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REDUCTION OF α-TOCOPHEROLQUINONE TO α-TOCOPHEROLHYDROQUINONE IN RAT HEPATOCYTES

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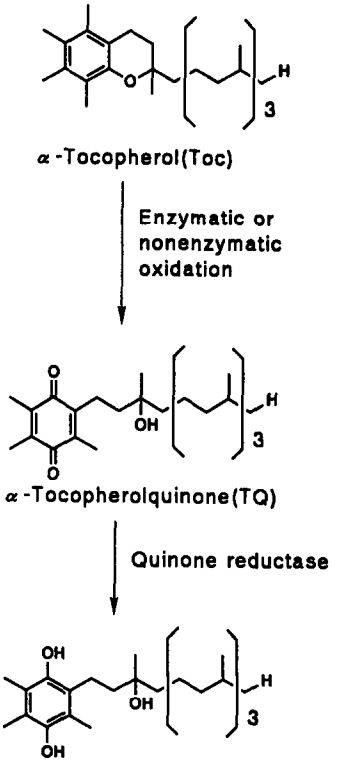
Abstract—The contents of α-tocopherolhydroquinone (TQH_2), α-tocopherolquinone (TQ) and α-tocopherol (Toc) in isolated rat hepatocytes and liver homogenates were determined by HPLC under anaerobic conditions, because TQH_2 easily autoxidizes to TQ under aerobic conditions. The viable hepatocytes were used for the determination without homogenization. The hepatocytes contained 3.1, ND and 5.0, 3.1–9.0, and 31.3–63.2 nmol of TQH_2, TQ and Toc/g liver, respectively. However, TQH_2 was not detected in liver homogenates because endogeneous TQH_2 autoxidizes to TQ during preparation of homogenates under aerobic conditions. The homogenates contained 2.0–23.5 and 36.5–54.9 nmol of TQ and Toc/g liver, respectively. Addition of TQ showed that TQ was reduced and converted into TQH_2 in isolated hepatocytes. The TQH_2 formation from TQ was also observed in liver homogenates in the presence of either NADPH or NADH. The formation was further analysed and confirmed by HPLC and mass spectrometry. The formation of TQH_2 was also found to occur in mitochondria, microsomes and cytosol. The specific activity of NADPH-dependent TQ reductase activity was in the order of mitochondria ≧ microsomes > cytosol. Furthermore, NADPH-cytochrome P450 reductase was found to catalyse TQH_2 formation from TQ.

α-Tocopherol (Toc§) is metabolized in animal tissues, or oxidized by oxygen and lipid peroxide radicals, to α-tocopherolquinone (TQ) (Scheme 1) [1, 2]. The metabolites of TQ in rat liver have been investigated previously [3–5]. Chow *et al.* [3] deduced that TQ would be present mainly in the reduced state in rat liver, from the detection of the conjugates of TQ in the feces. Bindoli *et al.* [4] reported the reduction of TQ to α-tocopherolhydroquinone (TQH_2) (Scheme 1) in rat liver submitochondrial particles, but they measured the TQH_2 formation from the increase in TQ after oxidation of TQH_2 to TQ with KOH treatment. In these papers, TQH_2 formed from TQ could not be identified directly, because TQH_2 easily autoxidizes to TQ under aerobic extraction conditions. On the other hand, Hughes and Tove [5] reported that the contents of TQ and TQH_2 in rat liver were 90 and 124 nmol/g tissue, respectively. However, Bieri and Tolliver [6] reported very small amounts of TQ (1–4 nmol/g tissue) in rat liver. Therefore, TQH_2 formation from TQ and the substantial contents of TQ and TQH_2 in animal tissues are still obscure.

In this paper we report the anaerobic extraction and subsequent HPLC determination of TQH_2, TQ and Toc contained in isolated hepatocytes and liver homogenates. This paper also describes the TQH_2 formation from TQ added to hepatocytes and the TQ reductase activity of subcellular fractions.



Scheme 1.

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§ Abbreviations: Toc, α-tocopherol; TQ, α-tocopherolquinone; TQH_2, α-tocopherolhydroquinone.

MATERIALS AND METHODS

Chemicals. Collagenase type I and bovine serum albumin (crystallized and lyophilized) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.) NADPH and NADH were obtained from the Oriental Yeast Co. (Tokyo, Japan). DL-α-Tocopherol (Toc) was obtained from the Wako Co. (Osaka, Japan). Other chemicals were of the highest quality commercially available.

Toc was further purified by HPLC, and obtained as a colorless oil.

TQ was obtained by ferric chloride oxidation of Toc [3] and purified by HPLC: pale brown oil; IR ν_{max}^{neat} cm^{-1}: 3450, 2900, 1630; UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 263 (4.20), 270 (4.20); EI-MS (70 eV) *m/z*: 446 (M^+), 413, 269, 221, 203, 178, 165, 150; ^{1}H-NMR (270 MHz, $CDCl_3$, TMS as internal standard): δ 2.547 (2H, t, J = 8.4), 2.038 (3H, s), 2.010 (6H, s), 1.497 (2H, t, J = 8.4), 1.237 (3H, s), 1.0–1.5 (21H, m), 0.865 (9H, d, J = 6.2), 0.842 (3H, d, J = 6.2).

TQH_2 was obtained by sodium borohydride reduction of TQ[4] and precipitated several times from petroleum ether until it showed a single peak in HPLC analysis: colorless waxy solid; IR ν_{max}^{neat} cm^{-1}: 3200; UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 290 (3.53); EI-MS (70 eV) *m/z*: 446 (M^+-2H), 413, 269, 221, 203, 178, 165, 150; ^{1}H-NMR (270 MHz, $CDCl_3$, TMS as internal standard): δ 2.720 (2H, t, J = 7.0), 2.185 (3H, s), 2.179 (3H, s), 2.163 (3H, s), 1.687 (2H, t, J = 7.0), 1.241 (3H, s), 1.0–1.5 (21H, m), 0.869 (6H, d, J = 6.6), 0.851 (3H, d, J = 6.2), 0.841 (3H, d, J = 5.9).

Conditions of HPLC. HPLC was performed on a Hitachi model 655 liquid chromatograph. A Radial Pak 8NVC18 column (8 mmϕ × 10 cm, Waters) was used for analysis, determination and fractionation. The mobile phase was CH_3CN-H_2O(93:7). The flow rate was 5.0 mL/min. The peaks were detected at 280 nm. Retention time: TQH_2, 3.5 min; TQ, 8.8 min; Toc, 15.0 min. External standards of TQH_2, TQ and Toc were used to determine the amount of the products in the extracts.

Animals. Male Wistar rats were obtained from Clea Japan (Tokyo, Japan). The rats were acclimatized to the animal house in our laboratory for a week. They were given a standard diet CMF which was obtained from the Oriental Yeast Co. (Tokyo, Japan) and tap water *ad lib.* This crude diet contained 17.2 mg Toc/100 g.

Preparation of rat hepatocytes. Hepatocytes were isolated from rats weighing 180–250 g by the collagenase perfusion method of Okajima and Ui [7]. The cell suspension was diluted to a final concentration of 3–5 × 10^7 cells/mL in Krebs–Ringer buffer, pH 7.4. Cell viability, as determined by Trypan blue exclusion, was 85–95%.

Subcellular fractionations. Rats weighing 230–250 g were used and the livers were homogenized manually in 0.25 M sucrose and 10 mM Tris–HCl buffer (pH 7.4) with a Potter–Elvehjem homogenizer. Mitochondria were prepared by the method of Schneider [8]. Respiratory control ratio of mitochondria was 2.5. After preparation of mitochondria, the supernatant was centrifuged at 105,000 *g* for 60 min. The pellet was washed twice with 0.15 M KCl. The microsomal pellet was finally suspended in 0.1 M phosphate buffer (pH 7.4). The amount of cytochrome P450 in microsomes measured by the method of Omura and Sato [9] was 1.0 nmol/mg protein. In order to prepare cytosol, the liver was homogenized in 2 vol. of 0.25 M sucrose. The homogenates were centrifuged directly at 105,000 *g* for 60 min and the supernatant was used as cytosol. Protein was determined by the method of Lowry *et al.* [10] using bovine serum albumin as a standard.

Preparation of NADPH-cytochrome P450 reductase. NADPH-cytochrome P450 reductase was a gift from Dr H. Kojima, Hokkaido Institute of Public Health. The enzyme was prepared from rat liver microsomes by the method of Yasukochi and Masters [11]. The specific activity was 40 U/mg protein.

Determination of TQH_2, TQ and Toc in isolated hepatocytes and liver homogenates. After isolation of hepatocytes, the viable hepatocytes was used for determination of TQH_2, TQ and Toc without homogenization. Liver homogenates obtained in the course of subcellular fractionations were used for the determination.

A 5.0-mL suspension of hepatocytes (3–5 × 10^7 cells/mL) or liver homogenates (20–30 mg protein/mL) was taken in a 50-mL test tube, then 5.0 mL of 1N HCl and 25 mL of $CHCl_3$-MeOH (2:1, containing 0.01% butylated hydroxytoluene were added. After the atmosphere in the tube was replaced with N_2 gas to prevent autoxidation, the tube was capped and shaken for 5 min, and then centrifuged (3500 rpm, 10 min). The $CHCl_3$ layer was evaporated to dryness under reduced pressure, then the reduced pressure was canceled with N_2 gas. The residue was dissolved in an adequate volume of AcOEt, and an aliquot of the solution was immediately injected to HPLC for the determination of TQH_2, TQ and Toc.

Formation of TQH_2 from TQ in rat isolated hepatocytes. Reaction mixture (30 mL, 3–5 × 10^7 cells/mL; equivalent to 20–30 mg protein of the hepatocytes/mL), containing rat isolated hepatocytes, 0.55 mM TQ, 2.5% bovine serum albumin, 5 mM glucose, 5 mM pyruvate and Krebs–Ringer buffer (pH 7.4) was incubated aerobically at 37° with shaking. At 30-min intervals, 2.0-mL samples were taken in test tubes to check the formation of TQH_2, then placed in ice. The samples were acidified with two drops (*ca.* 0.1 mL) of conc. HCl and then 5.0 mL of $CHCl_3$-MeOH (2:1) containing 0.01% BHT were added. These extracts were prepared by the same procedure as described above, then analysed by HPLC. The peak of TQH_2 was fractionated and TQH_2 was obtained as a colourless solid.

After incubation for 120 min, cell viabilities were 79–87%.

Formation of TQH_2 from TQ in homogenates and subcellular fractions of rat liver. A 1.0-mL reaction mixture containing rat liver homogenates or subcellular fractions (15–20 mg protein), various amounts of TQ and 0.1 M phosphate buffer (pH 7.4) was incubated with either 4.0 mM NADPH or NADH in N_2 gas at 37°. When intact mitochondria were used, 5 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 10 mM KCl and 5 mM $MgCl_2$ was used

instead of 0.1 M phosphate buffer under the above conditions. Reduction of TQ in mitochondria was also examined with 10 mM sodium succinate and 0.7 mM ADP instead of NADPH or NADH. In order to determine the specific activity of TQ reductase, 100 nmol of TQ were added to the reaction mixture which was then incubated for 10 min. At the end of the incubation period, the samples were acidified with one drop (*ca.* 0.05 mL) of conc. HCL and then 2.5 mL of $CHCl_3$-MeOH (2:1) containing 0.01% BHT were added. For comparison with isolated hepatocytes, extracts were also prepared by the same procedure using rat isolated hepatocytes, then analysed by HPLC.

Formation of TQH_2 from TQ by NADPH-cytochrome P450 reductase. A 1.0-mL reaction mixture containing 1% bovine serum albumin and NADPH-cytochrome P450 reductase (0.5 U), 0.1 mM TQ, 4.0 mM NADPH and 0.1 M phosphate buffer (pH 7.4) was incubated in N_2 gas at 37° for 15 min with shaking. The TQH_2 formed was analysed by the same procedure as described above.

In order to measure the rate of TQ reduction, the oxidation of NADPH was followed spectrophotometrically at 340 nm, using an extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. A 3.0 mL reaction mixture consisted of NADPH-cytochrome P450 reductase (0.11 U/mL), 0.2 mM NADPH, 10 μM TQ, 0.1 M phosphate buffer (pH 7.4) and 0.08% Triton X-100. The reaction was carried out at 25°.

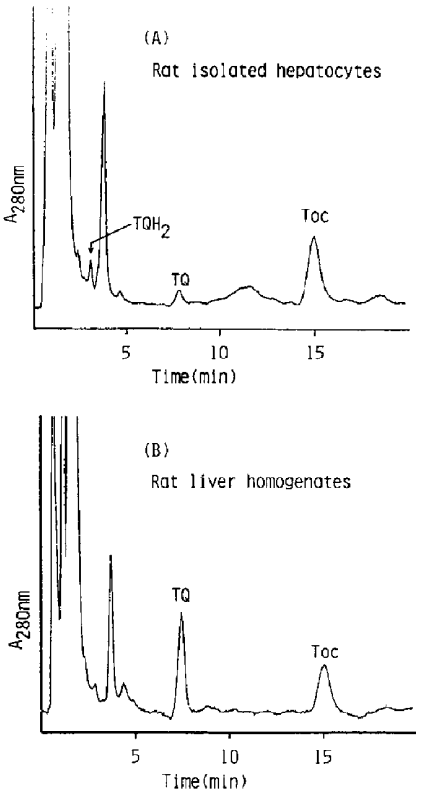

Fig. 1. HPLC analysis of isolated hepatocytes (A) and liver homogenates (B). Total lipids of samples were extracted with $CHCl_3$-MeOH under anaerobic conditions, as described in Materials and Methods. The extracts were immediately injected to HPLC.

RESULTS

Anaerobic extraction made it possible to protect TQH_2 against autoxidation, although TQH_2 easily autoxidizes under aerobic conditions. TQH_2 was detected in isolated hepatocytes by HPLC (Fig. 1A), but TQH_2 was not detected in liver homogenates (Fig. 1B). The contents of TQH_2, TQ and Toc are shown in Table 1. Isolated hepatocytes contained 1.7, ND and 2.8, 1.7–5.0, and 17.4–35.1 nmol of TQH_2, TQ and Toc/100 mg protein (N = 3), respectively. The contents in rat livers, calculated from these results and the protein contents of liver homogenates (mean 180 mg protein/g liver) are 3.1, ND and 5.0, 3.1–9.0, and 31.3–63.2 nmol of TQH_2, TQ, and Toc/g liver, respectively. Liver homogenates contained 36.5–54.9 and 2.0–23.5 nmol of Toc and TQ/g liver (N = 3), respectively. The TQ contents in the homogenates varied widely and did not relate to the Toc contents.

When isolated hepatocytes were incubated with added TQ, the amount of TQH_2 increased with incubation time (Fig. 2). After incubation for 2 hr, the concentration of TQH_2 increased to 30 nmol/100 mg protein of the hepatocytes (N = 3). The peak of TQH_2 was fractionated by HPLC and identified by comparing its mass spectrum with that of synthetic sample.

Additional TQ was converted to TQH_2 in liver homogenates by incubation with NADPH (Fig. 3A), but the formation of TQH_2 occurred hardly at all without NADPH (Fig. 3B). The TQH_2 formed was identified by HPLC and mass spectrometry.

The TQH_2 formation was observed in all subcellular fractions of rat liver by incubation with NADPH. The specific activity was determined from the increase in TQH_2. When TQ was used at 100 nmol/20 mg protein/mL in each subcellular fraction with NADPH, TQH_2 increased linearly with incubation time for 0–15 min. The specific activity was assayed from the amounts of TQH_2 formed during incubation for 10 min (Table 2). The specific activity in each subcellular fraction was in the order of mitochondria ≧ microsomes > cytosol. TQ was also converted to TQH_2 by incubation with NADH in mitochondria or microsomes, and the specific activity with NADH was equal to that with NADPH. In addition, the formation of TQH_2 from TQ was observed in mitochondria with succinate and ADP, similarly as with NADH.

NADPH-cytochrome P450 reductase prepared from rat liver microsomes catalysed the reduction of TQ to TQH_2. The specific activity, assayed from the amount of formed TQH_2 which was determined by HPLC (anaerobic conditions), was 27.3 nmol/min/U. On the other hand, the specific activity, measured spectrophotometrically from the oxidation of NADPH (aerobic conditions), was 252 nmol/min/U which reflected the reoxidation of TQH_2 under aerobic conditions.

DISCUSSION

Quinones, widely distributed in natural products

Table 1. TQH_2, TQ and Toc in isolated hepatocytes and liver homogenates

Samples	Rat	TQH_2 nmol/100 mg protein	TQH_2 nmol/g liver	TQ nmol/100 mg protein	TQ nmol/g liver	Toc nmol/100 mg protein	Toc nmol/g liver
Isolated rat hepatocytes	1	2.8	5.0*	2.0	3.6*	20.1	36.2*
	2	ND	ND*	5.0	9.0*	17.4	31.3*
	3	1.7	3.1*	1.7	3.1*	35.1	63.2*
Rat liver homogenates	1	ND	ND	12.5	23.5	26.6	49.9
	2	ND	ND	3.6	5.8	22.7	36.5
	3	ND	ND	1.6	2.0	44.0	54.9

ND, not detected: <0.5.
* Calculated from the protein content of rat liver homogenates (mean: 180 mg protein/g liver, N = 3)

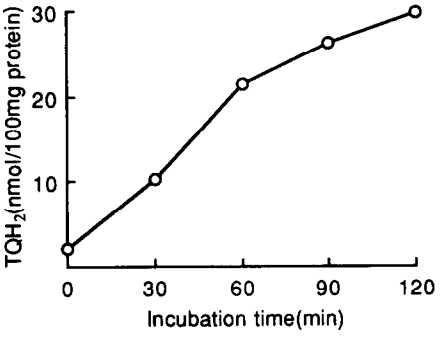

Fig. 2. Time-course of formation of TQH_2 in isolated hepatocytes. The reported results are the means of three experiments obtained with three different isolated hepatocytes.

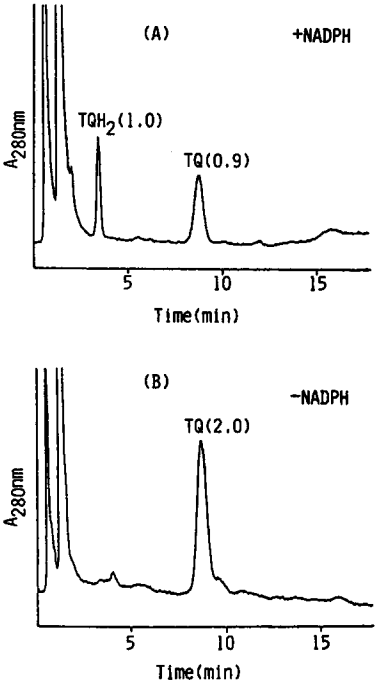

Fig. 3. Reduction of TQ to TQH_2 in liver homogenates. The reaction mixture (1.0 mL) consisted of liver homogenates (15 mg protein), 30 nmol of TQ and 0.1 M phosphate buffer (pH 7.4), in the presence of 4.0 mM NADPH (A) or in the absence of NADPH (B). Incubation was carried out at 37° for 15 min. The reaction mixture was extracted and analysed as described in Materials and Methods. The values in brackets indicate the concentration of each compounds expressed as nmol/mg protein. The values are the means of three experiments obtained with three different liver homogenates.

or used as anticancer therapeutic agents, are reduced by enzyme-catalysed redox cycles in biological systems [12, 13]. For example, menadione, benzoquinones and naphthoquinones are converted to their corresponding semiquinones or hydroquinones by NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, NADH-ubiquinone oxidoreductase and the flavoenzyme [NAD(P)H:(quinone acceptor) oxidoreductase], known as DT-diaphorase [14–17]. TQ has a structure analogous with that of ubiquinones and has been assumed to be converted to TQH_2 in mitochondria [4, 5]. The TQH_2 formation from TQ in mitochondria with either NADPH or NADH was confirmed in this paper. Moreover, TQH_2 formation from TQ was also observed in microsomes and cytosol. Since the reduction of TQ to TQH_2 occurred in all subcellular fractions (Table 2), the reduction of TQ might be catalysed by the hepatic enzymes discussed above. Actually, TQ was converted to TQH_2 by purified microsomal NADPH-cytochrome P450 reductase. The lower activity in cytosol than in mitochondria and microsomes could be ascribed to the low activity of DT-diaphorase with quinones having long carbon-chain substituents [15] such as TQ. In this study, not only NADPH- but also NADH-TQ reductase activity was found to be present in mitochondria, microsomes and homogenates of rat liver, and the two activities were present equally.

TQH_2 formation was observed in isolated hepatocytes by the addition of TQ, and TQH_2 accumulated in the hepatocytes (Fig. 2). Therefore,

Table 2. Rate of reduction of TQ to TQH_2 in liver homogenates and tissue fractions

Tissue fractions	TQH_2 formation (nmol/min/mg protein)
Homogenates	0.089 ± 0.016
Mitochondria	0.100 ± 0.016
Microsomes	0.083 ± 0.011
Cytosol	0.015 ± 0.003

TQ (100 nmol) and 4 mM NADPH were added to liver homogenates and tissue fractions (20 mg protein), and the reaction mixtures were incubated at 37° for 10 min. The amounts of TQH_2 formed were determined as indicated in Materials and Methods. Values are means ± SD for four determinations.

the amounts of TQH_2 detected in isolated hepatocytes (Table 1, Fig. 1) must have been endogeneously produced from TQ. However, endogeneous TQH_2 was not detected in liver homogenates. Because TQH_2 easily autoxidizes to TQ under aerobic conditions, endogeneous TQH_2 autoxidizes to TQ during the aerobic procedure for the preparation of homogenates. Thus, the amount of TQ in liver homogenates (2.0–23.5 nmol/g tissue) is considered to be the total amount of endogenous TQH_2 and TQ.

Although TQ itself is not considered to be an effective antioxidant [18], it was reported that it behaved as an effective antioxidant similar to Toc in cell cultures [19, 20]. On the other hand, Bindoli *et al.* [4] reported the antioxidative effect of TQH_2. Since, TQ was converted to TQH_2 in isolated rat hepatocytes, it is suggested that the TQH_2 formed acts as an antioxidant in biological systems.

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