AROMATIZATION OF HEXAHYDROBENZOIC ACID BY MAMMALIAN LIVER MITOCHONDRIA

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Since the original observation by LAUTEMANN[1] that orally administered quinate is excreted as HA** by man, many cyclohexane ring compounds have been found to be oxidized by animals[2]. More recently, BEER, DICKENS AND PEARSON[3] studied the aromatization of HHBA in detail using tissue slices. However, they were unable to demonstrate this reaction in tissue extracts which could vigorously oxidize straight chain fatty acids.

In the present report the enzyme system for oxidizing HHBA to form HA is described and some of its properties are discussed.

EXPERIMENTAL

Materials

ATP and cytochrome *c* were products of the Sigma Chemical Company. CoA was obtained from the Pabst Laboratories. HHBA was purchased from the Eastman Organic Chemicals. Amino acids and αKGA were products of either California Foundation for Biochemical Research or Nutritional Biochemical Corporation. Sodium-HHBA-1-^{14}C (1.2 μc/μmoles) was purchased from Nuclear Chicago Corporation.

Hexahydro-HA was prepared by the method of GODCHOT[4]. Hexahydrobenzohydroxamic acid was prepared as described by WILSON *et al.*[5]. HHBCoA was prepared by the procedure described for the synthesis of benzoyl CoA[6]. The acid anhydride used in this preparation was synthesized from HHBA by the method of LUMSDEN[7]. The HHBCoA solution after ether extraction (to remove unreacted acid anhydride) contained 2.6 μmoles of acyl mercaptan per ml when estimated by the procedure of GRUNERT AND PHILLIPS[8] with glutathione as the standard and 2.1 μmoles per ml by the modified procedure of LIPMANN AND TUTTLE[9] with the corresponding hydroxamic acid as the standard. This solution was used for enzyme studies. When a portion of this solution was chromatographed on Whatman No. 3 paper under the conditions described by STADTMAN[10] for acyl mercaptans, a compound with an R_F of 0.84 was detected under the ultraviolet lamp in addition to the two CoA spots (R_F's of 0.52 and 0.71). After elution from the paper this compound gave a positive hydroxamic acid test[8]. The difference spectrum between the untreated sample and a base hydrolyzed sample showed an absorption peak at 236 mμ.

Enzyme preparation and assay

Fresh guinea pig liver was homogenized in 9 volumes of 0.25 *M* sucrose solution containing 10^{-3} *M* EDTA and the mitochondria were isolated by the method of HOGEBOOM *et al.*[11]. The mitochondria were then washed twice and resuspended in 1.15% KCl solution containing 10^{-3} *M* EDTA. Incubation was carried out for 1 hour at 37° in 20 ml beakers using a Dubnoff metabolic shaker. The composition of the various incubation mixtures is described under each table. Benzoic acid or HA was estimated by a modification of the colorimetric method of DICKENS AND PEARSON[12].

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** The following abreviations are used in this paper: HA for hippuric acid, HHBA for hexahydrobenzoic acid or cyclohexanecarboxylic acid, ATP for adenosinetriphosphate, CoA for coenzyme A, αKGA for α-ketoglutaric acid, HHBCoA for hexahydrobenzoyl CoA and EDTA for ethylenediaminetetraacetic acid.

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RESULTS

Requirements for the enzyme system

During the fractionation studies of the guinea pig liver homogenate, it became evident that the overall enzyme system for aromatization was localized in the mitochondrial fraction. However, the mitochondria alone were inactive unless fortified by the boiled supernatant fraction. Systematic studies led to the findings that the supernatant could be completely replaced by αKGA, $MgSO_4$, EDTA, glycine, ATP and cytochrome *c* (Table I). The addition of CoA had no effect. The enzyme system is extremely sensitive to heavy metals; thus, EDTA was routinely added to sucrose and KCl solutions in preparing the mitochondria. The need for EDTA could be obviated by using distilled water passed through a demineralizer in preparing all of the reagents used in incubation. The system is also dependent on oxygen, since incubation of HHBA under nitrogen yielded less than half of the aromatic product.

TABLE I

REQUIREMENT FOR ENZYMIC AROMATIZATION

	μmoles of benzoic acid or HA formed	*Relative activity*
Complete	3.6	100%
No ATP	2.7	75
No cyt. *c*	2.7	75
No glycine	1.2	33
No EDTA	0.8	22
No $MgSO_4$	0.3	8
No αKGA	0.2	6

The complete system contained 0.5 ml of 0.5 *M* pH 7.4 phosphate buffer, 1 ml of mitochondrial preparation equivalent to 0.7 g of liver, 10 μmoles of HHBA, 0.3 μmoles ATP, 0.03 μmoles cyt. *c*, 30 μmoles glycine, 1 μmole EDTA, 15 μmoles $MgSO_4$ and 15 μmoles of αKGA in a final volume of 3 ml. The incubation was carried out for 1 hour in air.

Nature of the product

The requirement for glycine in the oxidation of HHBA indicated that HA is the major product in this reaction. This was confirmed by comparing the absorption spectrum of an authentic sample of HA with that of the extracted product in 0.1 *N* HCl or NaOH. Upon hydrolysis, the product acquired an absorption peak characteristic of benzoic acid at 275 mμ in 0.1 *N* HCl. Further evidence for the formation of HA was obtained by ascending paper chromatography on Whatman No. 1 paper. The solvent system used was 1-butanol–ammonium carbonate buffer* in which the R_F values for HA, benzoic acid and HHBA were 0.15, 0.24, and 0.38, respectively. When 4 μ*M* of HHBA-1-^{14}C were incubated under the condition described in Table I, the radioautogram of the paper chromatogram showed an almost quantitative conversion of HHBA to HA. If the incubation mixture was hydrolyzed before applying it to paper, a spot arose which corresponded to benzoic acid. The radioautogram from the incubation mixture without glycine showed that the major portion of the substrate was unmetabolized and only a trace of HA and a small spot for benzoic

* 1-Butanol is saturated with an aqueous solution which is 1.5 *N* NH_3 and 1.5 *N* $(NH_4)_2CO_3$.

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acid were detected. Thus, it is apparent that aromatization of HHBA is favored by the removal of benzoic acid as HA. CHELDELIN AND BEINERT[13] also have found that the oxidation of phenyl fatty acids is stimulated by the addition of glycine. They attributed this fact to the inhibitory effect of benzoic acid on the fatty acid oxidizing system.

Participation of HHBCoA

From the foregoing experiments one cannot conclude whether the condensation of glycine with the ring compound takes place before or after the aromatization reaction. In order to establish this point hexahydro-HA was used as a substrate. Before incubation, a sample of this was hydrolyzed by autoclaving overnight in a saturated $Ba(OH)_2$ solution and was then neutralized with H_2SO_4. The control sample also received $Ba(OH)_2$ and H_2SO_4 but was not autoclaved. Results in Table II show that only the free HHBA is oxidized.

TABLE II

AROMATIZATION OF FREE HHBA

	μmoles of HA formed
Hexahydro-HA, control, 10 μmoles	0
Hexahydro-HA, hydrolyzed, 10 μmoles	1
HHBA, 10 μmoles	2

Incubation condition was the same as described under Table I, except that 10 μmoles of glycine were added to each beaker. The amount of mitochondria used was equivalent to 0.5 g of liver.

TABLE III

AROMATIZATION OF HHBCoA

	μmoles of benzoic acid or HA formed
10 μ*M* HHBA, unfortified	0.08
1 μ*M* HHBCoA, unfortified	0.36
10 μ*M* HHBA, fortified	2.62

Incubation condition was as described under Table I. Mitochondria equivalent to 0.5 g of liver were used.

Since the aromatization reaction is similar to the dehydrogenation of fatty acids, the activation of HHBA by the formation of HHBCoA as in fatty acid oxidation[14] was considered likely as the initial step. Thus, HHBCoA was prepared and incubated with the unfortified mitochondria.

As shown in Table III, HHBCoA was oxidized under conditions where negligible amount of HHBA was oxidized. The presence or absence of glycine did not alter the magnitude of benzoic acid formation from HHBCoA. This may be because the amount of benzoic acid formed was not large enough to inhibit the aromatization reaction. It is clear that the requirements for the energy generating factors in this reaction are for the formation of HHBCoA which is then dehydrogenated and conjugated with glycine to form HA. The latter step has been studied recently by SCHACHTER AND TAGGART[6].

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TABLE IV

SPECIFICITY FOR SUBSTRATE AND ACCEPTOR

Substrate	*Acceptor*	*μmoles of benzoic acid or HA formed*
HHBA	None	1.1
HHBA	Glycine	4.6
Quinic acid	Glycine	0.0
Shikimic acid	Glycine	0.0
HHBA	Sarcosine	3.4
HHBA	Betaine	0.0
HHBA	β-Alanine	0.0
HHBA	Alanine	0.0
HHBA	Taurine	0.0
HHBA	Serine	0.0
HHBA	Ornithine	0.0
HHBA	Arginine	0.0
HHBA	Glutamine	0.0

Incubation condition was as described under Table I. 10 μmoles of substrate, 30 μmoles of acceptor, and mitochondria equivalent to 0.5 g of liver were used.

Specificity for substrate and acceptor

Table IV summarizes the specificity of the present system for compounds which can serve as substrates and for compounds which act as acceptors in stimulating the oxidation of HHBA.

The stimulation of HHBA oxidation by sarcosine was not surprising since sarcosine is known to give rise to glycine by the action of sarcosine oxidase in the mitochondria[15]. The oxidation of quinic acid could not be demonstrated even with a human liver homogenate although feeding experiments indicated a fairly rapid oxidation of this compound in man[3].

Other properties of the system

The aromatization of HHBA by guinea pig liver mitochondria took place at a rate of 4–8 μmoles per g of liver per h. The reaction proceeded linearly up to 1 hour and showed an optimal pH range of 7.0–8.0. Under the incubation conditions already described 8 μmoles of HHBA were required to saturate the enzyme system. Guinea pig kidney mitochondria showed considerably less activity than the liver mitochondria. Rabbit liver showed activity comparable to that of guinea pig but was not consistently active. A very weak activity was detected with rat liver mitochondria, but the mitochondria from cat, mouse, dog, monkey, toad, human, chicken, and pigeon livers did not oxidize HHBA. Replacing glycine with ornithine in the case of chicken or pigeon liver, experiments still gave negative results.

DISCUSSION

The data presented above establish the following sequence of reactions in the formation of HA from HHBA:

(a) HHBA + CoA + ATP $\xrightarrow{Mg^{++}}$ HHBCoA

(b) HHBCoA ⟶ benzoyl CoA

(c) benzoyl CoA + glycine ⟶ HA

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Steps (a) and (b) are quite analogous to the activation and β-oxidation of fatty acids. The question arises as to what extent the present system overlaps with the fatty acid oxidase system. BEER *et al.*[3] have shown that the fatty acid dehydrogenase system did not dehydrogenate HHBA. It is possible that the HHBA activating enzyme is particularly labile and that their preparation was lacking in this enzyme. We have found that kidney oxidizes HHBA to one-eighth the extent of an equivalent weight of liver. This may be attributed to difference in the amount of HHBA activating enzyme in the two organs, since kidney oxidized 1/2 as much HHBCoA as the liver. The inability of heart, another organ rich in the fatty acid oxidase system[16], to oxidize HHBA may also be attributable to lack of the HHBA activating system. Liver and kidney mitochondria and heart extract catalyzed the decolorization of 2,6-dichlorophenolindophenol by HHBCoA.

The failure to demonstrate the oxidation of HHBA with human and dog liver preparations despite the positive *in vivo* findings by BERNHARD[17] points to the lability of this enzyme system.

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SUMMARY

1. The enzyme system which aromatizes cyclohexanecarboxylic acid has been shown to be localized in the mitochondrial fraction of guinea pig liver.

2. The sequence of reactions has been shown to be the formation of the CoA derivative of the acid, dehydrogenation and the conjugation with glycine to form hippuric acid.

3. The removal of the oxidation product, benzoic acid, as hippuric acid greatly accelerated the oxidation reaction.

4. Analogy between the present system and the fatty acid oxidase system has been discussed.

REFERENCES

1 E. LAUTEMANN, *Liebigs Ann. Chem.*, 125 (1863) 9.
2 F. DICKENS, *Biochem. Soc. Symposia (Cambridge, Engl.)*, No. 5 (1950) 66.
3 C. T. BEER, F. DICKENS AND J. PEARSON, *Biochem. J.*, 48 (1951) 222.
4 M. GODCHOT, *Bull. soc. chim., France*, [4] 9 (1911) 261.
5 I. B. WILSON, S. GINSBERG AND E. K. MEISLICH, *J. Am. Chem. Soc.*, 77 (1955) 4286.
6 D. SCHACHTER AND J. V. TAGGART, *J. Biol. Chem.*, 203 (1953) 925.
7 J. S. LUMSDEN, *J. Chem. Soc.*, 87 (1905) 90.
8 R. R. GRUNERT AND P. H. PHILLIPS, *Arch. Biochem.*, 30 (1951) 217.
9 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
10 E. R. STADTMAN, *J. Biol. Chem.*, 196 (1952) 535.
11 G. H. HOGEBOOM, W. C. SCHNEIDER AND G. E. PALLADE, *J. Biol. Chem.*, 172 (1948) 619.
12 F. DICKENS AND J. PEARSON, *Biochem. J.*, 48 (1950) 216.
13 V. H. CHELDELIN AND H. BEINERT, *Biochim. Biophys. Acta*, 9 (1952) 661.
14 G. R. DRYSDALE AND H. A. LARDY, *J. Biol. Chem.*, 202 (1953) 119.
15 C. G. MACKENZIE, *J. Biol. Chem.*, 186 (1950) 351.
16 D. E. GREEN AND H. BEINERT, *Symposium on Phosphorus Metabolism*, 1 (1951) 330.
17 K. BERNHARD, *Hoppe-Seylers Z. physiol. Chem.*, 248 (1937) 256.

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