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Reduction of a-tocopherylquinone Model Compound With Various Reductant

Indrajati Kohar
Fakultas Farmasi Universitas Surabaya

Abstract

*In order to study the possibility of tranformation of a-tocopherylquinone (**TQ**) into a more oxidiseable compound and also to find out the recycling effect in the cells, an experiment was conducted by reducing the model compound 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**PQ**) with various reductants. In the experiment it was shown that glutathione did not reduce **PQ**,nor NADH by itself, so the effective reductant in the NADH/FAD combination must have been $FADH_2$. Thus there is a probability that in a biological system, the most probable reductant for **TQ** would be a flavin enzyme rather that ascorbic acid or glutathione. The non-physiological dithiothreitol was as effective as NADH/FAD which is interesting because of its similarity to the physiologically important reduced lipoic acid.*

*The reactivity of the various reductants used in this experiment decrease in the order of dithiothreitol ~ NADH/FAD (8/10) > sodium dithionite > NADH/FAD (2:10) > sodium ascorbate > ascorbic acid (Fig.**8**).*

***Key words**: a-tocopherylquinone, reduction, Vitamin E, oxidation product.*

Abstrak

Dalam usaha untuk mempelajari kemungkinan adanya transformasi dari α-tocopherylquinone (TQ) menjadi senyawa yang dapat dioksidasi dan juga untuk mengetahui efek daur ulangnya dalam sel, telah dilakukan suatu percobaan dengan mereduksi senyawa modelnya yaitu 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (PQ) dengan berbagai reduktan. Pada percobaan ini tampak bahwa glutathione tidak mereduksi PQ, demikian juga NADH tanpa kombinasi, maka reduktan yang efektif dalam kombinasi NADH/FAD adalah $FADH_2$. Jadi bisa diduga bahwa dalam sistem biologis, reduktan yang paling mungkin untuk TQ adalah enzim flavin dan bukan asam askorbat ataupun glu-

tathione . Penemuan menarik lainnya adalah reduktan non-fisiologis dithiothreitol sama efektifnya dengan NADH/FAD karena kemiripannya dengan asam lipoat tereduksi yang penting secara fisiologis.
Reaktivitas dari reduktan-reduktan yang digunakan dalam penelitian ini menurun menurut urutan berikut: dithiothreitol ~ NADH/FAD (8/10) >natrium dithionite > NADH/FAD (2:10) > natrium askorbat > asam askorbat (Gb.8).

Kata kunci: α-tocopherylquinone, reduksi, Vitamin E, hasil oksidasi.

Introduction

Vitamin E (α-tocopherol) in the unesterified form is slowly oxidised by atmospheric oxygen to form quinones, such as α-tocopherylquinone which Machlin (1984) concluded, from a review of the literature, were biologically inactive. However, this conclusion is in dispute (Emerson et al., 1939; Mackenzie et al., 1950; Cox et al., 1980; Gavino et al., 1981; Liepkalns et al., 1982).

Many experiments have been performed to observe the oxidation of α-tocopherol and its analogues. Inglet and Mattill (1955) studied the oxidation of α-tocopherol and its model compound, 2,2,5,7,8-pentamethy l-6-chromanol (**PH**) and also γ-tocopherol by benzoyl peroxide in benzene which resulted an excellent yield of 6-hydroxychroman benzoate and a small amount of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**PQ**) on the oxidation of **PH**.

The membrane concentration of vitamin E is very low, usually less than 0.05-0.1 mmol/mg protein (less than 1 per 1000 - 2000 membrane phospholipid molecules) and yet it is the major, if not the only chain-breaking antioxidant in membranes. Under normal conditions, “rancidification”, that is oxidation of membrane lipids and proteins, does not occur and it is difficult to rneder animals deficient in vitamin E, and vitamin E deficiency is seldom found in adult humans. So there must be an efficient mechanism for permitting low concentrations of

vitamin E to have such high efficiency in protecting membranes against damage and in supporting normal biological activity.

Parker et al. (1979) discovered a rapid free radical interaction between vitamin C and vitamin E and concluded that such a rapid interaction may be relevant to protection from free radical-mediated damage in vivo: the recycling of vitamin E at the expense of vitamin C. Moreover in certain conditions the vitamin C radical itself can be enzymatically reduced back to vitamin C by a NADH-dependent system.

To study the possibility of tranformation of α-tocopherylquinone into a more oxidiseable compound and at the same token finding out the recycling effect in the cells, an experiment was conducted using the model compound (**PQ**) and reduced it with various reductants.

Materials and Method

1. Reduction without air exclusion.

All of the following reactions were performed in methanol/water (1:1, v/v) hereafter called solvent.

1a. <u>Reduction of PQ (10 mM) by dithiothreitol (DTT, 50 mM and 200 mM)</u>

To 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzo-quinone **(PQ,** 2mmol) in solvent (0.1 ml) was added dithiothreitol (**DTT,** 10 mmol and 40 mmol) in solvent (0.1 ml), the mixture stirred at room temperature for 2 min and HPLC solvent (0.6 ml) added. The hexane layer (0.5 ml) was taken and dried (Na_2SO_4), then filtered and an aliquot (40 ml) chromatographed by Normal Phase Liquid Chromatography (NPLC).The reaction was repeated 7 times to give the time points shown in **Fig. 1**.

NPLC conditions: solvent: hexane/ethyl acetate= 3:2, separations were performed isocratically on a Lichrosorb Si 60, 10 μm column (300 x 4.6 cm) at a flow rate of 1 ml/min.

1b. Reduction of PQ (10 mM) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).:

The procedure was as above, but the sodium dithionite was dissolved in water.

1c. Reduction of PQ by glutathione :

The procedure was as above, except that the amount of glutathione used was greater than the other reductants and it did not dissolved in the usual amount of water. To **PQ** solution (0.05 ml) was added glutathione solution (0.15 ml) to give the final concentrations of **PQ** (20 mM) and glutathione (800 mM).

1d. Reduction of PQ (10 mM) by NADH (200 mM).

The procedure was as above.

1e. Reduction of PQ with NADH in the presence of FAD (Singer and Kearny, 1950) :

In the following reactions **PQ** was dissolved in methanol/0.05M phosphate buffer (pH 7.3; 1:1, v/v) and the reductants (NADH and FAD) were dissolved in phosphate buffer.

To **PQ** (2 mmol) in methanol/phosphate buffer (1:1, v/v, 0.1 ml) was added NADH (2 mmol) in phosphate buffer (0.1 ml) and the mixture stirred for 10 sec. Then FAD (2 mmol) in phosphate buffer (0.1 ml) was added and the protocol was performed and analysed as above. (The final concentration of **PQ**, NADH and FAD were 7 mM, 7 mM and 7 mM respectively). This experiment gave 65% reduction.

The above experiment was repeated using different concentrations of NADH plus FAD. Reduction by NADH (14 mM) plus FAD (7 mM) in 30 min was 100%. Other results of reduction of **PQ** (7 mM) by several combination of NADH and FAD were given in Fig. **3**.

2. Reduction under nitrogen

The following protocol describes the reduction of **PQ** by DTT for 5 min. Reduction for other time intervals and with other reductants were performed in the same way.

To **PQ** (2 mmol) in methanol/0.05 M phosphate buffer pH 7.3 (1:1,v/v, 0.1 ml) was added DTT ((20 mmol (except otherwise stated) in 0.05 M phosphate buffer (pH 7.3) or water (for sodium dithionite), 0.1 ml)) in a long, narrow tube (8 x 0.5 cm). The solution was mixed by leading a gentle stream of nitrogen through a capillary to the bottom of the tube for 5 min, then NPLC solvent (0.5 ml) was added and the mixture stirred with nitrogen for another 20 seconds. The hexane layer was removed, and dried

(Na_2SO_4), filtered and an aliquot (20 ml) chromatographed with NPLC, solvent. This protocol showed better (more) reduction than the previous protocol (stirring without nitrogen).

2a. Reduction of PQ (10 mM) by dithiothreitol (20 and 100 mM)

Results were recorded in Fig. **4**.

2b. Reduction of PQ (10 mM) by sodium dithionite (20 and 100 mM).

Results were recorded in Fig. **5**.

2c. Reduction of PQ (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

Results were recorded in Fig. **6**.

2d. Reduction of PQ (7 mM) by NADH (14 mM) plus FAD (different concentrations).:

PQ was mixed with NADH first by leading a gentle stream of nitrogen for 20 sec, then FAD was added and the remaining protocol was as above. Results were recorded in Fig. **7**. NADH (56 mM) plus FAD (70 mM) gave a very fast reaction, which was completed in 5 minutes.

2e. <u>Reduction of PQ (7 mM) by NADH (14 mM) plus FAD (7, 14 and 70 mM).</u>

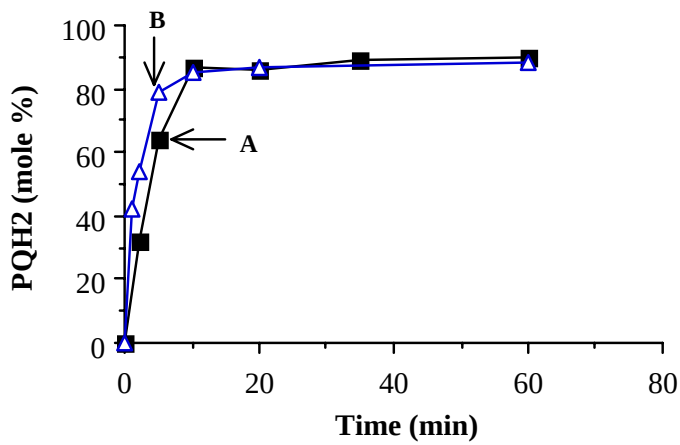
Results were recorded in **Fig 7**.

Result and discussion

In order to gain an appreciation of the ease of reduction of **TQ**, its model compound, **PQ** was subjected to reaction with several physiological and non-physiological reductants. **PQ** was used first because both the reductants and **PQ** could be dissolved in a methanol-buffer solution giving a homogeneous system. By contrast **TQ** could hardly be reacted in a homogeneous system due to its insolubility in water.

The experiments without air exclusion and using glutathione (up to the concentration of 40:1) showed no reduction and neither did the one using NADH.

Fig.1. Formation of **PQH_2** (mole %) from the reduction of **PQ** (10 mM) by dithiothreitol (**DTT**) (**A** = 50 mM and **B** = 200 mM).



In air, **DTT** still gave progressive reduction, it reached the maximum (about 90%) in 10 min for both concentration.

Fig. 2. HPLC analysis of the reduction of **PQ** (10 mM) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).

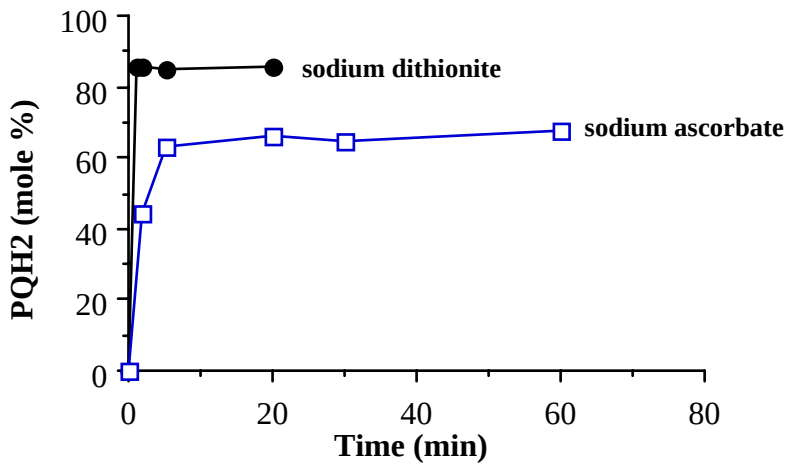
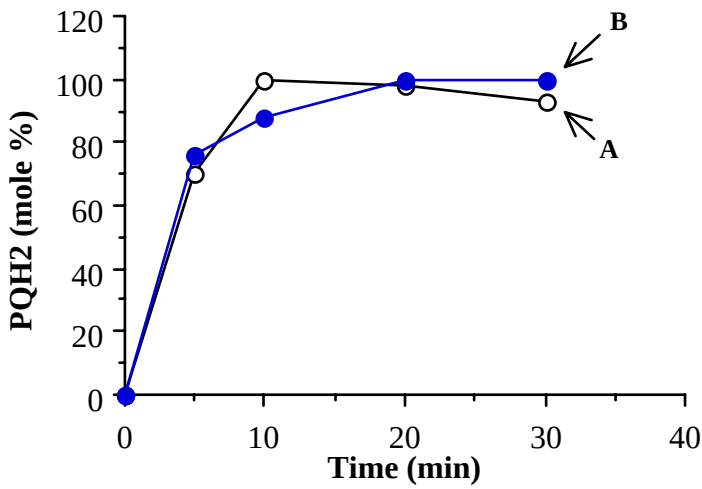


Fig. **2** showed that like **DTT**, sodium dithionite and sodium ascorbate were still capable to reduce **PQ** into **PQH_2** in air and sodium dithionite reduced faster than sodium ascorbate.

In the experiments using NADH and FAD in various concentrations, the results were as follows: Reduction of **PQ** (7 mM) by:

1) NADH (7 mM) plus FAD (7 mM): 30 min reaction gave 63% reduction.
2) NADH (14 mM) plus FAD (17.5 mM): 5 min and 30 min reaction gave 52.0% and 100% reduction respectively.
3) NADH (14 mM) plus FAD (35 mM) and NADH (14 mM) plus FAD (70 mM): results were presented in Fig. **3**.

Fig. 3. Formation of **PQH_2** (mole %) from the reduction of **PQ** by various concentration of NADH plus FAD. **A** = NADH (14 mM) plus FAD (35 mM), **B** = NADH (14 mM) plus FAD (70 mM).



The reduction by NADH plus FAD was slower than those by **DTT**. Sodium dithionite and sodium ascorbate at the earlier stage, however, it also reached similar maximum reduction at 10 min (NADH (14 mM) plus FAD (35 mM)) and 20 min (NADH (14 mM) plus FAD (70 mM)).

Results of Reduction of PQ performed under a gentle stream of nitrogen.

Fig. 4. Formation of **PQH_2** (mole %) from the reduction of **PQ** (10 mM) by dithiothreitol (**A** = 20 mM and **B** = 100 mM).

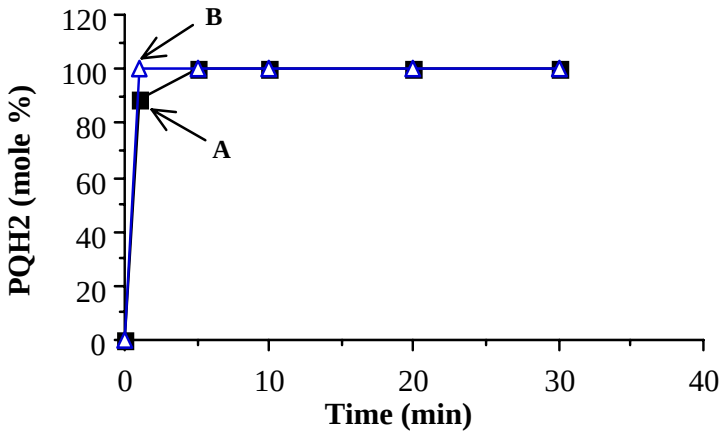


Fig. **4** showed that under N_2, even with lower concentrations (20 mM and 100 mM), **DTT** reduced **PQ** to **PQH_2** faster than in the reactions in air (compared with **Fig 1**).

Fig. 5. Formation of **PQH_2** (mole %) from the reduction of **PQ** (20 mM) by sodium dithionite (**A** = 20 mM and **B** = 100 mM).

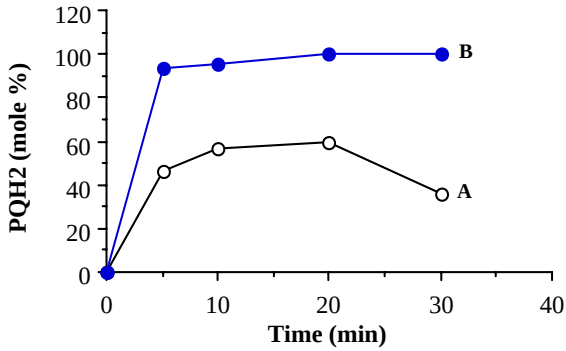


Fig. **5** showed the rate of reduction of **PQ** by sodium dithionite in different concentration

Fig. 6. Formation of **PQH_2** (mole %) from the reduction of **PQ** (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

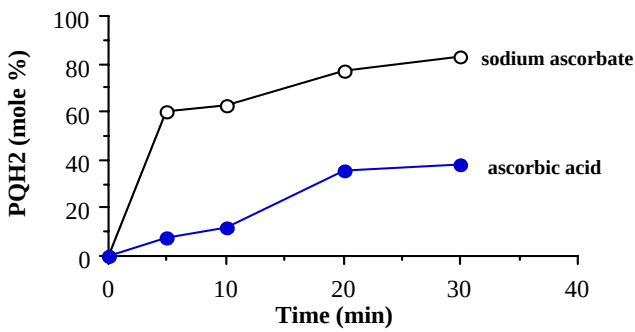
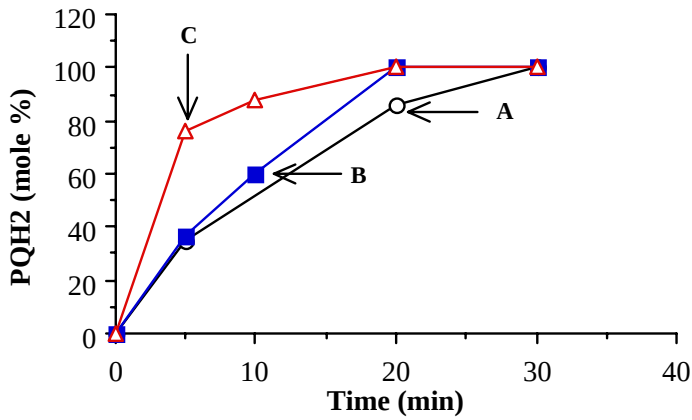


Fig. **6** showed in the reaction under N_2 ascorbic acid was capable to reduce **PQ** to **PQH_2** and the reduction of sodium ascorbate was faster than the one in air (compared with **Fig 3**).

Reduction of PQ (7 mM) by NADH plus FAD.

As there was no reduction on the reaction between **PQ** and NADH alone, the experiments were performed using NADH plus FAD in various concentration

Fig. 7. Formation of **PQH_2** (mole %) from of the reduction of **PQ** (7 mM) by NADH (14 mM) plus FAD (**A** = 7 mM, **B** = 14 mM, **C** = 70 mM).



It was obvious that the reductions by dithiothreitol, sodium dithionite and sodium ascorbate were affected by oxygen and that reactions under nitrogen would show a difference. Reduction by dithiothreitol in air never reached 100% attaining a maximum (88%) in 10 min and then remaining constant (Fig.**1**, line **A**,). Even when the ratio of dithiothreitol to **PQ** had been increased to 20:1 it did not make much difference.(Fig.**1**, line **B**). When the reduction was performed under nitrogen, with the smallest ratio of 2:1, dithiothreitol gave a total reduction at 5 min (Fig.**2**, line **A**), while a 10-fold molar ratio gave a total reduction at 1 min (Fig.**2**, line **B**).

Sodium dithionite showed a similar pattern to that of dithiothreitol. Reduction by a 20:1 molar ratio of sodium dithionite in air reached the maximum of 86% in 1 min and then remained constant (Fig.**2**). Reduction by 2:1 sodium

dithionite under nitrogen reached 51% in 5 min, gradually increasing to 60% in 20 min, followed by an autoxidation of **PQH_2** into **PQ** at 30 min, which was probably because there was no excess of sodium dithionite to protect the **PQH_2** (Fig.**5**, line **A**). A 10:1 molar ration of sodium dithionite under nitrogen gave rapid reduction (94% at 5 min) and 100% in at 20 min which remained constant until 30 min (Fig.**5**, line **B**).

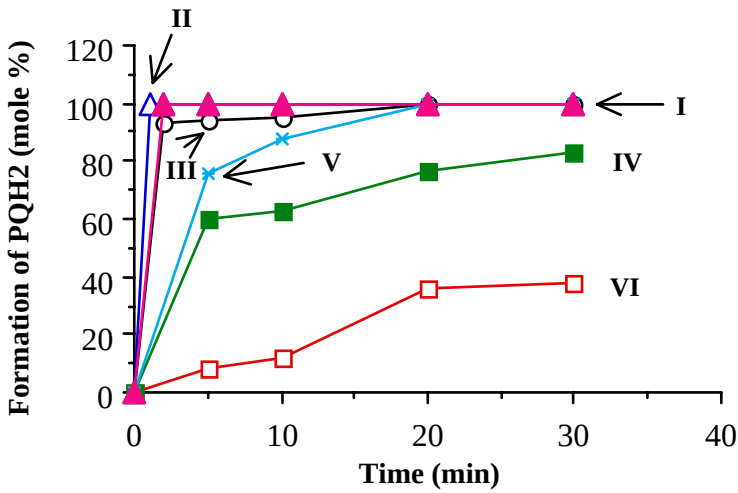
A 20:1 molar ratio of sodium ascorbate in air gave 44% reduction at 2 min and then gradually progressed to 68% at 60 min (Fig.**2**). However a 10:1 molar ratio of sodium ascorbate under nitrogen gave a slightly faster reaction which reached 84% reduction in 30 min (Fig. **6**).

NADH plus FAD gave total reduction (100%) at 30 min even in air (Fig.**3**). While reduction under nitrogen was much faster (Fig.**7**). However, at composition of NADH/FAD (14 mM/70 mM) the results were very similar whether the reduction was or was not performed under nitrogen (Fig. **3**, line **B** and Fig. **7**, line **C**).

Glutathione, at four times the concentration of the other reductants, did not reduce **PQ**. Since NADH by itself did not reduce **PQ** the effective reductant in the NADH/FAD combination must have been $FADH_2$. Thus there is a probability that in a biological system, the most probable reductant for **TQ** would be a flavin enzyme rather that ascorbic acid or glutathione. The non-physiological dithiothreitol was as effective as NADH/FAD which is interesting because of its similarity to the physiologically important reduced lipoic acid.

The reactivity of the above mentioned reductants decrease in the order of dithiothreitol ~ NADH/FAD (8/10) > sodium dithionite > NADH/FAD (2:10) > sodium ascorbate > ascorbic acid (Fig.**8**).

Fig. 8. Rate of reduction of **PQ** (10 mM, except in NADH/FAD reaction the concentration of **PQ** was 7 mM) by several reductants (100 mM). Presented as formation of **PQH_2** (mole %).



Legends **I** : Dithiothreitol (100 mM); **II**: NADH (56 mM) plus FAD (70 mM);**III**: sodium dithionite (100 mM): **IV**: sodium ascorbate (100 mM); **V**: NADH (14 mM) plus FAD (70 mM); **VI**: ascorbic acid (100 mM).

It can be concluded that the mode of action of α-tocopherol (**TH)** as an antioxidant is believed to involve a one electron redox cycle between **TH** and its radical **T**·. However the ease of oxidation of **PH** to **PQ** in aqueous system, the facile *in vitro* reduction of **PQ** to **PQH_2** by blood, the *in vivo* reduction of **TQ** to **TQH_2** after ingestion and also by a mixed population of leucocytes suggest that **TH** (Kohar, 1994) may be able to function in a completely different manner as antioxidant *in vivo*. If, for some reason, **T·** is not completely reduced to **TH** by ascorbic acid or another reductant, further oxidation of **TQ** may occur. There is also evidence that rats can biosynthesise **TQ** in the absence of **TH** (Hughes and Tove, 1980). A membrane-bound flavin enzyme may then reduce **TQ** to **TQH_2**.

Additional lipid peroxyl radicals could then reoxidise the **TQH_2** back into **TQ** thus establishing a quinone-hydroquinone antioxidant redox system similar to that of ubiquinone/ol-10. Thus the antioxidant activity of **TH** may be extended by its oxidation products in several ways. In some cases the oxidation products may themselves be antioxidants (Suarna and Southwell-Keely, 1991). Compound **TQ**, although of little use as an antioxidant in a non-initiated autoxidation (Golumbic, 1941 and 1942, Suarna and Southwell-Keely, 1991), may be converted into an antioxidant by reduction in a biological system. A get a more detailed explanation, some experiments of the reduction of **PQ** and **TQ** *in vitro* as well as *in vivo* are also carried out.

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