The Regulation of the Biosynthesis of Ubiquinone in the Rat

By SUBRAMANIAN RANGANATHAN and TANGIRALA RAMASARMA
Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

(Received 1 October 1974)

The urinary excretion of *p*-hydroxybenzoate was not altered by ubiquinone feeding, but, although decreased considerably, was not eliminated in protein deficiency. The incorporation of *p*-hydroxy[U-¹⁴C]benzaldehyde into ubiquinone *in vivo* increased in cold-exposed and *p*-chlorophenoxyisobutyrate (clofibrate)-fed rats, and these changes were parallel with the changes in the incorporation of [2-¹⁴C]mevalonate under these conditions. Starvation, cholesterol feeding and cholic acid feeding resulted in the decreased incorporation of *p*-hydroxy[U-¹⁴C]benzaldehyde into ubiquinone, confirming the decreased ubiquinone synthesis. Feeding exogenous ubiquinone increased the hepatic ubiquinone concentration, but did not cause any decrease in the incorporation of *p*-hydroxy[U-¹⁴C]benzaldehyde into ubiquinone, indicating the absence of a feedback control.

The biosynthesis of ubiquinone involves two pathways, one for the formation of the benzoquinone nucleus and the other for the formation of the isoprene side chain. The precursor for the benzoquinone nucleus is *p*-hydroxybenzoate, derived from tyrosine. The pathway for the formation of *p*-hydroxybenzoate, however, has not been fully established. Rats excrete *p*-hydroxybenzoate and other phenolic acids in the urine, and the excretion of these is enhanced by the administration of excess of tyrosine (Booth *et al.*, 1960).

The amount of *p*-hydroxybenzoate excreted by normal rats, however, is far in excess of its possible requirement in ubiquinone biosynthesis, its only known metabolic involvement.

The isoprene pathway, probably up to the formation of isopentenyl pyrophosphate, is shared by ubiquinone and sterols, although with vastly differing rates (Ramasarma, 1972). The rate of synthesis of sterols from mevalonate is several times greater than that of ubiquinone. The question arises as to which component is limiting for the biosynthesis of ubiquinone, the side chain or the ring precursor. The biogenesis of ubiquinone from mevalonate has been shown to be altered under various conditions (Ramasarma, 1972). A comparative study on the metabolism of the aromatic part of the molecule is described in the present paper.

Experimental

Materials

DL-[2-¹⁴C]Mevalonic acid lactone (5.6mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-[U-¹⁴C]Tyrosine (190mCi/mmol) was obtained from the Bhabha

Atomic Research Centre, Bombay, India. Ubiquinone-9 and α -*p*-chlorophenoxyisobutyrate (clofibrate) were gifts from Hoffmann-La Roche, Basle, Switzerland, and Dr. J. M. Thorp, Imperial Chemical Industries, Macclesfield, Cheshire, U.K., respectively. *p*-Hydroxybenzoic acid was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals were of AnalaR grade, obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals and diets

Male albino rats (weighing about 150 g) from the stock colony were used. The diet consisted of vitamin-free casein (20%), peanut oil (5%), sugar (10%), salt mixture (U.S. Pharmacopeia, 1947) (5%) and starch (60%). Vitamins were provided according to U.S. Pharmacopeia (1947). Food and water were given *ad lib*. Where supplements were given, they were mixed with the diet as specified. The diet was completely withdrawn for 48 h for starvation experiments. The casein was replaced by starch in the protein-free diet (Joshi *et al.*, 1963). Where mentioned, rats were exposed to a low environmental temperature of 0–5°C in a refrigerated chamber which was provided with a slow stream of fresh air.

Methods

Determination of *p*-hydroxybenzoate in the urine. Rats were kept in metabolic cages and urine was collected in flasks containing 1 ml of 2M-HCl, to prevent bacterial growth; faecal contamination in the urine was avoided during the collection. The cages and the funnels were rinsed with water before collection of samples. The volume was made up to

10ml and samples were acidified to a final concentration of 4M-HCl and heated in a boiling-water bath for 1h to hydrolyse conjugated phenolic acids. The samples were cooled to room temperature (about 25°C) and extracted three times with 3ml portions of diethyl ether. The ether extracts were evaporated to dryness, the residue was dissolved in a small volume of ethyl acetate, and *p*-hydroxybenzoate was separated by paper chromatography. The details of the methods of separation and determination are as described earlier (Ranganathan & Ramasarma, 1971).

Preparation of *p*-hydroxy[U-¹⁴C]benzaldehyde and its incorporation into ubiquinone. Tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5; tyrosine transaminase) was purified from rat liver by the procedure of Granner *et al.* (1968), up to the step of DEAE-cellulose column chromatography. The fraction containing enzyme was freeze-dried at this stage. L-[U-¹⁴C]Tyrosine (10μCi) was incubated with the purified transaminase, in the presence of 2-oxoglutarate (2μmol) and pyridoxal phosphate (0.1μmol), at 37°C for 15min. At the end of the incubation the mixture was made alkaline by adding 0.5ml of 1M-NaOH, to convert *p*-hydroxyphenylpyruvate formed during the incubation into *p*-hydroxybenzaldehyde. After 30min the mixture was extracted three times with 3ml portions of diethyl ether. The ether extracts, which contained *p*-hydroxy[U-¹⁴C]-benzaldehyde, were chromatographed on paper with aq. 2% (v/v) formic acid as developing solvent. *p*-Hydroxybenzaldehyde, which moved as a single spot, was eluted with ethanol, evaporated to dryness and stored for incorporation studies.

To study the incorporation of *p*-hydroxy[U-¹⁴C]-benzaldehyde into ubiquinone, 0.5μCi of this compound was injected intraperitoneally into each rat in 0.2ml of water. After definite time-intervals the rats were killed, the livers removed and ubiquinone was isolated. The incorporation was linear with time and reached a maximum at 8h. For all the experiments a 2h period was chosen to compare the changes in the rates of synthesis. Results of preliminary experiments showed that over 90% of the radioactivity in the non-saponifiable fraction was associated with ubiquinone obtained mainly in the 5%-ether fraction after chromatography on a deactivated-alumina column. The specific radioactivity was not altered significantly when this ubiquinone was purified by repeated t.l.c. [silica gel G; developing solvent: acetone–light petroleum (1:9, v/v)]. Therefore in all the experiments where ubiquinone synthesis was studied under various conditions the radioactivity in the 5%-ether fraction, after alumina column chromatography, was measured and taken as the measure of ubiquinone synthesis. The methods for the isolation and determination of ubiquinone are as described by Ramasarma & Jayaraman (1971).

Incorporation of [2-¹⁴C]mevalonate into ubiquinone in the rat liver slices. Freshly obtained rat liver was cut into slices of 0.5mm thickness with a tissue slicer (Arthur Thomas and Co., Philadelphia, Pa., U.S.A.). The slices were suspended in 5ml of Krebs–Ringer phosphate buffer (pH7.4) (Krebs & Henseleit, 1932) and incubated with 0.2μCi of [2-¹⁴C]mevalonate at 37°C for 2h. At the end of the incubation, the contents were saponified and ubiquinone was isolated. Carrier ubiquinone (1mg) was added before saponification and ubiquinone was purified by column chromatography and t.l.c.

Results

Urinary excretion of *p*-hydroxybenzoate

p-Hydroxybenzoate derived from the catabolism of tyrosine, an essential amino acid, is primarily used for the biosynthesis of ubiquinone. Two conditions, of feeding ubiquinone (Krishnaiah *et al.*, 1967) or protein-free diet (Joshi *et al.*, 1963), known to decrease biosynthesis of ubiquinone, have been tested for the changes in urinary excretion of *p*-hydroxybenzoate. As shown in Fig. 1, there was no significant change in the quantity of *p*-hydroxybenzoate excreted in the urine of the rats fed with exogenous ubiquinone for 15 days. This is not unexpected in view of the large excess of excretion of this compound relative to the requirement for biosynthesis of ubiquinone. On the other hand, the amount of *p*-hydroxybenzoate excreted decreased

Fig. 1. Excretion of *p*-hydroxybenzoate in ubiquinone-fed rats

Two rats were kept in each metabolic cage and urine was collected as described in the text. Ubiquinone was given at 1mg/day per rat, dissolved in 0.1ml of peanut oil. Each point represents the average of 12 rats kept in six cages and processed individually. The vertical lines indicate s.d.

Fig. 2. Excretion of *p*-hydroxybenzoate in protein deficiency

Urine was collected and analysed as described in the text. Two rats were kept in each metabolic cage and were given a protein-free diet. In protein re-feeding, complete synthetic diet containing casein was fed to the rats. Each point represents the average of three individual analyses with two rats in each.

gradually over a period of 10 days when the rats were fed on a protein-free diet (Fig. 2), indicating dependence on the availability of tyrosine. When protein was re-fed for about 10 days almost the normal extent of excretion was restored.

Incorporation of p -hydroxy[U - ^{14}C]benzaldehyde into ubiquinone under various conditions

The biosynthesis of ubiquinone, as measured by the incorporation of [2 - ^{14}C]mevalonate, was increased on exposure to low environmental temperature (Aithal *et al.*, 1968) and on feeding Clofibrate (Krishnaiah & Ramasarma, 1970) during the initial phases of the treatments. The incorporation of *p*-hydroxy[U - ^{14}C]benzaldehyde into ubiquinone was also higher in cold-exposed and Clofibrate-fed rats, compared with that in the normal rats (Table 1). It should be pointed out that under the conditions tested the increase in ubiquinone content was not highly significant. It has been shown (Aithal *et al.*, 1968; Krishnaiah & Ramasarma, 1970) that the ubiquinone content increased 2–3-fold, only at later stages of these treatments, whereas the increased rate of synthesis from mevalonate was observed during the initial 10 days. The present studies with the precursor of the aromatic ring confirm the increased synthesis within this initial period.

There are some situations where the biosynthesis of ubiquinone from acetate is known to be lowered. Under these conditions the rate-limiting step in isoprene synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase, is decreased to a very low activity and the synthesis of mevalonate is impaired. Incorporation of *p*-hydroxybenzaldehyde was tested under some of

Table 1. Incorporation of *p*-hydroxy[U - ^{14}C]benzaldehyde into ubiquinone under various conditions

The rats were injected with 0.5 μ Ci of *p*-hydroxy[U - ^{14}C]benzaldehyde (148 mCi/mmol) intraperitoneally, and killed after 2 h. Clofibrate, cholesterol and cholic acid were mixed with the diet at 0.5%, 1.0% and 2.0% (w/w) respectively. Four rats were used in each group. Means $\pm$ s.d. are given. All the changes are statistically significant at $P < 0.02$ as compared with the control.

Treatment of rats	Ubiquinone incorporation	
	(nmol/g of fresh liver)	(c.p.m./g of fresh liver)
Control	121 $\pm$ 11	1950 $\pm$ 350
Clofibrate-fed (10 days)	150 $\pm$ 13	3900 $\pm$ 1400
Cold-exposed (10 days)	147 $\pm$ 16	2980 $\pm$ 580
Starved (48 h)	150 $\pm$ 34	650 $\pm$ 220
Control	113 $\pm$ 20	1650 $\pm$ 340
Cholesterol-fed (10 days)	82 $\pm$ 12	660 $\pm$ 90
Cholic acid-fed (3 days)	58 $\pm$ 26	600 $\pm$ 220

Table 2. Incorporation of *p*-hydroxy[U - ^{14}C]benzaldehyde into ubiquinone in ubiquinone-fed rats

The rats were injected with 0.5 μ Ci of *p*-hydroxy[U - ^{14}C]benzaldehyde (148 μ Ci/ μ mol) intraperitoneally, and killed after 2 h. Ubiquinone-9 was fed to the rats orally (1.5 mg/day), dissolved in peanut oil, for 15 days. The control rats were given an equal volume (0.1 ml) of peanut oil. Four rats were used in each group. Means $\pm$ s.d. are given. The changes in the concentrations are significant (P values < 0.05) but not those for incorporation.

Expt. no.	Treatment	Ubiquinone incorporation	
		(nmol/g of fresh liver)	(c.p.m./g of fresh liver)
1	Control	121 $\pm$ 11	1950 $\pm$ 350
	Ubiquinone-fed	268 $\pm$ 22	1920 $\pm$ 410
2	Control	128 $\pm$ 38	678 $\pm$ 220
	Ubiquinone-fed	226 $\pm$ 26	605 $\pm$ 120
3	Control	152 $\pm$ 15	535 $\pm$ 175
	Ubiquinone-fed	245 $\pm$ 16	730 $\pm$ 60
4	Control	105 $\pm$ 15	840 $\pm$ 110
	Ubiquinone-fed	143 $\pm$ 14	710 $\pm$ 40
Mean of Expts. 1–4			
	Control	126	1000
	Ubiquinone-fed	221	991

these conditions. Table 1 shows that the biosynthesis of ubiquinone from *p*-hydroxybenzaldehyde is lowered in starved, cholesterol-fed and cholic acid-fed rats. These studies also confirm the decreased synthesis of ubiquinone under these conditions.

The above set of conditions for increased or decreased synthesis of ubiquinone, previously observed with mevalonate as the precursor for the

Table 3. *Effect of addition of p-hydroxybenzoate on the incorporation of [2-¹⁴C]mevalonate into ubiquinone*

Liver slices (300–400 mg) from a normal rat were incubated in 5 ml of Krebs–Ringer phosphate buffer (pH 7.4) with 0.2 μ Ci of [2-¹⁴C]mevalonate (5 μ Ci/ μ mol) for 2 h at 37°C. *p*-Hydroxybenzoate was added as a neutral solution, where indicated. The reaction was stopped by adding 15 ml of ethanol and 0.5 ml of 10M-NaOH. The method for the isolation of ubiquinone is as given in the text.

<i>p</i> -Hydroxybenzoate added (mM)	Incorporation (c.p.m./mg of ubiquinone)
—	2700
—	2480
0.1	2380
0.1	2600
0.4	2480
1.0	3050

isoprene side chain, are now confirmed with *p*-hydroxybenzaldehyde as the tracer for the benzoquinone nucleus.

Incorporation of p-hydroxy[U-¹⁴C]benzaldehyde into ubiquinone in ubiquinone-fed rats

Krishnaiah *et al.* (1967) found that feeding ubiquinone to the rat caused a marginal decrease in the incorporation of [2-¹⁴C]mevalonate into ubiquinone. Regulation of the biosynthesis of ubiquinone at a step after the branching in the isoprene pathway was suggested, especially during cold exposure (Aithal *et al.*, 1968). When the precursor of the ring, *p*-hydroxybenzaldehyde, was used to study the biosynthesis of ubiquinone, it was found that the incorporation was not altered in rats fed on ubiquinone (Table 2). The extent of increase in the content of ubiquinone in the liver varied in different experiments, but even when it was doubled there was no change in the incorporation of *p*-hydroxy-[U-¹⁴C]benzaldehyde into ubiquinone.

Effect of exogenous p-hydroxybenzoate on the biosynthesis of ubiquinone from [2-¹⁴C]mevalonate in rat liver slices

Aiyar & Olson (1972) have shown that the addition of exogenous mevalonate enhanced the incorporation of [¹⁴C]benzoate into ubiquinone in rat liver slices. This effect was very prominent when starved rats were used for the experiment. It was decided to test the converse experiment to see whether exogenous *p*-hydroxybenzoate has any effect on the incorporation of [2-¹⁴C]mevalonate into ubiquinone in the liver slices. The results given in Table 3 show that the incorporation of [2-¹⁴C]mevalonate into ubiquinone is not stimulated by the presence of exogenous

p-hydroxybenzoate. It has been shown that *p*-hydroxybenzoate and other phenolic acid intermediates inhibited the isoprene synthesis at a step before the formation of isopentenyl pyrophosphate, when added *in vitro* to cell-free systems from rat liver (Ranganathan & Ramasarma, 1973). Although 1 mM-*p*-hydroxybenzoate inhibited biosynthesis of sterols from mevalonate (Ranganathan & Ramasarma, 1973) by about 50% in the cell-free system, no inhibition of biosynthesis of ubiquinone was observed in the present experiment. The lack of stimulatory effect of exogenous *p*-hydroxybenzoate on ubiquinone synthesis from labelled mevalonate may not be due to the lack of entry of *p*-hydroxybenzoate into the cells because labelled *p*-hydroxybenzoate and benzoate are known to be incorporated into ubiquinone in rat liver slices. However, the concentration of *p*-hydroxybenzoate built up inside the cells may not be sufficient for inhibition. In view of these considerations and of its large excretion, *p*-hydroxybenzoate does not appear to be limiting for the biosynthesis of ubiquinone.

Discussion

Large excesses of *p*-hydroxybenzoate and other phenolic acids are excreted in the urine. The amount of *p*-hydroxybenzoate produced in the animals is therefore severalfold in excess of what is required for the biosynthesis of ubiquinone. The rate of biosynthesis of ubiquinone in the liver of the rat is about 0.05 μ mol/day (calculated from the present experiments), and this forms the major portion of ubiquinone synthesized in the animal, whereas ten times this quantity (0.5 μ mol) of *p*-hydroxybenzoate is excreted per day in the urine. Even when the animals were fed on a protein-free diet, the excretion of *p*-hydroxybenzoate was not abolished, although it was decreased to a low value. There was no change in the quantity of *p*-hydroxybenzoate excreted by the rats when fed on the end product, ubiquinone. There appears to be no relationship between the hepatic content or rate of synthesis of ubiquinone and the production and excretion of its ring precursor, *p*-hydroxybenzoate. These observations, however, do not give any clues to the regulation of the pathway except to indicate that there may be no rate-limiting step in this pathway and that *p*-hydroxybenzoate may be made use of at a constant rate dependent on the rate of synthesis of the side chain and the excess being excreted.

If *p*-hydroxybenzoate is considered to be formed by the pathway involving the non-enzymic formation of *p*-hydroxybenzaldehyde from *p*-hydroxyphenylpyruvate (Doy, 1960; Pitt, 1962), no regulation may be expected in the system. Excretion of the excess of product may be the only mechanism to prevent the accumulation of the product. A large amount

of tyrosine is converted into *p*-hydroxyphenylpyruvate and other phenolic acids, and this appears to be a wasteful process of using an essential amino acid, unless some yet unknown functions exist for these phenolic products.

It is logical to expect the excess of end product (ubiquinone) to inhibit its own synthesis. It has been observed that the increase in ubiquinone synthesis in rats exposed to cold is prevented by giving them exogenous ubiquinone (Aithal *et al.*, 1968). Also in the later stages of clofibrate feeding, the rate of ubiquinone synthesis from [2-¹⁴C]mevalonate decreases, probably owing to the accumulation of excess of ubiquinone synthesized during the earlier stages (Krishnaiah & Ramasarma, 1970). Ubiquinone feeding was found to cause a marginal decrease in its rate of synthesis from mevalonate (Krishnaiah *et al.*, 1967), and in present experiments with *p*-hydroxybenzaldehyde no significant change was observed. Under normal conditions, therefore, enriching the liver with exogenous ubiquinone does not seem to elicit any effect on its synthesis in the liver. An analogous situation exists in the metabolism of cholesterol in human liver, where feeding cholesterol results in the expansion of its body pool, without inhibiting its own synthesis (Taylor *et al.*, 1960; Grundy *et al.*, 1969; Quintas *et al.*, 1971).

The biosynthesis of ubiquinone has been shown to be affected under various stress conditions and administration of drugs. When the animals were exposed to low temperature (0–5°C) or given clofibrate, an antihypercholesterolaemic drug, incorporation of [2-¹⁴C]mevalonate was increased during the initial 15–20 days. Studies on the incorporation of the ring precursor, *p*-hydroxybenzaldehyde, showed similar changes. Incorporation of [¹⁴C]acetate into ubiquinone is known to be decreased in starved and cholesterol-fed rats, because of the lowered activity of 3-hydroxy-3-methylglutaryl-CoA reductase under these conditions. However, the incorporation of [2-¹⁴C]mevalonate into ubiquinone showed a considerable increase in starvation and cholesterol feeding. This was thought to be due to a decrease in the endogenous mevalonate pool with

consequent higher specific radioactivity of the administered mevalonate in these rats. The present studies on the incorporation of *p*-hydroxy[U-¹⁴C]-benzaldehyde into ubiquinone in starved and cholesterol-fed rats showed about 70% decrease, resolving the discrepancy involved in the studies using mevalonate as tracer, and confirmed the expected decreased synthesis of ubiquinone.

S. R. is a Senior Research Fellow of the Department of Atomic Energy, Government of India.

References

- Aithal, H. N., Joshi, V. C. & Ramasarma, T. (1968) *Biochim. Biophys. Acta* **162**, 66–72.
Aiyar, A. S. & Olson, R. E. (1972) *Eur. J. Biochem.* **27**, 60–64.
Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O. H., Jones, F. T. & DeEds, F. (1960) *J. Biol. Chem.* **235**, 2649–2652.
Doy, C. H. (1960) *Nature (London)* **186**, 529–531.
Granner, D. K., Hayashi, S., Thompson, E. B. & Tomkins, G. M. (1968) *J. Mol. Biol.* **35**, 291–301.
Grundy, S. M., Ahrens, E. H. & Davignon, J. (1969) *J. Lipid Res.* **10**, 304–315.
Joshi, V. C., Jayaraman, J. & Ramasarma, T. (1963) *Biochem. J.* **88**, 25–31.
Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33.
Krishnaiah, K. V. & Ramasarma, T. (1970) *Biochem. J.* **116**, 321–327.
Krishnaiah, K. V., Joshi, V. C. & Ramasarma, T. (1967) *Arch. Biochem. Biophys.* **121**, 147–153.
Pitt, B. M. (1962) *Nature (London)* **196**, 272–273.
Quintas, E., Grundy, S. M. & Ahrens, E. H., Jr. (1971) *J. Lipid Res.* **12**, 233–247.
Ramasarma, T. (1972) *Biochem. Soc. Symp.* **35**, 245–256.
Ramasarma, T. & Jayaraman, J. (1971) *Methods Enzymol.* **18**, 232–237.
Ranganathan, S. & Ramasarma, T. (1971) *Biochem. J.* **122**, 487–493.
Ranganathan, S. & Ramasarma, T. (1973) *Biochem. J.* **134**, 737–743.
Taylor, C. B., Patton, D., Yogi, N. & Cox, G. E. (1960) *Proc. Soc. Exp. Biol. Med.* **103**, 768–771.