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Original Research Communication

Action of Pyrroloquinolinequinol As an Antioxidant Against Lipid Peroxidation in Solution

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ABSTRACT

The activities of pyrroloquinoline quinone (PQQ), a coenzyme of methanol dehydrogenase and amine oxidase, and its reduced form pyrroloquinoline quinol ($PQQH_2$) as an antioxidant have been measured in solution. $PQQH_2$ was stable in the absence of oxygen but rapidly auto-oxidized to PQQ in the presence of oxygen in water. $PQQH_2$ was stable in an aprotic solvent such as acetonitrile, even in air. PQQ did not exert appreciable antioxidant activity, whereas $PQQH_2$ exerted higher reactivity than α-tocopherol toward galvinoxyl radical and peroxyl radical. $PQQH_2$ acted as a potent antioxidant against the oxidation of methyl linoleate in acetonitrile induced by azo compound and produced a clear induction period, from which the apparent stoichiometric number was obtained as 1.1. $PQQH_2$ reduced the α-tocopheroxyl radical and spared α-tocopherol in the oxidation of methyl linoleate. These results suggest that $PQQH_2$ may act as a potent antioxidant, particularly in combination with α-tocopherol. Antiox. Redox Signal. 1, 547–554.

INTRODUCTION

THERE IS NOW INCREASING EXPERIMENTAL, clinical, and epidemiological evidence suggesting that the oxidative damage of biological molecules plays a pivotal role in the pathogenesis of various diseases, as well as cancer and aging (Papas, 1999). Lipids are vulnerable to oxidation, and lipid peroxidation has been accepted to be an important event in the development of several diseases, including atherosclerosis (Steinberg *et al.*, 1989). Consequently, the role of antioxidants, especially monophenolic and polyphenolic compounds such as vitamin E and flavonoids, has received much attention recently (Papas, 1999).

Pyrroloquinoline quinone (PQQ), 4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid (Fig. 1), is a coenzyme of methanol dehydrogenase and bovine serum amine oxidase (Duine *et al.*, 1979; Salisbury *et al.*, 1979). Trace amounts of free PQQ have been detected in several organs and the plasma and urine of humans (Kumazawa *et al.*, 1992). Various physiological or pharmacological effects of PQQ like vitamin or growth factor activity (Killgore *et al.*, 1989), enhancing effect of DNA synthesis in human fibroblasts (Naito *et al.*, 1993) and inhibitory effect on the formation of hydrocortisone-induced cataract in embryos (Nishigori *et al.*, 1989) have been reported. Its reduced form pyrroloquinoline quinol ($PQQH_2$; Fig. 1) is a catechol derivative and expected to exert an antioxidant function. In fact, the effect of PQQ as an antioxidant has been reported against the lipid peroxidation of rat brain homogenate (Hamagishi *et al.*, 1990). A protective effect of PQQ against experimental liver injury induced by hepatotoxins such as carbon tetrachloride, D-galactosamine, thioac-

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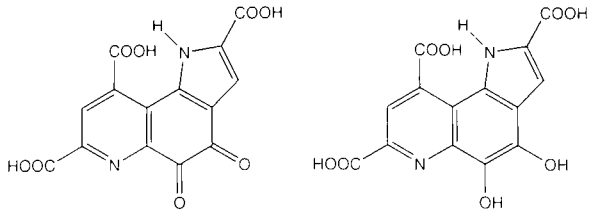


FIG. 1. Chemical structure of PQQ and $PQQH_2$.

etamide, and allyl formate has been observed (Watanabe *et al.*, 1988). The reduction-oxidation cycle of PQQ-$PQQH_2$ has been also studied (Itoh *et al.*, 1986; Sugioka *et al.*, 1988). Thus, the pharmacological application of PQQ (Davidson, 1992) and its esters (Urakami *et al.*, 1995/1996a, 1997) has received considerable attention. However, the basic kinetic data for PQQ and $PQQH_2$ as an antioxidant have been lacking. The present study was undertaken to obtain kinetic data on the antioxidant action of PQQ and $PQQH_2$ against lipid peroxidation in solution.

MATERIALS AND METHODS

Materials

PQQ was prepared by a method described previously (Urakami *et al.*, 1993, 1995/1996a,b) that uses *Hyphomicrobium denitrificans* TK 0411 (Urakami *et al.*, 1995). $PQQH_2$ was prepared by reducing PQQ with sodium borohydride (Itoh *et al.*, 1986). 2*R*,4′*R*,8′*R*-α-Tocopherol was supplied from Eisai Co. (Tokyo, Japan). Methyl linoleate obtained from Sigma (St. Louis, MO) was purified before use as described previously (Gotoh *et al.*, 1992). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was purchased from Wako Pure Chemical Inc. (Osaka, Japan) and used as received. Other chemicals were those of the highest grade available commercially.

Methods

The absorption spectra were measured with a spectrophotometer (Shimadzu UV-2200, Kyoto, Japan) at either 37°C or room temperature under air, unless otherwise specified. In some experiments, the absorption spectrum was taken under vacuum by mixing $PQQH_2$ powder and solvent after degassing the system using a vacuum line. The rates of interaction of antioxidants with galvinoxyl were measured by following the decrease in the maximum absorption of galvinoxyl at 429 nm using a rapid-mixing stopped-flow appratus (RX1000, Applied Photophysics, Letherhead, UK). The reactivity of $PQQH_2$ toward the peroxyl radical was estimated by a competition method using *N*,*N*′-diphenyl-*p*-phenylenediamine (DPPD) (Noguchi *et al.*, 1998).

The oxidation of methyl linoleate was carried out at 37°C in air in the presence and absence of the radical initiator AMVN and antioxidant. The formation of methyl linoleate hydroperoxides was measured with high-performance liquid chromatography (HPLC) apparatus equipped with a UV detector monitored at 234 nm. Samples were injected onto a reverse-phase column LC18 (particle size 5 μm; 4.6 × 250 mm; Supelco, Tokyo), methanol/H_2O (95/5, vol/vol) being used as an eluent with a flow rate of 1.0 ml/min.

The electron spin resonance (ESR) spectrum of the radicals was recorded on X-band ESR spectrometer (JEOL, Tokyo, Japan).

RESULTS

Reduction of PQQ to $PQQH_2$ and stability of $PQQH_2$

It is conceivable that PQQ exerts antioxidant capacity not *per se* but only when it is reduced to $PQQH_2$. PQQ was readily reduced *in vitro* to $PQQH_2$ by sodium borohydride, NADPH, and NADH, and the characteristic absorption spectrum of $PQQH_2$ was observed (Fig. 2). PQQ was stable at 37°C under air but $PQQH_2$ was unstable in water and in the presence of oxygen. In the absence of oxygen, $PQQH_2$ was stable even in water and the absorption spectrum of $PQQH_2$ did not change. However, the introduction of air or 2% oxygen–98% nitrogen gas induced a rapid change in absorption spectrum as shown in Fig. 2B, which suggests the autoxidation of $PQQH_2$ to PQQ. On the other hand,

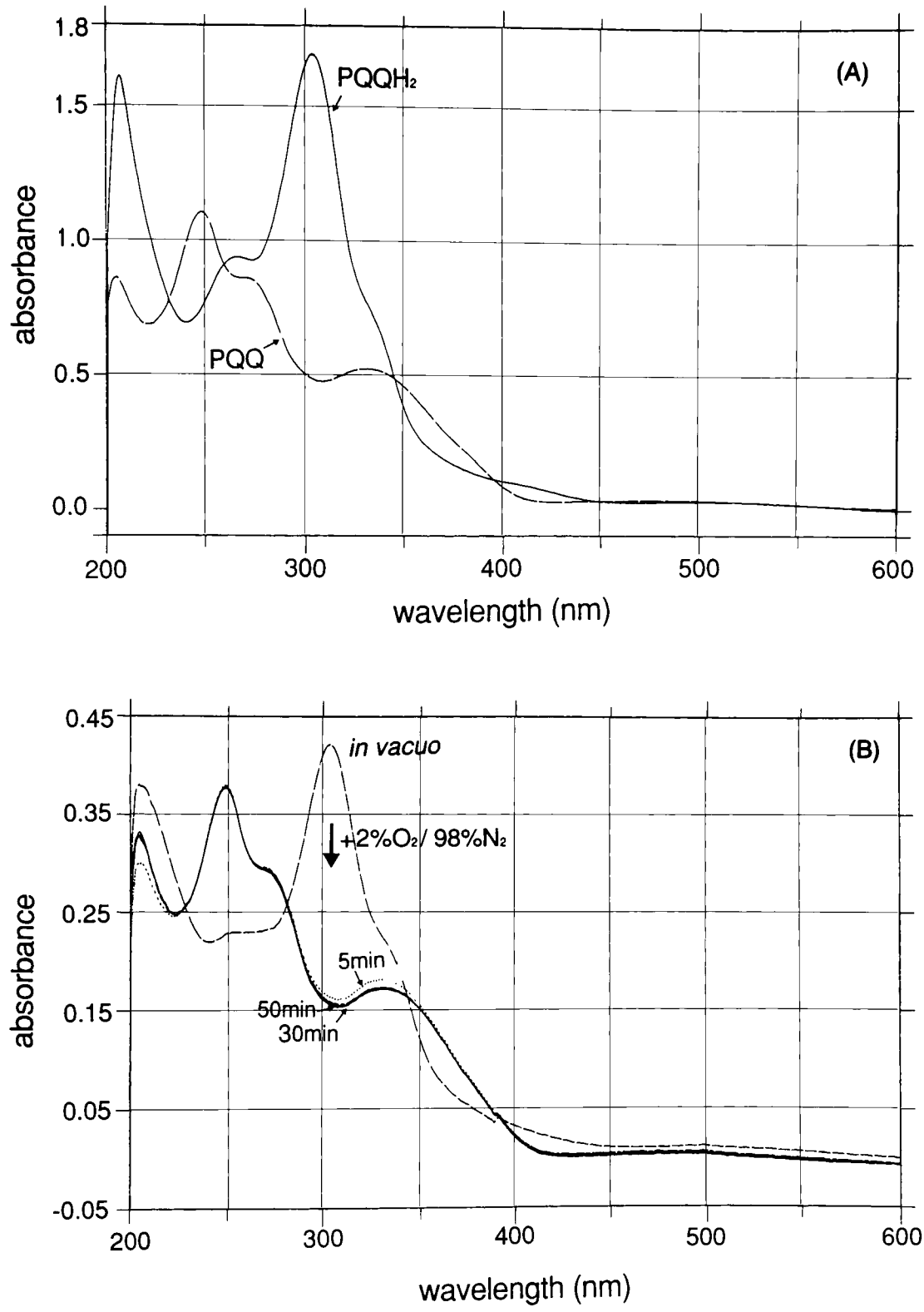


FIG. 2. A. Absorption spectra of PQQ and $PQQH_2$ in Tris-HCl buffer, pH 7.4. B. The change of absorption spectrum observed when 2% oxygen–98% nitrogen gas was introduced to an aqueous solution of $PQQH_2$ *in vacuo*.

$PQQH_2$ was found to be stable in organic aprotic solvent, even under air atmosphere.

Interaction of PQQ and $PQQH_2$ with galvinoxyl

To estimate the reactivities of PQQ and $PQQH_2$ toward radicals, the interaction of PQQ and $PQQH_2$ with galvinoxyl radical was studied spectrophotometrically. It is well known that the hydrogen-donating antioxidant reacts rapidly with galvinoxyl radical and that the rate correlates with the reactivity of the antioxidant toward peroxyl radicals, the chain-carrying species in lipid peroxidation. The rate of interaction between $PQQH_2$ and galvinoxyl was measured at 37°C in air by following the decrease in the absorption of galvinoxyl in acetonitrile-DMSO (93:2 by volume) solution. PQQ did not react with galvinoxyl at appreciable rate, whereas $PQQH_2$ did rapidly. Fig. 3 shows the decay of absorption at 429 nm of galvinoxyl caused by the reaction with $PQQH_2$ and also with α-tocopherol, a major lipophilic antioxidant *in vivo*, for comparison. It can be seen that $PQQH_2$ reacts with galvinoxyl radical faster than α-tocopherol.

The stoichiometric number of hydrogen of $PQQH_2$ that reacts with galvinoxyl was measured from a decrease in absorption at 429 nm

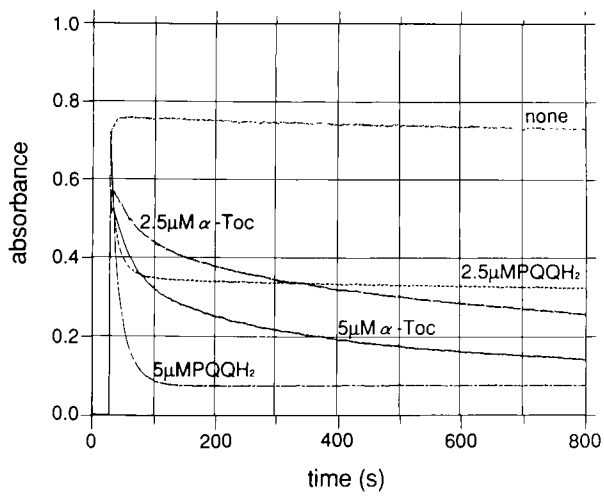


FIG. 3. Interaction of $PQQH_2$ with galvinoxyl. The rate of interaction between $PQQH_2$ and galvinoxyl (5.9 μM) was measured at 37°C in acetonitrile–DMSO (93:2 by vol) by following a decrease in the absorption at 429 nm by galvinoxyl as described in Materials and Methods. The results with α-tocopherol (α-Toc) are also included for comparison.

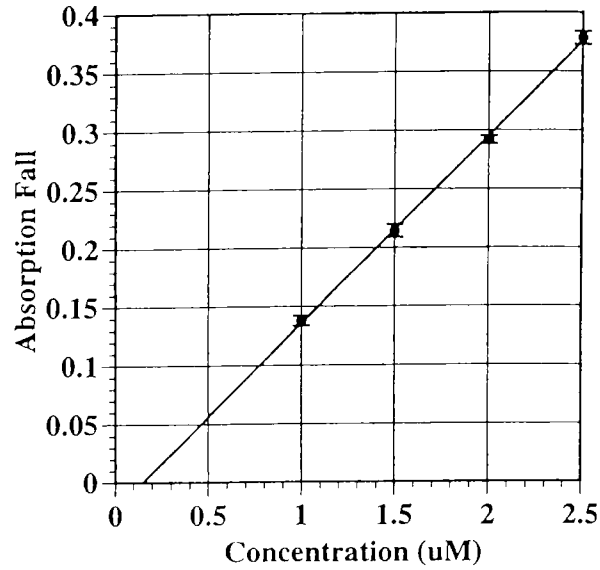


FIG. 4. Stoichiometric number for the reduction of galvinoxyl by $PQQH_2$. $PQQH_2$ (1–2.5 μM) was reacted with 5 μM galvinoxyl in acetonitrile at 37°C in air and the fall in the absorption at 429 nm was measured. The data points represent mean ± SD of five independent experiments. The slope of the plot of this absorption fall against $PQQH_2$ concentration gives the stoichiometric number as $n = 1.07$.

in the presence of excess galvinoxyl. As shown in Fig. 4, the absorption fall was proportional to $PQQH_2$ concentration and from the slope and extinction coefficient for galvinoxyl, $\epsilon = 1.50 \times 10^5\ M^{-1}cm^{-1}$, the stoichiometric number was obtained as $n = 1.07$.

Reaction of $PQQH_2$ with the peroxyl radical

To follow the reaction of $PQQH_2$ with the peroxyl radical, the change in the absorption spectrum during the incubation of $PQQH_2$ with AMVN was followed under air in acetonitrile–dimethylsulfoxide (DMSO) (93/2 by vol/vol) solvent. $PQQH_2$ was stable under air in this solvent, but the addition of AMVN induced the change in absorption spectrum as shown in Fig. 5. The absorption at 305 nm due to $PQQH_2$ decreased, while that at 330 nm by PQQ increased with time, suggesting that $PQQH_2$ was oxidized to PQQ by a reaction with the peroxyl radicals.

The reactivity of $PQQH_2$ toward peroxyl radicals was estimated by the competition method

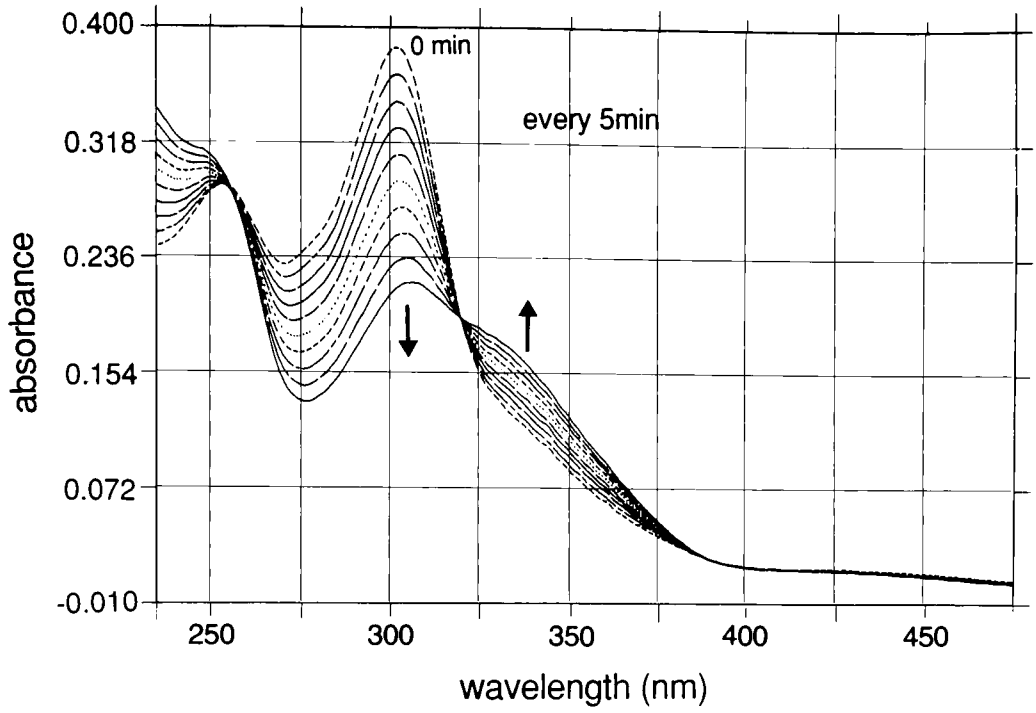
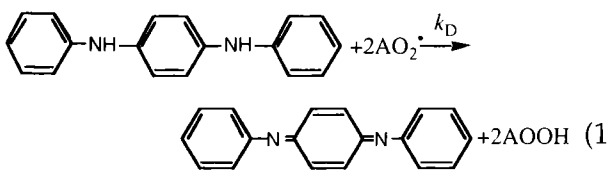


FIG. 5. Change in absorption spectrum during the oxidation of $PQQH_2$ by peroxyl radical. $PQQH_2$ (20 μM) was incubated in acetonitrile–DMSO (93:2 vol/vol) at 37°C under air in the presence of AMVN (1.0 mM).

using DPPD (Noguchi *et al.* 1998). It is known that DPPD reacts with two molecules of peroxyl radicals to give *N,N'*-diphenyl-*p*-benzoquinone diimine (DPBQ) (reaction 1).



(1)

DPBQ has a strong absorption at 440 nm and the rate of reaction 1 can be followed from its increase with time. When another antioxidant IH is added to this system, it scavenges peroxyl radicals in competition with DPPD (reaction 2),

$$AO_2^{\bullet} + IH \xrightarrow{k_I} AOOH + I^{\bullet} \qquad (2)$$

It was found as observed previously (Noguchi *et al.*, 1998) that the rate of increase in the absorption at 440 nm was directly proportional to AMVN concentration but independent of DPPD concentration. Because DPPD reacts with two radicals to give DPBQ, the rate of free radical flux from AMVN is obtained by multiplying the rate of DPBQ formation by 2. The rate of free radical generation from AMVN, Ri, is given in Equation (3),

$$Ri = 2ek_d[AMVN] \qquad (3)$$

where e and k_d are the efficiency of free radical formation and the rate constant for unimolecular decomposition of AMVN, respectively. The rate of DPBQ formation induced by 5.0, 10, and 20 mM AMVN was obtained as 0.840, 1.76, and 3.45 nM/sec, which gives ek_d as 1.72×10^{-6} s^{-1} as an average under the reaction conditions employed here. The effects of $PQQH_2$ and α-tocopherol on the formation DPBQ from DPPD (Fig. 6) show that $PQQH_2$ competes with DPPD more strongly than α-tocopherol, suggesting that $PQQH_2$ is more reactive toward peroxyl radicals than α-tocopherol, as observed toward galvinoxyl.

Inhibition of oxidation of methyl linoleate by $PQQH_2$

Methyl linoleate has been often used as a model substrate for assessing the antioxidant

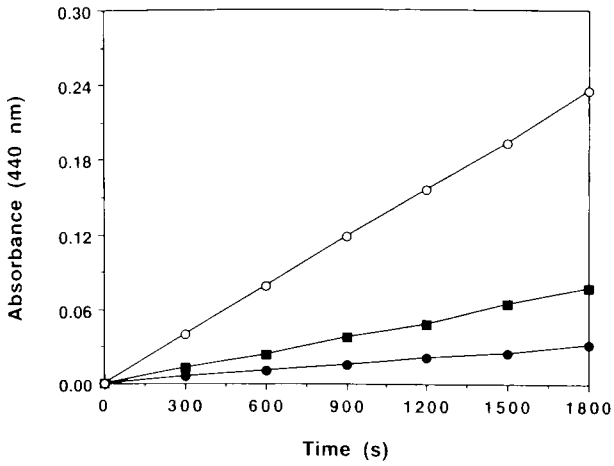


FIG. 6. Effect of $PQQH_2$ and a-tocopherol on the formation of DPBQ from DPPD. DPPD (0.1 m*M*) was incubated with AMVN (10 m*M*) at 37°C in acetonitrile–DMSO (93:2 vol/vol) in the absence (○) and presence of $PQQH_2$ (0.1 m*M*, ●) or α-tocopherol (0.1 m*M*, ■), and the formation of DPBQ was followed by an absorption at 440 nm.

activity against lipid peroxidation, as its oxidation mechanism and products have been well established and its oxidation rate can be easily and quantitatively measured. As shown in Fig. 7, $PQQH_2$ suppressed the oxidation of methyl linoleate and produced a distinct induction period in a dose-dependent manner. It has been known that the induction period t_{inh} is proportional to the antioxidant concentration and inversely proportional to the rate of chain initiation, Ri,

$$t_{inh} = \frac{n\ [\text{antioxidant}]}{\text{Ri}} \qquad (4)$$

where n is the stoichiometric number of peroxyl radicals scavenged by each antioxidant molecule (Niki *et al.*, 1984). It was found in the present study that the induction period was proportional to the $PQQH_2$ concentration and the stoichiometric number n was obtained as 1.1.

The antioxidant activity of $PQQH_2$ against the oxidation of methyl linoleate was also studied in the presence of α-tocopherol. The results are shown in Fig. 8. It shows that $PQQH_2$ extended the induction period and, more importantly, spared α-tocopherol. It was found by ESR study that $PQQH_2$ reduced α-tocopheroxyl radical (data not shown).

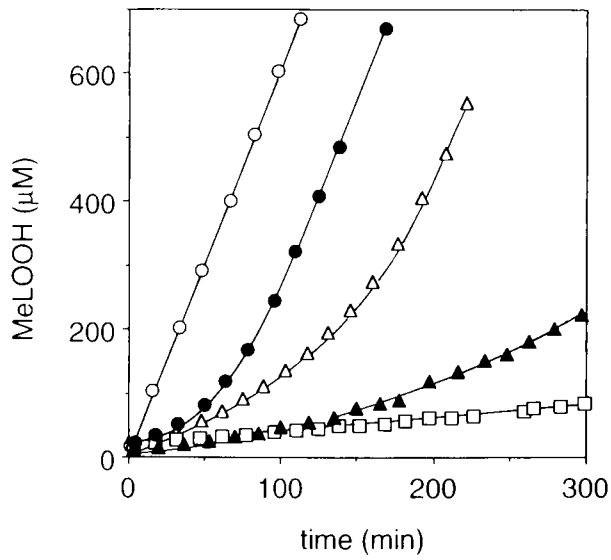


FIG. 7. Effect of $PQQH_2$ against the oxidation of methyl linoleate. Methyl linoleate (151 m*M*) was oxidized in acetonitrile–DMSO (93:2, vol/vol) in the presence of AMVN (0.10 m*M*) and $PQQH_2$ at 37°C in air and the formation of methyl linoleate hydroperoxide (MeLOOH) was followed with an HPLC. The concentrations of $PQQH_2$ were 0 (○), 2 (●), 4 (△), 10 (▲), and 20 μ*M* (□).

DISCUSSION

The antioxidant action of phenolic compounds has been well documented. Above all, monophenolic compounds such as tocopherol and butylated hydroxytoluene and polyphenols such as flavonoids have been studied extensively (Papas *et al.*, 1999). Dihydroxybenzenes are also known to act as a potent antioxidant. Ubiquinol and α-tocopheryl hydroquinone have been shown to act as a potent antioxidant in combination with α-tocopherol (Shi *et al.*, 1999a and papers cited therein). Catechols also act as an antioxidant by either chelating metal ions or scavenging radicals. For example, catecholestrogens such as 2-hydroxyestradiol have high antioxidant capacity (Lacort *et al.*, 1995). $PQQH_2$ is another type of catechol compound, but the activity of $PQQH_2$ as a radical-scavenging antioxidant has not been studied. The present study shows that $PQQH_2$ acts as a potent radical-scavenging antioxidant. As described above, $PQQH_2$ exerted higher reactivites than α-tocopherol toward galvinoxyl and peroxyl radicals and, more importantly, it

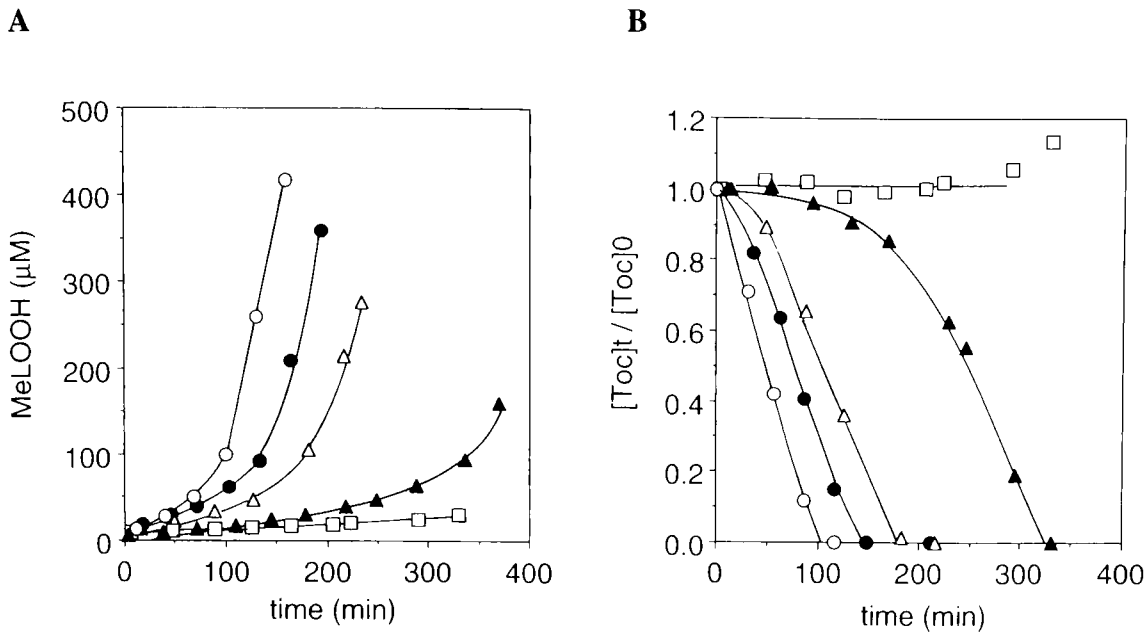


FIG. 8. Effect of $PQQH_2$ on the oxidation of methyl linoleate in the presence of α-tocopherol. Methyl linoleate (151 m*M*) was oxidized in the presence of AMVN (0.10 m*M*), α-tocopherol (2.0 μ*M*), and $PQQH_2$ of various concentrations at 37°C in air in acetonitrile–DMSO (93:2, vol/vol). The formation of methyl linoleate hydroperxide (MeLOOH) (**A**) and consumption of α-tocopherol (**B**) were followed. [Toc]t/[Toc]o denotes remaining fraction of α-tocopherol. The concentrations of $PQQH_2$ were 0 (○), 2 (●), 4 (△), 10 (▲), and 20 μ*M* (□).

is capable of reducing α-tocopheroxyl radical to regenerate α-tocopherol. This makes $PQQH_2$ particularly a potent antioxidant in combination with α-tocopherol. This may be also important under such conditions where α-tocopherol-mediated-peroxidation (Bowry and Stocker, 1993) is important.

$PQQH_2$ like ubiquinol and α-tocopheryl hydroquinone (Shi *et al.*, 1999a), is unstable, especially in the presence of water and air, and readily undergoes auto-oxidation to give PQQ, which makes the apparent stoichiometric number smaller. The instability of $PQQH_2$ in water implies the involvement of transition metal ions. The auto-oxidation of $PQQH_2$ is mediated by hydroperoxyl radical and/or superoxide, which may attack lipids as well and reduce the antioxidant efficacy of $PQQH_2$ as observed with ubiquinol and α-tocopherylhydroquinone (Shi *et al.*, 1999a,b). PQQ, like unbiquinone and α-tocopherylquinone, does not act as an antioxidant *per se*, but it may be reduced to $PQQH_2$ by a reducing system *in vivo*. In fact, it has been reported that the ratio of ubiquinol/ubiquinone is much higher than 1 *in vivo* (Aberg *et al.*, 1992), suggesting an efficient reducing capacity *in vivo*. In conclusion, $PQQH_2$ is a potent antioxidant and may play a role in the defense system *in vivo* against oxidative stress in combination with other antioxidants such as vitamin E.

ABBREVIATIONS

AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); DMSO, dimethyl sulfoxide; DPBQ, *N,N′*-diphenyl-*p*-benzoquinone diimine; DPPD, *N,N′*-diphenyl-*p*-phenylene diamine; ESR, electron spin resonance; PQQ, pyrroloquinoline quinone; $PQQH_2$, pyrroloquinoline quinol.

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