The Oxidation in Liver of L-Tyrosine to Acetoacetate through *p*-Hydroxyphenylpyruvate and Homogentisic Acid

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The picture of L-tyrosine metabolism obtained from isotope experiments and other studies on intact animals is still incomplete. Either *p*-hydroxyphenylpyruvate or 2:5-dihydroxyphenylalanine may be the first intermediate, since either could form the second postulated intermediate, 2:5-dihydroxyphenylpyruvate (Neubauer, 1909), and both are converted to homogentisic acid by alcaptonurics (Neuberger, Rimington & Wilson, 1947). *p*-Hydroxyphenylpyruvate is also known to be formed from tyrosine in scorbutic guinea pigs and premature infants (Sealock & Silberstein, 1940; Levine, Marples & Gordon, 1941) and was excreted by Medes's case of tyrosinosis (Medes, 1932). However, the evidence for the assumption that normal tyrosine metabolism goes through these homogentisic acid precursors and homogentisic acid itself is only indirect. Normal liver can oxidize homogentisic acid to acetoacetate (Edson, 1935*b*; Zorn, 1940; Ravdin & Crandall, 1950). Still more significant is the isotopic labelling of acetoacetate formed from labelled phenylalanine by liver slices (Schepartz & Gurin, 1949; Weinhouse & Millington, 1948), which shows that a molecular rearrangement of the side chain must have occurred. This rearrangement is similar to that which must be postulated for the intermediate formation of homogentisic acid from tyrosine (Meyer, 1901; Friedman, 1908).

Investigations of the oxidation of L-tyrosine in liver homogenates have not resolved any of the uncertainties about the reaction. There is agreement that the oxidation of L-tyrosine by liver homogenates uses four atoms of oxygen and produces one molecule each of carbon dioxide and acetoacetate but no ammonia (Bernheim & Bernheim, 1934; Felix, Zorn & Dirr-Kaltenbach, 1937; Zorn, 1940; Felix & Zorn, 1941; Sealock & Goodland, 1949). In liver slices fumaric acid (or malic acid) and acetoacetate are formed from L-tyrosine (Lerner, 1949). Felix & Zorn (1941) also found alanine to be a product of the reaction, and they thought that this arose from a splitting-off of the tyrosine side chain. Isotope experiments have failed to confirm this origin of the alanine (Lerner, 1949). The most thorough investigations of the intermediate steps (Felix *et al.* 1937; Zorn, 1940) have apparently excluded both *p*-hydroxyphenylpyruvate and homogentisic acid as

intermediates. Their investigations suggest a totally different pathway of L-tyrosine oxidation in liver than that deduced from intact animal experiments.

A more active L-tyrosine oxidation system than any hitherto used, freed from other reactions occurring in crude liver homogenates, was needed to investigate these discrepancies. Concentrated suspensions of liver homogenates have always been used to obtain the highest tyrosine oxidation activity (Bernheim & Bernheim, 1934; Sealock & Goodland, 1949), presumably to avoid dilution of unidentified dialysable components of the system. Using such suspensions, Rienits (1950) and Painter & Zilva (1950) have shown that addition of ascorbic acid gave a small but statistically significant increased oxidation of L-tyrosine in liver preparations from both scorbutic and normal guinea pigs. The recent identification of α -ketoglutarate and ascorbic acid as the major co-factors for L-tyrosine oxidation in liver homogenates (LeMay-Knox & Knox, 1951) has permitted a higher order of activity to be obtained. These additions to the reaction have also made possible some purification of the system and almost complete elimination of secondary reactions. With such preparations the pathway of tyrosine oxidation in liver has been re-investigated.

METHODS

Enzyme preparation. Rat livers were promptly chilled after removal from the animal and homogenized in a Waring blender with 3 parts of ice-cold 0.9% KCl, following the procedure used previously (Knox & Mehler, 1950). The soluble enzyme system was obtained free of mitochondrial elements by centrifuging at 6000 *g* for 20 min. and was then dialysed against tap water overnight. The preparations retained their activity for several days at 2°.

Enzyme reactions and assays. The oxidations were carried out in manometer cups or open flasks shaken in a water bath at 37° with air as the gas phase. The reaction mixture contained 1 ml. of the enzyme preparation and 1 ml. of 0.2M-phosphate, pH 7.5, in a total volume of 3 ml. When tyrosine and *p*-hydroxyphenylpyruvate were substrates, ascorbic acid was added, usually 0.1 ml. of a freshly made up and neutralized 0.5% solution. L-Tyrosine (1.5 mg./ml.) was dissolved in the buffer with heating, and the reaction started by tipping in from a side arm 0.3 ml. of 0.1M- α -ketoglutarate. The transaminase reaction was carried out in a mixture similar to that used for tyrosine oxidation, but without

ascorbic acid and under an atmosphere of N_2 . The *p*-hydroxyphenylpyruvate formation was followed in the Beckman spectrophotometer at 1 min. intervals by the increase in light absorption at 320 $m\mu$, or it was determined chemically after 20 min. incubation.

Analytical methods

The disappearance of *p*-hydroxyphenyl compounds was determined in 5% (w/v) metaphosphoric acid filtrates of the initial and final reaction mixtures by the Millon reaction, following the conditions of Medes (1932). The final colour, produced in the same degree by identical molar concentrations of tyrosine and *p*-hydroxyphenylpyruvate and not by the 2:5-dihydroxyphenyl compounds (Neuberger, 1947), was measured in the spectrophotometer at 480 $m\mu$. *p*-Hydroxyphenylpyruvate was determined chemically in 5% (w/v) trichloroacetic acid filtrates by the Briggs reaction (Neuberger, 1947). In the spectrophotometric assay at 320 $m\mu$ in 1 cm. cells, a change in optical density of 0.810 corresponds to 1.0 μ mol./ml. of *p*-hydroxyphenylpyruvate.

Acetoacetic acid was determined manometrically with aniline citrate (Edson, 1935*a*). L-Glutamic acid was determined by the specific decarboxylase method of Gale (1945), using suspensions of *Clostridium welchii* provided by Miss van Haltern.

The 2:4-dinitrophenylhydrazones of *p*-hydroxyphenylpyruvate was isolated from a trichloroacetic acid filtrate of a large-scale anaerobic reaction mixture by the procedure of Stumpf & Green (1944). The hydrazone, after chromatography in ethyl acetate on an alumina column (Datta, Harris & Rees, 1950) to separate the slower-moving hydrazone of unreacted α -ketoglutarate, was recrystallized from ethyl acetate and light petroleum.

The enzyme system oxidizing homogentisic acid was inactivated by adding 0.5M- NaH_2PO_4 containing 1% (v/v) acetic acid to pH 5, and after 15 min. at room temperature the mixture was neutralized to pH 7.2. For the isolation of homogentisic acid 50 ml. of a solution of this treated enzyme were incubated with tyrosine, α -ketoglutarate and ascorbic acid. The reaction was followed in a pilot run, and when this indicated completion of the reaction the mixture was deproteinized with metaphosphoric acid. Continuous extraction of the filtrate with peroxide-free ether, concentration and extraction back into water, taking advantage of the favourable partition of homogentisic acid (Neuberger, 1947), was repeated twice for purification. The solution obtained was chromatographed (descending solvent) using both *n*-butanol-water with a few drops of formic acid in the cabinet to prevent tailing, and the solvent C80 of Smith (1950) which consists of 80% saturated $(NH_4)_2SO_4$ containing 2% (v/v) isopropanol. The spots were located after 10 hr. runs by spraying with ammoniacal $AgNO_3$ (Bray, Thorpe & White, 1950). In *n*-butanol-water the isolated product and an authentic sample of homogentisic acid had the same R_F value of 0.5–0.6, very close to that of gentisic acid, but easily separated from *p*-hydroxyphenylpyruvic acid (R_F 0.1) and 2:5-dihydroxyphenylpyruvic acid (two spots, R_F 0.2 and 0.8). In solvent C80 the product and homogentisic acid had an R_F of 0.7 while gentisic acid was well separated (R_F 0.3) and the two keto acids less so (R_F values 0.60–0.65). The gentisic acid, but not the homogentisic acid spot, fluoresced in ultraviolet light.

Substrates. The compounds *p*-hydroxyphenylpyruvate, L-2:5-dihydroxyphenylalanine, the lactone of 2:5-dihydroxy-

phenylpyruvic acid, and homogentisic acid, without which this work could not have been done, were very kindly provided by Dr A. Neuberger. The lactone of 2:5-dihydroxyphenylpyruvic acid was hydrolysed to the free acid by the procedure of Neubauer & Flatow (1907), and neutral solutions of this acid were made up under N_2 immediately before use. Despite the lability of these compounds to oxidation, controls revealed no spontaneous oxidation nor was there darkening of the enzyme reaction mixtures containing them in the experiments at pH's of 7.5 or lower.

Sodium gentisate was obtained from Dr Severinghaus of Hoffman-LaRoche, Inc. Calcium pyridoxal-phosphate was a gift from Dr W. Umbreit of Merck and Co. D-isoAscorbic acid was obtained from Dr F. Bergel of Roche Products Ltd.

RESULTS

The oxidation of L-tyrosine in the presence of α -ketoglutarate and ascorbic acid

The oxygen uptake and L-tyrosine disappearance catalysed by rat-liver homogenates was increased approximately tenfold by additions of α -ketoglutarate and ascorbic acid (LeMay-Knox & Knox, 1951). The full activity of the homogenates was also obtained in the dialysed soluble fraction of liver used in the present studies when it was supplemented with both α -ketoglutarate and ascorbic acid. Little or no activity was found unless both compounds were added together (Figs. 1 and 2). Addition of these compounds also increased tyrosine oxidation in similar liver fractions from guinea pigs and rabbits.

The complete system will take up approximately four atoms of oxygen and form one molecule of acetoacetate for each molecule of L-tyrosine oxidized (Table 1). The use of four atoms of oxygen

Table 1. *Acetoacetate and glutamate formation during L-tyrosine oxidation by the rat-liver enzyme*

(1 ml. enzyme solution; 1 ml. 0.2M-phosphate, pH 7.5; 0.5 mg. ascorbic acid; 30 μ mol. α -ketoglutarate; approx. 1.5 mg. L-tyrosine. Incubation: 1 hr. at 38°.)

L-Tyrosine disappearing (μ mol.)	Oxygen consumed (μ mol.)	Acetoacetate formed (μ mol.)	L-Glutamate formed (μ mol.)
8.4	17.1	8.4	—
10.1	—	10.3	—
—	13.0	6.2	—
7.1	—	7.0	6.3
6.6	—	5.5	6.5

—, not determined.

instead of the theoretical value of five (to form acetoacetate and fumarate), and the requirement for α -ketoglutarate, suggest that a transaminase reaction replaces the oxidative deamination. This was confirmed by the formation of L-glutamate during the reaction (Table 1).

Fig. 1. Effect of addition of α -ketoglutarate on the oxidation of L-tyrosine. All vessels contained 1 ml. of enzyme solution, 1 ml. 0.2M-phosphate, pH 7.5, and 0.5 mg. ascorbic acid, in a total volume of 3.0 ml. A, without additions; B, +8.4 μ mol. of L-tyrosine; C, +30 μ mol. α -ketoglutarate; D, + both L-tyrosine and α -ketoglutarate. --- theoretical O_2 uptake of 4 atoms/mol. of tyrosine.

Fig. 2. Effect of additions of ascorbic acid and of glutathione on the oxidation of L-tyrosine. All vessels contained enzyme solution and phosphate as in Fig. 1, and 30 μ mol. of α -ketoglutarate. A, +0.1 μ mol. of ascorbic acid; B (●), +10 μ mol. of L-tyrosine; C (×), +L-tyrosine and 1 mg. of glutathione; D, +L-tyrosine and ascorbic acid. The O_2 uptakes of enzyme alone and enzyme + glutathione (not shown) and of enzyme + ascorbic acid (A) have been subtracted respectively from curves B, C and D.

Pyruvate could replace α -ketoglutarate in a crude homogenate (LeMay-Knox & Knox, 1951), but not in the dialysed soluble enzyme unless a small amount of α -ketoglutarate was also added. When tyrosine

was oxidized in this way with pyruvate present, the accumulation of alanine instead of glutamate could be demonstrated chromatographically.

Transamination reaction

The absence of oxygen uptake or any diminution in the Millon reaction with the enzyme plus tyrosine, unless α -ketoglutarate is present, suggests that the transamination of tyrosine is an obligatory initial reaction. An initial oxidation of tyrosine to 2:5-dihydroxyphenylalanine has been directly excluded in the dialysed soluble system, since L-2:5-dihydroxyphenylalanine in contrast to *p*-hydroxyphenylpyruvate was not oxidized in this system.

A tyrosine-glutamic transaminase similar to that of Hird & Rowsell (1950) and Cammarata & Cohen (1950) was detected in liver. The activity was found to be distributed nearly equally between the insoluble and soluble fractions of liver obtained by high-speed centrifugation. The reaction in the soluble fraction could be separated from the subsequent oxidation steps of tyrosine under anaerobic conditions. The transamination of L-tyrosine proceeded at a linear rate proportional to the concentration of the enzyme until over half of the added α -ketoglutarate was converted to glutamate. *p*-Hydroxyphenylpyruvate, also formed by the reaction, was identified by isolation as the 2:4-dinitrophenylhydrazone.

Dialysed liver supernatant (15 ml.) was incubated for 2 hr. under N_2 with 29 ml. of 0.1M-phosphate, pH 7.2, containing 37 mg. of L-tyrosine and 29 mg. of α -ketoglutarate. The *p*-hydroxyphenylpyruvate formed (27 mg.) as determined colorimetrically represented a 75% conversion of the α -ketoglutarate to glutamate. The recrystallized 2:4-dinitrophenylhydrazone obtained had a m.p. 170–171°. The mixed m.p. with an authentic sample (m.p. 171°), prepared in the same way, was 170–171° (all m.p.'s uncorr.).

If transamination is the obligatory initial reaction of L-tyrosine oxidation, the rate of transamination, if sufficiently slow, should determine the rate of the overall oxidation of tyrosine. The rate of transamination, generally faster than the overall tyrosine oxidation in fresh enzymes, could be decreased by certain treatments. Dialysis, or better, ammonium sulphate precipitation followed by dialysis, partially removed the coenzyme of the transaminase. After such treatments the slower rate of the transaminase reaction did limit the rate of the aerobic tyrosine oxidation reaction (Fig. 3). The transaminase activity lost by such treatments could be largely restored by addition of boiled fresh enzyme or by addition of pyridoxal phosphate. The addition of pyridoxal phosphate to an enzyme in which the transaminase reaction was the limiting step of tyrosine oxidation increased the rate of the transamination reaction, and by the same effect, increased the rate of the tyrosine oxidation (Fig. 4).

Fig. 3. Limitation of the rate of L-tyrosine oxidation by the rate of tyrosine transamination. All vessels contained 1 ml. of enzyme solution which had been precipitated between 30 and 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ and dialysed, 1 ml. 0.2M-phosphate, pH 7.5, 20 μmol . α -keto-glutarate and 8.5 μmol . L-tyrosine, in 3.2 ml. total volume. Anaerobic formation of *p*-hydroxyphenylpyruvate (○), determined by the Briggs reaction, + 0.5 mg. of ascorbic acid, and the aerobic disappearance of tyrosine (and *p*-hydroxyphenylpyruvate) measured by the Millon reaction (●).

Fig. 4. Effect of pyridoxal phosphate on the rates of tyrosine transamination and oxidation. Tyrosine oxidation (left, ○) was determined manometrically with an enzyme solution, and additions the same as in Fig. 3. Tyrosine transamination (right, ○) was determined in a similar system, but with one-half the enzyme concentration (0.5 ml. in 3.2 ml. total vol.), by the anaerobic formation of *p*-hydroxyphenylpyruvate measured at 320 $\text{m}\mu$. The optical density has been drawn to twice the scale of the O_2 uptake. Pyridoxal phosphate (50 μg .) added as indicated.

Ascorbic acid requirement for *p*-hydroxyphenylpyruvate oxidation

Ascorbic acid has no effect on the initial deamination of tyrosine to *p*-hydroxyphenylpyruvate and is required only for the next step, the oxidation of *p*-hydroxyphenylpyruvate (Fig. 2). In some preparations, like that used in Fig. 2, as little as 0.1 μmol . of ascorbic acid permitted the oxidation of an additional 5 μmol . or more of tyrosine, i.e. an amount 50 times the equivalent of that of the added ascorbic acid. The amount of ascorbic acid required for full activity of the system varied somewhat in different preparations. Generally, more ascorbic acid was required to obtain the same activity before dialysis than after dialysis (see Table 2). This catalytic effect of ascorbic acid on the oxidation of tyrosine is specific in so far as it was not shown by other reducing agents such as glutathione, cysteine, or dihydroxymaleic acid. Ascorbic acid can also form peroxide, but peroxide generated during the reaction by notatin and glucose will not replace the ascorbic acid requirement. Folic acid, which is reported to restore tyrosine metabolism to normal in scorbutic guinea pigs (Woodruff & Darby, 1948), is also inactive. *iso*Ascorbic acid had the same activity as ascorbic acid (Table 2).

Table 2. Comparison of the effects of D-ascorbic and D-isoascorbic acids on L-tyrosine oxidation

(Experimental details as in Table 1 except for ascorbic acid additions. Tyrosine disappearance was measured after 1 hr. although the reactions with suboptimal amounts of ascorbic or *iso*ascorbic acid did not necessarily continue for this length of time (cf. Fig. 2).)

Substance and amount (mg./3 ml.)	L-Tyrosine oxidized (μmol . in 1 hr.)	
	Undialysed enzyme	Dialysed enzyme
—	2.95	2.30
Ascorbic acid 0.02	—	2.29
<i>iso</i> Ascorbic acid 0.02	—	2.35
Ascorbic acid 0.05	3.96	—
<i>iso</i> Ascorbic acid 0.05	4.00	—
Ascorbic acid 0.10	4.97	9.46
<i>iso</i> Ascorbic acid 0.10	5.13	9.67
Ascorbic acid 0.30	8.97	—
<i>iso</i> Ascorbic acid 0.30	8.49	—
Ascorbic acid 0.50	9.34	9.66
<i>iso</i> Ascorbic acid 0.50	9.58	9.65

—, not determined.

In the absence of ascorbic acid an initial oxidation of tyrosine to acetoacetate nearly always occurred, and at a rate equal to that with ascorbic acid. However, the reaction without ascorbic acid soon stopped abruptly. With suboptimal amounts of ascorbic acid longer periods of reaction at the initial

rate were obtained, and with optimal amounts of ascorbic acid the reaction continued at the same rate for several hours. Ascorbic acid and *iso*ascorbic acid appeared to prolong tyrosine oxidation rather than to increase the rate of the reaction.

Pathway of p-hydroxyphenylpyruvate oxidation to acetoacetate

The system oxidizing L-tyrosine will not oxidize tyramine, L-2:5-dihydroxyphenylalanine, the lactone of 2:5-dihydroxyphenylpyruvic acid or gentisic acid. Several possible reaction pathways are therefore eliminated, and a degree of specificity can be attributed to the system. This specificity increases the probability that the compounds which can be oxidized by the system, *p*-hydroxyphenylpyruvic acid, 2:5-dihydroxyphenylpyruvic acid and homogentisic acid, are indeed successive intermediates in L-tyrosine oxidation. These compounds are all oxidized to acetoacetate with the theoretical oxygen uptakes and at rates comparable with that of the overall oxidation of L-tyrosine (Table 3).

The identification of *p*-hydroxyphenylpyruvate formed from L-tyrosine by the transaminase in this enzyme preparation has already been described.

The system in the enzyme preparation which oxidizes homogentisic acid can be preferentially inactivated while the initial tyrosine oxidation reactions are preserved. Losses of the homogentisic acid oxidizing activity noted during fractionation procedures could be attributed to the low pH produced in ammonium sulphate solutions during dialyses (Gilbert & Swallow, 1950). It was more con-

venient to expose the enzyme to pH 5 at room temperature for 15 min. This inactivated the system oxidizing homogentisate to acetoacetate and, though the initial tyrosine oxidation reactions were reasonably well preserved, the formation of acetoacetate from tyrosine was also stopped (Table 4). In this way, preparations were readily obtained that catalysed only two steps of the oxidation of tyrosine, the further oxidation to acetoacetate being eliminated simultaneously with the suppression of the system oxidizing homogentisic acid.

The partial oxidation of tyrosine by an enzyme treated at pH 5 yielded a product which resembled homogentisic acid. It had an ultraviolet absorption curve similar to that of the 2:5-dihydroxyphenyl compounds, darkened when shaken with air in the presence of alkali, and reduced ammoniacal silver nitrate in the cold. This product was isolated from two large-scale runs by continuous ether extraction and partition between water and ether, and then chromatographed in two different solvents. The product moved in a single spot identically with authentic homogentisic acid in both solvents, and could be separated from gentisic acid, *p*-hydroxyphenylpyruvic acid and 2:5-dihydroxyphenylpyruvic acid.

DISCUSSION

It is possibly fortuitous that all of the enzymes in liver catalysing the successive reactions of L-tyrosine to acetoacetate occur in the soluble fraction. The same overall reaction occurring in the homogenate was obtained intact with this fraction alone,

Table 3. *Oxidation of proposed intermediates by the L-tyrosine oxidizing system*

(Experimental details generally similar to those described in Table 1. In experiments with *p*-hydroxyphenylpyruvate no α -ketoglutarate, and in experiments with 2:5-dihydroxyphenylpyruvate and homogentisate no α -ketoglutarate and no D-ascorbic acid was added.)

Exp. no. ...	L-Tyrosine		<i>p</i> -Hydroxyphenylpyruvic acid			2:5-Dihydroxyphenylpyruvic acid		Homogentisic acid	
	1	2	1	2	3	1	2	1	2
Rates of oxidation (μ l. O ₂ /ml. enzyme/first 10 min.)	55	49	53	51	70	32	37	50	80
Atoms O ₂ /mol. acetoacetate formed	4.3	4.2	3.8	4.2	4.0	3.1	3.2	2.1	2.1

Table 4. *Elimination of the formation of acetoacetate from L-tyrosine by destroying the system oxidizing homogentisic acid*

Treatment of enzyme	Substrate			
	L-Tyrosine		Homogentisic acid	
	Oxygen uptake (μ mol./30 min./ml.)	Acetoacetate formed (μ mol./30 min./ml.)	Oxygen uptake (μ mol./30 min./ml.)	Acetoacetate formed (μ mol./30 min./ml.)
Dialysed	21.6	5.2	13.4	6.4
Dialysed and exposed to pH 5 for 15 min.	5.9	<0.1	0.3	<0.1

and its analysis was greatly simplified by even this degree of purification. The separation of the mitochondria avoided the further metabolism of the products, acetoacetate and fumarate (see below), and of α -ketoglutarate and glutamic acid. With moderate dialysis of the supernatant it was possible to reduce the blank reactions and to demonstrate clearly the requirements for α -ketoglutarate and ascorbic acid. It will be desirable, now that the overall reaction has been clarified, to have more detailed analyses of the individual steps than was possible in the preparations used here.

The first reaction, the deamination of L-tyrosine to *p*-hydroxyphenylpyruvate with its transaminase, is the most clearly characterized. It is an obligatory initial reaction in the L-tyrosine oxidation as determined by the absence of any reaction without α -ketoglutarate, and by the failure of the possible alternative intermediate, 2,5-dihydroxyphenylalanine, to be metabolized in the system. The formation of *p*-hydroxyphenylpyruvate from L-tyrosine by the transaminase in the system has been demonstrated, as has the further oxidation of this keto acid. The further reaction of *p*-hydroxyphenylpyruvate is the same as that of tyrosine, that is, both form the same product, acetoacetate, and at comparable rates. The ultraviolet absorption of this aromatic keto acid provides a convenient measure of the transamination, a method which may be of more general application. Additional treatments of the enzyme preparation, such as ammonium sulphate precipitation and/or dialysis, partially remove the coenzyme of this tyrosine-glutamic acid transaminase. The activity can then be restored by addition of pyridoxal phosphate, in agreement with the results of Cammarata & Cohen (1950), and of Feldman & Gunsalus (1950) for the bacterial enzyme. Liver preparations, partially deficient in pyridoxal phosphate, were used by adding or withholding the coenzyme to demonstrate that the rate of the transaminase reaction does, in fact, control the rate of the overall oxidation of tyrosine. Only the transaminase reaction occurs in the system anaerobically and substantially only this reaction in the absence of ascorbic acid aerobically. *p*-Hydroxyphenylpyruvate then accumulates, as it does in scorbutic guinea pigs fed tyrosine.

The oxidation of *p*-hydroxyphenylpyruvate does not take place in the side chain, since the product, *p*-hydroxyphenylacetate, is not a precursor of homogentisic acid (Neubauer, 1904). The oxidation, in the ring, must form 2,5-dihydroxyphenylpyruvate. This unusual reaction involves a migration of the pyruvyl side chain, in addition to the oxidation of the ring, and is of interest apart from the catalytic action of ascorbic acid in this step. The oxidation of *p*-hydroxyphenylpyruvate with an oxygen uptake of three atoms per mol. instead of

four, and the formation of 0.67 mol. of acetoacetate instead of one (Felix *et al.* 1937), was apparently due to incomplete oxidation.

2,5-Dihydroxyphenylpyruvate is oxidized by the system to the same product and at a similar rate to L-tyrosine. However, no attempt was made to accumulate this compound from the reaction of L-tyrosine or *p*-hydroxyphenylpyruvate, a necessary step before it is definitely identified as an intermediate. Oxidative decarboxylation of this probable intermediate would give rise to the carbon dioxide formed during tyrosine oxidation and to homogentisic acid.

Homogentisic acid was identified with reasonable certainty as the product formed by the oxidation of L-tyrosine with two atoms of oxygen and accumulated when the enzyme oxidizing homogentisic acid was destroyed. This compound, previously known to be formed only in alcaptonurics and in certain abnormal states, can now be accepted as a normal intermediate in tyrosine metabolism. It is oxidized to acetoacetate by liver at a more rapid rate than is tyrosine, and ascorbic acid is not required. Two enzymes responsible for the disappearance of homogentisic acid in liver have been identified (Raydin & Crandall, 1950). One oxidizes the homogentisic acid to fumaryl-acetoacetate, and the second hydrolyses this new intermediate to fumaric acid and acetoacetate. It has also been reported that Fe^{++} and phosphate are required respectively for the oxidation and hydrolysis reactions (Suda & Takeda, 1950). The hydrolytic enzyme is said to be the one previously known as acyl-pyruvase (Meister & Greenstein, 1948) and as the triacetic acid hydrolysing enzyme (Connors & Stotz, 1949).

Felix *et al.* (1937) excluded *p*-hydroxyphenylpyruvate and homogentisic acid as intermediates in tyrosine oxidation, although they also found both these compounds to be oxidized by their system. Being unaware of the tyrosine transaminase reaction, and having eliminated the possibility of oxidative deamination of L-tyrosine, they concluded that *p*-hydroxyphenylpyruvate was not formed from tyrosine. The metabolism of tyrosine through homogentisic acid to acetoacetate entirely by oxidative reactions was not considered possible, because this conversion would require five atoms of oxygen instead of the observed four. The transamination, replacing an oxidative deamination, negates this objection to homogentisic acid as an intermediate. The presence of the transaminase, which so alters their conclusions, was actually excluded by them on the apparently good evidence that α -ketoglutarate and tyrosine in a fresh liver homogenate did not form glutamic acid. However, they subsequently found that alanine was formed during tyrosine oxidation (Felix & Zorn, 1941). Lerner (1949) has demonstrated that the carbon skeleton of

alanine formed during tyrosine oxidation does not come from the tyrosine side chain, as Felix & Zorn suggested. These results can be explained by two successive transaminase reactions now known to occur in liver:

(1) L-Tyrosine + α -ketoglutarate $\rightarrow$ *p*-hydroxyphenylpyruvate + L-glutamate.

(2) L-Glutamate + pyruvate $\rightarrow$ α -ketoglutarate + L-alanine.

By these reactions tyrosine oxidation can occur in homogenates which contain pyruvate and only catalytic amounts of α -ketoglutarate. The accumulation in this way of alanine instead of glutamate has also been demonstrated chromatographically in the dialysed soluble system.

From the present work, and that of Ravdin & Crandall (1950), the oxidation of L-tyrosine in liver can be finally formulated as follows:

(1) L-Tyrosine + α -ketoglutarate $\rightarrow$ *p*-hydroxyphenylpyruvate + L-glutamate.

(2) *p*-Hydroxyphenylpyruvate + $\frac{1}{2}$ O₂ $\rightarrow$ 2:5-dihydroxyphenylpyruvate.

(3) 2:5-Dihydroxyphenylpyruvate + $\frac{1}{2}$ O₂ $\rightarrow$ homogentisate + CO₂.

(4) Homogentisate + O₂ $\rightarrow$ fumaryl-acetoacetate.

(5) Fumaryl-acetoacetate $\rightarrow$ fumarate + acetoacetate.

This formulation is consistent with the other work quoted, and describes the tyrosine metabolism of at least several species. Interruptions of the appropriate reactions in this pathway can account for the excretion of *p*-hydroxyphenylpyruvate in Medes's case of tyrosinosis (Medes, 1932) and in scorbutic guinea pigs and premature infants, and for the excretion of homogentisic acid by alcaptonurics.

Rienits (1950) and Painter & Zilva (1950) demonstrated definite, but much smaller, effects of ascorbic acid on L-tyrosine oxidation in liver homogenates than the effect which has been demonstrated for the oxidation of *p*-hydroxyphenylpyruvate in the present work. Only a small increase in tyrosine oxidation was observed, because the reaction in homogenates without added α -ketoglutarate was largely limited by the transaminase step. Also much larger amounts of ascorbic acid were necessary to produce an effect, probably due in part to the destruction of added ascorbic acid by the cytochrome system and in part to the fact that even undialysed soluble preparations require more ascorbic acid than do the dialysed ones. In the present study with dialysed preparations no reaction occurred after the first few minutes in the absence of added ascorbic acid, and though the requirement varied, only catalytic amounts of ascorbic acid were needed for long-continued reactions. 'Supersaturated levels' of ascorbic acid were not necessary.

The effect of ascorbic acid on *p*-hydroxyphenylpyruvate oxidation is not produced by other com-

pounds which variously share similar reducing or oxidizing properties (glutathione and peroxide) or a similar reductone structure (dihydroxymaleic acid). Of the compounds tested this quite specific effect is shared only by *iso*ascorbic acid, which also possesses some antiscorbutic activity.

Much greater amounts of ascorbic acid are required in guinea pigs to maintain a normal tyrosine metabolism than are required to prevent scurvy. For this reason it has been stated that the interaction between D-ascorbic acid and L-tyrosine does not indicate a connexion between the normal function of ascorbic acid and the normal metabolism of tyrosine. However, the derangement of the tyrosine metabolism is tested for by feeding abnormally large amounts of tyrosine (0.5 g./day). When less tyrosine is given (0.3 g.) the metabolic derangement is manifested only after greater ascorbic acid depletion (14 days instead of 8) (Painter & Zilva, 1947). When very large doses of tyrosine were given, there were indications that *p*-hydroxyphenylpyruvate was excreted even by animals saturated with ascorbic acid. The amount of ascorbic acid needed to assure the metabolism of tyrosine appears to be related to the amount of tyrosine administered. The lower antiscorbutic levels of ascorbic acid may then suffice for the metabolism of the much smaller physiological amounts of tyrosine and this action in tyrosine metabolism may then be a part of the vitamin function of ascorbic acid. The concentrations of ascorbic acid that were necessary for tyrosine oxidation in the present study were not greater than those which are only antiscorbutic in guinea pigs.

A connexion between these two physiological actions of ascorbic acid also follows from the studies with *iso*ascorbic acid. This compound is antiscorbutic and also acts in tyrosine metabolism in guinea pigs (Sealock & Silberstein, 1940). In both of these actions it is one-twentieth to one-fiftieth as effective as ascorbic acid (Dalmer & Moll, 1933; Demole, 1934). The suggestion has been made that ascorbic and *iso*ascorbic acids are really of equal activity, and that the apparent effect of *iso*ascorbic acid is reduced by its poor retention in the body (Painter & Zilva, 1950). That they do have equal activity, at least in tyrosine oxidation, has been demonstrated in the present *in vitro* system. Similarly, D-glucoscorbic acid is not retained at all in the body and has no antiscorbutic activity (Zilva, 1935), and yet also showed activity in the oxidation of tyrosine by liver homogenates (Painter & Zilva, 1950).

SUMMARY

1. A series of enzymes in the soluble fraction of liver homogenates catalyses the oxidation of L-tyrosine to acetoacetate. In this reaction one

molecule of L-tyrosine is oxidized with uptake of four atoms of oxygen and the formation of one molecule of acetoacetate.

2. Additions of α -ketoglutarate and catalytic amounts of ascorbic acid are required for the activity of the dialysed enzyme.

3. *p*-Hydroxyphenylpyruvate, 2:5-dihydroxyphenylpyruvate, and homogentisic acid are also oxidized to acetoacetate by this system, at rates comparable with that of tyrosine oxidation. *p*-Hydroxyphenylpyruvate and homogentisic acid are formed from tyrosine in the system, and have been identified as intermediates in the reaction.

4. *p*-Hydroxyphenylpyruvate is formed by the first enzyme in the series, an L-tyrosine-glutamic acid transaminase. This step can be made to limit the rate of the overall reaction in the absence of its

coenzyme, pyridoxal phosphate. *p*-Hydroxyphenylpyruvate accumulates anaerobically, or, in the absence of ascorbic acid, aerobically.

5. Homogentisic acid is formed by the further oxidation of *p*-hydroxyphenylpyruvate, probably through 2:5-dihydroxyphenylpyruvate as an intermediate. Catalytic amounts of ascorbic acid are required for this reaction. Homogentisic acid can be accumulated in an enzyme mixture treated at pH 5, which destroys the system oxidizing homogentisic acid.

6. Ascorbic and isoascorbic acids are equally effective in promoting the oxidation of *p*-hydroxyphenylpyruvate, and so far could not be replaced by other compounds tested. The possibility that the action in tyrosine metabolism of ascorbic acid is part of its vitamin action is discussed.

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