

# THE NATURE OF THE DEFECT IN TYROSINE METABOLISM IN ALCAPTONURIA

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(Received for publication, July 29, 1957)

Alcaptonuria is a rare, hereditary, metabolic disorder characterized by a defect in the oxidation of tyrosine (1-3). In this condition homogentisic acid, an intermediary product of tyrosine degradation in mammalian liver, is excreted in the urine. This disorder has been attributed to an abnormality of the enzyme system, homogentisic acid oxidase, but the exact nature of the abnormality has not been determined. The recent opportunity to perform a liver biopsy during abdominal surgery on a patient with alcaptonuria made it possible to determine more precisely the nature of the defect in homogentisic acid oxidase. In addition, the activities of the other enzymes known to participate in tyrosine catabolism were assayed and compared with those of non-alcaptonuric liver preparations. It was possible to establish with reasonable certainty that the defect in alcaptonuria is limited to the enzyme, homogentisic acid oxidase, that there is a failure to synthesize active enzyme, and that the metabolic block is essentially complete.

## *Methods*

*Preparation of Liver Homogenate*—A 2.8 gm. sample of liver removed during surgery on a 57 year-old male alcaptonuric patient (for repair of an esophageal hiatus hernia) was chilled immediately and homogenized with a Potter-Elvehjem type glass homogenizer in 11.2 ml. of cold 0.9 per cent KCl solution in a cold room at 5°. The homogenate was centrifuged at  $12,000 \times g$  for 20 minutes, and the resulting supernatant fraction containing 24.0 mg. of protein per ml. was used in the experiments described below.

Similar preparations were made from liver obtained at surgery on a non-alcaptonuric patient and at autopsy about 12 hours after death of another non-alcaptonuric patient.

*Preparation of Rat Liver Homogentisic Acid Oxidase*—Homogentisic acid oxidase was precipitated from a homogenate of rat liver by treatment with 32 per cent ethanol as described by Knox and Edwards (4). Re-

precipitation with 32 per cent ethanol removed most of the glutathione and maleylacetoacetic acid isomerase activity.

*Materials*—*p*-Hydroxyphenylpyruvic acid, *m*-hydroxyphenylpyruvic acid, 2,5-dihydroxyphenylpyruvic acid, and homogentisic acid were synthesized as previously described (5). In some experiments homogentisic acid isolated from the urine of the alcaptonuric patient was used. 2,5-Dihydroxyphenyl-DL-alanine was obtained from Dr. H. B. Gillespie (6).

Maleylacetoacetic acid was prepared enzymatically from homogentisic acid with rat liver homogentisic acid oxidase as described by Knox and Edwards (7). The product contained about 10 per cent fumarylacetoacetic acid. Fumarylacetoacetic acid was obtained from maleylacetoacetic acid by allowing an acidified solution of the latter to stand several days in the cold. The extent of isomerization was followed spectrophotometrically (7).

Homogentisic acid-C<sup>14</sup> labeled in the carboxyl group was kindly synthesized by Dr. E. M. Gal, University of California. The preparation contained approximately 1  $\mu$ c. per mg.

*Analytical Methods*—*p*-Hydroxyphenylpyruvic acid and homogentisic acid were determined colorimetrically following acidification of the incubation mixture and extraction into ether as described previously (5).

Acetoacetic acid was determined manometrically by decarboxylation at pH 5.2 with 4-aminoantipyrine at 37° (8). The rate of decarboxylation of acetoacetic acid by 4-aminoantipyrine is very rapid, and the reaction is essentially complete within 15 minutes, whereas the rates of decarboxylation of fumarylacetoacetic acid and maleylacetoacetic acid are much slower.

Ascending paper chromatography of homogentisic acid with *n*-butanol-water-formic acid was carried out as previously described (5).

*Estimation of C<sup>14</sup>O<sub>2</sub>*—After the incubation of 2  $\mu$ moles of labeled homogentisic acid-C<sup>14</sup> with the liver homogenate preparations, the Warburg flasks were removed from the bath, and the alkali in the center well was replaced by 0.2 ml. of 0.16 M Hyamine base in methanol and toluene (9). The contents of the main compartment were adjusted to pH 5.2 with 1 M acetic acid followed by the addition of 0.5 ml. of 0.5 M acetate buffer, pH 5.2. 0.2 ml. of 0.2 M 4-aminoantipyrine, dissolved in the acetate buffer, was added to the side arm, and the flasks were returned to the bath at 37°. Following temperature equilibration, acetoacetic acid was decarboxylated by tipping in 4-aminoantipyrine, and the CO<sub>2</sub> liberated was trapped by the Hyamine base in the center well. Suitable aliquots of the base were then removed, mixed with 10 ml. of 0.4 per cent diphenyloxazole in toluene, and counted in a liquid scintillation counter (9).

*Enzyme Assay Methods*—*p*-Hydroxyphenylpyruvic acid oxidase activity

was measured manometrically as previously described (5). Since the rate of tyrosine transamination is slower than the rate of *p*-hydroxyphenylpyruvic acid oxidation, tyrosine transaminase activity could be estimated manometrically when L-tyrosine was used as the substrate in the above assay method supplemented with  $\alpha$ -ketoglutarate and pyridoxal phosphate at pH 7.5. Homogentisic acid oxidase activity was determined manometrically by the method of Knox and Edwards (4). Maleylacetoacetic acid isomerase and fumarylacetoacetic acid hydrolase activities were determined by the spectrophotometric methods of Knox and Edwards (7).

### Results

The results of our studies indicate that the pathway of tyrosine oxidation in human liver homogenate preparations proceeds by the same sequence of reactions as it does in other mammalian species; that is, L-tyrosine  $\rightarrow$  *p*-hydroxyphenylpyruvic acid  $\rightarrow$  homogentisic acid  $\rightarrow$  maleylacetoacetic acid  $\rightarrow$  fumarylacetoacetic acid  $\rightarrow$  acetoacetic acid and fumaric acid (10). The enzymes involved were localized in the soluble protein fraction of the liver cell. As with other mammalian liver preparations (11–13),  $\alpha, \alpha'$ -dipyridyl completely blocked the oxidation of homogentisic acid, and this inhibitor could be used to study the enzymatic formation of homogentisic acid from *p*-hydroxyphenylpyruvic acid or from tyrosine. In view of these similarities, the methods developed to study tyrosine oxidation in animal liver were used to study and compare the tyrosine oxidation system of non-alcaptonuric and alcaptonuric human liver.

*Tyrosine Transaminase in Non-Alcaptonuric Liver Homogenate*—In the presence of  $\alpha$ -ketoglutarate tyrosine was converted to *p*-hydroxyphenylpyruvic acid by transamination, and the optimal pH for this reaction was near 7.8, as was previously found for rat and dog liver preparations (5). However, in balance studies on the over-all pathway of tyrosine oxidation, it was more convenient to use *p*-hydroxyphenylpyruvic acid as the substrate because of the relatively slow rate of the tyrosine transaminase step. In addition, the optimal pH range for *p*-hydroxyphenylpyruvic acid oxidase was from pH 6.3 to 7.8; thus a lower pH could be used to accumulate homogentisic acid.

*Oxidation of p-Hydroxyphenylpyruvic Acid to Homogentisic Acid in Non-Alcaptonuric Liver Homogenate*—In the absence of  $\alpha, \alpha'$ -dipyridyl, *p*-hydroxyphenylpyruvic acid was oxidized quantitatively to acetoacetic acid, and the theoretical amount of oxygen, 4 atoms, was consumed in this oxidation (Table I). In the presence of 0.001 M  $\alpha, \alpha'$ -dipyridyl, a quantitative accumulation of a product identified chemically as homogentisic acid resulted, and this oxidation consumed 2 atoms of oxygen as theoreti-

cally required (Table I). Additional evidence that the product was homogentisic acid was obtained by paper chromatography. Both the product and authentic homogentisic acid migrated as a single spot with an  $R_F$  of 0.75. No evidence for the presence of gentisic acid was found.

In accord with studies of other mammalian liver preparations (5, 14), neither 5  $\mu$ moles of 2,5-dihydroxyphenyl-DL-alanine nor 5  $\mu$ moles of 2,5-dihydroxyphenylpyruvic acid were oxidized under the same conditions in which 5  $\mu$ moles of *p*-hydroxyphenylpyruvic acid were completely oxidized to homogentisic acid.

TABLE I

*Oxidation of p-Hydroxyphenylpyruvic Acid to Homogentisic Acid and to Acetoacetic Acid in Non-Alcaptonuric Liver Homogenate*

The main compartment of the Warburg vessels contained 0.7 ml. of 0.2 M sodium phosphate buffer, pH 6.5, 200  $\gamma$  of 2,6-dichlorophenolindophenol, 20  $\mu$ moles of neutralized glutathione, and 0.5 ml. of liver homogenate supernatant fraction. 2  $\mu$ moles of  $\alpha, \alpha'$ -dipyridyl were added to the flasks when the accumulation of homogentisic acid was desired. 2.5  $\mu$ moles of *p*-hydroxyphenylpyruvic acid were present in the side arm. The total fluid volume was 2.0 ml.

	With $\alpha, \alpha'$ -dipyridyl	Without $\alpha, \alpha'$ -dipyridyl
	$\mu$ moles	$\mu$ moles
Net oxygen uptake.....	2.5	5.0
Decrease in <i>p</i> -hydroxyphenylpyruvic acid.....	2.4	
Apparent formation of homogentisic acid by chemical determination*.....	2.5	
Formation of acetoacetic acid†.....		2.4

\* The contents of three flasks were pooled and extracted with ether, and aliquots were analyzed chemically for *p*-hydroxyphenylpyruvic acid and homogentisic acid as described previously (5).

† The acetoacetic acid formed was determined by chemical decarboxylation with 4-aminoantipyrine as described under "Methods." The theoretical amount of acetoacetic acid expected was 2.5  $\mu$ moles.

The *p*-hydroxyphenylpyruvic acid oxidase activity of human liver was found to be similar to that of dog liver in regard to its sensitivity to several inhibitors. Diethyldithiocarbamate inhibited 80 per cent at  $1 \times 10^{-4}$  M, and *m*-hydroxyphenylpyruvic acid inhibited over 50 per cent at  $2.5 \times 10^{-4}$  M and was not oxidized as a substrate. In addition, the unusual type of inhibition produced by excess *p*-hydroxyphenylpyruvic acid, which could be prevented, in part, by ascorbic acid or 2,6-dichlorophenolindophenol, was observed with human liver preparations, just as with dog liver (5).

*Oxidation of Homogentisic Acid to Acetoacetic Acid in Non-Alcaptonuric Liver*—The conversion of *p*-hydroxyphenylpyruvic acid to acetoacetic acid

was demonstrated in the above experiments in which  $\alpha, \alpha'$ -dipyridyl was omitted (Table I). Although these data indicated the presence of homogentisic acid oxidase, a direct manometric assay of this enzyme activity was made by employing homogentisic acid as a substrate. The spectrophotometric assay method of Knox and Edwards (4) was unsatisfactory because too much glutathione remained in the homogenate, even after dialysis for 2 hours, to permit quantitative accumulation of maleylacetoacetic acid. Spectrophotometric assays for maleylacetoacetic acid isomerase and fu-

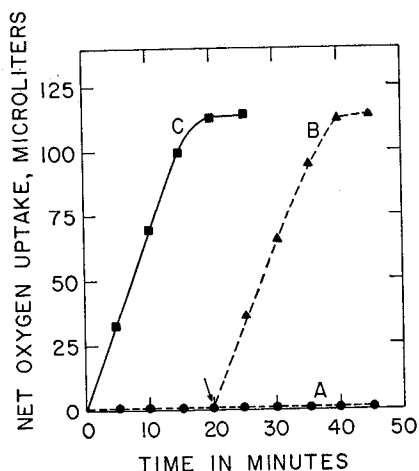


Fig. 1. Homogentisic acid oxidase activity in alcaptonuric liver homogenate. The flasks contained 20  $\mu$ moles of neutralized glutathione, 0.2  $\mu$ mole of  $\text{Fe}^{++}$ , liver preparations as described below, 0.4 ml. of 0.2 M phosphate buffer, pH 6.5, and 5  $\mu$ moles of homogentisic acid in the side arm. Total volume, 2.0 ml. A, 0.5 ml. of 20 per cent alcaptonuric liver homogenate. B, as in A with the delayed addition of 0.5 ml. of rat liver homogentisic acid oxidase ( $\downarrow$ ). C, 0.5 ml. of rat liver homogentisic acid oxidase.

marylacetoacetic acid hydrolase activities demonstrated the presence of both enzymes in high concentration in human liver. However, the isomerase activity was considerably lower in the autopsy liver specimen, and this was probably due to the instability of the enzyme during the post-mortem period.

*Homogentisic Acid Oxidase Activity in Alcaptonuric Liver Homogenate*—5  $\mu$ moles of homogentisic acid were incubated with a homogenate of liver from the alcaptonuric patient supplemented with  $\text{Fe}^{++}$ , the only known cofactor of homogentisic acid oxidase (11). No measurable oxygen uptake occurred in 45 minutes (Fig. 1).

The possibility was considered that alcaptonuric liver might contain an inhibitor of homogentisic acid oxidase. However, evidence against this

possibility was obtained by adding a preparation of rat liver homogentisic acid oxidase to an incubation mixture containing alcaptonuric liver homogenate which had failed to oxidize substrate during the preceding 20 minutes. The resulting rate of oxygen consumption in the presence of the rat liver homogentisic acid oxidase was exactly the same as when the latter was assayed alone (Fig. 1).

Although alcaptonuric liver appears to have no homogentisic acid oxidase activity demonstrable by the manometric assay, this method could not detect a rate of 0.1  $\mu$ mole of homogentisic acid oxidized per hour per

TABLE II

*Oxidation of C<sup>14</sup>-Carboxyl-Labeled Homogentisic Acid*

Complete oxidation of 336  $\gamma$  of homogentisic acid-C<sup>14</sup> with excess rat liver homogenate yielded 304,700 c.p.m. (910 c.p.m. per  $\gamma$  of homogentisic acid) after decarboxylation of the acetoacetic acid formed with 4-aminoantipyrine as described under "Methods." The reaction rate as measured by O<sub>2</sub> consumption was proportional to the amount of rat liver homogenate when 6.0 mg. and 1.25 mg. of protein were present, and the yield of C<sup>14</sup>O<sub>2</sub> corresponded to that expected from the manometric data. The flasks contained 2.0  $\mu$ moles of homogentisic acid-C<sup>14</sup>, 20  $\mu$ moles of neutralized glutathione, 0.2  $\mu$ mole of Fe<sup>++</sup>, 1.0 ml. of 0.2 M phosphate buffer, pH 6.5, and liver homogenate. The total fluid volume was 2.0 ml.; the flasks were shaken at 37° under air. Incubation time, 20 minutes.

Enzyme preparation	Protein*	C <sup>14</sup> O <sub>2</sub> from acetoacetic acid formed
	mg.	c.p.m.
No enzyme.....	0	618
Alcaptonuric liver homogenate.....	6	460
Rat liver homogenate.....	1.25	175,800

\* 6 mg. of protein equivalent to 50 mg. wet weight of liver.

0.1 gm. of wet weight of liver. Assuming a liver weight of approximately 1500 gm., this low rate would theoretically permit the oxidation of about 6 gm. of homogentisic acid per day in man, an amount approximately equivalent to the average daily intake of phenylalanine and tyrosine. Therefore, a more sensitive method was employed to test for homogentisic acid oxidase activity in alcaptonuric liver.

2  $\mu$ moles of homogentisic acid-C<sup>14</sup> labeled in the carboxyl group were incubated for 20 minutes with alcaptonuric liver homogenate and with various dilutions of rat liver homogenate. The resulting acetoacetic acid was decarboxylated with 4-aminoantipyrine, and the radioactivity in the liberated CO<sub>2</sub> was measured. Incubation of the radioactive substrate with the alcaptonuric liver homogenate gave no more radioactive CO<sub>2</sub> than a control incubation carried out without enzyme (Table II).

TABLE III

*Oxidation of p-Hydroxyphenylpyruvic Acid to Homogentisic Acid in Alcaptonuric Liver Homogenate*

The main compartment of the Warburg vessel contained 0.7 ml. of 0.2 M sodium phosphate buffer, pH 6.5, 200  $\gamma$  of 2,6-dichlorophenolindophenol, 20  $\mu$ moles of neutralized glutathione, and 0.5 ml. of alcaptonuric liver homogenate supernatant fraction. 2.5  $\mu$ moles of *p*-hydroxyphenylpyruvic acid were present in the side arm. The total fluid volume was 2.0 ml.

	Oxidation $\mu$ moles
Net oxygen uptake.....	2.6
Decrease in <i>p</i> -hydroxyphenylpyruvic acid.....	2.4
Apparent formation of homogentisic acid* by chemical determination...	2.6
“ “ “ “ “ “ enzymatic oxidation to maleylacetoacetic acid.....	2.5

\* The contents of three flasks were pooled and extracted with ether, and aliquots were analyzed chemically for *p*-hydroxyphenylpyruvic acid and homogentisic acid as described previously (5). Another portion was incubated with rat liver homogentisic acid oxidase to yield maleylacetoacetic acid. The latter was determined spectrophotometrically as described under "Methods."

TABLE IV

*Activity of Tyrosine Oxidation Enzymes in Alcaptonuric and Non-Alcaptonuric Human Liver*

Enzyme	Activity*		
	Non-alcaptonuric liver, autopsyt	Non-alcaptonuric liver, biopsy†	Alcaptonuric liver, biopsy
Tyrosine transaminase.....	0.67	3.6	3.2
<i>p</i> -Hydroxyphenylpyruvic acid oxidase..	4.3	6.7	4.6
Homogentisic acid oxidase.....	11.8	26.8	<0.0048
Maleylacetoacetic acid isomerase§.....	14.5	960	780
Fumarylacetoacetic acid hydrolase....	14	29	22

\* Activity calculated as micromoles of substrate oxidized per hour per 0.1 gm. wet weight of liver.

† 36 year-old male with Hodgkin's disease.

‡ 5 year-old female with congenital hemolytic anemia; splenectomy performed.

§ Units calculated as  $\Delta$  log optical density per hour per 0.1 gm. wet weight of liver (7).

*Formation of Homogentisic Acid from L-Tyrosine and p-Hydroxyphenylpyruvic Acid*—The quantitative formation of homogentisic acid from L-tyrosine with  $\alpha$ -ketoglutarate present, or from *p*-hydroxyphenylpyruvic acid (Table III), could be demonstrated in alcaptonuric liver homogenate.

The product was also identified as homogentisic acid by paper chromatography. Both the apparent and authentic homogentisic acids migrated as a single spot with an  $R_F$  of 0.75. For further identification, 2.5  $\mu$ moles of the apparent homogentisic acid, which had been extracted with ether from the alcaptonuric liver incubation mixture, were incubated with rat liver homogentisic acid oxidase. The resulting maleylacetoacetic acid was identified by its absorption spectrum and by its disappearance upon the addition of glutathione and excess maleylacetoacetic acid isomerase and fumarylacetoacetic acid hydrolase.

As was observed with the non-alcaptonuric human liver, 5  $\mu$ moles of 2,5-dihydroxyphenylpyruvic acid were not oxidized to homogentisic acid.

*Metabolism of Maleylacetoacetic Acid and Fumarylacetoacetic Acid in Alcaptonuric Liver*—Maleylacetoacetic acid isomerase and fumarylacetoacetic acid hydrolase activities were demonstrated in alcaptonuric liver homogenate. With the exception of homogentisic acid oxidase, all the enzymes involved in tyrosine oxidation were present in the alcaptonuric liver at levels comparable to that of the non-alcaptonuric liver (Table IV).

#### DISCUSSION

The results presented define more precisely and confirm previous assumptions on the nature of the metabolic block in alcaptonuria. Normal levels of activity were demonstrated for all but one of the enzymes of the tyrosine oxidation system in the liver of an alcaptonuric patient. However, with a very sensitive method of assay, no homogentisic acid oxidase activity could be detected, even in the presence of glutathione and  $Fe^{++}$ . Considered together with the inability to demonstrate an inhibitor, these findings lead to the conclusion that the disorder is due to the essentially complete absence of active homogentisic acid oxidase. Whether this defect represents failure to synthesize enzyme protein, or modification of the catalytic site on the protein, is still to be determined. The finding that maleylacetoacetic acid isomerase is as active in alcaptonuric liver as in non-alcaptonuric liver indicates that the synthesis of this enzyme is not dependent on the presence of its substrate, since the only known pathway for the formation of maleylacetoacetic acid is from homogentisic acid.

The association of alcaptonuria, a known hereditary disease, with the complete absence of activity of a single enzyme follows the pattern of other clinical disorders such as galactosemia (15, 16) or phenylketonuria (17, 18), and provides further evidence for a relationship in the human between individual genes and single enzymes. It would be of interest to learn whether a single gene controls the production of a given enzyme in several organs, and examination of renal tissue from an alcaptonuric patient for homogentisic acid oxidase activity might help to answer this question.



The pathway of tyrosine oxidation in human liver has been shown here to be similar to that of other mammals. This similarity applies also to the inability of 2,5-dihydroxyphenyl-DL-alanine and 2,5-dihydroxyphenylpyruvic acid to give rise to homogentisic acid under conditions in which the latter compound is produced from tyrosine or *p*-hydroxyphenylpyruvic acid. Even though feeding these compounds to alcaptonuric subjects results in increased excretion of homogentisic acid (19, 20), it is unlikely that they are intermediates in tyrosine metabolism.

#### SUMMARY

1. No detectable homogentisic acid oxidase activity was present in liver homogenate prepared from an alcaptonuric patient. Evidence is presented that the lack of activity was not due to the presence of an inhibitor or the absence of any known cofactor. The defect in alcaptonuria appears to be a failure to synthesize active homogentisic acid oxidase.

2. All of the other enzymes involved in tyrosine oxidation have essentially the same activity in alcaptonuric and non-alcaptonuric human liver.

3. The sequence of reactions of tyrosine oxidation appears to be the same in human liver as in the other mammalian species.

4. 2,5-Dihydroxyphenylpyruvic acid and 2,5-dihydroxyphenyl-DL-alanine are not oxidized in human liver homogenate preparations, and it is unlikely that these compounds are intermediates in the oxidation of tyrosine to homogentisic acid.

We wish to acknowledge the surgical care of the patients provided by Mr. Philip R. Allison, Nuffield Professor of Surgery, Oxford, England, and Dr. Andrew G. Morrow, Chief, Clinic of Surgery, National Heart Institute, and the medical care provided by Dr. Joseph S. McGuire and Dr. Ted Clemens, Jr., Clinical Associates of the National Institute of Arthritis and Metabolic Diseases.

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