BIOGENESIS OF UBIQUINONE IN RATS - AN ALTERNATE ORIGIN OF THE BENZOQUINONE MOIETY

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The addition of cyclohexane carboxylic acid (CCA) to the incubation medium results in a dilution of the radioactivity incorporated into ubiquinone-9 (UQ-9) from 1-^{14}C-benzoate by rat liver slices. This effect is more pronounced when the slices are pre-incubated prior to addition of the labeled precursor. A similar dilution by CCA of label incorporation, is observed using U-^{14}C-tyrosine, but not either CH_3-^{14}C-methionine or 2-^{14}C-mevalonate, as precursors. UQ-9, but not cholesterol, isolated from liver slices incubated with ring-U-^{14}C-CCA is found to be labeled. The extent of labeling of UQ-9 by this precursor is enhanced by the presence of an excess of mevalonate in the incubation medium and decreased by the addition of p-hydroxybenzoate. These results suggest that aromatisation of cyclohexane derivatives may serve as a possible source of the benzoquinone nucleus of UQ-9 in the rat.

INTRODUCTION

The benzoquinone ring of ubiquinone-9 (UQ-9) has been shown to be derived from the phenyl amino acids, by a pathway involving benzoate derivatives as intermediates, in the rat (1-4). Studies in several laboratories have provided evidence that benzoic acid may also arise in the animal body as a result of aromatisation reactions. Thus, many species of animals, including the rat, have been shown to be capable of oxidising cyclohexane carboxylic acid (CCA) to benzoic acid, both *in vivo* and *in vitro* (5-10). It has also been reported that the ingestion of quinic or shikimic acids, or of edible plant materials known to contain

appreciable amounts of these acids, leads to increased excretion of benzoic acid (6, 7, 11-13). Such aromatisations have generally been believed to be of no significance other than that of detoxification of exogenous compounds of dietary origin. In the present study evidence has been obtained suggesting the possibility of hydroaromatic compounds serving as precursors of the benzoquinone nucleus of UQ-9 in the rat.

MATERIALS & METHODS

Mevalonolactone, D-quinic acid and shikimic acid were obtained from Mann Research Laboratories (New York) and cyclohexanol and CCA were products of Eastman Organic Chemicals (Rochester). Among the radioactive chemicals used, 1-^{14}C-cyclohexanol and U-^{14}C-L tyrosine were purchased from New England Nuclear Corporation (Boston), while ring-U-^{14}C-benzoic acid, CH_3-^{14}C-L methionine and 2-^{14}C-DL mevalonic acid were obtained from Nuclear Chicago Corporation (Des Plaines). Ring-U-^{14}C-CCA was synthesised from ring-U-^{14}C-benzoic acid by reduction with metallic sodium in boiling isoamyl alcohol (14) and purified by repeated crystallisations of the benzylamine salt from ethyl acetate (9). The benzylamine salt was treated with dilute hydrochloric acid and the free acid was obtained by extraction with ether.

Female rats of the Wistar strain, maintained on a laboratory stock diet and weighing between 100 and 150 g were employed. The animals were killed by decapitation, thoroughly bled, and the livers after quick chilling in cold Krebs-Ringer phosphate buffer were cut into thin uniform slices with the help of a Stadie-Riggs slicer Liver slices were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing the radioactive precursor in an atmosphere of O_2 in a Dubnoff metabolic

shaking incubator at 37.5°C for 3 h. The incubation was terminated by saponifying the liver slices with 10% methanolic KOH in the presence of pyrogallol and the non-saponifiables were extracted with light petroleum ether. UQ-9 was isolated from the non-saponifiables by chromatography on alumina and, after further purification by thin layer chromatography and crystallisation, was assayed spectrophotometrically (15). Cholesterol was precipitated as the digitonide and determined colorimetrically (16). Radioactivity in the samples was measured in a Beckman LS-100 liquid scintillation spectrometer.

TABLE I

Incorporation of ^{14}C-labelled benzoate into UQ-9 by rat liver slices

Addition (2 mM)	Preincubation period h.	Radioactivity in UQ-9 DPM/mg
-	0	13464
Cyclohexanol	0	12878
CCA	0	10440
"	1	9866
"	2	7442
"	3	4928
Shikimic acid	3	13926
Quinic acid	3	11830

Two g liver slices were incubated in 10 ml of Krebs-Ringer phosphate buffer, pH 7.4, at 37.5°C for 3 h with 5 μC of labelled precursor.

RESULTS AND DISCUSSION

In the first set of experiments, the effect of some cyclohexane derivatives on the incorporation of radioactivity from 1-^{14}C-benzoate into UQ-9 by liver slices was investigated. Cyclohexanol, CCA, quinic acid and shikimic acid were added at a concentration of 2mM to the incubation medium either along with or prior to the addition of the labeled precursor as indicated in Table I. Among the compounds tested, CCA alone significantly lowered the labeling of UQ-9. This decrease in labeling was more marked when the slices were pre-incubated with CCA prior to addition of 1-^{14}C-benzoate in the medium. While the decrease in labeling of UQ-9 in the presence of CCA may indicate its aromatisation to benzoic acid and consequent dilution of the specific activity of the labeled precursor, these studies do not rule out a general inhibitory effect.

The latter possibility has been investigated by studying the effect of CCA on the biosynthesis of UQ-9 as evidenced by the incorporation of label from 2-^{14}C-mevalonate into the polyisoprenoid side chain of the molecule. Additionally, the labeling of UQ-9 by other known precursors such as U-^{14}C-tyrosine and CH_3-^{14}C-methionine in liver slices pre-incubated with CCA has also been assessed. In all these experiments, the radioactivity in cholesterol has also been determined and the results are summarised in Table II.

With 2-^{14}C-mevalonate as precursor, CCA did not exhibit any appreciable effect on the labeling of either UQ-9 or cholesterol. Likewise, the incorporation of radioactivity from CH_3-^{14}C-methionine, which has been shown to label exclusively, the O- and C-methyl groups of the benzoquinone ring (17), into UQ-9 was not decreased by pre-

TABLE II

Incorporation of ^{14}C-labeled precursors into UQ-9 and cholesterol by rat liver slices

Precursor	Addition (2 mM)	Radioactivity in UQ-9 DPM/mg	Radioactivity in Cholesterol DPM/mg
2-^{14}-C Mevalonate	-	7267	45680
"	CCA	7048	38940
"	Quinic acid	7566	43620
"	Shikimic acid	6935	39730
U-^{14}C-Tyrosine	-	6442	458
"	CCA	2186	392
CH_3-^{14}C-Methionine	-	17682	-
"	CCA	16896	-

Experimental details as in footnote to Table I. The activities of the labeled precursors added were: mevalonate - 2 μC; tyrosine - 10 μC; methionine - 5 μC.

incubation with CCA. Since the labeling of UQ-9 by 2-^{14}C-mevalonate and CH_3-^{14}C-methionine could be considered as a measure of the rates of biosyntheses of the side chain and ring moieties of the molecule, respectively, CCA does not appear to inhibit the biosynthesis of UQ-9 in liver slices. With U-^{14}C-tyrosine as the precursor, CCA was without appreciable effect on the labeling of cholesterol but was found to decrease the labeling of UQ-9 to an even greater extent than that observed earlier with 1-^{14}C-benzoate. It is conceivable that tyrosine being a more distal precursor of the ring moiety, there occurs a greater dilution due to aromatisation of CCA. These results strongly suggest that the

decrease in the labeling of UQ-9 by 1-^{14}C-benzoate and U-^{14}C-tyrosine in the presence of CCA, is due to dilution arising out of its oxidation yielding an intermediate involved in the biogenesis of the quinone ring of UQ-9.

To test this possibility more directly, the incorporation of radioactivity from 1-^{14}C-cyclohexanol and ring-U-^{14}C-CCA into UQ-9 by liver slices has been studied and the results are presented in Table III. No labeling of either cholesterol or UQ-9 was observed with 1-^{14}C-cyclohexanol; in the dilution experiments (Table I) also this compound did not show any effect on the labeling of UQ-9 by 1-^{14}C-benzoate. It is likely that cyclohexane derivatives lacking the carboxyl group are also aromatised (18), but the resulting benzenoid compounds may not serve as precursors of the benzoquinone moiety of UQ-9 since the presence of the

TABLE III

Incorporation of ^{14}C-labeled CCA into UQ-9 in rat liver slices

Precursor	Addition (5 mM)	Radioactivity in UQ-9 DPM/mg	Radioactivity in Cholesterol DPM/mg
1-^{14}C-cyclohexanol	MVA	-	-
U-^{14}C-CCA	-	6462	-
"	MVA	10875	-
"	p-OH benzoate	547	-

Experimental details as in footnote to Table I. The activities of the labeled precursors added were: cyclohexanol - 10 μC; CCA - 20 μC.

carboxyl group appears to be essential for this purpose (1, 17).

Using U-^{14}C-CCA as a precursor, a significant amount of radioactivity is incorporated into UQ-9, but cholesterol is not labeled. The absence of radioactivity in cholesterol would further suggest that the side chain of UQ-9 is also not labeled since both these moieties arise from a common isoprenoid pool; U-^{14}C-CCA may, therefore, be inferred to label only the ring portion of UQ-9. This is also supported by the finding that the addition of an excess of mevalonate to the incubation medium enhances the incorporation of radioactivity into UQ-9, an effect similar to that observed with other labeled precursors of the benzoquinone ring of UQ-9 such as 1-^{14}C-benzoate, U-^{14}C-p-hydroxy benzoate and CH_3-^{14}C-methione (1, 17). Further, the addition of p-hydroxy benzoate to the medium greatly decreases the incorporation of label into UQ-9 from U-^{14}C-CCA and is presumably due to dilution since p-hydroxybenzoate is an intermediate in the biosynthesis of the aromatic ring of UQ-9 (1-4).

The present studies clearly show that CCA can give rise to benzoate in rat tissues and can thus serve as a precursor of the benzoquinone moiety of UQ-9; this is the only indication so far of an alternate origin for the benzoquinone ring of UQ-9 in the rat. It is our belief that this is also the first reported instance where an aromatisation reaction in the animal body has been shown to have a physiological significance other than mere detoxification. While this pathway may be of little or no importance in the normal animal, in which the synthesis of the polyisoprenoid side chain of the molecule appears to be the rate-limiting factor, it could assume greater significance under conditions in which the formation of benzoate intermediates from phenyl amino acids is lowered. It is interesting to note in this context that rats maintained on

a protein deficient diet for upto thirty days still contained significant amounts of UQ-9 in liver (19) and that phenylketonuric subjects have been reported to have normal urinary output and, by inference, also tissue levels of UQ-10 (20).

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