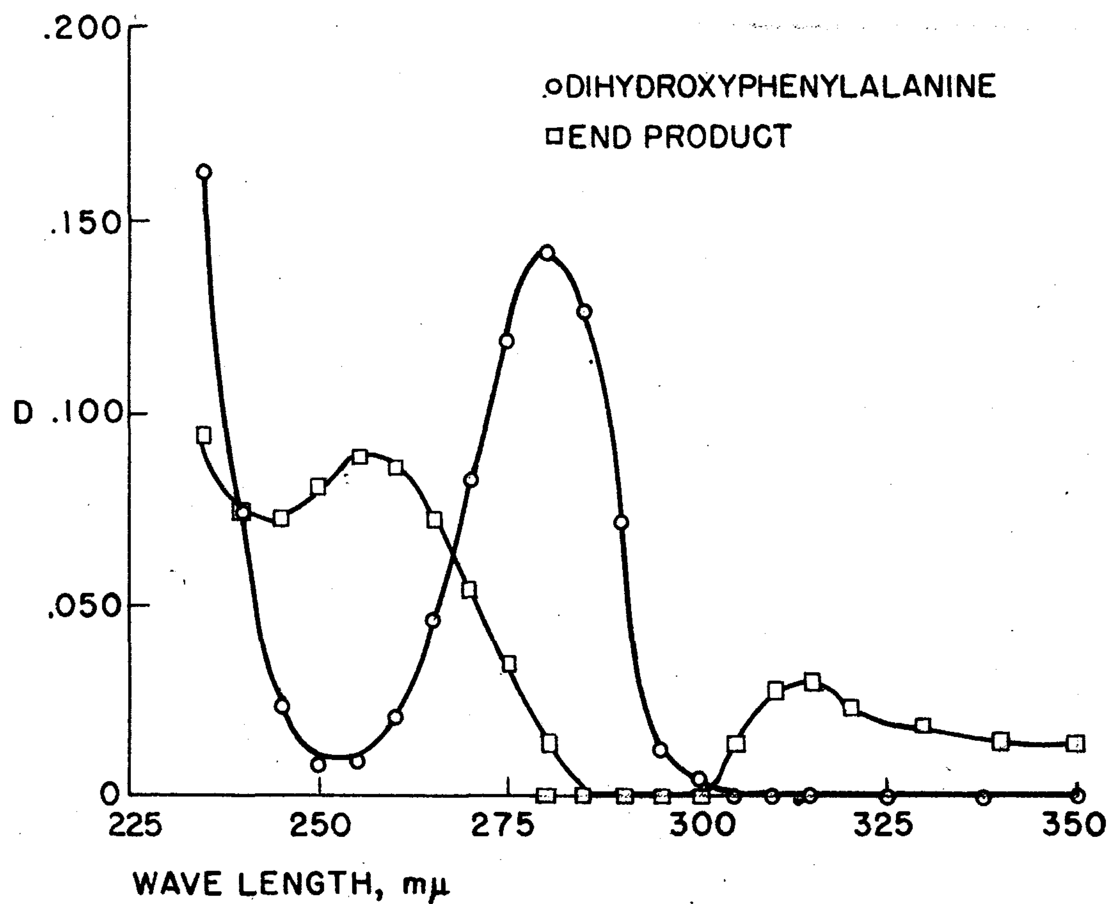


FIGURE 17. ULTRAVIOLET ABSORPTION SPECTRA OF DIHYDROXYPHENYLALANINE AND THE END PRODUCT IN 0.1 N HYDROCHLORIC ACID

One ml. of the butyl alcohol layer was diluted to 10 ml. with butyl alcohol for use in the instrument. Both the control and experimental metaphosphoric filtrates were treated in the same manner and the absorption values of the controls subtracted from the absorption values of the experimental to eliminate the tissue background. The curve representing the ultraviolet absorption of the butyl alcohol solution is shown in Figure 18 and, although the shape of the curve differs somewhat from that obtained with a metaphosphoric acid filtrate, in the actual wave lengths at which maximum absorption occurs, it is similar.

The butyl alcohol was then removed by vacuum distillation and the residue dissolved in 0.1 N hydrochloric acid. The procedure was as follows: Twenty-eight ml. of metaphosphoric acid filtrate were extracted five times with 10 ml. of n-butyl alcohol. The butyl alcohol extract was then evaporated to dryness under carbon dioxide, in vacuo. The residue and flask were cleared of any residual butyl alcohol by repeating the evaporation twice with 10 ml. of distilled water and once with 10 ml. of 0.1 N hydrochloric acid. The residue, when dissolved in 50 ml. of 0.1 N hydrochloric acid, did not give a positive Arnow test. This is indicative of the absence of the 3,4-dihydroxyphenyl group. Two ml. of the above 0.1 N hydrochloric acid solution were added to 20 ml. of 0.1 N hydrochloric acid and the absorption of this solution measured in the Beckman spectrophotometer. Twenty-

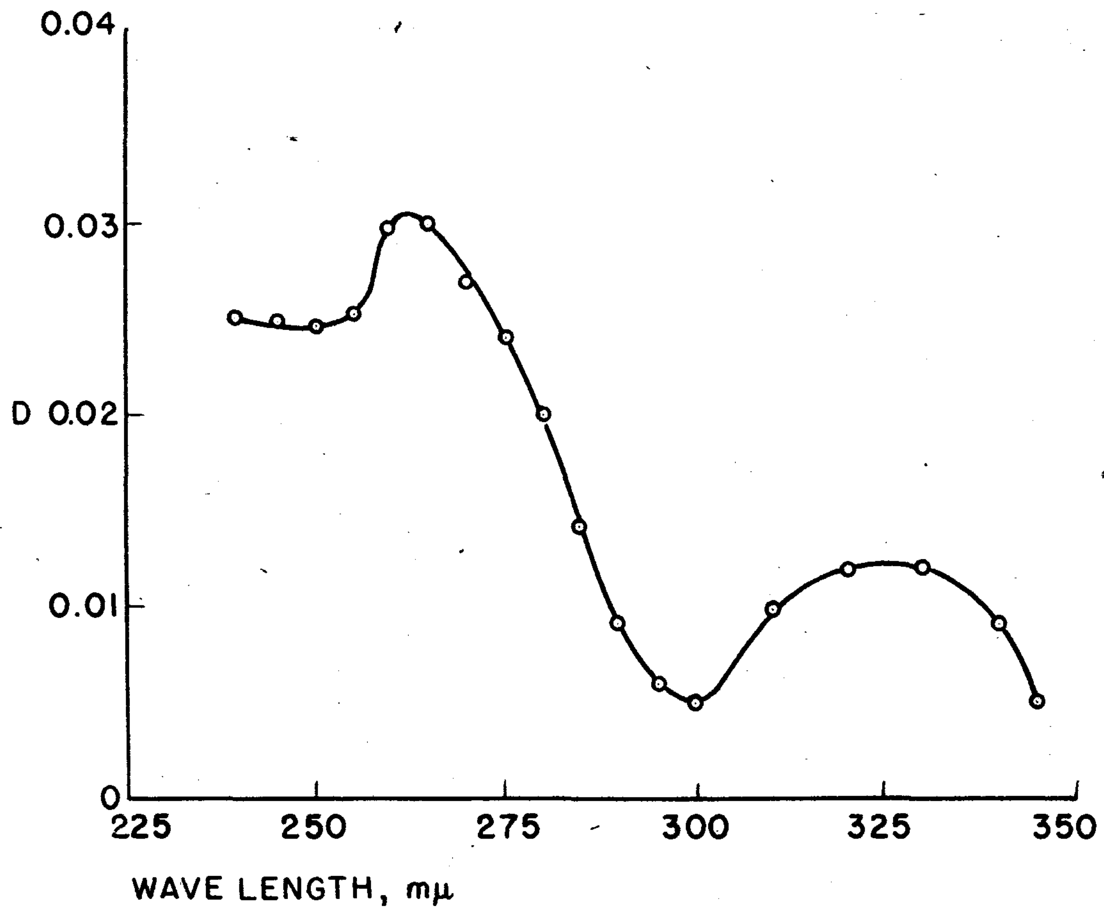


FIGURE 18. ULTRAVIOLET ABSORPTION SPECTRUM OF THE BUTYL ALCOHOL EXTRACT

eight ml. of metaphosphoric acid filtrate from the control flask were treated in the same manner.

The optical densities of these solutions were determined and the difference between the control and the experimental samples are plotted in Figure 19. Values were also obtained for the optical densities of these solutions after adjustment to pH 7.0 and pH 11.6. As shown in Figure 19 the curves exhibit maxima at approximately 260 millimicrons. Above 300 millimicrons there is appreciable variation of the density with variation in pH. As the pH increases the maximum at 320 millimicrons increases. However, the three curves agree essentially with the experimental curve shown in Figure 17, and the results are entirely indicative of the elimination or extensive modification of the dihydroxyphenyl group of the amino acid, for the maximum representative of the catechol nucleus has disappeared and a new maximum has appeared at 255 - 260 millimicrons. These findings support the data obtained with the Arnow analytical procedure and also furnish strong evidence for the production of another compound (or compounds) with characteristic absorption spectrum in the incubated extracts.

Mason (138) followed the oxidation of dihydroxyphenylalanine by "mammalian dopa oxidase" by the change in the ultraviolet absorption of the reaction mixture, and, although he obtained a maximum absorption at 305 - 310 millimicrons,

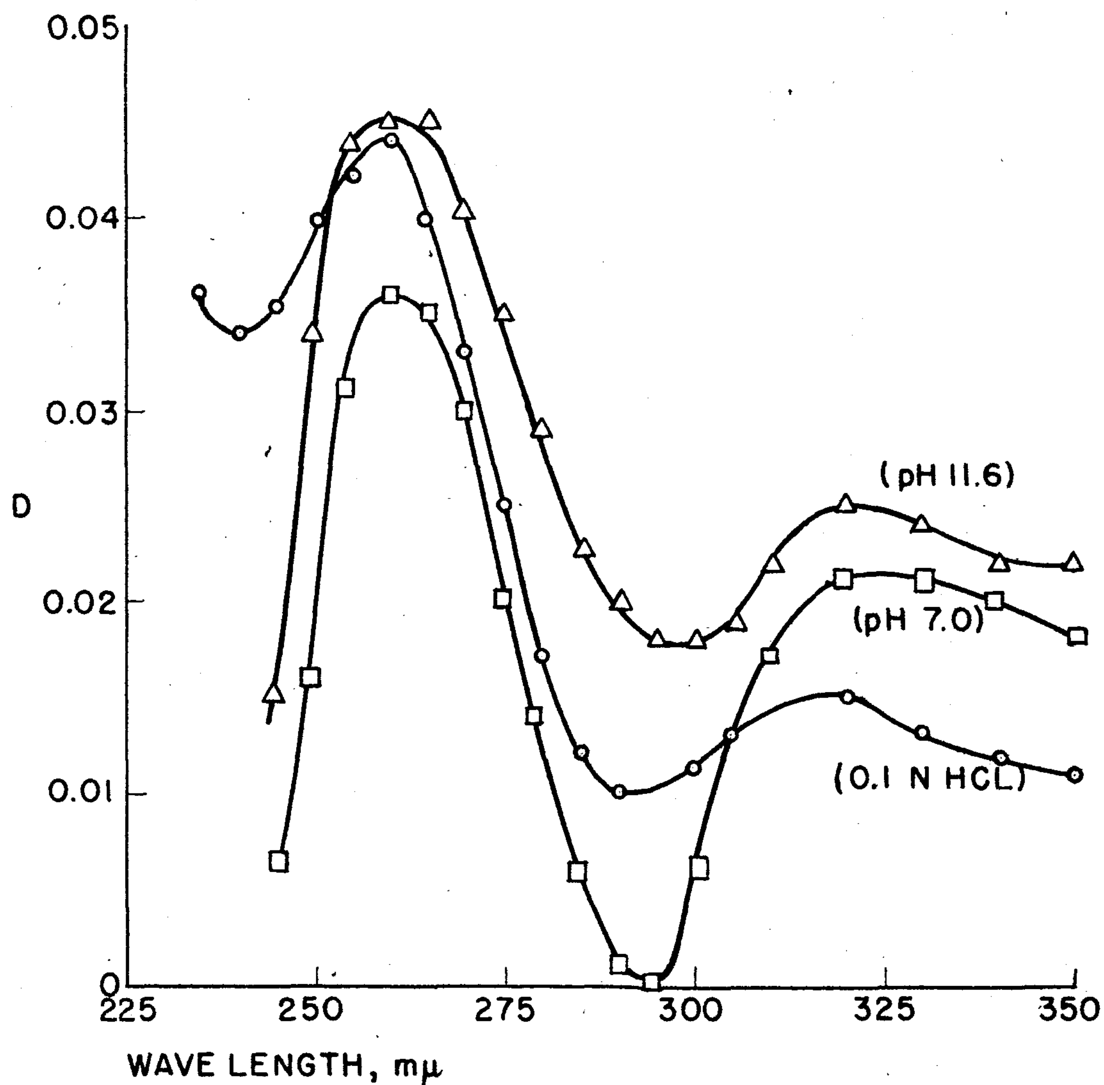


FIGURE 19. ULTRAVIOLET ABSORPTION SPECTRUM OF BUTYL ALCOHOL EXTRACT IN 0.1 N HYDROCHLORIC ACID, AND AT pH 7.0 AND pH 11.6

he did not observe a point of maximum absorption at 255 - 260 millimicrons. The maximum value at 310 - 315 millimicrons observed in this study may have originated from miscellaneous oxidation of the tyrosinase type, but in any case the maximum at 260 millimicrons is not comparable to the results obtained by Mason. As has been shown by Raper (109), the tyrosinase type reaction involves the formation of an o-quinone and, as Mason collected his data while the reaction was in progress, the lack of a maximum at 260 millimicrons in his experiments would indicate that the reaction initiated by kidney extract was not similar to the tyrosinase reaction. Therefore, it may be concluded that the product of the metabolism of dihydroxyphenylalanine by guinea-pig kidney extracts is not similar to the product obtained by Mason. In addition, the characteristic ultraviolet absorption of the product may be used as an indicator in future investigations involving the isolation and identification of the compound formed in the metabolism of l-3,4-dihydroxyphenylalanine by guinea-pig kidney extracts.

IV. DISCUSSION

The investigation of the metabolism of l-3,4-dihydroxyphenylalanine by kidney tissue has been undertaken to obtain additional information on the metabolism of the amino acid. The presence and activity of dihydroxyphenylalanine decarboxylase and the amine oxidase has been demonstrated. Warburg respirometer studies in conjunction with the Arnow analytical method for the determination of dihydroxyphenylalanine have demonstrated that as a final reaction in the enzymatic series the dihydroxyphenyl, or catechol, nucleus disappeared. Further, it has been shown that oxygen is not directly responsible for the disappearance of the dihydroxy groups of the amino acid. Conjugation of the type usually expected for phenols has also been eliminated. Ultraviolet absorption studies have demonstrated that either the catechol ring disappeared or was modified. In spite of the observation by Lan and Sealock (126) that vitamin C was very definitely related to the oxygen consumption of surviving kidney slices in the presence of dihydroxyphenylalanine, kidney extracts did not exhibit such a relationship. The inability of the kidney extracts to exhibit this difference in activity suggests the necessity of the intact cell in this phenomenon. Other factors cannot be excluded, for in the preparation of ex-

tracts the enzyme systems are disarranged and either the destruction or diluting-out of an unknown compoment of the system cannot be disregarded in the metabolism of dihydroxyphenylalanine.

The fact that dihydroxyphenylalanine is very easily converted by kidney extracts to a sympathomimetic amine has made the problem of its disposal of great importance. The action of the highly specific decarboxylase and of the amine oxidase is clearly defined, but the problem of the metabolism of the aromatic nucleus has not been answered. In intact animals a certain amount of the catechol nucleus can be recovered as a conjugate but the metabolism of the remainder is a mystery at the present time. The ability of the hydroxy groups in the 3,4-position to greatly increase the activity of these aromatic amines has been observed by numerous investigators, and the disappearance of the hydroxy groups in kidney extracts in a manner other than oxidation by the atmosphere of the flask demands consideration of another system in the kidney which functions in the metabolism of this amino acid.

The indication of a system involved in the disappearance of catechol-type compounds can also be considered of great importance in the broad picture of essential hypertension. In the case of experimental essential hypertension a chemical agent coming from the kidney produces peripheral vasoconstriction which after a while and for reasons unknown subsides

and a sympathetically mediated hypertension is established. Essential hypertension in man is usually diagnosed after the chronic stage has been developed. Therefore, the picture of the early stages is not evident. However, failure to find this early picture does not disprove the renal and metabolic origin of essential hypertension.

One of the very important, and as yet unanswered, questions involves the possibility of a "metabolic clamp" occurring in the organism comparable in its effect on the kidney metabolism to the application of a mechanical Goldblatt clamp in animals. The production of a chemical agent anywhere in the body which would act as such a chemical clamp and initiate the disease is not unreasonable. In view of the demonstrated ability of the kidney to produce the pressor substances, the function of the kidney in initiating the disease seems well founded. Perhaps disease or old age, in some manner, upsets the metabolism of the kidney and causes it to release rather than destroy pressor substances responsible for the onset of essential hypertension. The marked pressor effect of hydroxytyramine and the ability of the highly specific decarboxylase in kidney tissue to convert dihydroxyphenylalanine to this substance makes the participation of the kidney very likely. If kidney dysfunction should be prolonged, then the sympathetically mediated hypertension might become established.

Basic research on the enzyme systems of the kidney may eventually lead to an understanding of the cause of this disease and such work is one of the prerequisites for a rational therapeutic approach.

V. SUMMARY

1. Dihydroxyphenylalanine metabolism in cell-free kidney extracts was studied by means of the Warburg respirometer technique coupled with the Arnow colorimetric method for the determination of the amount of substrate disappearance during the incubation.

2. The activity of extracts prepared from the kidneys of scorbutic guinea pigs was essentially the same as the activity of normal kidney extracts, and the addition of crystalline ascorbic acid to the scorbutic extracts resulted in no additional activity.

3. The interrelationships of the dihydroxyphenylalanine decarboxylase and the amine oxidase enzyme systems were shown by the activity of the extracts in the presence of _l_-3,4-dihydroxyphenylalanine and hydroxytyramine.

4. Balanced experiments, in which the oxygen consumption, carbon dioxide evolution and ammonia formation were correlated, demonstrated the failure of the oxygen consumed to account for the disappearance of the _o_-dihydroxy groups of the amino acid during the incubation.

5. Hydrogen peroxide formed as a result of amine oxidation failed to account for the disappearance of the dihydroxy

portion of the amino acid and, in addition, the stability of the dihydroxyphenyl group of the amino acid was observed in the presence of the hydrogen peroxide formed by the action of a d-amino acid oxidase preparation on d-methionine.

6. Furthermore, inhibition studies, with dihydroxyphenylalanine as a substrate, demonstrated the ability of cyanide and semicarbazide to inhibit the diphenolic disappearance even when the amine oxidation was proceeding at a nearly normal rate. The data obtained by the use of other known enzyme inhibitors was not as informative.

7. The destruction or modification of the 3,4-dihydroxyphenyl group of the amino acid was manifested by the reduction of the diphenolic value and the change in the character of the ultraviolet absorption spectrum of the deproteinized extracts; the maximum of approximately 280 millimicrons, characteristic of the catechol nucleus, was eliminated and a new maximum at approximately 255 - 260 millimicrons was observed.

8. By the use of l-3,4-dihydroxyphenylalanine and hydroxytyramine the decarboxylase was shown to be much less active in the liver extracts than in the kidney extracts. However, the activity of the amine oxidase system in the liver compared favorably with the activity of this system in the kidney.

9. On the other hand, dialysis did not reduce the activity of the kidney extracts, and ammonium sulfate frac-

tionation followed by isoelectric separation did not result in a stable and active enzyme preparation.

10. Conjugation of the catechol nucleus was not observed.

11. The observation of the disappearance of the dihydroxy group of the amino acid in a manner other than oxidation by the oxygen consumed strongly suggests the existence of another enzyme system which operates in the metabolism of dihydroxyphenylalanine in tissue extracts.

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