ALTERNATE ROUTES FOR UBIQUINONE BIOSYNTHESIS IN RATS

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SUMMARY

Mitochondrial preparations from rat heart and liver were able to prenylate 3,4-dihydroxybenzoic acid (protocatechuic acid) and 3-methoxy-4-hydroxybenzoic acid (vanillic acid). Rat heart slices when incubated with 3,4-dihydroxy [U-^{14}C] benzoic acid could incorporate the label to ubiquinone. Rat heart slices were also able to convert 4-hydroxybenzoic acid to 3,4-dihydroxybenzoic and 3-methoxy-4-hydroxybenzoic acids, indicating alternate pathways for ubiquinone biosynthesis in mammals.

INTRODUCTION

Previous studies have shown that 4-HB is the precursor of the quinone ring of ubiquinone in mammalian systems and that an early step is the prenylation of the aromatic ring (1-6). In bacteria the next step is the formation of the polyprenyl phenol via decarboxylation. In mammalian systems an unresolved question concerns the stage at which decarboxylation of the prenylated 4-HB occurs. One of the alternate possibilities is decarboxylation of prenyl derivatives of PA and VA. In this communication we present evidence showing that an alternate ubiquinone biosynthetic pathway involving PA and VA does occur.

MATERIALS AND METHODS

Preparation and Incubation of Rat Heart Slices

Sprague-Dawley female rats (175-200 g) were anaesthetized with nembutal (35 mg/Kg body wt.). The hearts were suspended in modified calcium and glucose free Hank's buffer (7) gassed with 95% O_2 and 5% CO_2. Heart slices were prepared with a Stadie-Riggs slicer. The slices (2 g wet wt.) were suspended in 10 ml of Krebs improved Ringer-medium I (8) without Ca^{++} and glutamate, saturated with 95% O_2 and 5% CO_2. During the incubation the flasks were gassed with the same mixture. This incubation system was used for total synthesis of ubiquinone from PA and 4-HB.

Isolation and Incubation of Mitochondrial Preparations for Polyprenyltransferase Activity

Rat liver mitochondria were isolated as described by Momose and Rudney (4). Heart tissue was first homogenized with a polytron and mitochondria were then isolated the same way as described for the liver.

Abbreviations: 4-HB, 4-hydroxybenzoic acid; PA, protocatechuic acid; VA, vanillic acid; SAM, S-adenosyl-L-methionine.

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In a typical incubation, washed mitochondria were suspended in 1 ml of 10 mM potassium phosphate buffer, pH 7.3, supplemented with 10 mM $MgCl_2$, 0.01% Triton X-100, 1 ml of *Micrococcus lysodeikticus* and isopentenyl pyrophate mixture (9), and 10 mM of the respective aromatic substrates. Incubation was for 2 h with constant shaking at 37°, and the reaction was terminated by mixing with 3.75 volumes of chloroform-methanol (1:2 V/V) containing non-radioactive substrate (0.5 mg/ml). After allowing to stand overnight at room temperature, lipids were extracted according to Gaillard, *et al.* (10) and washed as described by Momose and Rudney (4). The extracts were then subjected to further analysis.

Lipophylic Sephadex (LH-20) Column Chromatography

Lipid fractions obtained from tissue incubations were dissolved in 1 ml of chloroform-methanol mixture (1:1) and chromatographed on Sephadex LH-20 column (1 cm x 58 cm) equilibrated with chloroform-methanol (1:1). 0.9 ml fractions of eluate were collected and aliquots (100 µl) from each fraction were counted for radioactivity.

Thin Layer Chromatography

The radioactive fractions from Sephadex LH-20 column eluates were pooled and subjected to thin layer chromatography on silica gel-G plates (250 microns) developed in acetone-petroleum ether (30:100) as solvent. Ubiquinone moved with an Rf of 0.9 in this system. Lipid fractions from mitochondrial incubations were chromatographed similarly using 15% acetone in petroleum ether. In this system prenylated products of 4-HB, PA and VA had Rfs of 0.58, 0.27 and 0.63 respectively.

Radioactivity Measurements

Radioactive areas on the thin layer plates were detected with Packard Radiochromatogram Scanner, Model 7201. Radioactivity was determined quantitatively using Beckman LS3145P liquid scintillation spectrometer. The scintillation fluid (10 ml/ml) consisted of 2,5-diphenyloxazole (5 g/l) and 1,4-bis[2-(5-phenyloxazolyl)] benzene (0.2 g/l) in toluene and Triton X-100 (1/3 V/V).

Isolation and Identification of PA and VA as Products from 4-4B

Heart slices were incubated with 2 µCi of 4-hydroxy[U-^{14}C] benzoate in Krebs Ringer-medium I (8) as described. The lipid fractions were extracted (4) and the non-lipid fractions and combined washings were acidified to pH 2 and stored at 0-4°C overnight. The precipitated salts were filtered out and the filtrate was saturated with NaCl. The free phenolic acids were extracted three times into equal volumes of ethylacetate, and then transferred into 1 M $KHCO_3$ (2 extractions with 20 ml each). The alkaline solution was brought to pH 2 with HCl and was extracted three times with 40 ml of ethylacetate. The combined extract was dried over anhydrous sodium sulfate and then reduced to a small volume and chromatographed bidimensionally on precoated cellulose plates (500 microns) with non-radioactive PA and VA as markers. The solvents were benzene-acetic acid-water (10:7:3 by volume; upper phase) in the first direction and 2% aqueous formic acid in the second direction. The Rf values for PA, VA and 4-HB respectively were 0.02, 0.72, and 0.46 in the first solvent system; and 0.57, 0.57, and 0.68 in the second solvent system. PA and VA were visualized under UV light and the spots were eluted and radioactivity determined.

Chemicals

4-hydroxy[U-^{14}C]benzoic acid (20 mCi/mmole) was synthesized by alkali fusion of L-[U-^{14}C]tyrosine by the method of Parson and Rudney (11).

TABLE I

Formation of PA and VA from 4-HB in Rat Heart Slices

Experiment	PA Formed (n moles)	VA Formed (n moles)
I	6.5	4.0
II	10.0	4.0

Rat heart slices (2g) were incubated at 37° C with 5 μM 4-hydroxy [U-^{14}C]benzoic acid in 10 ml of Krebs improved Ringer-medium I (8), without Ca^{++} and glutamate, saturated with 95% O_2 and 5% CO_2. During the incubation, the reaction vessels were gassed with the same gas mixture. After 3 h, the slices were homogenized with glass beads and mixed with 3.75 volumes of chloroform-methanol (1:2). After allowing to stand overnight at room temperature, lipids were extracted according to Gaillard, *et al.* (10) and washed 4 times as described by Momose and Rudney (4). The aqueous phase and the combined washings were pooled and analyzed for the formation of PA and VA.

4-hydroxy[carboxyl-^{14}C]benzoic acid (55 mCi/mmole) and vanillic acid [carboxyl-^{14}C] (53 mCi/mmole) was purchased from Schwartz/Mann, Division of Becton, Dickinson and Company, New York. Protocatechuate [U-^{14}C or carboxyl-^{14}C] was prepared enzymatically using p-hydroxybenzoate hydroxylase (E.C. 1:14:13:2), a gift from Dr. V. Massey. The non-radioactive chemicals were of the best grade available commercially.

RESULTS

Formation of PA and VA from 4-HB

Microorganisms are known to hydroxylate 4-HB to PA (12). However, this hydroxylation reaction has not been demonstrated in mammals. Analysis of the incubation mixtures for total synthesis of ubiquinone from 4-HB using heart slices, clearly indicated the formation of PA as a product from 4-HB (Table I). It may be noted that in addition to PA, its methylated derivative, VA also was detected in the medium.

Prenylation of PA and VA

PA and VA meet the structural requirements (13) for prenylation by the enzyme 4-HB:polyprenyl transferase. Our experiments were aimed not only to find out whether PA served as a substrate for prenylation by rat tissues, but also to assess how it compared with 4-HB as a substrate. The results are presented in Table II. It is evident from the data that both heart and liver mitochondrial systems can prenylate PA. Confirming our earlier finding (13) heart muscle proved to be more active with respect to polyprenyl transferase

TABLE II

Prenylation of 4-HB, PA, and VA by Rat Heart and Liver Mitochondrial Preparations

Substrate	p moles of Prenylated Products Formed		Relative Efficiency(%)	
	Heart	Liver	Heart	Liver
4-HB	710	500	100	100
PA	570	340	80	68
VA	320	100	45	30

1 ml of mitochondrial preparation containing 6 - 8 mg protein from the respective tissues were incubated with 10 mM (final concentration) each of 4-hydroxy[carboxyl-^{14}C]benzoic acid, 3,4-dihydroxy[U-^{14}C]benzoic acid, and 3-methoxy-4-hydroxy[carboxyl-^{14}C]benzoic acid at 37° for 2 h. Other conditions were the same as given under "MATERIALS AND METHODS".

activity; however, 4-HB appeared to be more efficiently prenylated than PA. Evidence for prenylation is based on the following observations (data not shown). Bacitracin, a known inhibitor of the enzyme (14) prevented the formation of the prenylated derivatives of PA and VA. The products were labeled when unlabeled PA and VA were used in the reaction mixture along with [^{14}C] isopentenyl pyrophosphate preincubated with *Micrococcus lysodecticus* cell free extract (9).

Ubiquinone Synthesis from PA

Rat heart slices provided a suitable system for studying complete synthesis of ubiquinone. We could demonstrate incorporation of radioactivity into ubiquinone when 3,4-dihydroxy[U-^{14}C]benzoic acid was incubated with rat heart slices. Figure 1 gives the radioactivity elution pattern of the lipid fraction on LH-20 column. Radioactivity peaks from fractions 22-26, 27-31, 32-37, and 38-40 were pooled separately and subjected to thin layer chromatography. Fractions 22-26 contained ubiquinone (815 d.p.m.). Identities of the compounds eluting in radioactive peaks between fractions 27-31, 32-37, and 38-40 have not been established.

DISCUSSION

Though alkylation of 4-HB has been implicated as the initial step in ubiquinone biosynthetic pathway for microorganisms as well as mammals (1-6), the possibility of 4-HB being hydroxylated and/or methylated prior to

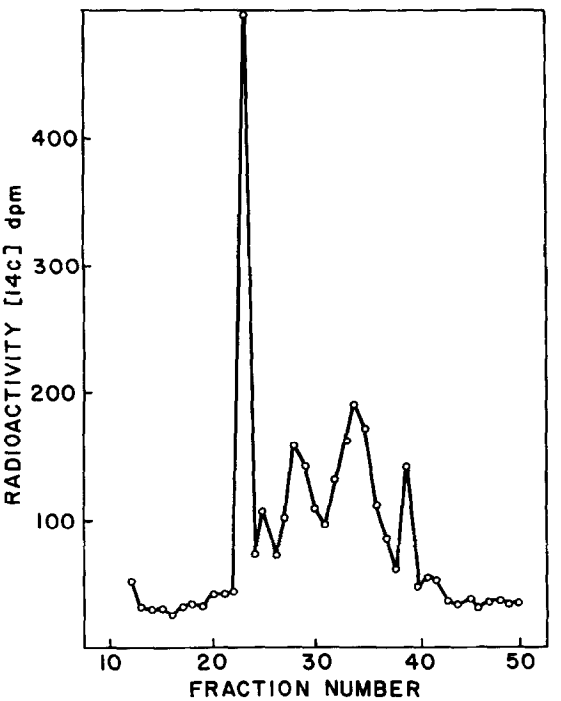

Figure 1. Sephadex LH-20 column chromatography of the lipid fraction from rat heart slices incubated with 3,4-dihydroxy[U-^{14}C]benzoic acid.

Heart slices (2g) were incubated at 37° with 3,4-dihydroxy[U-^{14}C] benzoic acid (5 μM) in 10 ml of Krebs improved Ringer-medium I (8) without Ca^{++} and glutamate, saturated with 95% O_2 and 5% CO_2. During the incubation, the reaction vessels were gassed with the same gas mixture. After 3 h the slices were homogenized and mixed with 3.75 volumes of chloroform-methanol (1:2) mixture. The lipids were extracted as described by Momose and Rudney (4). The extract was evaporated to dryness under nitrogen and taken in 1 ml of chloroform-methanol mixture (1:1) and chromatographed on Sephadex LH-20 column (1 cm x 58 cm) equilibrated with chloroform-methanol (1:1).

alkylation was not envisaged in these studies. Hydroxylation of the benzene ring is one of the steps involved in the degradative metabolism of aromatic hydrocarbons by microorganisms (12). We have shown that 4-HB can be hydroxylated to PA and subsequently O-methylated to yield VA in mammals. Aromatic ring hydroxylases have been purified from bacterial sources and are found to be flavin-containing monoxygeneases (15). The nature of the mammalian enzyme remains to be studied. The subsequent methylation step is presumably catalyzed by catechol-O-methyl transferase or an enzyme with similar properties.

The metabolic sequence for ubiquinone biosynthesis in bacterial system is well documented (6). Thus, in bacteria the prenylated 4-HB is first

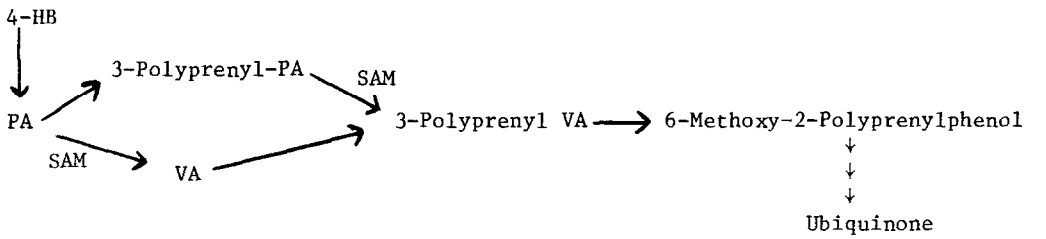

Figure 2.

decarboxylated to yield 2-polyprenyl phenol. However, this compound has not yet been detected in any animal systems, and there is no clear cut evidence to indicate the stage at which decarboxylation of 4-HB occurs. Our finding that 4-HB can be converted to PA and VA by rat tissue and these compounds can be prenylated by mitochondrial preparations indicates the possibility of an alternate pathway for ubiquinone biosynthesis in mammals. We have also found that PA could be incorporated into ubiquinone. This strengthens the hypothesis that alternate pathways exists for ubiquinone biosynthesis in mammals as presented in Figure 2. This hypothesis is also supported by the early observation that PA inhibited the incorporation of 4-HB into ubiquinone in rat kidney (11). The extent to which this pathway plays a role in ubiquinone synthesis remains to be investigated.

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