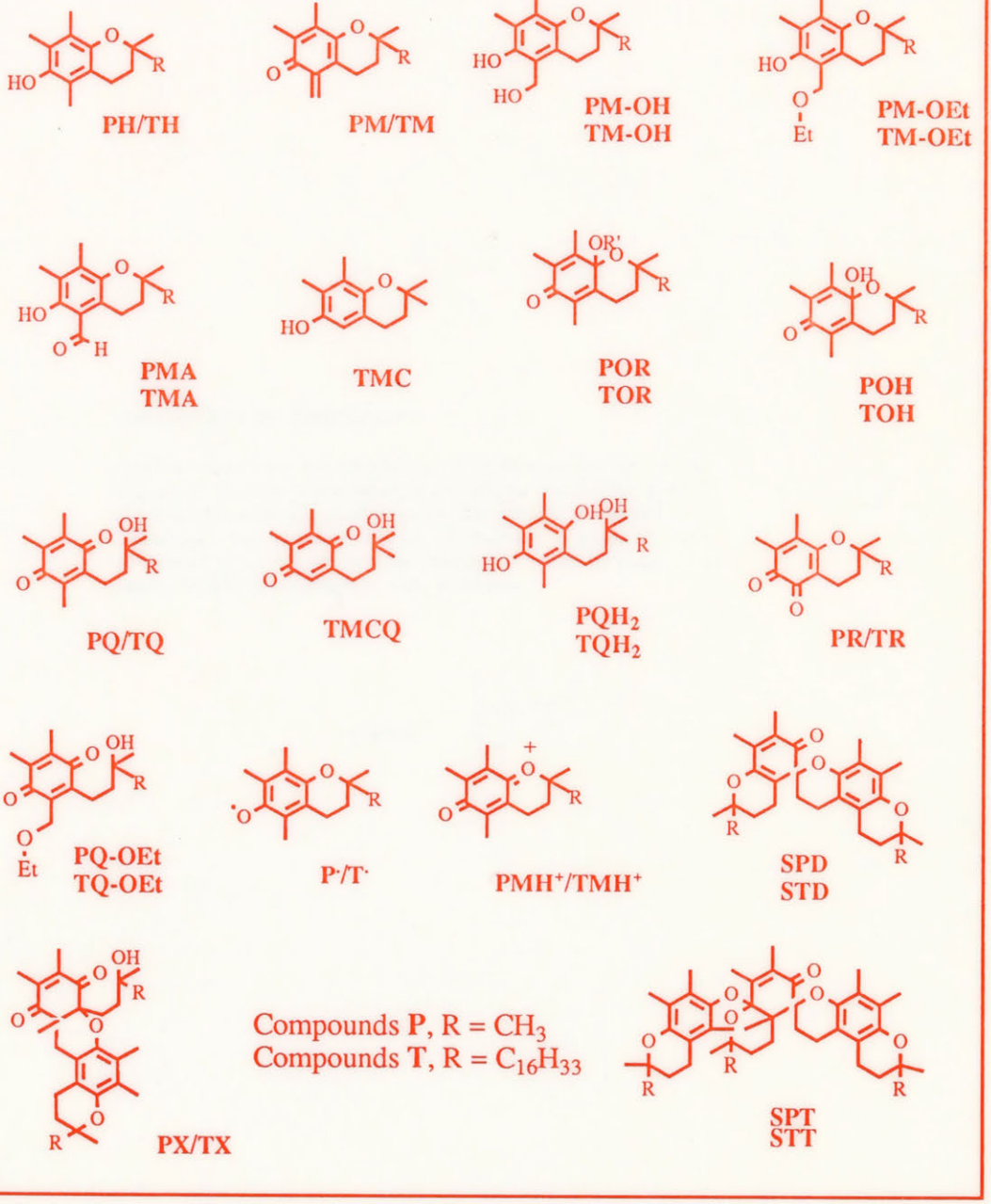
PH/TH
PM/TM
PM-OH
TM-OH
PM-OEt
TM-OEt
PMA
TMA
TMC
POR
TOR
POH
TOH
PQ/TQ
TMCQ
PQH2
TQH2
PR/TR
PQ-OEt
TQ-OEt
P·/T·
PMH+/TMH+
SPD
STD
PX/TX
Compounds P, R = CH3
Compounds T, R = C16H33
SPT
STT

STUDIES ON THE MODE OF ACTION OF VITAMIN E

by

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Dra. (The University of Surabaya)

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

at

THE UNIVERSITY OF NEW SOUTH WALES

March 1994

ACKNOWLEDGEMENTS

I would like to thank my Alma Mater, The University of Surabaya and also The Rector, Prof. Soebijono Tjitrowinoto, The Vice Rector I, Mr. Anton Prijatno, S.H., The Dean of The Faculty of Pharmacy, Drs. H. Ma'mur Apt. and all the members of The University and The Faculty for the opportunity given to me to achieve this Ph. D. degree.

I wish to express my sincere gratitude to my supervisor Dr. P.T. Southwell-Keely for his invaluable guidance, his understanding and patience and all he has done to help me throughout the years to complete this thesis, and most of all for his frienship.

My special thanks go to Drs. Handoyo, Apt., Dr. Harry K. Lasmono, my best friends Drs. R. Soediatmoko Sudiman, Mrs. B. Basuki and her late husband, Mr. S. Basuki for all their support prior to my departure and all the years I have been spending abroad.

I am very grateful to Prof. D. St.C. Black (Head of Department of Organic Chemistry) and Prof. P.S. Clezy for their help and suggestions and to Assoc. Prof. N. Cheetham and Dr. E. Mashimba for helping me with HPLC experiments.

Many thanks are extended to the staff of The School of Chemistry, in particular Mrs. H. Stender and Dr. Naresh Kumar (NMR Unit), Dr. J. Brophy (Mass Spectroscopy), Dr. H. P. Pham (Elemental Analysis), Dr. R. Haines (Physical Chemistry), Dr. L.Cherkson, Mr. J. Morgan, Mr. C. Bone and Mr. A. Skoblin (Electronic Workshop), Mr. R. Burgess and Mr. P. Sykes (Glass Workshop), Mr. P. Hallahan and his staff (Mechanical Workshop), Mrs. L. Fitzpatrick, J. Araya, Mrs. Thanh Vo Ngoc, J. Narayanan, Mrs. B. Wallington and Mrs. B. Litvak (Technical Staff) who have helped me in every possible way and all the staff in the Department of Physical Chemistry for allowing me to use their facilities in part of my experiments.

Special thanks to Dr. C. Suarna for all his patience and guidance from the beginning until the end of my course, to my very good friend and colleague, Mr. Harry Rose for his assistance to overcome my difficulties with the instrumentation. Also to all

my friends in the school of Chemistry for a very congenial atmosphere, in particular Mr. K. Lam and Mr. Don Rajasinghe.

I would also like to express my thanks to Fr. J. Bosman and Fr. P. Rijanto (The University Chaplains) and all the members of the Cathsoc. for all their help and moral support, and to my friends in St. Andrew's House, Prizzy and Dimitra, who strived with me in the final stage of my thesis writing.

I remember my parents and my sister and brothers and all their families, especialy my nephews Alex and Doni, with the highest gratitude for their continuous moral support while I have been overseas.

Finally, and the most importantly, my deepest and greatest gratitude to my Creator, whose loving care I always feel, Who always guides me and has sent me those wonderful people to help me in my difficult times.

Sydney, 27th March, 1994.

This thesis is dedicated to my father and mother,

Sundi and Sari Setiawati Kohar

and to my supervisor,

Dr. Peter T. Southwell-Keely.

ABSTRACT

Overoxidation of α-tocopherol by silver nitrate produces tocored as a major product. The first part of this thesis attempts to elucidate the pathway of formation of tocored using the α-tocopherol model compound, 2,2,5,7,8-pentamethyl-6-chromanol (**PH**), Oxidation of **PH** by silver nitrate in ethanol produces 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**PQ**) and 2,2,7,8-tetramethylchroman-5,6-dione (**PR**, the model compound of tocored) as major products. Formation of **PQ** is rapid and is accompanied by an equally rapid fall in pH. Formation of **PR** only occurs after **PQ** has reached maximum concentration and has begun to decline. It appears that acid promotes the dehydration and recyclization of **PQ** into a quinone methide (**PM**), which is then rehydrated into 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**), the phenolic isomer of **PQ**. Oxidative deformylation of **PM-OH** leads to **PR**. It is also demonstrated that **PQ** heated in ethanol in the presence of acid and in the absence of any oxidising agent, is converted into **PR**, **PH**, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OEt**) and 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (**PQ-OEt**). It seems that dehydration and recyclisation of **PQ** into **PM-OH** occurs as above and that **PQ** oxidises **PM-OH** into **PR**, while being reduced into the hydroquinone of **PQ** (PQH_2). PQH_2 then cyclises in acid to **PH**. A possible alternative pathway from **PQ** to **PR** that does not involve **PM-OH** is also discussed. These results suggest that **PQ** and, by implication, α-tocopherylquinone is not a stable compound and, in the presence of acid, is readily oxidised to **PR**. Thus it was observed that **PR** can be formed from **PH**, **PM-OEt**, **PM-OH** and **PQ** and was formed most rapidly from **PM-OH** suggesting that **PM-OH** is the immediate precursor of **PR**.

The second part of this thesis deals with HPLC analysis of the ease of reduction of α-tocopherylquinone (**TQ**) and its model compound **PQ** with several biological and nonbiological reductants, such as ascorbic acid, sodium ascorbate, sodium dithionite, dithiothreitol and NADH plus FAD. The experiments were performed under nitrogen and in neutral conditions (phosphate buffer pH 7), after several experiments in air gave

unsatisfactory results. **PQ** was reduced to PQH_2 faster than **TQ** to its hydroquinone (TQH_2) by all the above reductans. In **PQ** reduction, the reactivity of these reductants decreased in the order of dithiothreitol ~ NADH/FAD (8:10) > sodium dithionite > NADH/FAD (2:10) > sodium ascorbate > ascorbic acid. In contrast to **PQ**, **TQ** was not reduced by dithiothreitol, sodium ascorbate and ascorbic acid. In all these reductions, no formation of **PH** or **TH** was observed.

The third part of the thesis attempts to stimulate biological oxidation of **TH** and biological reduction of **TQ**. Incubation of **TH** with haemoglobin for 4 h produced a very small (12 %) conversion into **TQ** whereas a substantial amount (28 %) of **PH** was oxidised to **PQ** by haemoglobin in the same time. **PQ** was readily reduced to PQH_2 in blood whereas **TQ** was not reduced at all under the same conditions. However **TQ** was reduced to TQH_2 after ingestion and also by a mixed population of leucocytes.

The oxidation products **PQ** and **TQ** are formed by rearrangement of the unstable intermediates 8a-hydroxy-2,2,5,7,8-pentamethyl-6-chromanone (**POH**) and 8a-hydroxytocopherone (**TOH**) respectively. The fourth part of the thesis compares the ease of reduction of **POH** and **TOH** with that of **PQ** and **TQ** by a variety of reductants, both biological and non biological.

POH and **TOH** can be prepared from **PH** and **TH** by oxidation with N-bromosuccinimide. Ascorbic acid reduces **POH** and **TOH** to **PH** and **TH** respectively, the **POH** reaction being the faster and more complete. By contrast, ascorbic acid is a poor reducing agent for **PQ**, with PQH_2 the only product and does not reduce **TQ** at all. Sodium ascorbate does not reduce **POH** and **TOH** at all, but effectively reduces **PQ** and **TQ** to PQH_2 and TQH_2 respectively. Sodium dithionite reduces **POH** and **TOH** to mixtures of **PH** and PQH_2 and **TH** plus TQH_2 respectively, but reduces **PQ** and **TQ** to PQH_2 and TQH_2 only. NADH plus FAD reduces **POH** and **PQ** to PQH_2 and **TOH** to TQH_2 respectively.

TABLE OF CONTENT

PUBLICATIONS

Journals.

1. Kohar, I., Suarna, C. and Southwell-Keely, P.T. (1993) Oxidations of the α-tocopherol model compound 2,2,5,7,8-pentamethyl-6-chromanol. Formation of 2,2,7,8-tetramethylchroman-5,6-dione. Lipids 28, 1015-1020.
2. Kohar, I., Suarna, C., Southwell-Keely, P.T. and Stocker, R. (1993) Is α-tocopherol a reservoir for α-tocopherylquinone? Free Radical Res. Commun. (1993) (submitted for publication).

Posters.

1. Kohar, I., Suarna, C. Baca, M. and Southwell-Keely, P.T. (1992) A psossible role for α-tocopherylquinone as an in vivo antioxidant in *"Oxygen Radiacls" ed. Yagi, K. et al., Amsterdam: Excerpta Medica*, pp 617-20.
2. Kohar, I., Suarna, C., Baca, M. and Southwell-Keely, P.T. (1991) Is α-tocopherylquinone an in vivo antioxidant? Proc. Soc. Free Radical Res. (A'asia), Lorne, Vic., Nov. 1991: poster and abstract.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

I.1 Introduction.

The possibility of a relationship in human subjects between diet and chronic or acute disease has been a major preoccupation of nutritional and medical scientists for a long period of time. That free radical events and their disorders may be involved in a number of diseases has interested many scientists (Diplock, 1991). Since oxygen is required for cell viability and oxygen containing reactive molecules (some of which are free radicals) are most critical to biological systems, it is essential that mechanisms are available to control the reactive oxygen intermediates generated during cellular respiration (Bendich, 1990). An enzymatic system that requires adequate intake of selenium, copper, zinc and manganese, is needed to control the unwanted effect of the free radicals. If the level of intake of the minerals is low, proliferation of active oxygen metabolites may occur. Targets for attack are DNA, proteins, and polyunsaturated phospholipids, and peroxidation of polyunsaturated phospholipids will result in disruption of membrane architecture. Vitamin E, perhaps with ascorbic acid, can prevent this, and so do vitamin A and β-carotene (Diplock, 1991). These reactions have attracted the interest of many scientists and resulted in many researches and discoveries on free radicals and vitamin E and its interaction with other reductants and/or nutrients.

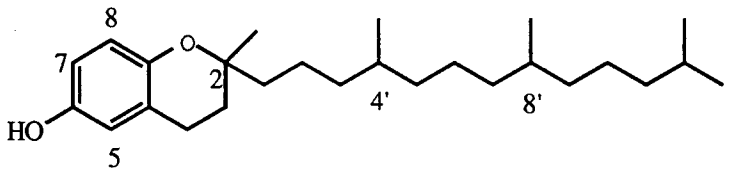
Since the early 1920s, after Evans and Bishop (1922) investigated influences of nutrition on reproduction in rats, a great deal of research on vitamin E has been performed. Evans suggested use of the letter E to designate the factor following the then recognised vitamin D. The Emersons were successful in purifying the factor affecting reproduction and suggested to Evans that it be given the name tocopherol from the Greek

tocos meaning childbirth and the verb *pherein* to bring forth. The suffix *ol* was added to indicate the alcohol (Evans et al., 1936). Vitamin E is now accepted as the generic name for a group of tocol and tocotrienol derivatives possessing some degree of vitamin activity and the most active compound is α-tocopherol (Machlin, 1991).

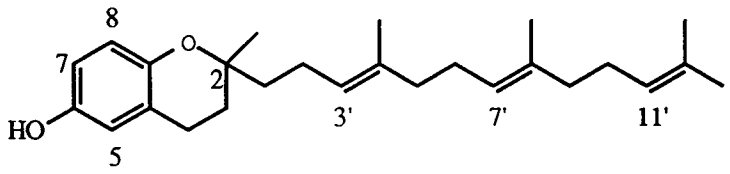
I.2.Chemical structure.

At least eight compounds have been isolated from plant sources that have vitamin E activity. Tocopherols are methyl-substituted derivatives of tocol, and comprise a chroman-6-ol nucleus attached at C-2 to a side chain composed of three saturated isoprene units. Tocotrienols are similar structures whose side chains contain three double bonds at 3', 7' and 11' positions of the side chain. The tocopherols and tocotrienols are designated alpha-(α), beta-(β), gamma-(γ) and delta-(δ), and differ according to the number and position of the methyl groups in the chromanol nucleus (**Fig.1**), and the β- and γ- forms are positional isomers. Of these, the principal tocopherols found in human and animal diets are α- and γ-tocopherol (Machlin, 1991).

Fig. 1 Chemical structures of the E vitamers.



Tocopherol



Tocotrienol

Position of Methyl Groups	Trivial Names (Abbreviations)	
	Tocopherols	Tocotrienols
5, 7, 8	α-tocopherol (α-T)	α-tocotrienol (α-T-3)
5, 8	β-tocopherol (β-T)	β-tocotrienol (β-T-3)
7, 8	γ-tocopherol (γ-T)	γ-tocotrienol (γ-T-3)
8	δ-tocopherol (δ-T)	δ-tocotrienol (δ-T-3)

I.3. Solubility.

The vitamins E are insoluble in water, readily soluble in alcohol and other organic solvents (including acetone, chloroform and ether), and in vegetable oils. Vitamin E acetates are less readily soluble in ethanol than the unesterified vitamin (The Merck Index, 1983).

I.4. Ease of oxidation of vitamin E.

Vitamin E (α-tocopherol, **TH**) in the unesterified form is slowly oxidised by atmospheric oxygen to form mainly biologically inactive quinones, such as α-tocopherylquinone (**TQ**)(**Fig.2**) (Machlin, 1984).

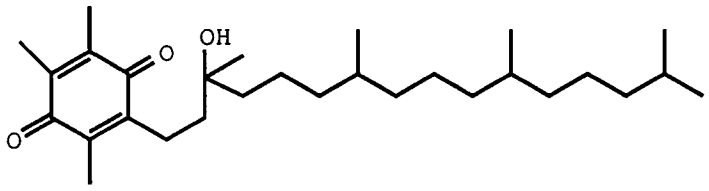
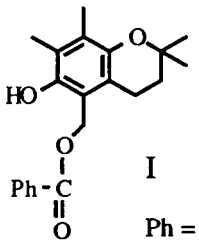


Fig.2. α-tocopherylquinone

Oxidation of α-tocopherol (**TH**) is accelerated by exposure to light, heat, alkaline pH conditions and the presence of certain trace minerals, such as iron (Fe^{3+}) and copper

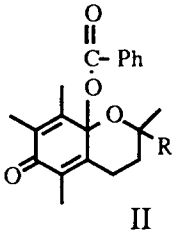
(Cu^{2+}). Ferrous and cuprous ions, and ground-state copper, do not react with **TH**. Ascorbic acid, if present, completely prevents the catalytic effect of ferric and cupric ions by maintaining the metals in their lower oxidation states. The oxidation products of **TH** in natural products include quinones, hydroquinones and tocopheroxides, as well as dimers and trimers (Machlin, 1984). Unlike the free tocopherol, the acetate ester is practically unaffected by the oxidising influence of air, light and UV light (Merck Index, 1983).

Many experiments have been performed to observe the oxidation of **TH** and its analogues. Inglet and Mattill (1955) studied the oxidation of **TH** and its model compound 2,2,5,7,8-pentamethyl-6-chromanol (**PH**) and also γ-tocopherol by benzoyl peroxide in



I

Ph = phenyl



II

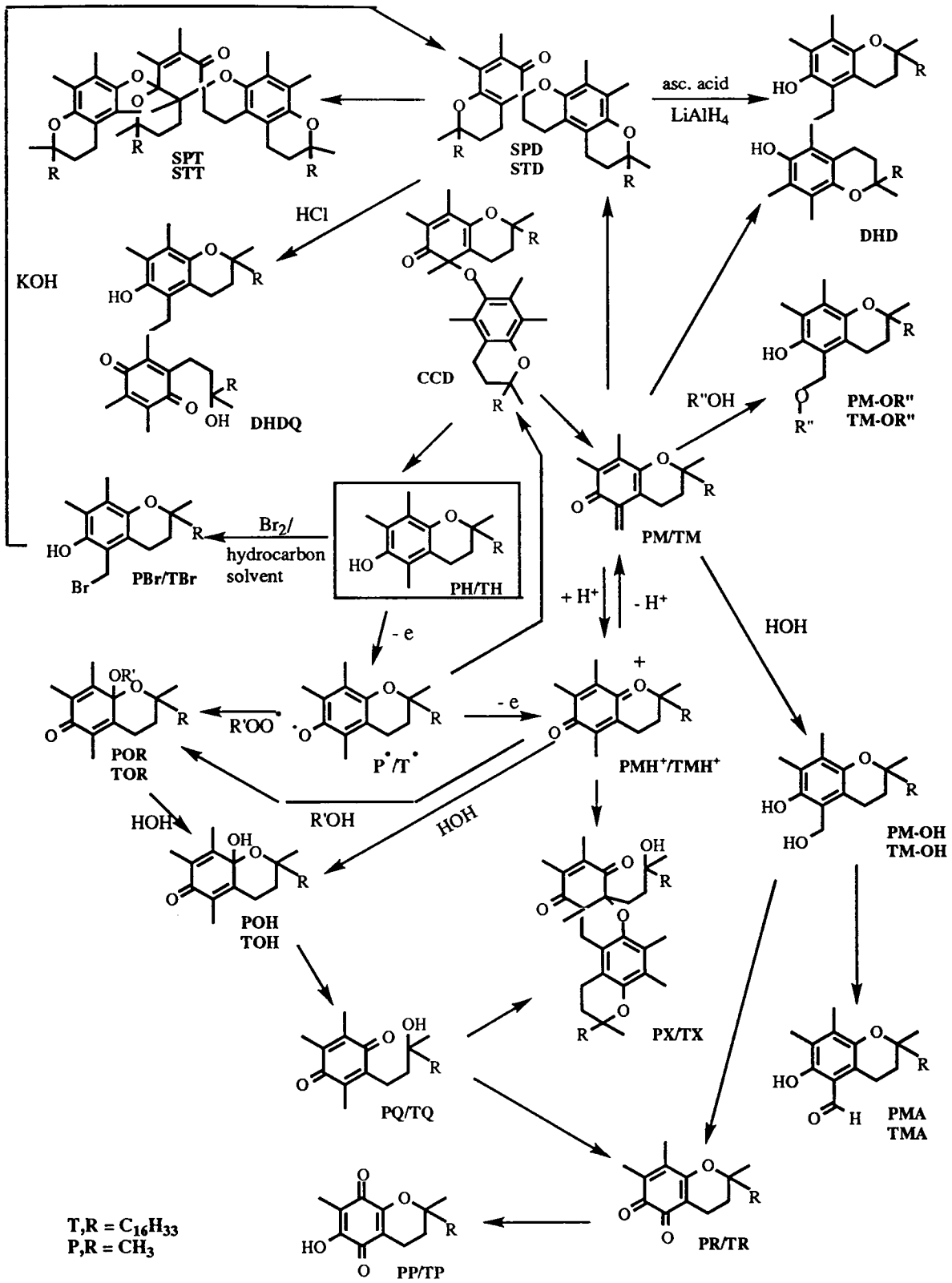
benzene which resulted an excellent yield of 6-hydroxychroman benzoate (I) and a small amount of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**PQ**) on the oxidation of **PH**. The oxidation of **TH** yielded 23-53% α-tocopherylquinone (**TQ**) and an appreciable amount of an intermediate compound (II). They proposed that products I and II could be accounted for best by assuming an intermediate formation of a 6-hydroxychroman free radical (**P•** or **T•**), see scheme **I**. The chromanoxyl radical could terminate itself by the formation of an hydrolysis-labile intermediate II, or by further oxidation to give **TQ** or **PQ**. On oxidation of γ-tocopherol, it gave the "red quinone", 2,7,8-trimethyl-2-(4',8',12'-trimethyltridecyl)-chroman-5,6-dione (**TR**). This illustrates the vulnerability of the C-5 position of tocopherol molecules. Goodhue and Risley (1965) oxidised **TH** with benzoylperoxide in anhydrous alcohols under nitrogen to find 8a-alkoxy-α-tocopherones (**TOR**), but when **TH** was reacted with bromine in hexane solution at room temperature, 5-bromomethyl-γ-tocopherol (**TBr**) was formed and converted rapidly to the spiranedienone dimer (**TX**) of tocopherol when treated with 1N KOH (Goodhue &

Risley, 1964). When reacted with bromine at -67°C in alcoholic solution **TH** produced 8a-alkoxy-α-tocopherones (**TOR**). They suggested that oxidation of **TH** in solvents such as hydrocarbons which can not donate groups to the 8a position mainly yields products substituted on the 5-methyl group of **TH**.

Frampton et al. (1952, 1954, 1960) performed some oxidation studies of **TH** and its model compound, **PH** with methanolic ferric chloride and they found four coloured oils (purple, blue-grey, orange and yellow) and a colourless wax. These were identified as 2,7-dimethyl-6-hydroxy-2-(4',8',12'-trimethyltridecyl)-5,8-chromandione (tocopurple, **TP**) and 6-hydroxy-2,2,7-trimethylchroman-5,8-dione (**PP**), **TQ** and **PQ**, 2,7,8-trimethyl-2-(4',8',12'-trimethyltridecyl)-chromane-5,6-dione (**TR**) and 2,2,7,8-tetramethylchroman-5,6-dione (**PR**), a dimer (**STD**) and trimer (**STT**) of α-tocopherol or **SPD** and **SPT** on the oxidation of **PH** (Skinner and Alaupovic, 1963, Skinner & Parkhurst, 1964), See scheme **1** (p. 6).

TH in petroleum ether was oxidised with alkaline ferric cyanide to find product (**STD**) which they reacted with ascorbic and hydrochloric acids to get 2 other products (**DHD** and **STT**, see scheme **1**). **DHD** can also be formed by reduction of **STD** with lithium aluminium hydride. When measured by the gestation-resorption bioassay of Mason and Harris, the activity of these 3 products showed less than 1/50 of the vitamin E activity of d-α-tocopheryl acetate (Nelan and Robeson,1962, Skinner and Alaupovic, 1963).

When oxidising **TH** with tetrachloro-o-quinone in aqueous acetonitrile or with N-bromosuccinimide in aqueous buffer-acetonitrile, Durckheimer and Cohen (1962, 1964) found a highly labile substance, the 8a-hydroxytocopherone (**TOH**) and 8a-hydroxy-2,2,5,7,8-pentamethyl-chroman-6-one (**POH**).



Scheme 1. Oxidation products of **PH** and **TH**.

Suarna et al. (1988, 1988a) found that oxidation of **TH** and its model compound, **PH**, by *t*-butyl hydroperoxide in chloroform containing alcohol produced 5-alkoxymethyl-7,8-dimethyltocol (**TM-OR**) or 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OR**) as the major products. In water-saturated chloroform **TH** and **PH** were oxidised by *t*-butyl hydroperoxide to **TQ** and **PQ**, 5-formyl-7,8-dimethyltocol (**TMA**), or 5-formyl-2,2,7,8-tetramethyl-6-chromanol (**PMA**), the spirodimer (**STD** or **SPD**) and spirotrimer (**STT** or **SPT**) of **TH** or **PH**, 5-(2,2,5,7,8-pentamethyl-6-chromanoxy)methyl-2,2,7,8-tetramethyl-6-chromanol, 5-hydroxymethyl-7,8-dimethyl-tocol (**TM-OH**) or 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**) and 3-hydroxymethyl-2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (**PQ-OH**). While in the presence of alcohol the major product was 5-alkoxymethyl-2,2,7,8-tetramethyl-6-chromanol.(Suarna, 1992) and in the absence of alcohol, the major products were **SPD** and **SPT** of **TH** and **PH**, also 1H,2,3-dihydro-3,3,5,6,9,10,11a(R)-heptamethyl-7a(S)-(3-hydroxy-3-methylbutyl)-pyrano[2,3-a]xanthene 8(7aH), 11(11aH) dione (**TX**). Different solvents and oxidants formed different products (Suarna, 1988, 1988a, 1989).

Liebler and Burr (1992) oxidised phosphatidylcholine liposomes containing **TH** with xanthine, xanthine oxidase and $FeCl_2$, and identified **TQ** and a highly labile **TOH** and other 8a-compounds.

I.5. Physiology of vitamin E (Vitamin E in vivo)

α-Tocopherol from natural sources (R,R,R-α-tocopherol) is the most biopotent form of vitamin E (Machlin, 1991). However a study on the activity against (Fe^{2+} + ascorbate)- and (Fe^{2+} + NADPH)-induced lipid peroxidation in rat liver microsomal membranes showed that *d*-α-tocotrienol has 40-60 times higher antioxidant activity and 6.5 times better protection of cytochrome P-450 against oxidative damage than *d*-α-tocopherol (Serbinova et al., 1990). They concluded that the higher antioxidant potency

potency of *d*-α-tocotrienol is due to (1) its higher recycling efficiency from chromanoxyl radicals, (see p.) (2) its more uniform distribution in membrane bilayers, and (3) its stronger disordering of membrane lipids which make interaction of chromanols with lipid radicals also more efficient.

Burton et al, (1983) showed that **TH** preferentially orients its long axis parallel to the hydrocarbon chains in a phospholipid layer, but indicated that the average depth of the head group and the ease with which the molecule could invert (ie., change from a 'head-up' to a 'head-down' position) had not been determined. Another problem relating to the physical location of **TH** in an organism has to do with the quite different vitamin E/lipid ratios found in different tissues - for example, plasma lipids as compared to erythrocyte membrane lipids, and normal liver cell lipids compared to Novikoff hepatoma lipids. Tocopherols are absorbed with other fats from the small intestine, transported through the lymphatic system within chylomicrons, and secreted from the liver in lipoproteins. **TH**-binding proteins found in hepatic cytosol may mediate intermembrane transport in cells (Catignani et al., 1977, Murphy et al., 1981, Mowri et al., 1981, Behrens et al., 1982, Verdon et al., 1988). Some recent reports have suggested that **TH** distribution between tissues may be affected by in vivo exposure to chemical toxicants (Liebler, 1993). The inhalation exposure to ozone enhanced pulmonary **TH** content and represented a mobilisation of **TH** from other tissues stores to the lung (Elsayed et al., 1990). Warren and co-workers also postulated that hepatic **TH** depletion in rats treated with methyl ethyl ketone peroxide or 1,2-dibromoethane represented mobilization of hepatic **TH** for distribution to other tissues (Warren & Reed, 1991 and Warren et al., 1991). Apparent **TH** half-life values ranged from approximately 5 to 7 days for **TH** turnover in rat or guinea pig liver, plasma, erythrocytes and spleen to nearly 30 days in rat brain and over 100 days in guinea pig brain. Ingold and co-workers used an innovative stable isotope dilution method to monitor the consumption and replacement of **TH** in rat and guinea pig tissues *in vivo*. Because turnover as measured in these experiments may reflect both the

oxidation and physical loss of unoxidised **TH** from tissues, the extent to which these processes contribute to **TH** turnover in different tissues remains to be investigated (Ingold et al., 1987 and Burton et al., 1990). According to Machlin (1991), people with plasma levels of less than 0.5 mg/dL can be classified as vitamin E deficient. He also stated that vitamin E levels in tissues varied considerably with no consistent relationship to lipid parameters and the highest vitamin E concentrations were found in the adrenal and pituitary glands, testis and platelets. The vitamin E is most concentrated in cell fractions rich in membrane, such as the mitochondria and microsomes.

One of the structural characteristics of **TH** is its long phytyl side chain. While the chromanol group is responsible for the antioxidant activity of the vitamin, the phytyl side chain apparently plays an important role in the incorporation and retention of **TH** in the membranes (Niki, 1987). He studied the inhibition of oxidation of soybean phosphatidylcholine (PC) liposomes using tocopherol analogous (E_n where n is the number of methylene groups in the phytyl side chain) as antioxidants and found that the length of inhibition period, the rate of oxidation during the inhibition period, and the rate of consumption of E_n were substantially independent of n, suggesting that the phytyl side chain of the chromanol had little effect on the inhibition of peroxidation of the liposome in which they are incorporated. When **TH**, **PH**, 2,6-di-*tert*-butyl-4-methylphenol and stearyl 3-(3,5,-di-*tert*-butyl-4-hydroxyphenyl)propionate were incorporated in soybean phosphatidylcholine liposomes in water dispersion, they showed similar antioxidant activities. However, when they were incorporated into dimyristoyl-phosphatidylcholine liposomes, only **PH** and 2,6-di-tert-butyl-4-methylphenol could suppress the oxidation of soybean phosphatidylcholine liposomes dispersed in the same aqueous system (Niki, 1985, Niki et al., 1988). This proved that the antioxidative properties of **TH** and its model compound without the phytyl chain are quite similar within micelles and liposomes as well as in homogenous solution but that the phytyl side chain enhances the retention of **TH** in liposomes and suppresses the transfer of **TH** between liposomal membranes, thus

can not suppress the oxidation of different membranes. While chromanols with short side chains were not retained in the liposomal membranes firmly and were able to suppress oxidative hemolysis in RBCS (erythrocytes).(Niki, 1987).

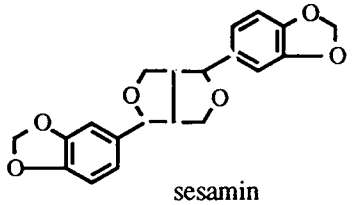
According to Urano and Matsuo's (1987) investigation, the antioxidant activity of **TH** against vitamin A-induced hemolysis of rabbit erythrocytes was higher than those of the other naturally occurring tocopherols, and was almost the same as those of chromanols having two isoprene units or a straight chain instead of an isoprenoid chain. It is also of interest that α-tocopheryl acetate and nicotinate inhibit hemolysis much more than α-tocopherol itself.

Again, Niki and co-workers (1988) studied the inhibition of **TH** on radical induced, oxidative hemolysis of rabbit erythrocytes. They observed that the erythrocytes from vitamin E-deficient rabbits underwent haemolysis faster than those from normal rabbits, but in the absence of 2,2'-azobis (amidinopropane)dihydrochloride (AAPH) there was little hemolysis in the vitamin E-deficient rabbit erythrocytes. Their study also showed that **TH** was consumed linearly with time during the incubation with AAPH, but that haemolysis took place before all the **TH** was depleted, and the shorter the side chain, the more effective the analogues in suppressing the erythrocyte haemolysis.

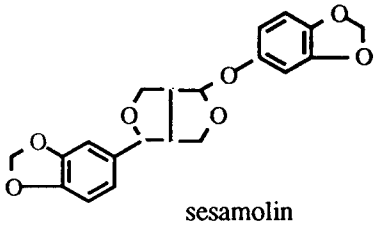
Norkus et al. (1993) suggested that UV-B irradiation enhances both the absorption of topically applied α-tocopheryl acetate and its bioconversion to free **TH.**

Among the eight natural forms of tocopherol having vitamin E activity, **TH** has the greatest physiological activity, however, γ-tocopherol (γ-**T**) is the most abundant form of vitamin E found in the lipid fraction of many seeds and nuts as soybean, walnuts and corn but its vitamin E activity in the prevention of liver necrosis in rats and in other indications of vitamin E activity is estimated to be 6-16% that of **TH** (Bieri & Evart, 1974). Even though humans ingest larger quantities of γ-tocopherol than that of **TH**, the concentration of γ-**T** in blood plasma is only -10% that of **TH**. Baker and co-workers discovered that the γ-**T** level in plasma decreased rapidly with the increase of **TH** level

due to supplementation with either *all-rac*-α-tocopheryl acetate or RRR-α-tocopheryl acetate, remained constant as along as the supplement was administered and rebounded several days after the cessation of supplementation (Baker et al., 1986). They also suggested that the γ/α plasma tocopherol ratio might serve as a sensitive index of **TH** ingestion. Yamashita et al. (1992) discovered that γ-**T** in sesame seeds exerted vitamin E activity equal to that of **TH** through a synergistic interaction with sesame seed lignans (sesamin and sesamolin).



sesamin



sesamolin

Cooney et al. (1993) noted that γ-**T** formed 5-nitroso γ-**T** in the presence of HONO and suggested that the superiority of γ-T to **TH** in the detoxification of nitrogen dioxide was due to the lack a C-5 methyl group and that was more reactive than **TH** at this position, by contrast **TH** reacted with NO_2 to form a nitrosating agent.

I.6. Biological Activity of Vitamin E.

Autoxidation (or lipid peroxidation) can be classified as a free radical chain reaction, which can be spontaneous and which many organic compounds undergo with atmospheric oxygen at ambient temperatures (Burton et al., 1983). It proceeds in three stages (Burton & Traber, 1990a). In the initiation phase, carbon-centered lipid radicals L• are produced by the abstraction from, or addition to, a polyunsaturated fatty acid, LH, of an oxy radical generated elsewhere (eg. by the decomposition of a hydroperoxide, LOOH).

Initiation : production of L• (carbon-centered radical).

$$HO\bullet + LH \text{ ----------> } H_2O + L\bullet \quad (1)$$

In the propagation phase (reaction 2 and 3), the carbon-centered radical reacts rapidly with molecular oxygen to form a peroxyl radical (LOO•), a chain-carrying radical that is able to attack another polyunsaturated lipid molecule. Although the initial peroxyl radical is converted to a hydroperoxide (LOOH), this process produces a new carbon-centered radical which is in turn rapidly converted into another peroxyl radical. This continues and can become a runaway process, consuming all polyunsaturated fat and forming a corresponding quantity of hydroperoxide (LOOH) (equation.3)(Porter, 1986).

$$L\bullet + O_2 \text{ ----------> } LOO\bullet \quad (2)$$

$$LOO\bullet + LH \text{ ----------> } LOOH + L\bullet \quad (3)$$

The chain reaction will terminate (termination phase) when the chain-carrying peroxyl (LOO•) meets and combines with another peroxyl radical to form inactive products.

Termination: $LOO\bullet + LOO\bullet$ ----------> inactive products.

Some compounds (which are called preventive antioxidants) can stop the initial production of radicals (Burton & Ingold, 1989). For example, catalase and glutathione peroxidase convert the free radical precursors hydrogen peroxide (H_2O_2) and fatty acid hydroperoxides (LOOH), respectively, into harmless products.

In the event that peroxyl radicals do form, a chain-breaking antioxidant is required to inhibit the propagation phase, thus reducing the rate of an autoxidation (Burton et al., 1983a; Burton & Traber, 1990a). Typically these antioxidants are sterically hindered phenols, of which **TH** is a special example. **TH** short-circuits the destructive propagative cycle and can intercept the peroxyl radical more rapidly than polyunsaturated fatty acids by donating its phenolic hydrogen atom to the radical and converting it to a hydroperoxide product (Burton & Traber, 1990).

Some authors have classified cellular antioxidants based on their physiological functions into three categories: preventive antioxidants, chain-breaking antioxidants, and repair and de novo compounds (Sies, 1985, Burton & Ingold, 1989, Niki, 1991).

- Preventive, or primary, antioxidants reduce the rate of initiation of free radical chains,

most commonly by converting the free radical-producing hydroperoxidic products of prior autoxidation, LOOH, to harmless products, such as the corresponding alcohols, ie.:

LOH <---------- LOOH ----------> LOO•, LO•, HO• (Burton et al., 1983a).

2. Chain-breaking, or secondary, antioxidants trap the chain-propagating peroxyl radicals, LOO•, and thereby reduce the length of the autoxidation chains. The majority of chain-breaking antioxidants, AH, are phenols or diarylamines and their mechanism of action can be described by the following reactions:

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet} \quad (4)$$

$$LOO^{\bullet} + A^{\bullet} \longrightarrow LOOA \quad (5)$$

Each molecule of AH stops two oxidation chains because the resonance-stabilised antioxidant radical, A•, is too unreactive to continue the chain (Burton et al, 1983a). The relative and absolute effectiveness of chain-breaking antioxidants depends primarily on their reactivity towards peroxyl radicals; that is, on the rate constant for the reaction (4), k_4. Reaction (5) is very rapid and does not affect the kinetics. The larger the magnitude of k_4 the better the antioxidant activity will be. (Howard & Ingold, 1963). L• is a lipid radical, which will react again and again with O_2 to form LOO• which in turn will react with LH until all the lipid is consumed and none left. In their kinetic studies on the inhibition of the autoxidation of styrene by a wide variety of synthetic phenols, Howard & Ingold (1963) showed that k_4 can be enhanced by substituting the aromatic ring with various electron-donating groups.

The preventive and chain-breaking antioxidants are considered as a first level of protection against oxidative damage. The second level of protection against oxidative injury in cells comes from a series of repair systems (Savanian, 1988). The repair systems include enzymes that attempt directly to restore biomolecules to their native conformation, as well as catabolic enzymes that specifically degrade non functional proteins, lipids, and nucleic acids (Niki, 1991, Davies, 1991).

TH is a hydrophobic, peroxyl radical-trapping, chain-breaking antioxidant found

in the lipid fraction of living organisms. Its principal function is to protect the unsaturated lipid materials of an organism from the undesirable effects of uncontrolled, spontaneous autoxidation (Burton et al., 1983).

TH will react with the lipid peroxyl radical to form the tocochromanol radical which is not a powerful radical and will not react with the lipid to form the lipid radical. It can also react with the lipid peroxyl radical to form molecular products (also called nonradical products) which terminate the sequence and is thus a chain breaking antioxidant by delaying or inhibiting the propagation of the free radical chain reaction (Howard et al, 1967, Burton, et al, 1986). The mechanism of prevention of lipid peroxidation by **TH** is through the transfer of hydrogen atoms from tocopherol to the lipid peroxyl radical LOO• (Willson, 1983). They react primarily with peroxyl radicals for three reasons. First, because propagation (reaction 4) is the slowest step in lipid peroxidation and peroxyl radicals accumulate to much higher concentrations than other radicals involved (Pryor, 1984). Second, peroxyl radicals react with **TH** several orders of magnitude faster than with most other molecules eg., polyunsaturated fatty acids (Howard & Ingold, 1967; Burton & Ingold, 1981). This kinetic rate advantage allows **TH** to suppress peroxyl radical-dependent reactions even at low concentrations. Third, **TH** and other phenols (AH) usually are poor inhibitors of reactions 1 and 2 because they do not compete effectively with other oxidisable substrates for initiating radicals such as HO• (Czapski, 1984 and Simic, 1991). Moreover, **TH** does not compete effectively with O_2 for reaction with carbon-centered radicals (Simic, 1991).

Interest in vitamin E is also increasing for its role in the prevention of certain disease states and the toxicities of a number of xenobiotics (Machlin, 1991).

Oxidative modification of low density lipoprotein (LDL) can lead to an increased and uncontrolled uptake of cholesterol by macrophages (Steibrecher et al., 1989). Formation of such high-uptake LDL *in vivo* is implicated as an early and perhaps crucial step in a cascade of cellular processes which leads to the formation of fatty streaks and

eventually atherosclerotic lesions in the artery wall (Steinberg et al., 1988). Since the formation of high uptake LDL is generally held to be preceded by, and to some extent caused by, peroxidation of the LDL lipid, much attention has been devoted to the inhibition of lipid peroxidation in LDL by endogenous antioxidant (Gey et al., 1991.)

There is growing clinical and biochemical evidence that free radical-mediated oxidative modification of low density lipoproteins (LDL) may be involved in the early stages in development of atherosclerotic lesions (Steinberg et al.,1989). Radical-trapping antioxidants will tend to protect LDL against oxidative modification and hence may diminish the risk and severity of atherosclerosis.

It was recently shown that, during a toxic chemical or oxidative insult to isolated hepatocytes, the cellular content of **TH** decreased dramatically, parallel with a stimulation of lipid peroxidation, and is followed by biochemical expressions of membrane damage and cell injury (Farris et al., 1985 , Pascoe et al., 1987). An increase in the **TH** content of these cells protects them against the oxidation-associated cytotoxicity. **TH** has been proposed to protect biological systems against oxidative damage by prevention of membrane lipid peroxidation (Willson, 1983), stabilisation of membrane structure, and through the maintenance of the redox balance of intracellular thiols (Pascoe et al, 1987). It is also accepted to function as a potent chain-breaking antioxidant in vivo by scavenging oxygen radicals and terminating free radical chain reactions and to protect biological molecules and tissues from oxygen toxicity. This has received much attention recently in connection with a variety of pathological events, rheumatoid arthritis, cancer and even aging process (Machlin, 1991). The order of *in vitro* antioxidant activities of the tocopherols is the same as that of their biological potencies, ie. $\alpha > \beta \geq \gamma > \delta$ (Century & Horwitt, 1965), and the antioxidant activity depends on the molar ratio of antioxidant to unsaturated lipid, with one molecule each of the α-, β-, γ - and δ- tocopherol protecting, respectively, 220,120,100 and 30 molecules of polyunsaturated fatty acid (Fukuzawa et al., 1982).

Barr and Crane (1977) studied the function of **TH** in the electron transport chain of spinach chloroplasts (Spinacia oleracea). They found that all quinones and **TH** could be recovered from untreated chloroplasts and no **TH** or plastoquinone C were found in chloroplasts treated with radicals (diphenylpicrylhydrazyl, DPPH). Also the recovery of increased amounts of **TQ** from DPPH treated chloroplasts suggested that some endogenous **TH** has been converted to the tocopherylquinone form.

Niki (1987a) studied the effect of inhibition of oxidation of liposomal and biomembranes by **TH**. He found that the liposomal membranes of phosphatidylcholine (PC) were oxidised by a free radical chain mechanism when they were attacked by free radicals in the presence of oxygen. Tocopherols and related chromanols incorporated into PC liposomes suppressed efficiently the oxidation which was induced by either 2,2'-azobis(2,4-amidinopropane) dihydrochloride (AAPH) or 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). His experiment also showed that **TH** produced a distinct inhibition period. It was consumed linearly with time and when it was depleted the inhibition period was over and a fast oxidation took place at the same rate as that without antioxidant and the length of inhibition period was proportional to the concentration of tocopherols. Also the rates of consumption of **TH** induced by AAPH were similar when it was incorporated into either soybean PC or 14:0 PC liposomal membranes, suggesting that **TH** can scavenge radicals, probably at the surface of the membrane, which attack membranes from the aqueous region. On the other hand, **TH** can also scavenge radicals within the lipid region of the membrane, since it suppresses the AMVN-initiated peroxidation where the free radical chain oxidation takes place. The high reactivity of tocopherols toward oxygen radicals stems from the weak O-H bond of chromanols and high resonance stabilisation of chromanoxyl radicals. Therefore, the esters of tocopherols such as tocopheryl acetate and tocopheryl nicotinate do not act as an antioxidant. One of the important questions on the activity of **TH** is its mobility in the membranes. The ratio of **TH** to lipids in biological membranes is small, usually below 1 to 1000. Therefore,

TH and/or lipid peroxyl radicals must move around rapidly to meet each other in order to suppress the peroxidation efficiently. Etsuo Niki (1987a) also stated that, when both α- and δ-tocopherols were incorporated into PC liposomal membranes and subjected to oxidation, only **TH** was consumed initially and d-tocopherol remained almost constant until after substantially all of **TH** was depleted. Niki et al , (1985) and Doba et al , (1985) proved that even in the liposomal membrane system there was a synergistic inhibition of peroxidation by the combination of vitamin E and C. This is because tocopheryl radical is reduced by vitamin C to regenerate **TH**.

The relative antioxidant activities of ubiquinol-10 vs. **TH** are ubiquinol-10 > **TH** in LDL, ubiquinol-10 < **TH** in homogeneous solution and ubiquinol-10 equals **TH** in aqueous lipid dispersion (Ingold et al., 1993). While in homogenous solution the antioxidant or radical scavenging activity of ubiquinol homologues does not depend on the length of their isoprenoid chain, in membranes ubiquinols with shorter isoprenoid chain (Q_1-Q_4) are much more potent inhibitors of lipid peroxidation than the analogues with longer isoprenoid chain (Q_5-Q_{10}) (Kagan, et al., 1990b).

I.7. Interaction of α-tocopherol and other cellular reductants and nutrients.

I.7.1. Interaction with ascorbate/TH recycling.

A synergistic action of ascorbate and **TH** was first reported in 1941 (Golumbic and Mattill, 1941), followed 20 years later by Tappel and coworkers (Tappel et al., 1961). Packer and co-workers used a pulse radiolysis experiment to observe this reaction directly in homogeneous solution (Packer et al., 1979; Leung et al., 1981) and also proved that in longer reactions (20-60 min.) the mixture of both vitamin C and E was much more effective in suppressing lipid peroxidation than the sum of both vitamins alone. This suggests that interaction between these vitamins yields an enhanced delivery of antioxidant protection. All these experiments provided evidence that a one-electron α-

tocopherol redox cycle exists in biological systems.

TH and ascorbate are synergistic antioxidants in many *in vitro* models for lipid peroxidation (Leung et al., 1981, Doba et al., 1985, Liebler et al., 1986, Wefers & Sies, 1988). In these systems, the onset of rapid lipid peroxidation in liposomal and microsomal membranes often is preceded by a lag phase, during which **TH** may be significantly depleted (Niki, et al, 1985a, Liebler et al, 1986, Scheschonka et al, 1990). In liposome oxidations initiated with lipid-soluble azo compounds, ascorbate prolonged the lag in rapid oxygen consumption (the index of lipid peroxidation used) and prevented **TH** depletion (Niki et al., 1985a, Doba et al., 1985). This prevention of **TH** consumption is often referred to as a "sparing effect", which may be defined as an ability of one antioxidant to slow or prevent the depletion of another (Liebler, 1993).

Ascorbate also extends the lag phase preceding rapid iron-catalysed lipid peroxidation in liposomes and microsomes (Fukuzawa, et al., 1985, Liebler et al, 1986, Wefers & Sies, 1988). As in liposome oxidations initiated by azo compounds, ascorbate prevents or slows **TH** depletion. The lag phase preceding rapid iron-catalysed lipid peroxidation is extended by increasing **TH** content through dietary supplementation (Hill & Burk, 1984). However, the lag phase preceding peroxidation of otherwise identical microsomal preparations may vary significantly depending on buffer type, metal chelators, and the presence of other oxidants (Scheschonka et al., 1990). Ascorbate also may control lag phases in iron-catalysed lipid peroxidation by altering the redox ratio of Fe^{2+}/Fe^{3+} (Miller & Aust, 1989).

Because it did not inhibit oxygen consumption in this system, ascorbate was considered unable to inhibit lipid peroxidation by directly trapping radicals in the lipid bilayer (Niki et al., 1985a; Doba et al., 1985), instead it was proposed to exert its antioxidant effect by recycling **TH**. Niki and co-workers (1984) studied the oxidation of methyl linoleate in homogeneous solution initiated with azo compounds in the absence and presence of vitamin C and vitamin E. Both vitamins acted as chain-breaking

antioxidants and they suppressed the oxidation and produced an induction period. In this study they found that vitamin E was consumed after vitamin C disappeared. Similar results were also observed when vitamin C was added during the induction period. Thus vitamin E decayed linearly with time at first, but then remained constant when vitamin C was added. They concluded that vitamin E which has higher K_{inh} than vitamin C, scavenges the peroxyl radical faster than vitamin C and the α-tocopheroxyl radical reacts with vitamin C to regenerate vitamin E. This sequence contributes to the regeneration and maintenance of vitamin E levels in tissue (Packer et al., 1979, Niki et al., 1984).

As vitamin E is oil-soluble and vitamin C is water-soluble, in biological systems these two vitamin must be located in separate phases. Doba and co-workers in 1985 developed a system, dilinolenoyl phosphatidyl choline (DPL) multilamellar liposomes to permit the differentiation of the effects of peroxidation initiated in the lipid phase from the effects of peroxidation initiated in the aqueous phase. This experiment led to the discovery that vitamin C is an excellent synergist with either **TH** or Trolox(-) for oxidation initiated in the lipid phase and that it is also a satisfactory co-anti oxidant with either Trolox(-) or **TH** for oxidation initiated in the aqueous phase. The inability of ascorbate to trap peroxyl radicals originating in the lipid phase, in contrast to aqueous peroxyl radicals, and the synergism with α-tocopherol indicate that α-tocoperoxyl radicals are more accessible to ascorbate than are membrane peroxyl radicals (Doba et al., 1985). In fact, ascorbate is a very powerful endogenous anti oxidant in plasma that can completely protect the lipoproteins from detectable peroxidative damage induced by aqueous peroxyl radicals. It traps the peroxyl radicals in the aqueous phase with a rate constant large enough to intercept virtually all these radicals before they can diffuse into the plasma lipids. The peroxyl radicals that escape the anti oxidants in the aqueous phase then diffuse into the plasma lipids to be trapped by the lipid anti oxidants like **TH** (Frei et al., 1990).

A similar explanation of different solubilities between vitamin C and E was suggested by Buettner, (1993). It is presumed that ascorbate recycles tocopherol via

tocopheroxyl radical, producing the ascorbate radical. Although ascorbate is a highly polar, lipid-insoluble anion, electron transfers from ascorbate to more lipid-soluble species may occur as a result of transient, reversible partitioning of electron acceptors into the hydrophilic surface region of the membrane (Sentjurc et al., 1990). In a membrane, the phenolic OH group of **TH** will be at the water-membrane interface, which is near the polar head groups of the phospholipids which compose the bilayer and the phytyl tail of tocopherol lies parallel to the phospholipid fatty acyl chains (Perly et al., 1985). Thus, the peroxidation and antioxidant process will proceed as follows:

1. The initiation process, the very fast reaction of the lipid radical with O_2 and the physical separation of the radical site on the polyunsaturated fatty acid (PUFA) and the "OH" of tocopherol exclude any significant reaction between PUFA radicals and tocopherol.

2. Once the lipid peroxyl radical is formed, a significant dipole is present, about 2.6 Debye (Barclay et al., 1981), that will allow the peroxyl radical moiety on the lipid chain to "float" (partition) to the membrane-water interface.

3. This provides a condition that allows the repair of PUFA radical by tocopherol, thereby preventing the chain propagation.

4. The relatively stable tocopheroxyl radical formed, is at the membrane-water interface, allowing the water-soluble ascorbate access to membrane-bound tocopheroxyl radical for the repair reaction, thereby recycling the tocopherol (Packer et al., 1979, Niki et al., 1984, Doba et al., 1985).

5. The potentially dangerous lipid hydroperoxide can be cleaved by phospholipase A_2, allowing glutathione peroxidase (GPx) to detoxify the hydroperoxide to a fatty acid alcohol or it can be converted directly to an alcohol by phospholipid hydroperoxide - glutathione peroxidase (PH-GPx) (Ursini et al., 1985).

Ascorbic acid-6-palmitate afforded significantly greater protection than α-tocopheroxyl acetate, but less than **TH** itself. Trolox is far less effective than ascorbic

acid at inhibiting thiobarbituric acid reacting substances (TBARs) formation. While in terms of interactive effects, **TH** showed synergism with both forms of ascorbic acid, α-tocopheryl acetate showed only an additive effect. The combination of Trolox(-) and ascorbic acid almost completely abolished lipid peroxidation. The superior efficiency of ascorbic acid-6-palmitate over ascorbic acid is probably due, at least in part to its lipid solubility, even though, however, in biological systems hydrolysis of either α-tocopheryl acetate or ascorbic acid-6-palmitate to the free vitamin might influence the effectiveness of the antioxidant (Record, et al. 1992).

The ability of ascorbate to exert antioxidant effects in iron-catalyzed lipid peroxidation appears highly dependent on the membrane content of **TH**. For example, ascorbate inhibited iron-catalyzed lipid peroxidation and prevented **TH** depletion only in liposomes containing **TH** at levels above the threshold (Liebler et al., 1986). Similarly, ascorbate extended the lag phase preceding NADPH and iron-induced lipid peroxidation in normal rat liver microsomes, but not in vitamin E-deficient microsomes (Wefers & Sies, 1988). This control of the ascorbate prooxidant/antioxidant balance by membrane **TH** content strongly suggests that ascorbate exerts its antioxidant effect principally by regenerating **TH** (Liebler, 1993). At **TH** levels above the threshold, a relatively low peroxyl radical flux may limit tocopheroxyl-peroxyl reactions and favour tocopheroxyl reduction by ascorbate. At **TH** levels below the threshold, greater peroxyl radical flux may favor tocopheroxyl-peroxyl radical reactions at the expense of the recycling reaction (Liebler, 1993). Probably the strongest evidence that ascorbate regenerates **TH** in these liposomal and microsomal systems comes from experiments in which ascorbate alone exerted either a prooxidant effect or no antioxidant effect, but in which inclusion of **TH** allowed ascorbate to exert an antioxidant effect (Liebler et al., 1986, Wefers & Sies, 1988, Thomas, et al, 1992).

Scarpa et al., (1984) monitored tocopheroxyl radicals formed from **TH** during Fe^{3+}-triethylenetetramine- and phospholipid hydroperoxide-induced liposome oxidation.

In the presence of ascorbate, the tocopheroxyl signal was suppressed and the semidehydroascorbate radical signal was observed instead. Upon complete consumption of the ascorbate, the tocopheroxyl radical signal reappeared. In similar studies, Packer and colleagues detected tocopheroxyl radicals in liposomes, submitochondrial particles (Packer et al., 1989, Mehlhorn et al., 1989) and microsomes (Mehlhorn et al., 1989, Packer et al., 1989, Kagan et al., 1990) treated with arachidonic or linoleic acids and lipoxygenase or exposed to UV irradiation. These observations may reflect ascorbate-dependent regeneration of **TH** from the tocopheroxyl radical. However, none of these experiments distinguish ascorbate-dependent tocopheroxyl radical reduction from direct antioxidant actions of ascorbate that prevent **TH** oxidation (Liebler, 1993). In UV-irradiated samples, the appearance of the semidehydroascorbate radical ESR signal does not necessarily indicate tocopheroxyl radical repair because the semidehydroascorbate radical also can be formed directly from ascorbate by photoinduced electron transfer (Buettner et al., 1987). In oxidations initiated by the lipoxygenase/arachidonic acid system, arachidonic acid-derived peroxyl radicals have been proposed as the initiating oxidants (Kagan et al., 1990 & 1990a). However, neither the identity of the oxidizing species nor its ability to react directly with ascorbate or other water-soluble reductants has been determined. In the absence of this information, it is reasonable to conclude that the semidehydroascorbate ESR signal results either from ascorbate repair of the tocopheroxyl radical or from direct reaction of ascorbate with the oxidant (Liebler, 1993). Although Packer et al., pointed out that the reductants did not inhibit the lipoxygenase-catalyzed formation of conjugated dienes from arachidonic acid (Packer et al., 1989), this does not exclude the possible direct reaction of ascorbate with arachidonate-derived oxidants once they are formed (Liebler, 1993). Also the claim that **TH** does not inhibit lipoxygenase (Packer et al., 1989) conflicts with another report that **TH** noncompetitively inhibits this enzyme (Reddana et al., 1985). Whether this inhibition involves **TH** oxidation is not known. Nevertheless, these experiments do not provide unambiguous evidence for

tocopheroxyl radical repair because they do not distinguish prevention of **TH** oxidation from regeneration of **TH** (Liebler, 1993). Ingold et al., (1993) reported that ascorbate is an extremely effective antioxidant for LDL containing **TH**.

When applied topically, **TH** acts not just as a sunscreen but affords more biological protection, possibly by reducing free radical-driven damage to the cell membrane and to the genome, while high dietary levels of the vitamin did not alter the degree of lipid peroxidation (Record et al., 1991). On the contrary, Mitchel and McCann (1993) claimed that topically applied **TH** may act as a complete tumor promoter in 7,12-dimethylbenz[*a*]-anthracene (DMBA)-initiated mouse skin. They suggested that since **TH** is a powerful antioxidant, reduction of cellular oxidant levels may trigger the tumor promotional process and it will be wise to be careful to avoid repetitive or prolonged topical exposure of human skin to antioxidants like **TH**.

The available data from *in vitro* model experiments do not adequately dissociate direct antioxidant effects of ascorbate from ascorbate-driven tocopheroxyl radical repair, although direct antioxidant effects are often assumed to be negligible (Liebler, 1993). The usual caveats about extrapolating *in vitro* observations to *in vivo* systems are particularly applicable. The sensitivity of the ascorbate-α-tocopherol interaction to variations in **TH** content, ascorbate concentration, catalytic metals and types of initiating radicals suggests that these variables would modulate the efficiency of an ascorbate-dependent, one-electron redox cycle *in vivo* . Indeed, the fate of tocopheroxyl radicals formed during "normal" turnover in unstressed tissues may be different than that in tissues exposed to a toxic oxidant stress (Liebler, 1993). The fate of the tocopheroxyl radical in a liposome model system appeared to vary with the rate of **TH** depletion (Liebler & Burr, 1992). This suggests that the rapid production of peroxyl radicals, as may be expected during a toxic oxidative stress, may drive tocopheroxyl-radical-peroxyl radical reactions that produce epoxides and other irreversibly oxidized products. In contrast, relatively slow formation of peroxyl radicals may allow the tocopheroxyl radical to participate in disproportionation

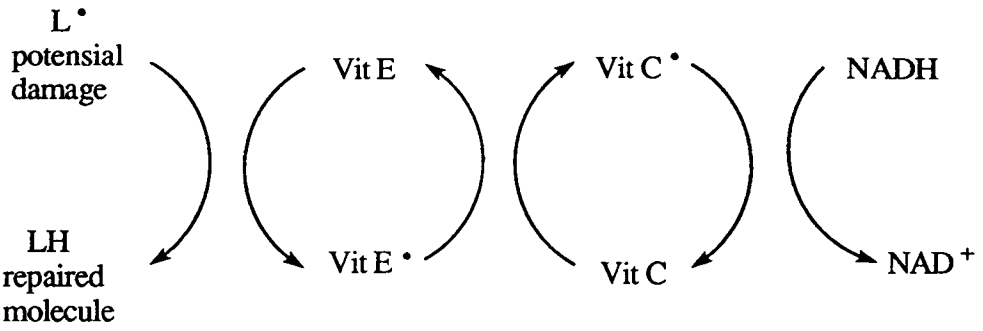
reactions or in reactions with reductants that result in recycling. Future product studies of α-tocopherol turnover *in vivo* under normal conditions and those of oxidative stress should provide a test of this hypothesis (Liebler, 1993).

Sharma & Buettner (1993) used electron spin resonance (ESR) spectroscopy to study the production and temporal relationship of ascorbate and tocopheroxyl radicals in plasma subjected to continuous free radical-mediated oxidative stress.

TH, β-carotene, NADH, reduced cytochrome c and several phenothiazine derivatives undergo rapid electron transfer reactions with a variety of electrophilic free radicals, while thiol-containing compounds undergo hydrogen atom transfer reactions, but thiyl radicals also readily undergo electron transfer reactions (Willson, 1983). Unlike **TH**, ascorbate reacts with the carbon-centered isopropanol radical, even though the reaction is relatively slow compared to the reaction of the radical with glutathione and presumably also occurs by a hydrogen atom transfer mechanism (Willson, 1983).

The membrane concentration of vitamin E is very low, usually less than 0.05 -0.1 nmol/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 render 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.

Packer 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 enzymatically to vitamin C by a NADH-dependent system (**scheme 2**).



Scheme 2

In 1990, Kagan et al. using ESR measurement observed the recycling and antioxidant activity of tocopherol homologues of differing hydrocarbon chain lengths in liver microsomes. The efficiency of recycling increased in the order: α-tocopherol (α-C16) < α-C11 < α-C6 < α-C1. The higher recycling efficiency of short hydrocarbon chain homologs of **TH** is responsible at least in part for their greater antioxidant potency. In 1989 Packer and co-workers suggested that the recycling of **TH** and other phenolic antioxidants by plasma reductants may be an important mechanism for the enhanced antioxidant protection of LDL (Packer & Landvik, 1989).

The most explicit *in vivo* test of the ascorbate-α-tocopherol recycling hypothesis was reported by Burton et al (1990). They measured the turnover kinetics of **TH** in several tissues from guinea pigs fed diets containing different levels of ascorbate. Because guinea pigs cannot synthesize ascorbate, rates of α-tocopherol turnover were expected to vary inversely with ascorbate status. These investigators reported that **TH** turnover kinetics were unaffected by ascorbate status in all tissues examined and they concluded that ascorbate-dependent recycling of **TH** was of very little importance *in vivo* in guinea pigs that were not oxidatively stressed. Additional studies with this methodology may ultimately resolve the question of whether ascorbate mediates **TH** regeneration to a significant extent *in vivo*. **TH** turnover studies in animal models of oxidative stress might identify circumstances where ascorbate status affects **TH** turnover (Liebler, 1993).

Tappel (1968) hypothesized that vitamins C and E work synergistically to protect

lipids from peroxidation. When plasma is subjected to free radical-mediated oxidative stress, there is a steady decline in the level of ascorbate (Frei et al., 1988 & 1989). Only after the complete consumption of ascorbate does the **TH** concentration begin to fall, coinciding with the appearance and steady increase in the plasma lipid hydroperoxide content (Frei et al., 1989). This study agrees with a study performed by Mulholland and Strain (1993) on the effects of supplementation of young healthy volunteers with large doses of **TH** and vitamin C. Their study was based on the total radical-trapping antioxidant potential (TRAP) of plasma and it also showed that when **TH** and vitamin C were supplemented together, the increase of vitamin C level in the plasma was significantly less than if vitamin C was supplemented alone. It is possible that a mechanism exists to conserve **TH** and that vitamin C may be oxidised to some extent in the digestive system to prevent oxidation of the high dose (1 g) of **TH**.

Kagan and co-workers (1992) using ESR observed the recycling of **TH** in human low density lipoprotein. In their study they demonstrated that :

1) chromanoxyl radicals of endogenous **TH** and of exogenously added α-tocotrienol, **TH** or its synthetic homologue with 6-carbon side-chain, chromanol-α-C_6, can be directly generated in human LDL by ultraviolet light, or by interaction with peroxyl radicals produced either by an enzymic oxidation system (lipoxygenase + linolenic acid) or by an azo-initiator, 2,2'-azo-bis(2,4-dimethylvaleronitrile) (AMVN).

2) ascorbate can recycle endogenous **TH** or exogenously added chromanols by direct reduction of chromanoxyl radicals in LDL.

3) dihydrolipoic acid (DHLA) is not efficient in direct reduction of chromanoxyl radicals but recycles **TH** by synergistically interacting with ascorbate (reduces dehydroascorbate thus maintaining the steady-state concentration of ascorbate).

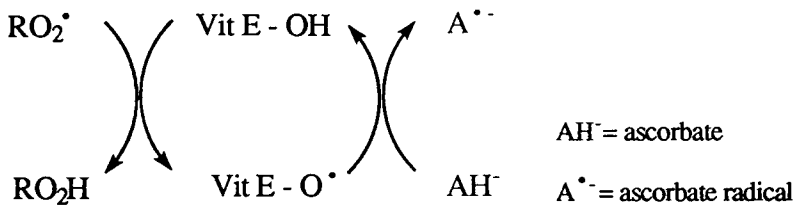
4) β-carotene is not active in **TH** recycling but may itself be protected against oxidative destruction by the reductants of chromanoxyl radicals.

By using submitochondrial particle membranes, Maguire et al., (1989) proved that NADH succinate and reduced cytochrome c-linked oxidation reduce the tocopheroxyl radical and **TH** consumption, and may have an important physiological role in recycling **TH**.

In 1992, Maguire, Kagan and co-workers proposed that reduced mitochondrial ubiquinones have a role in **TH** protection, presumably through efficient reduction of tocopheroxyl radical. In human erythrocyte membranes, **TH** recycling can be provided either by NADH-cytochrome b_5-dependent enzymatic recycling or by a nonenzymatic pathway involving ascorbate and dihydrolipoic acid (Constantinescu et al., 1993). Also in this study they observed that ascorbate and **TH** (but not **PH**) showed a synergistic effect in inhibiting lipid peroxidation produced by lipoxygenase and arachidonic acid in the erythrocyte membranes, while the combination of DHLA and **TH** (or **PH**) did not show the synergistic effect, but DHLA and ascorbate did.

A two-electron redox cycle was shown in the reaction of 8a-alkoxytocopherones (Durckheimer and Cohen, 1964) which decompose to **TQ** in aqueous acid but are easily reduced to **TH** by aqueous ascorbic acid. Following this hypothesis, Liebler and co-workers (1989) found that ascorbic acid reduces neither 8a-[(2,4-dimethyl-1-nitrilopent-2-yl)dioxy]tocopherone nor 8a-hydroxytocopherone directly but reacts instead with T^+. They also stated that the slow rates of these reactions at neutral pH suggest that biochemical catalysis may be required to complete a two-electron **TH** redox cycle in biological membrane.

In summary it is most probable that vitamin E radical can react with vitamin C involving a hydrogen and electron transfer mechanism although not proven unequivocally (scheme 3) (Willson, 1983).



Scheme 3.

Some reasons why ascorbate and **TH** are both well-suited to serve as small molecule chain-breaking donor antioxidants in biological systems:

1. Their radical are neither strongly oxidising nor strongly reducing.

2. Their radicals react poorly with oxygen, producing very little, if any, superoxide via electron transfer or peroxyl radicals by O_2 addition.

3. Kinetically, only relatively small amounts are required in order to be effective antioxidants.

4. They can be recycled, directly or indirectly, by enzyme systems.

I.7.2. Interaction with selenium.

The interrelationships of selenium and **TH** in animal nutrition suggest that the biological function dietary selenium must be analogous to that of **TH**. Selenium is known to follow the metabolic pathway of sulfur and is mainly incorporated into amino acids and proteins.The 4s and 4p electrons of selenium in comparison to 3s, 3p electrons of sulfur give unique oxidation reduction reactions in selenium compounds. The main oxidation-reduction reactions of selenium compounds which appeared related to its biological function can be classified as : i) lipid antioxidant inhibiting peroxidation, ii) peroxide decomposition, iii) free-radical scavenging, iv) repair of molecular damage sites (Tappel, 1965).

Buttriss and Diplock, (1988) discussed the interrelationship of **TH** and selenium using rats given a diet deficient in **TH** and selenium, or diets supplemented with either or both of these nutrients. Their research revealed (1) that all the organelles studied were

severely depleted of **TH** in rats deficient in **TH**, but that there was a large rise in the **TH** content in organelles of rats deprived of selenium but given adequate amounts of **TH**, suggesting an increased uptake or mobilisation of **TH** to compensate for the damaging effects of selenium deficiency. (2) Vitamin E deficiency caused a consistent fall of polyunsaturated fatty acid (PUFA) content (13-66% of the control level), selenium deficiency alone did not cause a consistent effect on PUFA in the fractions studied, while, paradoxically, **TH** and selenium deficiency together caused a consistent rise in the proportion of PUFA, ranging from 11 to 311% (the author did not explain this paradoxical phenomena). They also stated that the absence of **TH** and of the selenoenzyme glutathione peroxidase caused a more severe disruption in the membrane architecture than that in **TH** deficiency alone. As in the susceptibility of erythrocytes to haemolysis, **TH**, in contrast to selenium, decreased haemolysis whether or not glucose was present (Diplock, 1978).

Chen and co-workers (1993) investigated the effectiveness of **TH**, selenium, and β-carotene against oxidative damage in rat liver slices and homogenates. They stated that the effectiveness of antioxidants against oxidative damage to haeme proteins depended on the type of oxidation inducer. **TH** seemed to be more effective in protection against iron, while selenium was most effective against *t*-butyl hydroperoxide (TBHP) and β-carotene was effective against TBHP but not against iron. In general the combination of the three antioxidants gave the most effective protection against TBHP and iron. In 1990 Leibovitz et al. showed that in rat tissue slices, **TH** gave the greatest protection against lipid peroxidation in liver, heart and spleen, and selenium was most protective in kidney. The combination of them was either additive, less than additive or synergistic, depending on the concentration of the dietary antioxidants (Zamora et al., 1991).

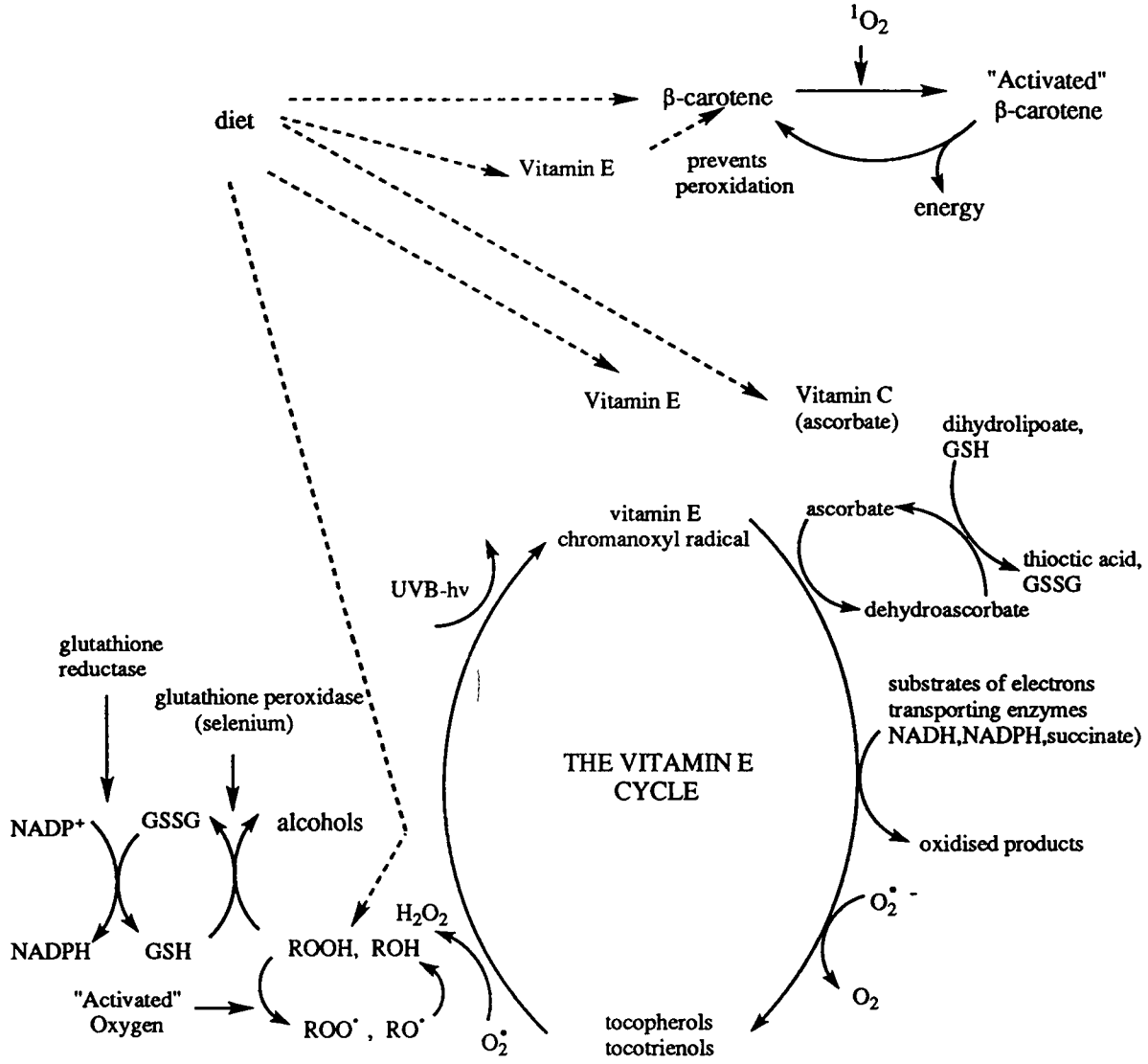
When tested against mammary carcinogenesis in female Sprague-Dawley rats, **TH** supplementation alone has been found to give no prophylactic effect, however, it can

potentiate the anticarcinogenic potency of selenium in the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumor model (Ip, 1988; Horvath & Ip, 1983).

I.7.3. Interaction with vitamin B_{12}.

According to Oski and co-workers (1966), there was an inverse relationship between levels of serum vitamin B_{12} and levels of serum tocopherol in the rat. It appeared that **TH** may play a role in the transport or metabolism of vitamin B_{12} and in its absence vitamin B_{12} accumulated extracellularly with the development of a possible intracellular deficiency.

Antioxidant interactions can be depicted as in scheme **4** (p. 31)(Bendich, 1990, Packer, 1992).



Scheme 4. Antioxidant interactions and recycling of **TH**.

I.8. α-tocopherylquinone (TQ) and α-tocopherylquinol (TQH_2).

Hughes and Tove (1980a) successfully reduced TQ to TQH_2 in the presence of NADH with a stochiometry of 1:1 catalysed by a partially purified soluble enzyme preparation from Butyrivibrio fibrisolvens which contained a relatively large amount of dithionite (Yamasaki & Tove, 1979). When the tocopherol derivatives were extracted aerobically, the ratio of TQ produced to fatty acid reduced was 2:1, whereas with anaerobic extraction it was 1:1 (Hughes & Tove, 1980a). This led to the hypothesis that

the reaction involved the oxidation of two TQH_2 to two TQH (the semiquinone). With aerobic extraction, the two semiquinones were oxidised to quinones, whereas, with anaerobic extraction, disproportionation occurred, yielding one TQ and one TQH_2. The equal levels of TQ and TQH_2 in most tissues and the equilibrium of the reduction of endogenous TQ by liver mitochondria may be similarly explained. While it is possible that the equality represents the true equilibrium between these two compounds, it is also possible that the mitochondrial reaction results in complete reduction of TQ to TQH_2 and that the major form of TQ in most tissues is the semiquinone. This would account for the high frequency of equal amounts of TQ and TQH_2 when extractions were performed anaerobically and why the presence of TQH_2 had been undetected in all the previous studies employing aerobic extractions (Hughes & Tove, 1980). They also discovered that the combination of TQ and TQH_2 varied in tissues of the rat from 20 nmol/g of adipose tissue to 215 nmol/g of liver and in each tissue except the brain, the TQ and TQH_2 were about equal. However neither compound was detected in blood.

The contents of TQH_2, TQ and TH (nmol/g) in rat hepatocytes determined by HPLC under anaerobic conditions were 3.1, ND and 5.0, 3.1-9.0 and 31.3 - 63.2 nmol respectively. However, TQH_2 was not detected in liver homogenates because endogenous TQH_2 autoxidised 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 TH/g liver respectively. Addition of TQ showed that TQ was reduced and converted to 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 (Hayashi et al., 1992).

I.9. Analyses and instrumentation.

Emmerie and Engel (1938) reported a colorimetric determination of TH based on the reducing properties of tocopherols towards ferric chloride. The ferrous salt which is formed in the reduction is determined colorimetrically with α:α'-dipyridyl.

Determination of tocopherols by paper chromatography was performed by Green et al. (1955) either by a single-dimensional separation on zinc carbonate-impregnated paper or in conjunction with a second-dimensional partition separation on paraffin-coated paper. Chromatographic runs are short and rapid and can be made without preliminary removal of carotenoids and moderate amounts of vitamin A or sterol.

Nair and Lura (1968) developed a method to identify **TH** from tissue by combined gas-liquid chromatography (GLC), mass spectrometry and infrared spectroscopy.

In 1970, Bieri et al. determined **TH** in erythrocytes by GLC. **TH** in a lipid extract was oxidised to **TQ** which was separated by thin layer chromatography (TLC), eluted and quantitated by GLC. Calculation was based on the recovery of added **TH**. Taylor (1970) purified the **TH** sample by column and thin layer chromatography and quantitated **TH** content in the rat liver microsomal fractions by spectrophotofluorometer. Then in 1971 Lehmann and Slover determined plasma tocopherol by GLC

In 1973, Kayden and co-workers performed a method of TLC followed by UV measurement ($FeCL_3$ reaction) to determine the **TH** content in red blood cells. Lovelady (1973) used a TLC-GLC system to separate the individual tocopherols for qualitative and quantitative determination in blood plasma and red blood cells.

Determination of **TH** in human serum had been performed by reverse phase chromatography (RPLC) with UV detection (292 nm) (De Leenheer et al., 1978, Bieri et al., 1979, Driskell et al., 1982) and also by normal phase chromatography (NPLC) with the same detection (280 nm) (Nilsson et al., 1978). RPLC with UV detection is applied to separate and analyse **TH**, tocopherol isomers, **TQ**, **STD**, retinol and cholesterol in red blood cells and human serum (Koskas et al., 1984, Stump et al. 1984, Cuesta Sanz and Castro Santa-Cruz, 1986).

McMurray and Blanchflower (1979) and also Hatam and Kayden (1979) performed a determination of **TH** in the plasma of cattle and pigs using RPLC with

fluorimetric detection while Lehmann and Martin (1983) used this method to determine α- and γ- tocopherol. Jansson et al. (1980) quantitated serum tocopherols by NPLC with fluorescence detection.

Pascoe et al (1987a) determined the **TH** and **TQ** content in small biological samples i.e. freshly isolated rat hepatocytes by RPLC with electrochemical detection.

Murphy and Kehrer (1987) performed a simultaneous measurement of tocopherols and tocopherylquinones in tissue fractions of chicken by RPLC with redox-cycling electrochemical detection.

The HPLC procedure has several advantages over other currently used methods for these vitamins. A simultaneous fluorometric procedure (Thompson et al., 1973) determined total retinol and total tocopherols whereas HPLC separates retinol from its esters and also distinguishes between α-tocopherol and β-,γ-tocopherol pair. Fluorometric analyses are often plagued by spurious results from contamination (Sinclair and Slattery, 1978). With HPLC, contamination is infrequent and if present is usually recognisable from the recording. Other procedures for analysing **TH** in plasma are usually time-consuming and involve inherent losses. In the HPLC method for plasma, no oxidative conditions or separation steps are included that would lead to losses. The use of internal standards eliminates errors from pipetting or the evaporation of solvents. Care must be taken, however, to see that the composition of the dilute working standards does not change from either decomposition or evaporation (Bieri et al, 1979).

The usual oxidative loss of **TH** during the preparation of red cells for analysis has been noted previously and various measures used to prevent it (Bieri et al, 1970, Kayden et al, 1973). Even though the cells were not saponified, significant formation of an oxidation product of **TH** occurred during haemolysis and extraction. Only the inclusion of 0.5% pyrogallol in the saline solution used to dilute the cells was found adequate to prevent oxidative loss (Bieri et al., 1987). The much smaller sample size and shortened

analysis time make the procedure a marked improvement over previous methods for red cell **TH** (Bieri et al 1970, Kayden et al, 1973).

In an effort to make biokinetic and bioavailability studies of vitamin E more convenient and accessible, Ingold and his research groups have developed the technique of stable isotope labelling and gas chromatography-mass spectrometry (GC-MS) to measure the absorption, transport, uptake and retention of tocopherols in humans and laboratory animals, as deuterated tocopherols may be ingested safely because deuterium is a stable isotope and has no deleterious effects. Furthermore, the deuterated tocopherols do not undergo any measurable, metabolically mediated exchange at the positions of substitution (Burton & Traber, 1990a).

I.10. Benefits of Vitamin E.

Some benefits of vitamin E are:

- possible anti cancer effects due to its capacity to down-regulate protein kinase C, which is very important in intracellular signalling. This would have the effect of inhibiting cell proliferation. Vitamin E and other antioxidants can function as anticarcinogens by quenching free radicals or reacting with their products, thus blocking formation of carcinogens, inhibiting carcinogens from reaching target sites and enhancing the immune system (Mahoney & Azzi, 1988; Mergens and Bhagavan, 1989; Boscoiboinik et al., 1991; Packer, 1992). Generally, tumor tissues peroxidise less easily than their normal counterparts and this resistance to peroxidation could be partially due to the greater concentration of tocopherol in the tumor tissue than in normal tissue (Swick and Bauman, 1951, Walton and Packer, 1980).

- hypocholesteremic effects: γ-tocotrienol is the most active as a hypocholesteremic agent by inhibiting human menopausal gonadotropin CoA reductase activity, the rate limiting step in the biosynthesis of cholesterol, even though the corresponding tocopherols do not show these effects (Packer, 1992).

- a lower risk to ischaemic heart disease mortality is significantly correlated with higher plasma levels of vitamins E and A (Packer, 1992).

- without adjustment for the various risk factors, plasma concentrations of vitamin E, vitamin C, and carotenoids were inversely related to an increased risk of angina pectoris, while after adjustment for the various risk parameters, only plasma vitamin E remained as a significant factor. Thus, populations with a high incidence of coronary heart disease might well benefit from diets rich in vitamin E (Packer, 1992).

- there is a high correlation between low plasma levels of vitamin E and the high risk of coronary artery disease due to the multifactorial actions of vitamin E, not only as an antioxidant but also as a biological response modifier. When polyunsaturated fatty acids, cholesterol and/or apoprotein B in low density lipoproteins are oxidatively modified, the damaged LDL are phagocytosed by macrophages and may result in the formation of foam cells. When the foam cells accumulate under the unbroken layer of endothelial cells, they aggregate to form a fatty streak which serves as a nucleus for the development of the atherosclerotic plaque. Higher concentrations of vitamin E are expected to hinder all of these processes (Packer, 1992).

- as lipid peroxidation may be an important factor in provoking premature aging, vitamin E may play an important role in delaying the aging process (Packer, 1992). The life span of small mammals is enhanced by the addition of vitamin E to the diet in some studies (Harman, 1980), but not in others (Blackett and Hall, 1981), however, many gerontologists remain sceptical of the true effect.

- it may also have an important role in arthritis, in relieving pain and improving mobility in patients with osteoarthritis, and also minimising or delaying cataract development in isolated animal lenses as well as protecting the lungs against the injurious effects of smog, smoke, and smoking (Packer, 1992).

As for the safety of vitamin E, animal studies show that vitamin E has low toxicity and is not mutagenic, carcinogenic or teratogenic (Machlin & Bendich, 1987). In human

studies using double-blind protocols, and in other large studies using oral vitamin E supplementation, few side effects have been reported even at doses as high as 3,200 IU/day. A variety of side effects have been reported in individual case reports or uncontrolled studies, but many of the effects have not been observed in larger, better controlled studies. So far, in conclusion, vitamin E appears to be remarkably safe when administered orally to adults (Machlin & Bendich, 1987).

I.11. Aims of the present work.

As has been mentioned before, a great deal of work has been performed in an attempt to prove the regeneration of **TH** through the recycling of tocopheryl radical (**T•**) by ascorbate or other enzymatic systems. An unanswered question is, what happens if the oxidation goes beyond **T•** or in other words if there is insufficient ascorbate.

Much work had been done in oxidising **TH** (or its model compound, **PH**) with various oxidising agents such as silver nitrate, *t*-butyl hydroperoxide, $FeCl_3$ etc. and a large number of products had been found. Some of these products are phenolic, and have been shown to posses antioxidant activity in a bulk phase system (Suarna and Southwell-Keely, 1991). Thus there is a possibility that the role of tocopherol is not ended after it is oxidised, but that some of its oxidation products will take over and continue the task. Surprisingly the product called tocored (**TR**) still has antioxidant activity against safflower oil. Tocored was not predicted to have antioxidant activity, because like the **TQ**, it is an oxidised compound and **TQ** is definitely not an antioxidant in this bulk phase system (Suarna and Southwell-Keely, 1991).

Tocored is an interesting compound because it is known to be a major oxidation product of γ-T. There have been conflicting data in the literature for many years concerning the antioxidant activity of α– and γ -T. When reacted with a radical, **TH** (or its model compound, **PH**) reacts faster than γ-T (or its model compound, 2,2,7,8-tetramethyl-6-chromanol, **TMC**) (Kunkel, 1950, Grams and Eskins, 1972, Burton and Ingold, 1981, Niki et al., 1986), but in autoxidation inhibition, **TMC** is more potent than

PH (Suarna and Southwell-Keely, 1991) . It was decided to investigate whether this contradiction was due to the oxidation products formed by **TH**- and γ-T (**TQ** and **TR**). The major oxidation product of **TH** is **TQ** which has no antioxidant activity whereas the major oxidation product of γ-T was believed to be tocored which does have antioxidant activity (Suarna & Southwell-Keely, 1991). Thus, perhaps tocored was contributing to the antioxidant activity of γ-T whereas **TQ** could not contribute to the antioxidant activity of **TH**. One of the aims of this work was to determine the route of formation of tocored from **TH** and also to test wether some of the oxidation products were intermediates in the formation of tocored.

Additional aims of this work were to investigate other reactions which may extend the antioxidant activity of **TH**.

There is at least one other way in which the activity of **TH** may be extended. If **TH** is oxidised to **TQ** in a biological system, it is quite possible that various reductants may be capable of reducing **TQ** to its hydroquinone (TQH_2) which would be expected to be a very good antioxidant. Thus an antioxidant redox cycle may be established between **TQ** and TQH_2 with **TH** acting merely as a reservoir for these compounds. The immediate precursor of **TQ** is the unstable compound 8a-hydroxy-tocopherone (**TOH**). It is possible that **TOH** may be reduced to either TQH_2 or **TH** in biological systems.

II.CHAPTER TWO

EXPERIMENTAL DETAIL

II.1 INSTRUMENTATION

II.1.1. Ultraviolet and Infrared spectrophotometry.

Infrared (IR) spectra were determined on KBr disc using a Perkin Elmer 580 N spectrophotometer. Ultraviolet (UV) spectra were determined on a Hitachi U-3200 spectrophotometer

II.1.2. ^{13}C and ^{1}H nuclear magnetic resonance.

^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectra were determined on a Bruker AM 500 spectrometer. NMR spectra were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard.

II.1.3. Mass spectrometry.

Electron impact mass spectra were determined on an A.E.I. MS 902 spectrometer. High resolution molecular weights were determined on a Bruker Spectrospin Fourier Transform Ion Cyclotron Resonance Spectrometer.

II.1.4. Elemental analysis.

Elemental analyses were determined on a Perkin Elmer Autoanalyser 240 and melting points (uncorrected) were determined on an Electrothermal melting point apparatus (England).

II.1.5. Thin layer chromatography (TLC).

Preparation of preparative TLC plates: to Kieselgel 60 GF_{254} (Merck, 100 g) was added demineralised water (200 ml) and the mixture was shaken for 40 seconds until it

was homogenised and thickened. The slurry was then spread over 6 plates (20x20 cm) to a thickness of 1 mm, using a spreader. The plates were let dry at room temperature and were activated before use by heating in an oven at 100°C for about 30 minutes and then cooling in a desiccator. In regard to TLC solvent, the light petroleum used has boiling point 60-80°C unless otherwise stated

II.1.6. High performance liquid chromatography (HPLC).

The instrumentation used in HPLC was:

1. Pumps : - Waters Model M-6000 A,
2. Columns: - normal phase, Waters μPorasil (250 x 4.6 mm)
   - reverse phase, C_{18} 10 μ Alltech/Applied Science, (250 x 4.6 mm).
   - Supleco C_{18}, (250 x 4.6 mm)
   - cyano, Millipore Waters RC (100 x 8 mm)
3. Detector : A. Waters Model 440 Absorbance Detector (fixed wavelength: 280 nm).
   B. ETP Kortec UV-VIS Detector (variable wavelength).
   C. ICI, SD 2100 UV-VIS Detector (variable wavelength).
   D. BAS dual electrode LC-4B electrochemical detector (Bioanalytical Systems).
4. Integrator: A. SIC Chromatocorder 12 : attenuation : 4, chartfeed : 5, end time : 12 min, minimum area : 1000, minimum height : 10, minimum width : 0.100, twice time : 0.000, calculation method : 00.
   B. Hewlett-Packard, HP 3394A Integrator
   C. Data acquisition interface: SMAD (Morgan-Kennedy Research).
   D. Shimadzu C-RuA Chromatopac.
5. Recorder: National Pen Recorder VP-6513 A
6. Solvent systems: A. Hexane/ethyl acetate = 4:1, B. Hexane/ethyl acetate = 3:2, C. Hexane/isopropanol = 98:2, D. Hexane/isopropanol = 96:4, E. Hexane/isopropanol = 95:5, F. Solvent a = hexane:chloroform = 9:1 and solvent b = hexane:chloroform:ethyl

acetate = 4:1:5, G. Methanol/Milli-Q water/glacial acetic acid = 12:7:1, H. Methanol/Milli-Q water = 92.5:7.5, I. Methanol/Milli-Q water = 95:5, J. Methanol/Milli-Q water = 98:2, K. Methanol/50 mM sodium perchlorate = 96:4

Unless otherwise stated the flow rate was 1 ml/min.

II.2. MATERIALS AND SOLVENTS

The following commercial materials and solvents were used without further purification: trimethylhydroquinone and 2,3-dimethyl-1,4-hydroquinone (Tokyo Kasei, TCI), *t*-butyl hydroperoxide (purification see p.39, Ega-Chemie), paraformaldehyde (Aldrich-Chemie), glutathione, NADH, FAD, ubiquinone-10, papain, dithiothreitol (Sigma), L-ascorbic acid and benzoic acid (May & Baker), N-bromosuccinimide (purification see **p.40**, Ajax), boric acid, sodium dithionite, glacial acetic acid (Ajax), sodium ascorbate, absolute alcohol (CSR), phosphate buffered saline (Dulbecco 'A'), HPLC grade hexane, ethyl acetate, methanol and isopropanol (Mallinckrodt), spectroscopic grade methanol (Mallinkrodt), spectroscopic grade ethanol (BDH), TLC-sheets, silica gel 60 F_{254}, 0.2 mm (Riedel-de Haen).

II.2.1. Purification of Chloroform (Vogel, 1961).

Double distilled chloroform was shaken with concentrated sulfuric acid (5%), then washed thoroughly with distilled water until the washings were pH 6 (pH meter), dried (anhydrous $CaCl_2$) and distilled. It gave pure chloroform bp. 61°C, which was used immediately.

II.2.2. Purification of *tert*-butylhydroperoxide (Barnard and Hargrave, 1951).

To commercial *t*-butylhydroperoxide (50g, 65% material) was slowly added with stirring 10N NaOH (16 g in 40 ml water) and the precipitated sodium salt was redissolved in a minimum quantity of water. The solution was then extracted with ether (3 x 75 ml). The ether layer was discarded and the aqueous layer was then acidified (to pH 2-3) by the slow addition of HCl, keeping the temperature below 5°C, then extracted again with ether (3 x 25 ml). The ether phase was dried (Na_2SO_4) and the solvent removed *in vácuo*. The residue was distilled to give 3 fractions:

1. Fraction I : 2.47 g, bp. 31-32°C/20-23 mm Hg.
2. Fraction II : 3.61 g, bp. 27-31°C/20 mm Hg.
3. Fraction III : 0.61 g, bp. 25-27°C/20 mm Hg.

The purity of the samples was checked iodometrically as in **II.2.3.**below.

II.2.3. Analysis of purified *tert*-butylhydroperoxide.

(Kokatnur and Jelling, 1941).

To an iodine flask was added isopropanol (25 ml), *t*-butylhydroperoxide (0.1 g), saturated potassium iodide solution (1 ml) and glacial acetic acid (1 ml). The mixture was heated to *incipient* boiling for 5 minutes with occasional swirling and, without cooling, it was titrated with 0.1 M sodium thiosulfate solution to the disappearance of the yellow colour.

The results were: fraction I: 94.6 %; fraction II : 99.3 % and fraction III : 95.3 %.

II.2.4. Purification of dl-α-tocopherol.

dl-α-Tocopherol (Roche Products) was purified by TLC using hexane-ethyl acetate 4:1 as the developing solvent.

II.2.5. Purification of N-bromosuccinimide (Fieser & Fieser, 1970).

The yellow N-bromosuccinimide (2.3 g) was dissolved in bidistilled water (23 ml) and heated at 90°C while stirring until it dissolved. The solution was cooled to room temperature (the yellow coloured disappeared gradually) and white crystals were formed. The crystals were filtered by suction and dried in vacuo over P_2O_5 for 2 days. The yield was 1.02 g with mp 162-163°C (Fieser & Fieser 173-175°C).

II.3. SYNTHESIS

II.3.1. PREPARATION OF 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL (PH) (Smith et al., 1939).

Isoprene (15 ml) was distilled and the fraction boiling at 34°C was collected.

Trimethylhydroquinone (2g) and zinc chloride (0.2 g) were dissolved in glacial acetic acid (20 ml) and the solution heated to 100°C. The freshly distilled isoprene (2 g) was slowly added and the mixture was refluxed for 2 hours (118-120°C). One drop of concentrated sulfuric acid was added and the solution refluxed for a further 1 hour. The mixture was poured into water and the solid product was crystallised from ethanol. The white crystalline product (0.64 g) with mp. 94-95°C (lit., Smith et al., 1939, 94-95°C) was identified as 2,2,5,7,8-pentamethyl-6-chromanol and had:

Elemental analysis calc. for $C_{14}H_{20}O_2$: C = 76.36, H = 9.05.
Found: C = 77.00, H = 9.43

UV (λ_{max}, n-hexane) : 297 nm, ε = 4000.

IR (KBr)(cm^{-1}): 3280 (OH), 2983, 2933 (CH), 1462, 1385, 1372, 1088 (C-O-C), 927.

^{1}H NMR ($CDCl_3$): δ 1.28 (s, 6H, 2 x 2C-CH_3), 1.77 (t, 2H, 3-CH_2, J = 6.9 Hz), 2.12 (s, 6H, Ar-CH_3), 2.16 (s, 3H, Ar-CH_3), 2.63 (t, 2H, 4-CH_2, J = 6.9 Hz), 4.20 (s, 1H, OH).

^{13}C NMR ($CDCl_3$) : δ 145.69, 145.60, 122.60, 121.10, 118.56, 117.12, 72.48, 33.08, 26.71, 21.08, 12.22, 11.80, 11.29.

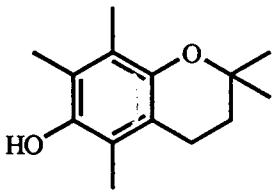
DEPT. EXPERIMENT ($CDCl_3$):

CH,CH_2 and CH_3 : 11.29, 11.80, 12.22, 21.08, 26.71 and 33.08.

CH_3 positive : 211.29, 11.80, 12.22 and 26.71.

CH_2 negative : 21.08 and 33.08.

The chemical structure:



$C_{14}H_{20}O_2$, M.W. 220.

II.3.2. PREPARATION OF 2,2,7,8-TETRAMETHYL-6-CHROMANOL (TMC). (Smith et al., 1939, modified).

To 2,3-dimethyl-1,4-hydroquinone (5.0 g) in glacial acetic acid (40 ml) was added $ZnCl_2$ powder (0.51 g) and the volume of the solution was made to about 100 ml with glacial acetic acid.

The mixture was heated to about 100°C to dissolve 2,3-dimethyl-1,4-hydroquinone and $ZnCl_2$ and to the solution was added isoprene (5.5 g) slowly for about 30 minutes. The mixture was heated under reflux for one hour and was allowed to stand for another one hour. Two drops of concentrated sulfuric acid were added and the reaction was stopped after 45 minutes. The mixture was cooled and poured into about 200 ml cold water. A white emulsion was formed with insoluble material floating on the surface.

The emulsion was extracted with ether (3 x 50 ml) and the combined ether layers washed with bidistilled water (4 x 30 ml) and extracted with Claisen's alkali until the ether layer became colourless. After the extraction, the Claisen's alkali was almost black.

[Claisen's alkali: KOH (20 g) was added to bidistilled water (14.3 ml), the solution cooled and methanol (57.1 ml) added].

The colourless ether layer was washed with bidistilled water (4 x 30 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was recrystallised from light petroleum (bp 40-60°C) to give colourless crystals (0.79 g) which had mp. 102-103°C (Frampton et al, 1960 found mp. 101-102°C) and was identified as 3,3,5,6,8,8-hexamethylbenzoyl[1,2-b,4,3-b']dipyran (bichroman of γ-tocopherol model compound) and had the following spectral data:

Elemental analysis calc. for $C_{18}H_{26}O_2$: C = 78.83 and H = 9.49
Found : C = 78.97 and H = 9.69.

UV (λ_{max}, n-hexane) : 300.2 nm., ε = 4399.

IR (KBr)(cm^{-1}): 3450, 2980, 2920, 1440, 1420, 1380, 1370, 1270, 1220, 1170, 1120, 1090, 960, 890, 840, 650.

MS (ei) m/z (rel.intensity) : 274 [M^+] (100); 219 (56); 218 (98); 203 (58); 174 (48).

^{1}H NMR : δ 1.29 (s, 12H, 4 x CH_3 on the chroman rings), 1.78 (t, 4H, 2 x CH_2 on the chroman rings), 2.10 (s, 6H, 2 x Ar-CH_3), 2.54 (t, 4H, 2 x CH_2 on the chroman rings).

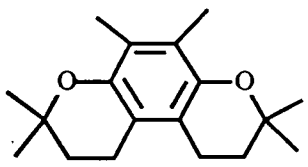
^{13}C NMR : δ 11.74, 20.09, 26.82, 32.98, 72.34 (2 peaks), 115.48, 123.35 and 144.72.

DEPT. EXPERIMENT ($CDCl_3$) :

CH and CH_3 positive : 11.74 and 26.82.

CH_2 negative : 20.09 and 32.98.

The chemical structure:



$C_{18}H_{26}O_2$, M.W. 274.

The black Claisen's alkali fraction was poured immediately into bidistilled water (300 ml) and glacial acetic acid (100 ml) added to ensure that the mixture was acidic which changed the solution to a white emulsion. The emulsion was extracted with diethyl ether (2 x 50 ml) and subsequently with light petroleum (bp 60-80°C)(2 x 30 ml). The organic layers were combined and washed with bidistilled water (4 x 30 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The black residue was cooled on ice and formed crystals which were filtered and rinsed with cool light petroleum (bp 40-60°C). The crystals (brownish) were recrystallised from light petroleum (bp 40-60°C) to yield a pale amber crystalline material which was then heated with charcoal (activated norit) for about 10 minutes and filtered. The filtrate was dried and cooled on ice. The crystals which formed were filtered and rinsed with cool light petroleum (40-60°C) and white crystals were obtained. The yield was 0.56 g and had mp. 81.0-83.0°C (lit. mp 72.5-73.5°C (Nakamura and Kijima 1972); 86-87°C (Frampton et al, 1960); 84.5-85.5°C (Smith and Tess, 1944)).

The compound was identified as 2,2,7,8-tetramethyl-6-chromanol by the following spectral data :

Elemental analysis calc. for $C_{13}H_{18}O_2$: C = 75.84, H = 8.95.
Found : C = 75.73, H = 8.74.

UV ($\lambda_{max,\ n\text{-hexane}}$) : 299.7 ($\varepsilon$=3,900) and 294.3 nm. Nilsson et al 1968, found 300 and 294 nm.

IR (KBr)(cm^{-1}): 3280 (OH), 2990, 2925, 2873 (CH_3), 1640, 1500, 1450, 1425, 1390, 1370, 1350, 1340, 1260, 1250, 1220, 1080 (C-O-C), 940, 930, 850, 750.

MS (ei) m/z (rel.intensity): 206 (M^+)(57), 191 (7), 177 (4), 163 (4), 151 (100), 135 (6), 122 (20).

^{1}H NMR ($CDCl_3$): δ 1.23 (s, 6H, 2 x CH_3), 1.75 (t, 2H, J = 6.8 Hz, 3-CH_2), 2.11 (s, 3H, ^{8}C-CH_3), 2.14 (s, 3H, ^{7}C-CH_3), 2.69 (t, 2H, J = 6.8 Hz, 4-CH_2), 4.31 (s, 1H, OH, ^{6}C-O<u>H</u>), 6.38 (s, 1H, =^{5}CH-).

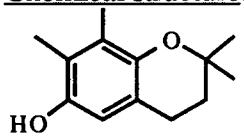
^{13}C NMR ($CDCl_3$) : δ 11.89, 11.92, 22.65, 26.96 (2 peaks), 32.92, 73.43 (quat.C), 112.10, 116.08, 121.70, 125.79, 145.86 and 146.30.

DEPT. EXPERIMENT ($CDCl_3$) :

CH and CH3 positive : 11.89, 11.92, 26.96 and 112.10.

CH2 negative : 22.65 and 32.93.

Chemical structure:



$C_{13}H_{18}O_2$, M.W. 206

II.3.3. PREPARATION OF 5-HYDROXYMETHYL-2,2,7,8-TETRA METHYL-6-CHROMANOL (PM-OH) (Nakamura and Kijima, 1972).

To 2,2,7,8-tetramethyl-6-chromanol (173 mg, 0.84 mmole) in dry toluene (2.70 ml) were added glacial acetic acid (0.1 ml), boric acid (182 mg, 3 mmol)) and paraformaldehyde (170 mg). The mixture was stirred and heated under reflux at 93-97°C for 2.5 hours, cooled and filtered. The residue was rinsed with light petroleum (bp 60-80°C) and a white crystalline solid was formed immediately and stuck to the glass. To the filtrate plus crystals was added chloroform, which dissolved the crystals, and the solution was washed with bidistilled water (3 x 5 ml). The solution was then stirred with $NaHCO_3$ solution (15 ml) for 30 minutes, washed again with bidistilled water (4 x 5 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*.

The oily residue was dissolved in a little light petroleum (bp 40-60°C) which was then removed in a gentle stream of nitrogen gas to yield colourless crystals. The material was recrystallised from light petroleum (bp 40-60°C) and obtained as fine needles (yield 132.2 mg, 67%). The compound had mp 120 -121°C (lit. mp 124-125°C (Nakamura and Kijima, 1972)).

The compound was identified as 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol and had the following data :

Elemental analysis calc. for $C_{14}H_{20}O_3$: C = 71.19, H = 8.47.
Found : C = 70.90, H = 8.64.

UV ($\lambda_{max,n\text{-}hexane}$) : 305 nm and 300 nm (sh) and ε = 4416.

IR (KBr) (cm^{-1}): 3390(OH), 2982, 2940, 2888(CH_3), 1455, 1429, 1388, 1373, 1345, 1270, 1232, 1133, 1270, 1178, 1133, 1091(C-O-C), 1062, 976, 900, 854, 833, 748 and 602.

MS(ei.) m/z (rel.int.): 236 [M^+](23), 218(92), 203(88), 189(32) 175(100), 161(24), 147(27), 135(30), 123(28), 109 (41).

^{1}H NMR ($CDCl_3$) δ : 1.28 (s, 6 H, 2 x 2C-CH_3), 1.77 (t, 2H, 3-CH_2, J = 7.0 Hz), 2.05 (t, 1H,-CH_2-OH, J = 5.7 Hz), 2.11 (s, 3H, ^{8}C-CH_3), 2.16 (s, 3H, ^{7}C-CH_3), 2.64 (t, 2H, 4-CH_2, J = 7.0 Hz), 4.87 (d, 2H, (CH_2)-OH, J = 5.5 Hz), 7.09 (s, 1H, ^{6}C-OH).

D_2O EXCHANGE ($CDCl_3$) :

The singlet at 7.09 ppm and the triplet at 2.05 disappeared on exchange with D_2O, while the doublet at 4.87 changed to a singlet.

N.O.E. EXPERIMENT IN $CDCl_3$:

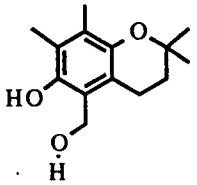
Irradiation of the doublet at 4.87 gave an NOE on the singlet at 7.09 and on the triplet at 2.64 ppm indicating that the -CH_2OH was on position 5 of the chromanol ring.

^{13}C NMR($CDCl_3$) δ : 11.75, 11.96, 20.09, 26.66 (2 peaks), 32.83, 59.96, 72.61, 115.10, 118.93, 123.00, 125.68, 145.01 and 147.26.

DEPT. EXPERIMENT ($CDCl_3$) :

CH, CH_2 and CH_3 : 11.75, 11.96, 20.09, 26.65, 32.83 and 59.96.
CH_3 positive : 11.75, 11.96 and 26.65.
CH_2 negative : 20.09, 32.83 and 59.96.

The chemical structure:



$C_{14}H_{20}O_3$, M.W. = 236

The yield is definitely improved by using dry toluene as a solvent.

II.3.4. PREPARATION OF 2,2,7,8-TETRAMETHYLCHROMAN-5,6,-DIONE (PR).

To a solution of 2,2,5,7,8-pentamethyl-6-chromanol (200 mg) in absolute ethanol (5 ml) was added silver nitrate (737 mg) and the mixture heated at 80°C for 2 hours. The mixture was filtered and the residue rinsed with chloroform. A white precipitate appeared in the filtrate which was refiltered and washed with bidistilled water (4 x 5 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was purified on thin layers of silica gel using solvent a (light petroleum/chloroform = 9:1)/solvent b (light petroleum/chloroform/ethyl acetate = 4:1:5) (1:1). Six bands were obtained with the following Rf_s: 0.10 (pink), 0.24 (colourless), 0.31 (colourless), 0.39 (orangish-yellow), 0.48 (red), 0.69 (colourless).

Compound Rf 0.48 (red, 38 mg) was purified (TLC, as above) and identified as **PR** by the following spectra:

Elemental analysis calc.for $C_{13}H_{16}O_3$: C = 70.91, H = 7.27.
Found : C = 71.22, H = 7.60.

UV.($\lambda_{max,\ n\text{-hexane}}$): 238.8, 270.0 and 436.8 nm.
($\lambda_{max,\ methanol}$): 240.4, 282.0 and 466.4 nm.

IR (KBr)(cm^{-1}): 2976, 1677 (C=O), 1647 (C=O), 1588, 1455, 1402, 1325, 1186, 1186, 923, 687, 642.

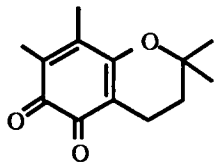
MS (ei.) m/z (rel.intensity): 220 [M^+] (47), 205 (100), 192 (43), 137 (60).

^{1}H NMR ($CDCl_3$): δ 1.36 (s, 6H, 2 x 2C-CH_3), 1.73 (t, 2H, 3-CH_2, J = 6.7 Hz), 1.93 (s, 3H, 8-CH_3), 2.02 (s, 3H, 7-CH_3), 2.42 (t, 2H, 3-CH_2, J = 6.7 Hz).

^{13}C NMR ($CDCl_3$): δ 11.56, 13.72, 15.66, 26.70 (2 peaks), 31.39, 79.07, 110.06, 134.15, 143.70, 163.29, 177.92, 180.82.

DEPT. EXPERIMENT ($CDCl_3$) :
CH, CH_2 and CH_3 : 11.56, 13.72, 15.66, 26.70 and 31.39.
CH_3 positive : 11.56, 13.72 and 26.70.
CH_2 negative : 15.66 and 31.39.

The chemical structure:



$C_{13}H_{16}O_3$, M.W. = 220.

II.3.5. PREPARATION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ)

To a solution of **PH** (95 mg) in absolute ethanol (50 ml) was added silver nitrate (293 mg) and the mixture heated for 8 minutes at 80°C. The resulting mixture was extracted with light petroleum (3 x 25 ml), the petroleum extract washed with water (4 x 50 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The fraction which did not dissolve in light petroleum was dissolved in chloroform, washed with water (5 x 50 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The total crude yield was 135 mg and was purified by TLC (silica) with solvent a/solvent b = 1:1 (as in **II.3.4.**) and the yield was 42 mg (44%).

A better result was obtained by oxidising **PH** with silver nitrate (1:3, weight ratio) in methanol at room temperature for 5 minutes (until the colour of the solution was bright yellow). In this case no **PR** was formed, it was easier to purify **PQ** and a better yield resulted. The extraction and purification method was the same as above. The oily product was crystallised by dissolving in a small amount of light petroleum and removing the solvent under a gentle stream of nitrogen. This process was repeated three times and finally bright yellow crystals (64%), mp. 57 - 58°C, (lit. mp 62°C, John et al., 1939) were obtained under vacuum with P_2O_5 and paraffin chips.

PQ had the following spectral data:

UV ($\lambda_{max,\ n\text{-hexane}}$): 258.6, 266.8 nm; ε_{266} = 19,034.

IR (KBr)(cm^{-1}) : 3471 (OH), 2975, 2932 (CH_3), 1647 (C=O), 1460, 1380, 1320, 1283, 1221, 1154, 1110 (C-O-C), 1062, 1027, 932 and 905.

MS (ei) m/z (rel. intensity): 236 [M^+](12), 221 (17), 203 (38), 180 (95), 178(40), 175 (40), 163 (30), 150 (47), 138 (32), 135 (48), 91 (27), 59 (100).

^{1}H NMR ($CDCl_3$): δ 1.27 (s, 6H, 2 x CH_3), 1.53 (m, 2H, 2'-CH_2), 2.00 (s, 6H, 2 x =C-CH_3), 2.03 (s, 3H, =C-CH_3), 2.55 (m, 2H, 1'-CH_2).

^{13}C NMR ($CDCl_3$) δ: 11.94, 12.28, 12.35, 21.66 (2 peaks), 29.08, 42.12, 70.73, 140.24, 140.43, 140.57, 144.35, 187.27, 187.67.

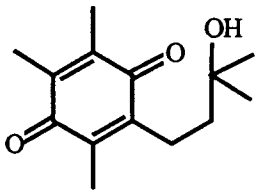
DEPT. EXPERIMENT ($CDCL_3$):

CH, CH2 and CH3 : 11.94, 12.28, 12.35, 21.66, 29.08 and 42.12

CH and CH3 positive : 11.94, 12.28, 12.35 and 29.08.

CH2 negative : 21.66 and 42.12.

The chemical structure:



$C_{14}H_{20}O_3$, M.W. = 236.

II.3.6. PREPARATION OF 1,4-DIHYDROXY-2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYLBENZENE (PQH_2).

To **PQ** (100 mg) in ether (25 ml) was added sodium dithionite (2 g) in water (2 ml). This solution was shaken vigorously for 30 minutes and the colour changed from yellow to colourless. The aqueous phase was discarded, then sodium dithionite (1 g) in water (4 ml) was added and the solution shaken for a further 15 minutes. The aqueous phase was discarded and the ether phase washed with water (5 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. White crystals were obtained, but after 3 hours in the refrigerator they became pink and did not dissolve in ether, chloroform, benzene or light petroleum. The yield was 77.4 mg and mp. 149-152°C.

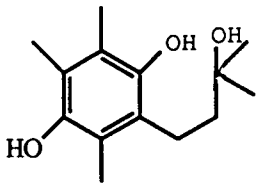
UV.(λ_{max}, methanol): 286.1 nm.

IR. (KBr)(cm^{-1}): 3381 (OH), 2929, 2972, 2929, 1645, 1462,1455, 1380,1327, 1250, 1213, 1082, 1054, 903, 635. [The hydroquinone was dissolved in acetone immediately after preparation, then dried under nitrogen. Unless the IR. spectrum run immediately, it still showed a strong peak at 1634 cm^{-1}, which indicated that there was still a quinone group in the compound, which also proved that the hydroquinone compound was very unstable.]

MS (ei) m/z (rel.intensity): 238 [M^+] (19); 220 (39); 165 (100); 136 (15).

^{1}H NMR (acetone & benzene d_6): δ 1.12 (s,); 1.56 (m,); 1.64 (t,); 2.20 (s,) 2.26 (s,) 2.33 (s,) 2.83 (t,). Because of the instability of the compound it was not possible to obtain satisfactory ^{1}H NMR spectra.

The chemical structure:



$C_{14}H_{22}O_3$, MW 238.

II.3.7. PREPARATION OF α-TOCOPHERYLQUINONE (TQ).

To dl-α-tocopherol (1 g) in methanol (50 ml) was added silver nitrate (3 g) and the mixture was stirred at room temperature for 4 minutes. The colour immediately turned yellow and gradually grey silver precipitated. After 4 minutes, when the colour was bright yellow, water (50 ml) was added to the solution and the products were extracted into chloroform (3 x 50 ml). The chloroform extract was washed with water (3 x 50 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The yellow product was purified using preparative TLC and light-petroleum/ethyl acetate (8:2) as the solvent. Three yellow major bands were obtained. The band with R_f 0.38 was identified after further purification as α-tocopherylquinone by the following data:

Elemental analysis calc. for $C_{29}H_{50}O_3$: C = 78.03, H = 11.21.
Found : C = 78.16, H = 11.48.

UV (λ_{max}, n-hexane) = 258 and 266.8 nm and $\varepsilon_{(266)}$ = 20,356.

IR (KBr)(cm^{-1}): 3509 (OH), 2955, 2928, 2869, 1645 (C=O), 1464, 1377, 1308, 1281, 1258, 715.

MS (ei) m/z (rel.intensity): 446 [M^+](7), 223 (3), 222 (15), 221 (100), 203 (72), 178 (80), 165 (85).

^{1}H NMR ($CDCl_3$): δ 0.82.- 0.86 (4d, 4 x CH_3 on the side chain), 1.00 - 1.39 (m, 21 H, CH and CH_2 on the side chain), 1.22 (s, 3H, 3'C-CH_3), 1.50 (m, 2H, 2'-CH_2), 2.00 (s, 6H, 2 x =C-CH_3), 2.03 (s, 3H, =C-CH_3), 2.55 (m, 2H, 1'-CH_2).

^{13}C NMR ($CDCl_3$) δ: 11.97, 12.29, 12.37, 19.69, 19.72, 19.75, 21.34, 21.43, 22.63, 22.73, 24.50, 24.81, 26.60, 27.98, 32.78, 37.30, 37.41, 37.63, 37.72, 39.38, 40.25, 42.31, 72.64, 140.19, 140.43, 140.54, 144.48, 187.23 and 187.67.

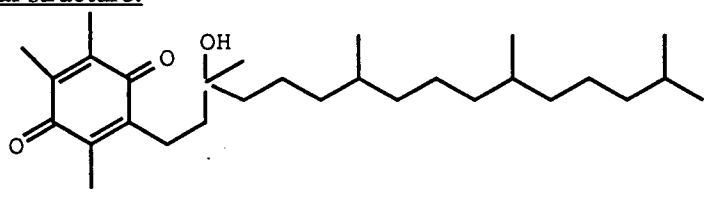
DEPT. EXPERIMENT ($CDCl_3$):

CH only : 27.98 and 32.78 (2 peaks).

CH and CH_3 positive : 11.97, 12.29, 12.37, 19.69, 19.72, 19.75, 22.63, 22.73, 26.60, 27.98 and 32.78.

CH_2 negative: 21.34, 21.43, 24.50, 24.81, 37.30, 37.41, 37.63, 37.72, 39.38, 40.25, and 42.31.

The chemical structure:



$C_{29}H_{50}O_3$, MW = 446

II.4. OXIDATION EXPERIMENTS.

II.4.1. OXIDATION OF 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL (PH) WITH *TERT.*-BUTYLHYDROPEROXIDE IN CHLOROFORM.

To **PH** (100 mg, 0.45 mmol) in chloroform (15 ml) was added *t*-butyl-hydroperoxide (41 mg, 0.46 mmol) in chloroform (15 ml) and the solution was heated to 60°C for three hours. The solution was then cooled to room temperature, washed with 5% ferrous sulphate (3 x 15 ml), followed by water (15 ml), dried (Na_2SO_4), and the solvent removed *in vacuo*. The residue (110 mg) was chromatographed on thin layers of silica gel using light petroleum/ethyl acetate (9:1) and five bands were obtained, $Rf_1 = 0.00$, $Rf_2 = 0.10$ (3 mg - oily, red-brown), $Rf_3 = 0.41$ (16 mg-solid, brown), $Rf_4 = 0.53$ (66 mg - solid, orange-yellow), $Rf_5 = 0.59$ (20 mg - solid-pale yellow). All of the bands from Rf_2 to Rf_5 were rechromatographed using chloroform as solvent.

Rf_2 gave 7 more bands with $Rf_{2.1} = 0.00$, $Rf_{2.2} = 0.03$, $Rf_{2.3} = 0.06$, $Rf_{2.4} = 0.13$, $Rf_{2.5} = 0.24$, $Rf_{2.6} = 0.28$, $Rf_{2.7} = 0.50$.

Only the major band $Rf_{2.4}$ was taken for further investigation.

Rf_3 gave 7 more bands: $Rf_{3.1} = 0.003$, $Rf_{3.2} = 0.06$, $Rf_{3.3} = 0.1$, $Rf_{3.4} = 0.56$, $Rf_{3.5} = 0.63$, $Rf_{3.6} = 0.77$, $Rf_{3.7} = 0.84$.

Bands $Rf_{3.3 - 3.7}$ were used for further investigation combined with bands from other separations.

Rf_4 gave 5 bands: $Rf_{4.1} = 0.06$, $Rf_{4.2} = 0.10$, $Rf_{4.3} = 0.60$, $Rf_{4.4} = 0.71$, $Rf_{4.5} = 0.86$.

Five bands were obtained from Rf_5: $Rf_{5.1} = 0.00$, $Rf_{5.2} = 0.10$, $Rf_{5.3} = 0.61$, $Rf_{5.4} = 0.70$, $Rf_{5.5} = 0.80$.

Bands $Rf_{5.2 - 5.5}$ were used for further analysis combined with other bands.

$Rf_{2.4}$, $Rf_{3.3}$, $Rf_{4.2}$ and $Rf_{5.2}$. were combined and chromatographed using light petroleum/ethyl acetate 9:1, giving a yellowish-brown compound (4 mg) which was

identified as 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone by the following spectra:
UV (λ_{max}, n-hexane): 259 and 268nm.

IR (KBr)(cm^{-1}) : 3519(OH), 2976 (CH), 1647 (C=O),1380.

MS (ei) m/z (rel.intensity): 236[M^+](18), 222(23), 203(42), 180(67), 150(47), 135(43), 91(33), 83(42), 59(100), 43(97).

^{1}H NMR ($CDCl_3$): δ 1.28 (s, 6 H, 2 x CH_3), 1.49-1.55 (m, 2H, 2'-CH_2), 2.00 (s, 6H, 2 x =C-CH_3), 2.04 (s, 3H, =C-CH_3), 2.54-2.60 (m, 2H, CH_2).

$Rf_{3.5}$, $Rf_{4.3}$ and $Rf_{5.3}$ were combined, extracted and rechromatographed as before to give 6 bands. The band with Rf 0.54 was the major band (15 mg) and was identified as the starting material **PH** by comparison of its UV, IR and ^{1}H NMR spectra with those of the authentic material.

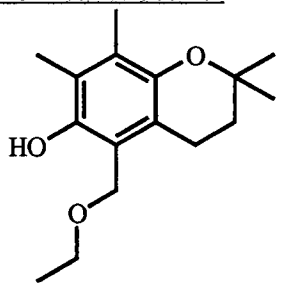
From band with $Rf_{5.5}$, 45 mg of solid compound was obtained and this compound was rechromatographed with chloroform yielding four bands. Band $Rf_{5.5.3}$ (35 mg) was identified as 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OEt**) by its spectral data:
UV (λ_{max}, n-hexane): 304 nm.

IR (KBr)(cm^{-1}): 3334 (OH), 2976, 2944 (CH), 1462, 1386, 1278, 1130, 1104, 651.

MS (ei) m/z (rel.intensity): 264 [M^+](44), 218 (100), 203 (71), 189 (20), 175 (53), 161 (7), 91 (14).

^{1}H NMR ($CDCl_3$): δ 1.28 (s, 6H, 2 x CH_3 + t, 3H, J = 7.0 Hz, CH_3-CH_2-O-), 1.76 (t, 2H, J = 6.9 Hz, 3-CH_2), 2.13 (s, 3H, 8C-CH_3), 2.16 (s, 3H, 7C-CH_3), 2.60 (t, 2H, J = 6.9 Hz, 4-CH_2), 3.60 (q, 2H, J = 7.0, CH_3-CH_2-O-), 4.71 (s, 2H, 5-CH_2-O), 7.68 (s, 1H, OH).

The chemical structure:



$C_{16}H_{24}O_3$ M.W. = 264.

II.4.2. pH OF REACTION OF 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL (PH) WITH *t*-BUTYL HYDROPEROXIDE IN CHLOROFORM-ETHANOL-WATER AT 60°C.

(a) To **PH** (54 mg, 0.25 mmol) in chloroform saturated with water (15 ml) was added *t*-butyl hyroperoxide (27 mg = 0.3 mmol) in the same solvent (15 ml) and the solution refluxed at 60°C. At various intervals an aliquot (3 ml) was taken, bidistilled water (7 ml) added, homogenised and the pH determined (pH meter). Results are recorded in Table **1** column (a), p. 58 and Fig. **3**, p. 125.

(b) **PH** (54 mg = 0.25 mmol) was dissolved in chloroform saturated with water (30 ml), the solution refluxed at 60°C and the pH determined at intervals as in **II.4.2.(a)**. Results are recorded in Table **1** column (b), p. 58 and Fig. **3**, p. 125.

(c) *t*-Butyl hydroperoxide (27 mg) was dissolved in chloroform saturated with water (30 ml), the solution refluxed at 60°C and the pH determined at intervals as in **II.4.2.(a)**. Results are recorded in Table **1** column (c), p. 58 and Fig. **3**, p. 125.

(d) Chloroform saturated with water (30 ml) was refluxed at 60°C and the pH determined at intervals as in **II.4.2.(a)**. Results are recorded in Table **1** column (d) and Fig. **3**.

(e) A solution of **PH** (54 mg = 0.25 mmol) and *t*-butyl hydroperoxide (27 mg, 0.3 mmol) in absolute ethanol (6 ml) and chloroform (24 ml) was treated with the same

protocol as in **II.4.2.(a)**. At 30 minutes the colour became slightly yellow, at 45 minutes pale yellow, at 75 minutes slightly orange and at 150 minutes orange. Results are recorded in Table **1** column (e), p. 58 and Fig. **4**, p. 126.

(f) *t*-Butyl hydroperoxide (27 mg) was dissolved in chloroform (24 ml) and absolute ethanol (6 ml) and the reaction performed as in **II.4.2.(a)**. Results are recorded in Table **1** column (f), p. 58 and Fig. **4**, p. 126.

(g) Chloroform (24 ml) was heated with absolute ethanol (6 ml) at 60°C and the reaction performed as in **II.4.2.(a)**. Results are recorded in Table **1** column (g) and Fig. **4**.

(h) To **PH** (54 mg, 0.25 mmol) in a mixture of bidistilled water (0.45 ml) and absolute ethanol (5.55 ml) was added *t*-butyl hydroperoxide (27 mg, 0.3 mmol) in purified chloroform (24 ml) and the reaction performed as in **II.4.2.(a)**. The yellow colour which formed immediately became intense at 30 minutes and very intense at 45 minutes. Results are recorded in Table **1** column (h), p. 58 and Fig. **5**, p. 127.

(i) **PH** (55 mg) was dissolved in a mixture of bidistilled water (0.45 ml), absolute ethanol (5.55 ml) and chloroform (24 ml) and the reaction performed as in **II.4.2.(a)**. Results are recorded in Table **1** column (i), p. 58 and Fig. **5**, p. 127.

(j) *t*-Butyl hydroperoxide (27 mg) was dissolved in a mixture of bidistilled water (0.45 ml), absolute ethanol (5.55 ml) and chloroform (24 ml) and the reaction performed as in **II.4.2.(a)**. Results are recorded in Table **1** column (j), p. 58 and Fig. **5**, p. 127.

(k) The pH of chloroform (3 ml) mixed with bidistilled water (7 ml) was 6.85. The pH of bidistilled water itself was 6.80.

Table 1. pH of reaction between **PH** and *t*-butyl hydroperoxide in various solvents (measured at 21-24°C).

Time (min)	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)
2	6.24	5.86	6.33	5.76	6.71	5.35	6.87	6.42	6.46	5.92
5	6.47	6.04	6.27	6.06	5.21	5.45		5.51	6.32	5.79
15	6.39	6.42	6.02	6.16	4.17	5.84	6.73	4.82	6.46	5.80
30	6.19	6.41	5.06	6.42	3.88	5.74	6.55	4.25	6.37	5.27
45	5.96	6.25	4.70	6.48	3.89	5.72	6.52	4.03	6.64	5.13
60	5.80	6.58	4.52	6.58	3.32	5.76	6.57	3.81	6.28	4.51
75	5.85	6.51	4.45	6.56	3.39	5.80	6.58	3.61	6.25	4.45
90	5.85	6.61	4.39	6.55	3.34	5.63	6.53	3.40	6.21	4.42
120	5.70	6.62	4.26	6.59	3.30	5.60		3.42	6.37	4.25
150	4.86	6.68	4.03	6.46	2.95	5.15	6.59	2.84	6.37	4.10
180	4.77	6.42	3.94	6.53					6.44	4.05

II.4.3 OXIDATION OF 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL WITH SILVER NITRATE IN ABSOLUTE ETHANOL AT 60°C.

A solution of 2,2,5,7,8-pentamethyl-6-chromanol (**PH**) (50 mg, 0.23 mmol) in absolute ethanol (50 ml) was heated to 60°C. From this solution was taken 2.0 ml (used as the control), then $AgNO_3$ (500 mg, 2.95 mmol) was added and the solution was stirred at 60°C. (At 2 minutes the colour of the solution changed to yellow and a silver mirror was formed. At 10 minutes a grey colour obscured the yellow; at 15 minutes it turned grey and yellow, and at 120 minutes was orange which it remained until the conclusion of the reaction). An aliquot (2 ml) was taken at intervals (2, 5, 10, 20, 30, 45, 60, 90, 120 and 180 min), water (5 ml) added and extracted with ether (5 ml). The ether extract was washed with water (2 x 5 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The dry residue was dissolved in a little light petroleum and the solvent removed

under a gentle stream of nitrogen. Residues from each time interval were kept dry and cool until analysis by HPLC. Prior to analysis, the samples were dissolved in hexane (1 ml) and an aliquot (20 µl) injected. Results are recorded in Table **2** and Fig. **6**, p. 131.

Table 2. HPLC analysis of the oxidation of 2,2,5,7,8-pentamethyl-6-chromanol (**PH**) by silver nitrate in absolute ethanol at 60°C.

Time (min)	**PH** (mM)	**PM-OEt** (mM)	**PQ** (mM)	**PR** (mM)
0	4.0	0.0	0.0	0.0
5	1.5	0.2	3.0	0.0
10	0.0	0.2	3.6	0.0
20	0.0	0.2	3.5	0.1
30	0.0	0.2	3.3	0.2
45	0.0	0.2	3.3	0.2
60	0.0	0.2	3.2	0.3
90	0.0	0.2	3.0	0.4
120	0.0	0.2	2.7	0.6
180	0.0	0.0	2.7	1.1

The pH of the reaction was also checked by taking an aliquot (3 ml) of a duplicate reaction at intervals (2, 5, 10, 20, 30 and 45 min) and adding it to ice cooled bidistilled water. After it was shaken thoroughly, the pH of the solution was measured (results are recorded in Table **3**, p. 60 and Fig. **6**, insert, p. 131). Some control reactions were also performed as below:

(a) **PH** (25 mg) in absolute ethanol (25 ml) was heated at 60°C. An aliquot (3 ml) was taken at intervals, diluted with cold water (7 ml) and the pH measured as in **II.4.2.**(a). (Results in Table **3**, column **II**).

(b) Silver nitrate (250 mg) was dissolved in absolute ethanol (25 ml), heated at 60°Cand the pH determined at intervals as in II.4.2.(a). (Results in Table **3**, column **IV**).

(c) A solution of nitric acid (10^{-3} M) in absolute ethanol (25 ml) was also heated at 60°C and the pH determined at intervals as in II.4.2.(a). (Results in Table **3**, column **III**).

Table 3. pH of the reaction between **PH** and silver nitrate in absolute ethanol at 60°C and also of the control reactions.

Time (mins.)	I	II	III	IV
0	-	6.27	3.36	6.07
1	2.92			
2	2.81	6.29	3.31	6.11
5	2.86	6.21	3.34	6.12
10	3.38	6.38	3.37	6.82
20	3.41	6.24	3.32	10.43
30	3.86	6.48	3.33	10.30
45	4.33	6.36	3.30	10.21

The pH of absolute ethanol (3 ml) in cold bidistilled water (7 ml) was 6.50.

The temperature of the pH measurements varied between 16 to 19°C.

II.4.4. OXIDATION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) WITH SILVER NITRATE IN ABSOLUTE ETHANOL AT 60°C.

PQ (25 mg, 0.11 mmol) in absolute ethanol (50 ml) was heated to 60°C and an aliquot (2 ml) was taken as a control. Then $AgNO_3$ (250 mg, 1.47 mmol) was added and the solution stirred at 60°C. An aliquot (2 ml) was taken at intervals for HPLC analysis as in **II.4.3.** In this reaction no change in colour occurred until 3 h. Analysis by HPLC showed that no reaction had occurred in 3 h.

II.4.5. OXIDATION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) WITH SILVER NITRATE IN BOILING ABSOLUTE ETHANOL.

PQ (100 mg, 0.45 mmol) in absolute ethanol (20 ml) was heated to boiling (78°C) and an aliquot (1 ml) taken as a control. Then silver nitrate (1 g, 5.89 mmol) was added and the solution stirred at 78°C. An aliquot (1 ml) was taken for analysis at intervals (5, 10, 15, 30, 60, 90, 120 and 180 min) and the protocol was the same as in **II.4.3.** After 50 min the colour turned orange and after 60 min red crystals appeared on the wall of the flask. The residue was treated as in **II.4.3.** The only significant product was **PR** whose identity was confirmed by its UV and ^{1}H NMR spectra. Results are given in Table **4** and Fig. **7**, p. 132.

Table 4. HPLC analysis of the oxidation of **PQ** by silver nitrate in boiling absolute ethanol.

Time (min)	**PQ** (mM)	**PR** (mM)
0	15.0	0.0
5	15.3	0.0
10	15.6	0.0
20	16.0	0.0
30	15.4	0.0
45	15.4	0.0
60	14.6	0.3
90	12.4	0.4
120	7.0	9.9
180	1.3	16.9

II.4.6. OXIDATION OF 2,2,7,8-TETRAMETHYL-6-CHROMANOL (TMC) WITH SILVER NITRATE IN ABSOLUTE ETHANOL AT 60°C.

2,2,7,8-Tetramethyl-6-chromanol (**TMC**) (50 mg, 0.24 mmol) in absolute ethanol (50 ml) was heated at 60°C and an aliquot (2 ml) was taken as a control. Then silver nitrate (50 mg, 2.95 mmol) was added and the remainder of the reaction performed as in **II.4.3.** The colour of the solution turned pink at 12 min, red at 18 min and then intensely red after 60 min. Results are recorded in Table **5**, p. 63 and Fig. **17**, p. 144.

After 3 h, water (25 ml) was added to the reaction mixture which was extracted with ether (15 ml). The combined ether fractions were washed with water (10 ml), dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was dissolved in a small amount of chloroform and chromatographed on thin layers of silica gel with solvent light petroleum (60-80°C)/ethyl acetate (1:1) to obtain the following 4 bands

Rf_1 : 0.34 - bright yellow (13.8 mg); identified as 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (**TMCQ**) by the following data:

UV (λ_{max}, n-hexane) : 254.8 nm, (shoulder at 261.2 mn), 322 (low, broad).

IR (KBr)(cm^{-1}) : 3420 - 3520 (OH), 2940, 2985, 1650 (C=O), 1380, 1320.

^{1}H NMR: δ 1.27 (s, 6H, 2 x CH_3), 1.63 (m, 2H, 2'-CH_2), 2.01 (q, 3H, =C-CH_3, J = 1.7 Hz and 2.02 (q, 3H, =C-CH_2, J = 1.7 Hz), 2.51 (m, 2H, -CH_2), 6.52 (t, 1 H, 3- CH, J = 1.5 Hz). (NOE spectra, see Fig. **18**, p. 146).

^{13}C NMR : δ 12.04, 12.38, 24.32, 29.27 (2 peaks), 41.82, 70.63, 132.16, 140.66, 141.09, 149.30, 167.60 (2 peaks).

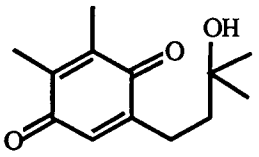
DEPT. EXPERIMENT ($CDCl_3$) :

CH only : 132.16

CH and CH_3 positive : 12.04, 12.38 and 29.27.

CH_2 negative : 24.32 and 41.82.

The chemical structure:



$C_{13}H_{18}O_3$, M.W. = 222.

Rf_2 : 0.53 - red (15 mg) was identified as **PR** by comparison of its UV and ^1H NMR spectra to those of the authentic compound.

Rf_3 : 0.72 - pink (2 bands overlapping) (1.1 mg) - was not analysed any further.

Rf_4 : 0.92 - yellow (1 mg) was not analysed any further.

Table 5. HPLC analysis of the oxidation of 2,2,7,8-tetramethyl-6-chromanol (**TMC**) by silver nitrate in absolute ethanol at 60°C.

Time (min)	**TMC** (mM)	**TMCQ** (mM)	**PR** (mM)
0	3.1	0.0	0.0
5	1.3	0.6	0.0
10	1.0	0.7	0.1
20	0.5	1.5	0.5
30	0.2	2.2	0.8
45	0.0	2.3	1.0
60	0.0	2.2	1.0
90	0.0	2.1	1.2
120	0.0	2.1	1.4
180	0.0	1.8	1.7

II.4.7. OXIDATION OF 5-HYDROXYMETHYL-2,2,7,8-TETRA-METHYL-6-CHROMANOL (PM-OH) WITH SILVER NITRATE IN ABSOLUTE ETHANOL AT 60°C.

5-Hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**) (50 mg, 0.23 mmol) in absolute ethanol (50 ml) was heated to 60°C and an aliquot (2 ml) taken as a control. Silver nitrate (500 mg, 3 mmol) was then added and the reaction performed as in **II.4.3**. The colour of the solution at 5 minutes turned grey, at 7 minutes light orange, at 8 minutes pink, at 10 minutes red and at 30 minutes intense red. After 3 h the reaction was stopped and treated as in **II.4.6**. Results are recorded in Table **6** (p. 65) and Fig. **16** (p. 142). The residue was chromatographed on thin layers of silica gel and the following compounds were identified - 2-(3-hydroxy-3-methylbutyl)-3-hydroxymethyl-5,6-dimethyl-1,4-benzoquinone (**PQ-OH**), **PR** and **PM-OEt**.

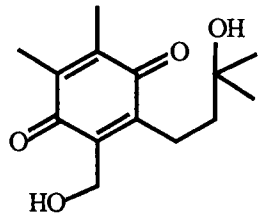
PQ-OH had $R_f = 0.04$ and was identified by the following spectra:

UV $\lambda_{max\ (hexane)}$: = 262 nm

^{1}H NMR ($CDCl_3$): δ 1.25 (s, 6H, 2 x CH_3), 1.59 (m, 2H, 2'-CH_2), 2.02 (s, 6H, 2 x =C-CH_3), 2.64 (m, 2H, 1'-CH_2), 2.81 (small & broad peak, 1H, =C-CH_2-O<u>H</u>), 4.56 (s, 2H, =C-C<u>H$_2$</u>-OH).

Because the yield was very small (1 mg), no other analyses could be done.

The chemical structure:



$C_{14}H_{20}O_4$, M.W. = 252.

PR and **PM-OEt** were identified by comparison of their UV, IR and ^{1}H NMR data with those of the authentic materials.

Table 6. HPLC analysis of the oxidation of 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**) by silver nitrate in absolute ethanol at 60°C.

Time (min)	**PM-OH** (mM)	**PM-OEt** (mM)	**PQ-OH** (mM)	**PR** (mM)
0	4.0	0.7	0.0	0.0
5	3.5	1.1	0.1	0.0
10	2.7	1.2	0.6	0.0
20	0.0	1.1	1.1	0.8
30	0.0	1.1	1.0	1.4
45	0.0	0.9	0.8	1.7
60	0.0	0.7	0.7	2.1
90	0.0	0.4	0.4	2.4
120	0.0	0.3	0.3	2.7
180	0.0	0.2	0.1	2.9

II.4.8. OXIDATION OF 5-ETHOXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OEt) WITH SILVER NITRATE IN ABSOLUTE ETHANOL AT 60°C.

5-Ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OEt**)(50 mg, 0.2 mmol) in absolute ethanol (50 ml) was heated to 60°C and then reacted with silver nitrate as in **II.4.3** (without collecting the 2 min fraction). Results are recorded in Table 7 (p. 67) and Fig. **14** (p. 139). After 3 h the reaction was stopped and treated as in **II.4.6** to obtain 5 bands.

Rf_1: 0.26 (yellow, 13.4 mg) was identified as the 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (**PQ-OEt**) by the following data:

UV. $\lambda_{max\ (n\text{-}hexane)}$: 259.9 nm (shoulder at 265 nm).

IR (KBr)(cm^{-1}): 3483, 3437 (OH), 2975, 2932, 2875, 1648 (C=O), 1304, 1279, 1237, 1219, 1153, 1124 and 1099.

MS (e.i) m/z (rel.intensity): 280 [M^+](1), 262 (7), 234 (34), 221 (50), 176 (100).

^{1}H NMR ($CDCl_3$): δ 1.20 (t, 3H, J = 7.0 Hz, -O-CH_2-CH_3), 1.25 (s, 6H, 2 x CH_3), 1.65 (m, 2H, 2'-CH_2), 2.01 (s, 6H, 2 x CH_3), 2.64 (m, 2H, 1'-CH_2), 3.55 (q, 2H, J = 7.0 Hz, -O-CH_2-CH_3), 4.40 (s, 2H, =C--CH_2-O-).

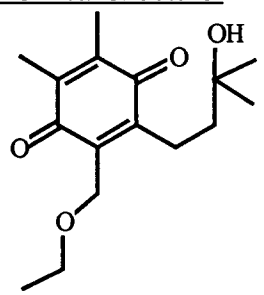
^{13}C NMR ($CDCl_3$) : δ 12.36, 12.45, 15.25, 21.98, 29.18 (2 peaks), 45.17, 62.60, 66.81, 70.84, 138.32, 140.69, 140.88, 148.48, 186.72 and 187.77.

DEPT. EXPERIMENT ($CDCl_3$) :

CH_3 positive: 12.36, 12.45, 15.25 and 29.18.

CH_2 negative: 21.98, 45.17, 62.60 and 66.81

The chemical structure:



$C_{16}H_{24}O_4$, M.W. = 280.

Rf_2: 0.39 (yellow, 13 mg) was identified as **PQ** (by its UV and ^{1}H NMR spectra)

Rf_3: 0.51 (red, 16 mg) was identified as **PR** (by its UV and ^{1}H NMR spectra).

Rf_4: 0.65 (colourless, 6 mg, but changed very readily to yellow-orange) and because of its instability it is still unidentified.

Rf_5: 0.82 (yellow, 1.2 mg) - unidentified.

Table 7. HPLC analysis of the oxidation of 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OEt**) by silver nitrate in absolute ethanol at 60°C.

Time (min)	**PM-OEt** (mM)	**PQ** (mM)	**PQ-OEt** (mM)	**PR** (mM)
0	3.5	0.0	0.0	0.0
5	3.5	0.0	0.0	0.0
10	3.5	0.0	0.0	0.0
20	3.4	0.3	0.0	0.0
30	2.8	1.0	0.2	0.0
45	1.8	2.3	0.7	0.4
60	1.4	2.7	0.6	0.8
90	0.8	3.1	0.5	1.3
120	0.8	2.7	0.5	1.7
180	0.6	2.4	0.2	2.3
240	0.6	1.7	0.1	2.6

pH OF THE REACTION OF 5-ETHOXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (**PM-OEt**)(20 mg) WITH SILVER NITRATE (200 mg) IN ABSOLUTE ETHANOL (20 ml) AT 60°C.

PM-OEt (20 mg) in absolute ethanol (20 ml) was heated to 60°C, silver nitrate (200 mg) was added and the reaction performed as in **II.4.3.** An aliquot (3 ml) of the reaction mixture was taken at intervals (1, 5, 10, 20, 40, and 60 min), diluted with bidistilled water (7 ml) and the pH measured. Results are given in Table **8**, p. 67 and.Fig. **14**, insert, p. 139.

Table 8. pH of oxidation of 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OEt**) with silver nitrate in absolute ethanol at 60°C

Time (min)	pH	Time (min)	pH
1	5.62	20	4.50
5	4.87	40	4.35
10	4.70	60	4.35

As a control, the pH of absolute ethanol (3 ml) in bidistilled water (7 ml) was measured as 6.24. Also the pH of 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol solution (1 mg/ml) in absolute ethanol was 6.28.

II.5. ACID EXPERIMENTS.

Analysis methods:

(1) TLC using solvent system: (i) solvent a (hexane/chloroform = 9:1) and solvent b (hexane/chloroform/ethyl acetate = 4:1:5), 1:1, (ii) light-petroleum/ethyl actate 8:2.

(2) HPLC system:

NPLC with solvent system C was used to measure **PQ**, **PM-OEt**, **PH** , **PQ-OEt** and **PR**. (flow rate 1 ml/min; after 4 minutes the flow rate was increased to 1.5 ml/min. The retention time for **PM-OEt** was 3.6 min; **PH**: 4.5 min.; **PQ**: 8.4 min; **PQ-OEt**: 9.7 min and **PR**: 10.5 min.

RPLC with solvent system G was used to measure **PM-OH**, **PM-OEt** and 5-formyl-2,2,7,8-tetramethyl-6-chromanol (**PA**). The retention time for **PM-OH** was 3.7 mins ; **PM-OEt** 4.6 mins and **PA** was 6.2 mins. **PM-OH** could not be detected with normal phase system most likely due to its lability in the silica column and unfortunately **PQ** and **PR** gave the same retention time as the **PM-OH** in reverse phase system. Therefore, in order to determine the concentration of **PM-OH**, duplicate samples were analysed by normal and reverse phase. The concentration of **PQ** and **PR**, determined by normal phase HPLC were converted into the corresponding areas for reverse phase system and these were subtracted from the reverse phase peak which contained all three compounds.

II.5.1. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN PURIFIED CHLOROFORM CONTAINING 20% ABSOLUTE ETHANOL AT 60°C.

The pH of purified chloroform was checked prior to the reaction by shaking chloroform (3 ml) with bidistilled water (7 ml) and the pH of the aqueous layer was 6.50. The pH of a solution of chloroform containing 20% absolute ethanol was determined the same way to be 6.70. **PQ** (10 mg) in chloroform containing 20% absolute ethanol (2 ml) was heated to 60°C and a small aliquot taken for TLC (solvent (i)) analysis at intervals (0, 5, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 120 and 240 min). No decomposition was evident up to 4 hours.

II.5.2. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.001M NITRIC ACID AT 60°C.

PQ (10 mg) in absolute ethanol containing HNO_3 (10^{-3}M, 2 ml) was heated at 60°C under nitrogen. At intervals (0, 5, 10, 20, 30, 45, 60, 75, 90, 105, 120, 150 and 165 min) a small aliquot was taken for analysis by TLC as in **II.5.1.** Appropriate standards were also spotted on the plate. Results are presented in Table **9.**

Table 9. TLC analysis of reaction between **PQ** and dilute nitric acid in absolute ethanol at 60°C.

Compound	Colour	Rf	Time of reaction (min)										
			0	5	10	20	30	45	60	75	90	105	120
dimerquinone	colourless	0.37				vs	vs	vs	vs	s	s	s	s
PQ	yellow	0.44	b	b	b	b	b	b	b	b	b	b	b
PR	pink							vs	vs	s	s	s	s
PM-OEt	colourless	0.85								s	s	s	s
PMA	colourless	0.89								s	s	s	s

vs = very small amount, s = small amount, b = big amount.

II.5.3. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.03M SULFURIC ACID AT 60°C

PQ (50 mg) in absolute ethanol (5 ml) was heated at 60°C under nitrogen and concentrated sulfuric acid (1 drop) in absolute ethanol (5 ml) was added. An aliquot (1 ml) was taken at intervals (10, 20, 30, 45, 60 and 90 min) and cold bidistilled water (3 ml) added. The aliquot was extracted with hexane/ethyl acetate 6:4 (2 x 3 ml) and the organic phase washed with cold bidistilled water (2 x 3 ml). The pH of the combined aqueous phases was measured. The organic phase was washed further with cold bidistilled water (3 x 3 ml), dried (Na_2SO_4), the solvent removed *in vacuo*, dissolved in a little hexane and analysed by TLC (solvent (ii)) and quantitated by NPLC (solvent C). Results are presented in Tables **10** (p. 70), **11** and **12** (p. 71) respectively, and Fig. **8**, p. 133. A control measurement was also carried out by dissolving **PQ** (50 mg) in absolute ethanol (10 ml) and heating to 60°C. An aliquot (1 ml) of the solution was taken, bidistilled water (10 ml) added and the pH measured. The remaining solution was treated as above.

The pH of absolute ethanol (1 ml) in bidistilled water (10 ml) was 6.27.

Table 10. pH of the reaction of **PQ** in absolute ethanol containing 0.03M sulfuric acid.

Time (min)	pH (control)	pH (reaction)	Time	pH (control)	pH (reaction)
0	5.21	2.57	45	5.16	2.75
10	5.18	2.62	60	5.19	2.77
20	5.12	2.68	90	5.19	2.86
30	5.09	2.65	120	5.19	2.84

Table 11. TLC analysis of the reaction between **PQ** and 0.03M sulfuric acid in absolute ethanol at 60°C.

Compound	Colour	Rf	Time of reaction (min)						
			0	10	20	30	45	60	90
PQ	yellow	0.16	b	b	b	b	s		
PR	red	0.18	s	s	b	b	b	b	b
unknown	colourless	0.37				tr	s	s	s
PH	colourless	0.53		tr	tr	tr	s	b	b
PM-OEt	colourless	0.67		vs	s	s	s	b	b
SPT	colourless	0.83					tr	tr	tr

tr = trace, vs = very small amount, s = small amount, b = big amount.

Table 12. HPLC analysis of the reaction between **PQ** and 0.03M sulfuric acid in absolute ethanol at 60°C.

Time (min)	**PQ** (mM)	**PM-OEt** (mM)	**PH** (mM)	**PQ-OEt** (mM)	**PR** (mM)
0	21.0	0.0	0.0	0.0	0.0
2	12.8	0.0	0.0	0.0	0.0
10	11.8	1.0	0.9	0.3	0.00
20	11.8	2.8	2.8	0.3	0.8
30	10.1	3.6	3.6	0.6	1.2
45	8.4	5.0	5.3	0.3	1.7
60	7.5	7.3	6.8	0.2	2.1
90	5.5	7.8	8.9	0.1	2.7

II.5.4. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C

PQ (50 mg) in absolute ethanol containing 0.001M sulfuric acid (10 ml) was heated at 60°C under nitrogen and the remaining experiment was the same as in **II.5.3.** Results are recorded in Tables **13** and **14** (p. 72) and Fig. **9** (p. 134).

Table 13. TLC analysis of the reaction between **PQ** and 0.001M sulfuric acid in absolute ethanol at 60°C.

Compound	Colour	Rf	Time of reaction (min)						
			0	15	30	45	60	90	120
unknown	colourless	0.06		s	s	tr	tr	tr	tr
unknown	colourless	0.12		s	s	tr	tr	tr	tr
PQ	yellow	0.16	b	b	b	b	b	b	b
PR	red	0,18						s	s
unknown	colourless	0.48			tr	tr	tr	tr	tr
PH	colourless	0.53			tr	s	s	s	s
PM-OEt	colourless	0.70				s	s	vs	vs

tr = trace, vs = very small amount, s = small amount, b = big amount.

Table 14. HPLC analysis of the reaction between **PQ** with 0.001 M sulfuric acid in absolute ethanol at 60°C.

Time (min)	**PQ** (mM)	**PM-OEt** (mM)	**PH** (mM)
0	21.2	0.0	0.0
2	14.0	0.2	0.4
5	13.5	0.2	0.5
15	12.3	0.2	0.4
30	12.7	0.3	0.5
45	12.9	0.3	0.7
60	13.5	0.7	1.3

II.5.5. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.03M PHOSPHORIC ACID AT 60°C

The procedure was the same as in **II.5.3.** Results are recorded in Table **15** (p. 73).

Table 15. TLC analysis of the reaction between **PQ** and concentrated phosphoric acid in absolute ethanol at 60°C.

Compound	Colour	Rf	Time of reaction (min)						
			0	15	30	45	60	90	120
PQ	yellow	0.19	b	b	b	b	b	b	b
unknown	colourless	0.50					s	s	s
SPT	colourless	0.87		b	b	b	s	s	s

s = small amount, b = big amount.

II.5.6. REACTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.03M HYDROCHLORIC ACID AT 60°C.

The experimental details were the same as in **II.5.3.** Results are recorded in Table **16** and Fig. **10** (p. 135).

Table 16. HPLC analysis of the reaction between **PQ** and 0.03M hydrochloric acid in absolute ethanol at 60°C.

Time (min)	**PQ** (mM)	**PM-OEt** (mM)	**PH** (mM)	**PA** (mM)	**PR** (mM)	**PQ-OEt** (mM)
0	21.0	0.0	0.0	0.0	0.0	0.0
2	11.4	0.0	0.0	0.0	0.0	0.0
10	11.8	0.7	0.7	0.0	0.0	0.5
20	10.0	4.4	2.7	0.1	1.2	0.7
30	6.4	9.3	3.7	0.1	2.4	0.6
45	2.9	11.6	5.1	0.2	2.4	0.3
60	1.3	15.6	5.5	0.3	2.4	0.2
90	0.5	16.6	6.9	0.3	2.2	0.0

II.5.7. REACTION OF 5-HYROXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OH) IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C

A solution of 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**)(12.5 mg, 0.06 mmol) in absolute ethanol containing 10^{-3}M sulfuric acid (5 ml) was refluxed at 60°C under nitrogen. Aliquots (0.5 ml) were taken at intervals (2, 5, 15, 30, 45, 60 and 90 min) and HPLC analysis performed as in **II.5.3**. Results are recorded in Table **17** (p. 74) and Fig. **13** (p. 138).

Table 17. HPLC analysis of the reaction between **PM-OH** and 0.001 M sulfuric acid in absolute ethanol under nitrogen at 60°C.

Time (min)	**PM-OH** (mM)	**PM-OEt** (mM)
0	2.7	0.0
2	2.6	0.0
5	2.2	0.8
15	0.7	2.5
30	0.1	2.5
45	0.1	3.0
60	0.0	2.7

II.5.8. REACTION OF 5-HYROXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OH) AND 2-(3-HYDROXY-3-METHYL-BUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C (I).

5-Hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**, 10.5 mg, 0.05 mmol) and **PQ** (10.5 mg, 0.05 mmol) were dissolved in absolute ethanol containing 0.001M H_2SO_4 (4.2 ml) and the mixture heated at 60°C under nitrogen as in **II.5.3**. Aliquots (0.5 ml) were taken at intervals and analysed by NPLC as in **II.5.3**. Results are recorded in Table **18** (p. 75) and Fig. **11** (p. 136).

Table 18. HPLC analysis of the reaction between **PM-OH** (10.5 mg) and **PQ** (10.5 mg) and 0.001 M sulfuric acid in absolute ethanol at 60°C.

Time (min)	PM-OH (mM)	PQ (mM)	PR (mM)	PH (mM)	PQ-OEt (mM)	PM-OEt (mM)
0	10.6	10.6	0.0	0.0	0.0	0.0
2	10.8	8.8	0.0	0.2	0.0	1.9
5	6.3	8.7	0.0	0.3	0.0	2.0
15	4.4	8.6	0.3	1.1	0.2	8.3
30	0.0	7.7	1.8	1.8	0.5	10.1
45	0.0	7.7	1.8	2.5	0.7	10.1
60	0.0	3.3	1.8	2.5	0.8	11.2

II.5.9. REACTION OF 5-HYROXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL AND 2-(3-HYDROXY-3-METHYL-BUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C (II).

PM-OH (25 mg, 0.11 mmol) and **PQ** (25 mg, 0.11 mmol) were dissolved in absolute ethanol containing 0.001M H_2SO_4 (5 ml), the solution heated at 60°C under nitrogen and the reaction analysed as in **II.5.3**. Results are recorded in Table **19** (p. 75) and Fig. **12** (p. 137).

Table 19. HPLC analysis of the reaction between **PM-OH** (25 mg) and **PQ** (25 mg) and 0.001 M sulfuric acid in absolute ethanol at 60°C.

Time (min)	PM-OH (mM)	PQ (mM)	PR (mM)	PH (mM)	PQ-OEt (mM)	PM-OEt (mM)
0	21.2	21.2	0.0	0.0	0.0	0.0
2	27.6	11.8	0.0	0.2	0.0	0.9
5	28.8	11.0	0.0	0.6	0.0	2.1
15	20.9	10.7	0.0	2.3	0.2	11.0
30	12.7	9.6	0.4	2.9	0.6	14.2
45	11.7	9.3	0.7	3.8	1.0	14.3
60	6.6	8.5	1.1	4.0	1.1	14.3
90	0.0	8.2	1.2	4.3	1.2	14.6

II.5.10. REACTION OF 5-ETHOXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OEt) AND 2-(3-HYDROXY-3-METHYL-BUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C.

PM-OEt (25 mg, 0.1 mmol) and **PQ** (25 mg, 0.11 mmol) were dissolved in absolute ethanol containing 0.001M H_2SO_4 (5 ml), the solution heated at 60°C under nitrogen and the reaction analysed as in was analysed as in **II.5.3.** Results are recorded in Table **20** (p. 76) and Fig. **15** (p. 141).

Table 20. HPLC analysis of the reaction between **PM-OEt** plus **PQ** and 0.001 M sulfuric acid in absolute ethanol at 60°C.

Time (min)	PQ (mM)	PM-OEt (mM)	PH (mM)	PR (mM)	PQ-OEt (mM)
0	21.2	18.9	0.0	0.0	0.0
2	11.2	15.6	0.4	0.0	0.0
5	12.0	15.6	0.8	0.0	0.3
15	12.7	17.1	2.1	0.0	1.0
30	11.3	15.4	3.1	0.2	1.1
45	10.7	14.4	3.6	0.5	2.0
60	11.7	16.9	5.2	0.8	2.6
90	10.7	16.1	5.7	1.3	2.4

II.5.11. REACTION OF 5-ETHOXYMETHYL-2,2,7,8-TETRAMETHYL -6-CHROMANOL (PM-OEt) IN ABSOLUTE ETHANOL CONTAINING 0.001M SULFURIC ACID AT 60°C.

A solution of **PM-OEt** (25 mg, 0.1 mmol) in absolute ethanol containing 0.001M H_2SO_4 was heated at 60°C under nitrogen and the analysis performed as in **II.5.3.** Results are recorded in Table **21**, p. 77.

Table 21. HPLC analysis of the reaction between **PM-OEt** and 0.001 M sulfuric acid in absolute ethanol at 60°C.

Time (min)	**PM-OEt** (mM)	**PH** (mM)
0	18.9	0.0
2	18.5	0.0
5	18.8	0.0
15	18.7	0.3
30	17.8	1.2
45	17.8	1.2
60	17.7	1.2
90	17.7	1.2

II.6. REDUCTION EXPERIMENTS

REDUCTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) AND α-TOCOPHERYLQUINONE (TQ) WITH VARIOUS REDUCTANTS.

Reduction of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (PQ) with various reductant.

II.6.1. Analysis by UV Spectrophotometry.

To **PQ** (1.4 mg, 6.4 μmol) in methanol/water (50:50, v/v, 0.3 ml) was added freshly prepared sodium dithionite (11.5 mg, 60 μmol)in water (0.3 ml). At various intervals (5, 10, 20, 40 & 60 min) aliquots (0.1 ml) were taken and diluted with methanol/water (50:50, v/v, 0.9 ml). An aliquot (0.1 ml) of this solution was taken and diluted to 2 ml with methanol/water (50:50, v/v) and the UV spectrum recorded from 400 to 200 nm. (Freshly prepared sodium dithionite becomes brown immediately and then the colour fades. It is essential to use the reagent immediately after preparation).

This experiment was repeated using dithiothreitol, ascorbic acid, sodium ascorbate and glutathione as reductants. Only sodium dithionite (10- and 25-fold molar ratio) and dithiothreitol (5-fold molar ratio) showed a rapid reduction by UV analysis. Neither sodium dithionite nor dithiothreitol by themselves showed any absorbance in the UV (Fig. **19** and **20**, p. 148 and 149). Reduction of **PQ** (10 mM) by ascorbic acid (10, 20 and 40 mM), sodium ascorbate (10 and 20 mM), glutathione (20 mM and 100 mM) and NADH (10 mM and 100 mM) did not show any formation of $\mathbf{PQH_2}$, even when NADH was dissolved in phosphate buffer pH 7.

These experiments were performed to check the quantitation of the reduction of **PQ** by UV analysis and proved to be unsatisfactory (Fig. **19** and **20**).

II.6.2.Analysis by TLC:

Reactions of **PQ** (10 mM) and dithiothreitol (**DTT**, 50 mM) or sodium ascorbate (100 mM) were repeated and after 5 and 10 min aliquots were taken for analysis by TLC (solvent system - light petroleum/ethyl acetate = 1/1). Spots were located with UV light. The spotting and drying of the spots were performed under a stream of nitrogen and the plate was run only for 15 min to reduce the possibility of the reoxidation of the hydroquinone (**PQH_2**) to **PQ** while it was on the plate. Results are recorded in Table 22.

Table 22. TLC analysis of the reduction of **PQ** by various reductants.

No	Reactions	Rf	Colour	Corresponding compounds
1	**PQ** (1 mmol) + **DTT** (5 mmol) (in aq.MeOH) for 5 min.	4 spots:		
		0.06	colourless (m)	unknown
		0.14	colourless (b)	**DTT**
		0.20	colourless then yellow (s)	**PQH_2**
		0.32	pale yellow (b)	**PQ**
2	**PQ** (1 mmol) + **DTT** (5 mmol) (in aq.MeOH) for 10 min.	4 spots		
		0.06	colourless (m)	unknown
		0.14	colourless (b)	**DTT**
		0.20	colourless (m)	**PQH_2**
		0.32	yellow (b)	**PQ**
3	**PQ** (1 mmol) + sod. ascorbate (10 mmol) for 5 min.	3 spots:		
		0.00	brown yellow (b)	sod. ascorbate
		0.19	colourless (vs)	**PQH_2**
		0.33	yellow (b)	**PQ**
4	**PQ** (1 mmol) + sod. ascorbate (10 mmol) for 10 min.	3 spots:		
		0.00	brown-yellow (b)	sod. ascorbate
		0.19	colourless (s)	**PQH_2**
		0.33	yellow (b)	**PQ**

vs = very small amount, s = small amount, m = medium amount, b = big amount.

In aqueous methanol the reaction between **PQ** and **DTT** occurred more rapidly than in pure methanol.

II.6.3. Analysis by HPLC.

II.6.3.1. Reduction performed in air.

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

II.6.3.1.1. Reduction of PQ (10 mM) by dithiothreitol (DTT, 50 mM and 200 mM).

To **PQ** (2 µmol) in solvent (0.1 ml) was added **DTT** (10 µmol and 40 µmol) in solvent (0.1 ml), the mixture stirred at room temperature for 2 min and HPLC solvent B (0.6 ml) added. The hexane layer (0.5 ml) was taken and dried (Na_2SO_4), then filtered and an aliquot (40 µl) chromatographed by NPLC (solvent B). The reaction was repeated 7 times to give the time points shown in **Table 23** and **Fig. 21**, p. 150.

Table 23. HPLC analysis of the formation of **PQH_2** (mole %) by dithiothreitol (50 mM and 200 mM)).

Time	Reductants	
(min)	**DTT** (100 mM)	DTT (400 mM)
0	0	0
1		42
2	32	54
5	64	79
10	87	85
20	86	87
35	89	
60	90	88

II.6.3.1.2. Reduction of PQ (10 M) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).

The procedure was as in **II.6.3.1.1.**, but the sodium dithionite was dissolved in water. Results appear in Table **24**, p. 81 and Fig. **22**, p. 150.

Table 24. HPLC analysis of the formation of PQH_2 (mole %) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).

Time (min)	Reductants	
	sodium dithionite (200 mM)	sodium ascorbate (200 mM)
0	0	0
1	86	
2	86	44
5	85	63
20	86	66
30		65
60		68

II.6.3.1.3. Reduction of PQ by glutathione.

The procedure was as in **II.6.3.1.1**, except that the amount of glutathione used was greater than the other reductants and it did not dissolved in the usual amount of water. In this case 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). There was no reduction of the **PQ** by glutathione.

II.6.3.1.4. Reduction of PQ (10 mM) by NADH (200 mM).

The procedure was as in **II.6.3.1.1** and no reduction occurred.

II.6.3.1.5. 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 μmol) in methanol/phosphate buffer (1:1, v/v, 0.1 ml) was added NADH (2 μmol) in phosphate buffer (0.1 ml) and the mixture stirred for 10 sec. Then FAD (2 μmol) in phosphate buffer (0.1 ml) was added and the protocol was performed and

analysed as in **II.6.3.1.1.** (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 are given in Table 25 and Fig. 23, p. 151.

Table 25. HPLC analysis of the formation of PQH_2 (mole %) from the reduction of **PQ** (7 mM) by various concentrations of NADH plus FAD.

Time (min)	Reductants		
	NADH (7 mM) plus FAD (14 mM)	NADH (14 mM) plus FAD (35 mM)	NADH (14 mM) plus FAD (70 mM)
0	0	0	0
5	52	70	76
10		100	88
20		98	100
30	100	93	100

II.6.3.2. 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 µmol) in methanol/0.05 M phosphate buffer pH 7.3 (1:1,v/v, 0.1 ml) was added DTT ((20 µmol (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 B (0.5 ml) was added and the mixture stirred with nitrogen for another 20 seconds. The hexane layer was removed, dried

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

II.6.3.2.1. Reduction of PQ (7 mM) by NADH (14 mM) plus FAD (different concentrations).

In NADH plus FAD experiment, **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 in **II.6.3.2**. Results are recorded in Table **26** below and Fig. **27**, p. 153.

Table 26. HPLC analysis of the formation of $\mathbf{PQH_2}$ (mole %) by various concentrations of NADH plus FAD.

Time	Concentrations of NADH (14 mM) and FAD (x mM)		
(min)	FAD (7 mM)	FAD (14 mM)	(70 mM)
0	0	0	0
5	35	37	76
10		60	88
20	86	100	100
30	100	100	100

NADH (56 mM) plus FAD (70 mM) gave a very fast reaction, which was completed in 5 minutes.

II.6.3.2.2. Reduction of PQ (10 mM) by sodium dithionite (20 and 100 mM).

Results are recorded in Table **27** below and Fig. **25**, p. 152.

Table 27. HPLC analysis of the formation of PQH_2 (mole %) from the reduction of **PQ** (10 mM) by various concentrations of sodium dithionite.

Time (min)	Concentration of sodium dithionite	
	20 mM	100 mM
0	0	0
5	46	94
10	57	95
20	60	100
30	36	100

II.6.3.2.3. Reduction of PQ (10 mM) by dithiothreitol (20 and 100 mM)

Results are recorded in Table **28** below and Fig. **24**, p. 152.

Table 28. HPLC analysis of the formation of PQH_2 (mole %) from the reduction of **PQ** (10 mM) by various concentrations of dithiothreitol.

Time (min)	Concentration of dithiothreitol	
	20 mM	100 mM
0	0	0
1	89	100
5	100	100
10	100	100
20	100	100
30	100	100

II.6.3.2.4. Reduction of PQ (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

Results are recorded in Table **29** below and Fig. **26**, p. 153.

Table 29. HPLC analysis of the formation of PQH_2 (mole %) from the reduction of **PQ** (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

Time (min)	Reductants	
	sodium ascorbate	ascorbic acid
0	0	0
5	60	8
10	63	12
20	77	36
30	83	38

Table 30. PQH_2 (mole %) formed from the reduction of **PQ** (10 mM or 7 mM for the NADH plus FAD reactions) by various reductants (reactions under nitrogen).

Time (min)	Sodium dithionite (100 mM)	Dithiothreitol (100 mM)	Ascorbic acid (100 mM)	Sodium ascorbate (100 mM)	NADH (14 mM) plus FAD (70 mM)	NADH (56 mM) plus FAD (70 mM)
0	0	0	0	0	0	0
1		100				
2	93					100
5	94	100	8	60	76	100
10	95	100	12	63	88	100
20	100	100	36	77	100	100
30	100	100	38	83	100	100

II.6.4. Reactions of α-tocopherylquinone (TQ) with various reductants.

The HPLC system was the same as in the **II.6.3** experiments, except for the solvent which was solvent system A. In **TQ** reductions, unless otherwise stated **TQ** was dissolved in methanol instead of methanol/buffer (1:1, v/v) and the protocol was as in **II.6.3.2.**

II.6.4.1.Reduction of TQ (10 mM) by sodium dithionite (100 and 200 mM).

At the moment the reductant solution was added to the **TQ** solution, it gave a milky appearence, due to the low solubility of the **TQ** in the polar solvent. Therefore, after nitrogen was blown into the mixture, it was stirred using a small magnetic bar. The remaining protocol was the same as in **II.6.3.2.** Results are recorded in Table **31**, p. 87 and Fig. **29**, p. 156.

II.6.4.2. Reduction of TQ (10 mM) by DTT (100 mM).

The procedure was as in **II.6.3.2.** This reaction gave 8% reduction after 5 min and 12% after 20 minutes, but it did not give any significant reduction after 30 min.

II.6.4.3. Reduction of TQ (10 mM) by DTT (200M).

The procedure was as in **II.6.3.2.** This reaction did not give any significant reduction until 60 min. However, it gave 76% reduction at 45 min when a small quantity of methanol was added during the process. It gave significant reduction when **TQ** was dissolved in hexane (heterogenous system), but was not reproducible. Several solvents were used in the trial to get the reduction of **TQ**, such as acetonitrile, acetone and glycerol, but it did not improve the situation. Also the use of a suspending agent such as xanthan (0.1% in buffer) was tried but still without success.

Reaction of **TQ** with sodium ascorbate and ascorbic acid (10-fold mol. ratio) did not give any reduction.

II.6.4.4. Reduction of TQ by NADH plus FAD in various ratios, in homogeneous and heterogeneous solution.

In the homogeneous reaction, **TQ** was dissolved in methanol, the NADH was dissolved in methanol containing 25% phosphate buffer (pH 7.3), while the FAD was dissolved in phosphate buffer alone and the remaining procedure was as in **II.6.3.2** However, in the heterogeneous reaction, **TQ** was dissolved in hexane, the NADH in methanol containing 25% phosphate buffer (pH 7.3) and the FAD in phosphate buffer (pH 7.3). The analysis was as in **II.6.3.2.1.** Results are recorded in Table **31**, p. 87 and Fig. **29**, p.156.

Table 31. TQH_2 (mole %) formed from the reduction of **TQ** (10 mM or 7 mM in NADH plus FAD reactions in homogeneous reaction or 20 mM in heterogeneous reaction) by various reductants.

Time (min.)	Dithionite (100 mM), homogeneous sol.	Dithionite (200 mM), homogeneous sol.	NADH (28mM) plus FAD (70 mM), homogeneous sol.	NADH (40 mM) plus FAD(100 mM), heterogeneous sol.	NADH (80 mM) plus FAD (100 mM), heterogeneous sol.
0	0	0	0	0	0
3					32
5	60	93	28	29	60
10	69	93	35	66	83
20	82	93	46	62	84
40	65	93	57		86
60			53	63	

II.6.5. Reduction of ubiquinone-10 (20 mM) by NADH (160 mM) plus FAD (200 mM).

Ubiquinone-10 (**UQ**, 17 mg, 0.02 mmol)was dissolved in hexane (1 ml), while the NADH (114 mg, 0.16 mmol) and FAD (166 mg, 0.2 mmol) were dissolved separately in 0.05 M phosphate buffer pH 7.3 (1 ml). Then **UQ** solution (0.1 ml) was mixed with NADH solution (0.1 ml) by a gentle stream of nitrogen for 20 sec and FAD solution added and the procedure was completed as in **II.6.3.2.1.** Results are recorded in Table **32** below and Fig. **30**, p. 158.

Table 32. HPLC analysis of the formation of ubiquinol-10 (mole %) from the reduction of **UQ** (20 mM) by NADH (160 mM) plus FAD (200 mM) in heterogeneous solution.

Time (min)	Ubiquinol-10 (UQH_2) (mole %)
0	0
3	40
5	44
10	48
20	55
40	78

II.6.6. Reduction of TQ (10 mM) and UQ (10 mM) by NADH (160 mM) plus FAD (200 mM).

TQ and **UQ** solutions in hexane were mixed, NADH added and the mixture stirred under nitrogen for 20 sec then the procedure was as in **II.6.3.2.1.** Results are recorded in Table **33** below and Fig. **31**, p. 158.

Table 33. HPLC analysis of the reduction of **TQ** (10 mM) and **UQ** (10 mM) by NADH (160 mM) plus FAD (200 mM) in heterogeneous solution.

Time (min)	**TQ** (mole %)	**TQH_2** (mole %)	**UQ** (mole %)	**UQH_2** (mole %)
0	100	0	100	0
3	93	7	88	12
5	82	18	74	26
10	50	50	35	65
20	38	62	14	87
40	18	82	16	84
		...		

II.7. BIOLOGICAL EXPERIMENTS

II.7.1. Reduction of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (PQ) by blood.

Standard **PQ**: 1.05 nmol/μl gave an area/nmol = 88860 units, retention time 10 min.

Standard PQH_2 was made by stirring **PQ** solution (1.05 μmol) in methanol (10 μl) with sodium dithionite (20 μmol) in water (40 μl)) for 10 min. An aliquot (5 μl) of this solution was diluted with methanol-water (50:50; 1 ml) and chromatographed by RPLC, solvent G, detector C, integrator B (area/nmol, 19829 units), retention time 4.1 min.

II.7.1.1. Choice of solvent to prevent red blood cell lysis.

DMSO, methanol and methyl linoleate (0.5 μl of each) were tried. To blood (400 μl) was added solvent (0.5 μl) and the mixture was homogenised and centrifuged at 3000 rpm for 2 min. With DMSO the plasma showed a trace of red colour, while with methanol and methyl linoleate there was no trace of red colour. As a result methanol was used for dissolving **PQ** and **TQ**.

II.7.1.2. Reduction of PQ by fresh blood (freshly drawn venous blood was immediately transferred into a vacutainer containing sodium heparin and was used within 1 h).

To fresh blood (2.5 ml) was added **PQ** (314 nmol) in methanol (3 μl). The blood was shaken gently to homogenise the **PQ** and then divided into 6 aliquots of 400 μl each in Eppendorf tubes (1.5 ml). These tubes were shaken at 37°C and 200 rpm for 2, 10, 20, 30, 45 and 60 min. After the shaking time was completed, the blood was centrifuged at 3000 rpm for 2 min at 4°C. To plasma (80 μl) was added methanol (200 μl) and the mixture vortexed for 2 sec and then centrifuged at 3000 rpm for 2 min at 4°C. The clear supernatant (40 μl) was chromatographed using RPLC (solvent G). The PQH_2 peak appeared at 4.1 min and the **PQ** peak appeared at 10.0 min. An additional sample of fresh blood without added **PQ** was treated in exactly the same way as a blank.

The blank extract also gave a peak at 4.1 min, which was then subtracted from the peak of the **PQH_2** in each of the samples. Results are recorded in Table **34**, p. 92 and Fig. **32**, p. 160.

II.7.1.3. Reduction of PQ by two day old blood (Each time point was carried out as a separate experiment.

To each portion of the blood (400 µl) in an Eppendorf tube (1.5 ml) was added **PQ** solution (52 nmol) in methanol (0.5 µl) and shaken for 2, 10, 20, 30, 45 and 60 minutes in a shaker (200 rpm.) with a water bath (37°C). After the appropriate time they were centrifuged at 3000 rpm for 2 min at 4°C, and the procedure was continued as in **II.7.1.2**. Results are recorded in Table **34**, p. 92 and Fig. **32**, p. 160.

II.7.2. REDUCTION OF α-TOCOPHERYLQUINONE (TQ) IN BLOOD.

Because of the heterogeneous nature of these reactions each time point was determined as a separate experiment.

α-Tocopherylquinol (**TQH_2**) standards:

To **TQ** (1 µmol) in methanol (10 µl) was added sodium dithionite (107 mg) in water (200 µl). Hexane (200 µl) was added and the mixture stirred for a certain periods of time. At a particular time an aliquot (10 µl) of the hexane layer was diluted with HPLC solvent A (400 µl) then an aliquot (40 µl) chromatographed by NPLC (solvent A, detector C, integrator B). This experiment showed that the optimum time for formation of **TQH_2** was 60 minutes. Therefore this protocol was used for making the **TQH_2** standards for all the experiments, as the **TQH_2** standards had to be freshly made each time.

Table 34. Reduction of **PQ** by fresh and 2 day old blood (calculated as % of **PQ** added).

Time (min)	Reduction by fresh blood (calculated as % of added **PQ**)						Reduction by 2 day old blood (calculated as % of added **PQ**)					
	First experiment		Second experiment		Average		First experiment		Second experiment		Average	
	PQ	**PQH_2**	**PQ**	**PQH_2**	**PQ**	**PQH_2**	**PQ**	**PQH_2**	**PQ**	**PQH_2**	**PQ**	**PQH_2**
2	71	7	80	5	76 ± 4.5	6 ± 1	90	3	92	8	91 ± 1	6 ± 2.5
10	47	28	57	9	52 ± 5	19 ± 9.5	63	20	53	30	58 ± 5	25 ± 5
20	42	28	43	22	43 ± 0.5	25 ± 3	54	55	64	45	59 ± 5	50 ± 5
30	25	32	36	35	31 ± 5.5	34 ± 1.5	44	59	29	26	37 ± 9	43 ± 16
45	24	43	31	37	28 ± 3.5	40 ± 3	32	55	35	40	34 ± 1.5	48 ± 7.5
60	50	24	56	28	53 ± 3	26 ± 2			57	27	57	27

II.7.2.1. Recovery of TQH_2 from bovine serum albumin.

To bovine serum albumin (15 mg) in phosphate buffered saline (100 µl) was added TQH_2 standard (5 µl, 6.3 nmol) and the mixture was shaken at 37°C for a certain period of time. Methanol (200 µl) was added, the mixture vortexed for 2 sec and centrifuged at 3000 rpm for 2 min at 4°C. An aliquot (40 µl) of the supernatant was chromatographed by RPLC (solvent J, detector C, integrator B). The recovery at 0, 10, 20, 30, 45 and 60 min was 42%, 60%, 82%, 63%, 48% and 57% respectively.

II.7.2.2. Recovery of TQ from blood analysed by NPLC (solvent A).

To blood (400 µl) was added **TQ** (50 nmol) in methanol (0.5 µl). After mixing well, it was centrifuged at 3000 rpm for 2 min at 4°C. To whole blood (before centrifugation) or plasma or packed cells (80 µl) was added hexane (200 µl), and the mixture vortexed for 15 sec and centrifuged at 3000 rpm for 2 min at 4°C and an aliquot (60 µl) chromatographed by NPLC. **TQ** could be recovered only from whole blood extraction (126% from trial 1 and 93% from trial 2). An additional sample of the same blood without addition of **TQ** treated in exactly the same way, served as a blank. The blank extract did not show any **TQ**.

II.7.2.3. Recovery of TQ from blood analysed by NPLC (solvent E).

To fresh or one day old blood (400 µl) was added **TQ** (50 nmol) in methanol (0.5 µl). After mixing well, it was incubated at 37°C for a certain periods of time. At a particular time the blood was centrifuged at 3000 rpm for 2 min at 4°C. To whole blood (before centrifugation) or plasma or packed cells (80 µl) was added solvent E (200 µl) and the mixture was vortexed for 15 sec and centrifuged at 3000 rpm for 2 min at 4°C, and an aliquot (60 µl) chromatographed by NPLC (solvent E, detector C, integrator B). The results are recorded in **Table 54**, p. 161.

II.7.2.4. Recovery of TQ from blood analysed by RPLC.

To blood (400 µl) was added **TQ** (50 nmol) in methanol (0.5 µl) and the mixture

shaken at 37°C and 200 rpm for a certain period of time. Hexane/isopropanol = 19/1 (400 µl) or hexane/isopropanol = 9:1 (6 ml) or hexane (6 ml) was added and the mixture vortexed for 5 sec. The hexane layer (200 µl or 5 ml if 6 ml of solvent was added) was collected, dried under a gentle stream of nitrogen or the solvent removed *in vacuo* (for 5 ml of solvent). The residue was redissolved in methanol (250 µl) and chromatographed (10 µl) by RPLC (solvent J, detector C, integrator B). Results appeared in **Table 54**, p. 161.

II.7.2.5. Recovery of TQ from red blood cells by replacing the plasma with phosphate buffered saline (PBS).

To blood (400 µl) was added **TQ** solution (0.5 µl, 50 nmol) and the mixture was shaken at room temperature for a certain period of time, then centrifuged at 3000 rpm for 3 min. Plasma (200 µl) was discarded and the volume replaced with PBS (200 µl). After shaking gently the mixture was centrifuged at 3000 rpm for 3 min at 4°C and the PBS layer (200 µl) was discarded. This volume was replaced by adding fresh PBS (200 µl) and the cells were suspended. Hexane (1 ml) was added, shaken and vortexed for 5 sec, then centrifuged at 3000 rpm for 2 min at 4°C. The hexane layer was collected (0.8 ml) and the analysis performed as in **II.7.2.4**. The results are recorded in **Table 54**, p. 161.

II.7.2.6. Incorporation of TQ into liposomes (Batzri & Korn, 1973).

Dipalmitoyl phosphatidyl choline (37 mg, 0.05 mmol) and **TQ** (11.2 mg, 0.03 mmol) were dissolved in absolute ethanol (1.5 ml). The solution was filtered through a 0.45 µm filter and rapidly injected (in a fine stream) into a well stirred flask of 0.016M KCl (1.19g/l, 30 ml). This solution then was concentrated to half volume in an ultrafiltration device (Amicon, Model 5400) fitted with membrane of MW cut-off 30KDa (Amicon PM 30) using nitrogen pressure of 15 psi and the concentration to half volume was repeated four times.

Analysis of **TQ** content of liposomes: Liposome suspension (2 ml) was freeze dried

(Dynavac, FD1 Freeze Drier, Temperature -50°C, vacuum: 3 Torr). The dry residue was dissolved in hexane/isopropanol 98:2 (1 ml), and centrifuged at 3000 rpm for 3 min (Beckman Model TJ-6), and an aliquot (0.5 and 1 µl) was chromatographed by NPLC (solvent E, detector C, integrator B). The liposomes contained 17% **TQ**.

II.7.2.7. Attempted reduction of TQ in liposomes by blood.

As previous attempts to reduce **TQ** in blood were unsuccessful, the suspension of **TQ** in liposomes was used on the assumption that the **TQ** in liposomes may be easier to disperse in blood and consequently be more subject to enzymatic reduction by the blood.

To blood (2 ml) was added liposome suspension (0.5 ml) which contained 0.9% sodium chloride. After shaking gently for 2 min the blood mixture was divided between several Eppendorf tubes (250 µl each tube) and the tubes shaken at 200 rpm at 37°C. After a certain time the **TQ** was extracted as in **II.7.2.3**. The recovery was very poor (1-2%) and no sign of $\mathbf{TQH_2}$.

II.7.2.8. *In vivo* reduction of TQ

TQ (350 mg) dispersed in milk (10 ml) was taken orally. The vessel which had contained the **TQ** dispersion was washed with milk (5 x 10 ml) to ensure removal of **TQ** and the washings were also taken orally. At intervals of 5 and 10 h a sample of blood (10 ml) was withdrawn into an evacuated tube containing sodium heparin and the plasma analysed for **TQ** and its $\mathbf{TQH_2}$. Cells and plasma were separated by centrifugation at 2300 rpm for 10 min. To plasma (200 µl) was added methanol (2 ml) followed by hexane (10 ml). The solution was mixed by vortex (3 sec) and the layers allowed to separate. The hexane layer was removed and taken to dryness *in vacuo* and the residue dissolved in methanol (180 µl). Aliquots of the methanol extract were analysed by RPLC (Supleco C_{18}, solvent K at a flow rate of 1.2 ml/min, detector D, integrator D). Results are recorded in Table **35**, p. 96 and Fig. **33**, p. 162.

Table 35. Concentration (μM) of **TQ**, **TQH_2** and **TH** in plasma after ingestion of **TQ**.

Time (hour)	TQ	TQH_2	TH
0	0.3	0.0	49.4
5	1.7	0.4	50.7
10	1.0	0.3	49.4

II.7.2.9. Reduction of TQ by human mononuclear cells.

Human peripheral blood mononuclear cells were isolated from blood and incubated in teflon containers as described by Geczy and Jones (1988): Venous blood from a normal healthy donor (9 vol) was mixed with 1 vol sodium citrate (3.8 w/v in 0.9% saline) and diluted with 2 parts of Ca^{2+}-free Hanks Balanced Salt Solution (HBSS). Peripheral blood mononuclear cells (PBM) were separated on pyrogen tested Lymphoprep, washed twice in Ca^{2+}-free HBSS and finally suspended in RPMI 1640 supplemented with 5 x 10^{-7} M mercaptoèthanol, 10 mM glutamine and 28 mM HEPES buffer.

To a suspension of mononuclear cells (5 x 10^7 cells) in RPMI 1640 medium (10 ml) was added bovine serum albumin/TQ complex (500 nmol/500 nmol in 1 ml distilled water) and the solution incubated at 37°C for 19 h. Aliquots of the suspension (2 ml) were removed at 1.5 and 19 h and centrifuged at 1400 rpm for 10 min at 4°C. The supernatant was removed and the mononuclear cell pellet washed with PBS (2 ml; treated with chelex). The mononuclear cells were lysed by addition of distilled water (500 μl) and vortexing for 2 min and then extracted with a solution of methanol/hexane (2 ml/10 ml). The hexane extract (9 ml) was taken to dryness *in vacuo* and the residue dissolved in methanol (450 μl). Aliquots of the methanol extract were analysed for **TQ** and **TQH_2** by RPLC as in **II.7.2.8.**

Results are recorded in Table **36**, p. 97 and Fig. **34**, p. 163.

Table 36. Reduction of TQ by human mononuclear cells.

Time (hour)	TH (nmol/10^7 cells)	TQ (nmol/10^7 cells)	TQH_2 (nmol/10^7 cells)
0.0	0.120	0.003	0.000
1.5	0.040	0.130	0.000
19.5	0.220	0.840	0.016

II.7.3. OPTIMISATION OF EXTRACTION OF α-TOCOPHEROL (TH) AND α-TOCOPHERYLQUINONE (TQ) FROM BLOOD.

II.7.3.1. Protocol 1.

To plasma (400, 300, 200, 100 or 50 µl) was added methanol (500 µl), the mixture shaken for a few sec, and hexane /isopropanol (99:1)(2 ml) added. The mixture was then vortexed for 3 sec and centrifuged at 3000 rpm for 2 min. An aliquot of the hexane layer (1.7 ml) was dried under a gentle stream of nitrogen. The dried sample was then dissolved in hexane (200 µl) and an aliquot (10 µl) chromatographed by NPLC (solvent C, detector C, integrator B). The results are recorded in **Table 55**, p. 164.

II.7.3.2. Protocol 2.

Plasma (fresh to 10 day old; room temperature or 4°C; 100 µl) was added to a solvent (methanol or ethanol or acetonitrile or isopropanol or hexane; room temperature or 4°C) and the mixture shaken or vortexed for several sec. Hexane/isopropanol (98:2, 2 ml, unless otherwise stated in the table) was added, the mixture vortexed for 4 sec and centrifuged at 3000 rpm for 2 min at 4°C. An aliquot of the hexane layer (1.7 ml) was dried under a gentle stream of nitrogen, the residue redissolved in hexane (200 µl) and an aliquot (10 µl) chromatographed by NPLC as in protocol **1**. Results are recorded in Table **55**, p. 164.

II.7.4. Solvent interference in NPLC.

Dichloromethane (2 ml) was dried under a gentle stream of nitrogen then hexane (200 µl) was added and an aliquot (10 µl) chromatographed by NPLC (as in **II.7.3.1**). It gave big peaks at 3.03 and 3.26 min which interfere with the **TH** peak.

Ether, treated the same way gave substantial peaks at 4.00, 4.66, 5.12, 5.74 and 6.40 min (very big) which interfere with the **TH** and **TQ** peaks.

Ether was refluxed with sodium hydroxide for 3 h and distilled, then an aliquot (2 ml) was dried under a gentle stream of nitrogen and dissolved in hexane (200 µl) and chromatographed as in **II.7.3.1**. It still gave substantial peaks at 2.07, 3.24 and 3.67 min and smaller peaks at 4.72, 5.28, 7.35 and 10.79 min which interfere with **TH** and **TQ** peaks.

Acetone was refluxed with sodium hydroxide for 3 h and distilled, then an aliquot (500 µl) was dried under a gentle stream of nitrogen, the residue dissolved in hexane (200 µl) and chromatographed as in **II.7.3.1**. It gave a variety of big peaks from 3 until 7.36 min which interfere with **TH** and **TQ** peak.

II.7.5. Analysis of plasma using the SMAD data acquisition interface.

II.7.5.1. Optimisation of detector wavelength.

An analysis of plasma (100 µl) using ethanol (500 µl) and hexane/isopropanol (98:2)(2ml) was performed using detector wavelength 280, 275 and 270 nm respectively. The protocol as in **II.7.3.1** chromatographed by NPLC (solvent C, detector B, integrator C) and results are recorded in **Table 56**, p. 165.

II.7.5.2. Other methods of analysis of **TH** and **TQ** in plasma were performed as below:

Protocol A. Plasma (100 µl, 7 day) was added to ethanol (500 µl, the mixture shaken for 5 sec. Then NPLC solvent C (3 ml) was added, vortexed for 20 sec and centrifuged at 3000 rpm for 2 min at 4°C. An aliquot (2.6 ml) was dried under a gentle stream of nitrogen, redissolved in solvent C (200 µl) and an aliquot (20 µl) chromatographed by

NPLC (at 275 nm) as in **II.7.5.1**. Results are recorded in **Table 57**, p. 166.

Protocol B. Plasma (100 µl, fresh) was added drop by drop to vigorously stirred ethanol (500 µl). NPLC solvent C (5 ml) was slowly added to the stirred mixture (in 1 min), then vortexed (4 sec) and the mixture was allowed to settle (3 min). An aliquot of the hexane layer (4.7 ml) was dried under a gentle stream of nitrogen, redissolved in solvent C (100 µl) and an aliquot (50 µl) rechromatographed as in **II.7.5.1**. Results are recorded in **Table 57**, p. 166.

Protocol C. Ethanol (500 µl) was added in small portion to plasma (100 µl, 7 day old). During this process the mixture was shaken gently. NPLC solvent C (2 ml) was added, the mixture vortexed (4 sec) and allowed to settle (1 min). An aliquot of the hexane phase (1.7 ml) was treated as in **Protocol B**. Results are recorded in **Table 57**, p. 166.

Protocol D. Ethanol (500 µl) was added drop by drop to the stirred plasma (100 µl, 1 day old). Extraction was performed by adding NPLC solvent C (2 ml) slowly to the stirred mixture, vortexing (4 sec) and allowing the mixture to settle (1 min). An aliquot of the hexane phase (1.7 ml) was collected and dried. This procedure was repeated twice more and 2 ml of hexane layers were collected each time instead of 1.7 ml. The hexane layers were combined and dried under a gentle stream of nitrogen, redissolved in solvent C (200 µl) and an aliquot (100 µl) chromatographed as in **II.7.5.1**. Results are recorded in **Table 57**, p. 166.

Protocol E. Analysis of plasma with or without surface active agent.
Plasma (7 day old, 100 µl) was added in small portion to ethanol (500 µl) containing (or without) a surface active agent (1% sodium lauryl sulfate or 1% tween), the mixture shaken for 5 sec and extracted (i) with solvent C (1 ml), vortexed (3 sec), more solvent C (1 ml) was added and vortexed (4 sec). The mixture was allowed to settle for 1 min,

then an aliquot of the hexane layer (1.7 ml) dried under a gentle stream of nitrogen. Stage (ii): to the residue of (i) was added solvent C (2 x 1 ml) as in (i) and an aliquot of hexane layer (1.8 ml) combined with the dried residue of (i) and the solvent removed under a gentle stream of nitrogen. Stage (iii): the aqueous plasma residue of (ii) was treated as in (ii), except an aliquot (2 ml) was collected instead of 1.8 ml. The final dried residue was redissolved in solvent C (100 µl) and chromatographed (20 µl) by NPLC as in **II.7.5.1**. Results are recorded in **Table 59**, p. 167.

Protocol F. Plasma (fresh or 7 day old, 100 µl) was added drop by drop to stirred ethanol (500 µl). Stage (i): solvent C or hexane or solvent E (2 x 1 ml) was added to the stirred (1 min) mixture, the mixture vortexed (4 sec) and allowed to settle (3 min). An aliquot of hexane layer (1.7 ml) was dried under a gentle stream of nitrogen. Stage (ii): the aqueous plasma residue of stage (i) was stirred for 25 sec and then treated as in **Protocol E** stage (ii). Stage (iii), to the stirred aqueous plasma residue of stage (ii) was added ethanol (250 µl) and the mixture stirred (30 sec), then treated as in **Protocol E** stage (iii). Results are recorded in Table **58**, p. 166.

II.7.5.3. Activation of papain (Scarf et al., 1982).

Enzyme stock solution: papain (20 mg) and dithiothreitol (4 mg) were dissolved in phosphate buffered saline (0.2 ml).

Dilute enzyme solution: the enzyme stock solution (20 µl) was diluted in buffer (1 ml).

Substrate solution: benzoyl arginine ethyl ester (BAEE)(80 mg) was dissolved in phosphate buffered saline (50 ml)

Substrate (3 ml) was placed in the sample and blank cuvettes and allowed to equilibrate for 35 min at room temperature. Then to the blank was added buffer solution (50 µl) and to the sample was added diluted enzyme solution (50 µl). Rate of hydrolysis of BAEE was measured at 253 nm. The measurement was taken once in every 30 seconds for 3 min and then it was taken once in every 2 min.

This experiment showed that the papain had been activated by dithiothreitol (specific

activity = 10.5 μmol/min/mg) and it was used to digest the protein in the blood samples.

II.7.5.4. Analysis of TH and TQ in blood using stock papain solution or proteinase K.

To plasma or packed cells or whole blood (100 μl) was added papain stock solution or proteinase K in PBS (different amounts), the mixture homogenised and incubated at 37°C for various time. Then ethanol (500 μl) was added in small portion into the blood mixture and the tube flicked for 15 sec. Extraction of TH and TQ was completed as in protocol D (p.99). Control extractions on plasma and also on whole blood without adding papain or proteinase K were performed in quadruplicate. Results are recorded in Table **60**, p. 168.

II.7.5.5. Analysis of TH and TQ in plasma and packed cells using stock papain solution and incubated for varying times.

To plasma or packed cells (100 μl, 4 day old) was added papain stock solution in phosphate buffered saline (40 μl, 8 mg), the mixture was shaken at 37°C for 15, 30, 45, 60 and 90 min. and the rest of the procedure was as in **II.7.5.4.** Results are recorded in Table **61**, p. 168.

II.7.5.6. Analysis of blood fractions for TH and TQ content.

1. To methanol (500 μl) was added whole blood (or plasma or white cells or packed cells or red cells (100 μl)) while stirring, then hexane (2 x 2 ml) was added and the solution vortexed for 5 sec and centrifuged at 3000 rpm for 1 min at 4°C. An aliquot of the hexane layer (3.5 ml) was dried under a gentle stream of nitrogen and then chromatographed by NPLC as in **II.7.5.1.** This experiment was performed in quadruplicate. Results are recorded in Table **62**, p. 169).

Confirmation of identity of TH and TQ in blood.

To confirm that the compounds appearing on the chromatogram were **TH** and **TQ**, whole blood was extracted as in **II.7.5.6** and the extract chromatographed on a preparative silica column (Waters μPorasil, 10 μm, 3.9 x 300 mm) at 2 ml per minute. The peaks which appeared at the retention time of **TH** and **TQ** were collected separately and their UV spectra recorded using hexane-isopropanol 2% as the reference solvent. The suspected **TH** peak gave a maximum wavelength at 291.8 nm, while the **TH** standard gave a maximum wavelength at 293.2 nm; whereas the suspected **TQ** gave a bicuspid type of peak at 268.2 and 259.4 nm, while the **TQ** standard had a characteristic bicuspid peak at 268.6 and 260.6 nm.

II.7.6. Reaction of TH and PH with haemoglobin (Hb) solution at room temperature and 37°C.

II.7.6.1. Reaction of TH with Hb or BSA (as a control) solution at room temperature.

Bovine serum albumin (BSA, 150 mg) or Hb (120 mg or 150 mg) was dissolved in PBS (1 ml). An aliquot (200 μl) of the BSA or (100 μl) of Hb solution was taken for each separate reaction. To each aliquot of BSA solution was added **TH** (7.7 nmol) in methanol (10 μl) and to each aliquot of Hb solution was added **TH** (5.4 nmol) in methanol (5 μl). After the required time, methanol (500 μl) was added to the sample which formed a solid white precipitate in the BSA reaction. The precipitate was broken with a glass rod, hexane (2 ml) was added, the mixture vortexed for 5 sec and centrifuged at 3000 rpm for 1 min at 4°C. An aliquot of the hexane layer (1.5 ml) was collected. To the aqueous supernatant residue was added hexane (2 ml), the mixture vortexed for 5 sec, centrifuged at 3000 rpm for 1 min at 4°C, and an aliquot of the hexane layer (2 ml) was collected and combined with the first one. The combined hexane extracts were dried under a gentle stream of nitrogen and the residue redissolved in hexane (200 ml) and chromatographed as in **II.7.5.1.** BSA solution without added

TH was treated the same way and served as a blank. The blank did not show any **TH** or **TQ**. Results are recorded in Table **37**, p. 103 and Fig. **36**, p. 170.

II.7.6.2. Reaction of TH with Hb or BSA solution at 37°C.

BSA and Hb solutions were made by dissolving BSA (150 mg) or Hb (150 mg) in PBS (1 ml). **TH** solution (2.5 µl, 16.0 nmol) was added to BSA solution (200 µl) or to Hb solution (100 µl) and the mixture incubated at 37°C for a certain period of time. Then to the reaction mixture was added bidistilled water (100 µl) and the whole mixture transferred with a pasteur pipette to stirred methanol (250 µl). To the residue was added bidistilled water (50 µl) and this also was transferred to the stirred methanol. The residue was also rinsed with methanol (250 µl) and transferred to the stirred mixture which was then extracted with hexane/isopropanol (98:2, 2 ml). The remainder of the experiment was completed as in **II.7.5.7.1.** Results are recorded in Table **37**, p. 103, Fig. **37**, p. 171.

Table 37. Reaction of **TH** with BSA or Hb solution at room temperature and 37°C (calculated as % of added **TH**).

Time (min)	Room temperature						37°C			
	BSA 15%		Hb 12%		Hb 15%		BSA 15%		Hb 15%	
	TH	**TQ**	**TH**	**TQ**	**TH**	**TQ**	**TH**	**TQ**	**TH**	**TQ**
0	96	0.1	75	10	39	2	99	0	87	6
5	74	0.3								
20	86	0.4								
30			63	17	50	2	91	0	88	7
40	94	0.6								
60			70	9	44	2	99	0	78	8
120			70	9	39	3	93	0.1	75	10
180			95	10	49	3	91	0.3	66	10
240			75	10	51	3	75	0.6	64	12

II.7.6.3. Reaction of PH with BSA and Hb solution at 37°C.

PH (20 nmol) in methanol (2.5 µl) was added to BSA (15%) or Hb (15%) solution (200 µl) in PBS, the solution mixed gently and shaken at 37°C at 200 rpm for a certain period of time. Bidistilled water (100 µl) was then added and the experiments were completed as in **II.7.6.2**, with the sample dried under a very gentle stream of nitrogen and only for a very short time until just dry (1 min). Results are recorded in Table **38** and Fig. **38**, p. 172.

Table 38. Reaction of **PH** with BSA or Hb solution calculated as % of added **PH** .

Time (hour)	In BSA solution		In Hb solution	
	PH	**PQ**	**PH**	**PQ**
0	71.0	0.0	73.5	0.8
0.5	73.9	0.0	62.9	12.0
1	71.3	0.0	61.2	14.4
2	73.5	0.0	35.5	19.9
3	74.5	0.0	35.9	21.1
4	76.7	0.0	27.8	27.5

II.7.7. REACTION OF BLOOD WITH CARBON MONOXIDE.

(Seligson, 1963)

Preparation of oxyhaemoglobin standard:

Blood (0.5 ml) was diluted to 50 ml with 0.007M ammonia solution and air was gently bubbled through the solution for 10 min. The visible spectrum of oxyhaemoglobin was taken and the peaks found to be at 576 and 541 nm with a valley at 560 nm. Thus the equation (1) in the above reference was modified to use the peak at 576 nm rather than 574 nm.

Equation 1: $$R_{oxyhaemoglobin} = \frac{A_{576} - A_{560}}{A_{494}} \quad \ldots\ldots \quad (1)$$

Absorbances at 576 nm (1.617), 560 nm (0.9028) and at 494 nm (0.5287) gave $R_{oxyhaemoglobin} = 1.226$.

Preparation of carboxyhaemoglobin standard.

Blood (1 ml) was diluted to 100 ml with 0.007M ammonia and carbon monoxide was bubbled gently through for 3 min and the visible spectrum determined as above. Absorbances at 576 nm (1.1321), 560 nm (1.2645) and 494 nm (0.5684) gave $R_{carboxyhaemoglobin} = -0.233$.

Equation 2: $$R_{carboxyhaemoglobin} = \frac{A_{576} - A_{560}}{A_{494}} \quad \ldots\ldots\ldots\ldots \quad (2)$$

Equation 3: $$R_X = \frac{A_{576} - A_{560}}{A_{494}} \quad \ldots\ldots\ldots\ldots \quad (3)$$

The percentage of carboxyhaemoglobin in the suspected sample is then given by

$$\frac{R_{oxyhaemoglobin} - R_X}{R_{oxyhaemoglobin} - R_{carboxyhaemoglobin}} \times 100 \quad \ldots\ldots\ldots\ldots \quad (4)$$

Preparation of carbonmonoxygenated blood.

A fine stream of carbon monoxide was passed into a sealed tube containing about 4 ml of blood. To avoid the bubbling of the blood, the gas was blown over the surface of the blood which was shaken gently. After 10 min of carbonmonoxygenation the blood was diluted (1:10) with 0.007M ammonia and the absorbances measured at 576 (1.5087), 560 (1.3593) and 494 nm (0.6719) giving an R value of 0.222 which corresponds to 68.8% carbonmonoxygenation. Using the absorbance at 574 rather than 576 nm gave 63.5% carbonmonoxygenation. Carbon monoxide was blown into the blood for a further 5 min giving a carboxyhaemoglobin level of 91%. Measurement at 574 nm rather than 576 nm gave carboxyhaemoglobin level of 91.7%. The packed cells of the treated blood were checked for carboxyhaemoglobin content 3 hours after the treatment and the the level found to be 90.3%.

Both the untreated and the treated blood were used for determination of **TH** and **TQ** content in the whole blood, plasma and packed cells.

Determination of **TH** and **TQ** content in the untreated and treated blood.

To untreated whole blood (260 mg) was added PBS (255 mg) and after gentle homogenization an aliquot (100 mg) of the diluted blood was added drop by drop to the

stirred methanol (500 µl). Hexane (2 ml) was then added and the mixture vortexed for 10 sec and centrifuged at 3000 rpm for 2 min at 4°C. An aliquot of the hexane (1.5 ml) was taken and dried under a gentle stream of nitrogen. The remaining aqueous residue was extracted with additional hexane (2 ml) and an aliquot (2 ml) combined with the first hexane extract and dried again under nitrogen. The dried residue was redissolved in hexane (100 µl) and an aliquot (80 µl) was chromatographed by NPLC solvent C. The experiment was performed in duplicate.

The rest of the untreated blood was centrifuged at 3000 rpm for 10 min, the plasma discarded and an aliquot (101 mg) of the packed cells was treated in the same way as the whole blood. The plasma was replaced by the same amount of PBS and after homogenisation was centrifuged again for another 10 min. The supernatant PBS was discarded and this washing was repeated one more time. To an aliquot of the packed cells (398 mg) was added PBS (1.2 g), and the analysis was performed as in the whole blood determination.

The above procedure was also carried out for the blood treated with carbon monoxide. The colour of the plasma from the treated blood was red.

As a comparison, another analysis on untreated blood was performed using RPLC solvent K. Results are recorded in Table **63**, p. 173.

II.7.8. ANALYSIS OF HEART MUSCLE AND LIVER FOR TH AND TQ

II.7.8.1. ANALYSIS USING THIN LAYER CHROMATOGRAPHY.

(Bieri, 1969).

To lamb heart muscle (2.3 g, cut into small pieces) in a glass stoppered test tube (16 x 150 mm) was added ethanol (4 ml; containing 2% pyrogallol) and the tube shaken in a water-bath (65-70°C). After 5 minutes, nitrogen gas was passed into the tube (30 sec) through a 1 ml pasteur pipette and 60% KOH solution (1.5 ml) was added. Then the nitrogen tube was removed, the test tube rapidly stoppered and the shaking continued for 20 min to digest the heart muscle. The test tube was cooled in an ice-bath,

an equal amount of water was added and then hexane (5 ml) containing BHT (0.001%) was also added, followed by vigorous shaking for 2 min. The hexane layer separated immediately.

The hexane layer and some of the saponification (brown-black) layer was transfered into two 5 ml centrifuge tubes and centrifuged at 1000 rpm for one min. The hexane layer (3.2 ml) was taken and dried under nitrogen at 50°C. The dried extract was dissolved in benzene (25 µl) and chromatographed by two dimensional TLC using **TH**, β-tocopherol (β-**T**), **TQ** and **UQ-10** as standards. Benzene/ethanol = 99:1 was used as the first solvent and hexane/ethanol = 9:1 as the second solvent. Spots were visualized under ultraviolet light. (Bieri, 1969). See results and discussion Fig. **39** and **40**, p. 174 and 175 respectively.

II.7.8.2. ANALYSIS USING HPLC.

Analysis of **TH** and **TQ** content in the heart and liver of dog.

Dog heart muscle (2.46 g) and liver (2.19 g) were digested with 60% KOH and extracted with hexane (2 x 5 ml) as described in **II.7.6.1**. A gentle stream of nitrogen was blown into the mixture during digestion and extraction. The combined hexane layers for each sample were then dried (Na_2SO_4) and the solvent removed *in vacuo*. Each dried extract was then dissolved in hexane (400 µl) and chromatographed by NPLC using solvent C, detector C (270 nm) and integrator B. Results are recorded in Table **63**, p. 175.

The compounds at retention time 4.08 and 5.22 min were confirmed to be **TH** and **TQ**, by re-running the chromatograms with the detector at 280 and 290 nm. At 280 nm the peak at 4.08 min became bigger and that at 5.22 min became very small, while at 290 nm the peak at 4.08 min became even bigger and that at 5.22 min disappeared.

II.8. EXPERIMENTS ON 8a-HYDROXY-2,2,5,7,8-PENTAMETHYL-6-CHROMANONE AND 8a-HYDROXYTOCOPHERONE. (Durckheimer and Cohen, 1964).

II.8.1. Preparation and U V analysis of 8a-hydroxy-2,2-5,7,8-pentamethyl-6-chromanone (POH).

Several methods were performed in order to find the best method of preparation of **POH**.

Method I: A solution of **PH** (11 mg; 0.05 mmol) in acetonitrile (10 ml) was cooled in an ice bath and added rapidly to a stirred, chilled solution of N-bromosuccinimide (NBS, 36 mg; 0.2 mmol) in a mixture of 0.1M phosphate (KH_2PO_4, 10 ml) and acetonitrile (10 ml). The cool solution was stirred for 1 minute then hexane (HPLC, 20 ml) added and the mixture shaken vigorously. The hexane layer was removed and washed with ice cold water (4 x 10 ml) and dried rapidly (Na_2SO_4). The whole process was accomplished in 6 min. At 10 min the UV analysis took place and the data of this observation were used to determined the rate of decomposition of **POH** (Fig. **54**, p. 89).

Method II: The same as in method **I** except that the time of the reaction was reduced to 30 seconds and the hexane layer washed only once and the UV analysis started at 8 min.

Mehtod III: The same as in method **II** with the reaction time 20 sec.

Method IV: The same as in method **II**, but using **PMC** (5.5 mg) in acetonitrile (5 ml) and NBS (18 mg) in a mixture of 0.1 M phosphate and acetonitrile (10 ml), extracted with hexane (10 ml) and washed with ice cold water (2 x 5 ml).

II.8.2. Estimation of POH concentration.

To estimate the yield of **POH**, the freshly prepared **POH** (100 µl freshly prepared by method IV) was reacted with ascorbic acid (10%) (100 µl) for 30 min, then hexane (0.5 ml) was added, the solution mixed well and the hexane layer filtered through a pad of tissue paper and dried (Na_2SO_4) and chromatographed by NPLC (solvent D, detector B, integrator C). The amount of **POH** formed in the starting material was estimated by the amount of **PH** formed in the reaction of **POH** and ascorbic acid. The yield ranged between 150 - 180 µM and contained 5-10% **PQ**.

Other attempts to gain higher yields were carried out as follows:

1. The preparation was carried out in a separatory funnel for 30 seconds, then extracted with hexane and washed with cold water once.
2. Extraction was performed with hexane/isopropanol = 9:1.

There was no improvement with these two other methods, instead extraction with hexane/isopropanol= 9:1 showed an additional big peak in the chromatogram (at 3.3 minutes, before the **PH** peak) probably due to the reaction of isopropanol and **POH**.

II.8.3. Reaction of 8a-hydroxy-2,2-5,7,8-pentamethyl-6-chromanone (POH) (150 µM) with various reductants.

II.8.3.1. Reactions in air.

A cold **PH** (2.2 mg) in acetonitrile (2 ml) was added to a chilled and stirred NBS (7.2 mg) in acetonitrile/0.1 M phosphate 1:1 (4 ml) and the solution was stirred for 30 sec, extracted with hexane (10 ml) and the hexane layer washed with ice cold water (2 x 4 ml). An aliquot of the **POH** containing, hexane layer (1 ml) was dried (Na_2SO_4), filtered and chromatographed as in **II.8.2**. Additional aliquots (100 µl) of **POH** were stirred with the reductants (3 mM, 100 µl) for various times (1, 10, 20 and 40 min) at room temperature, extracted with hexane-isopropanol (10%, 1 ml), dried (Na_2SO_4), filtered and chromatographed (20 µl) by NPLC as in **II.8.2**. Each reaction time was a separate experiment. A solution of **PQ** in hexane (same concentration as the **POH**) was also reacted with the same reductants under the same conditions.

Only ascorbic acid and sodium dithionite (3 mM) reduced the **POH** to **PH**, but no significant formation of PQH_2 was detected even from the **PQ** reaction (control) under the same conditions (Fig. **44**, p. 181).

Table 39. HPLC analysis of the reaction of **POH** (150 μM) with ascorbic acid (3 mM) and with sodium dithionite (3 mM) in air.

Time	Reaction products of **POH** (μM) ascorbic acid		Reaction products of **POH** (μM) with sodium dithionite	
(min)	**PH**	**POH**	**PH**	**POH**
1	113.0	20.0	63.0	61.0
10	124.0	13.0	116.0	24.0
20	120.0	14.0	119.0	23.0
40	123.0	14.0	118.0	27.0

Reaction of **PQ** (150 μM) with dithiothreitol (3 mM) showed a significant decrease of **PQ** but on the other hand did not show any formation of other compound. Even though the experiment was repeated several times and even by adding or extracting the reaction mixtures with a more polar solvent (such as hexane-isopropanol 10% or hexane-isopropanol 4%) to enhance the recovery of PQH_2, if it was formed during the reactions, the results were the same.

Reaction with NADH (0.3 mM) plus FAD (1.5 mM) and NADH (2.4 mM) plus FAD (3 mM) dissolved in water or in 0.05 M phosphate buffer pH 7 did not reduce **POH**. However the higher concentration of NADH and FAD caused a 27% reduction of **PQ** to PQH_2.

All the above experiments were performed in a small sample tube and no precaution was taken to exclude oxygen. The results were unsatisfactory except the reactions with ascorbic acid which formed **PH**, but the reactions with **PQ** gave hardly any product, whereas earlier results had shown formation of PQH_2 with certain

reductants (see Reduction Experiments). Because of this, the experiments were repeated using the long, narrow tube and the reactions were performed under nitrogen as in **II.6.3.2.**

II.8.3.2. Reactions of 8a-hydroxy-2,2,5,7,8-pentamethyl-6-chromanol (POH) with several reductants using a 1:20 molar ratio under nitrogen.

POH (100 µl aliquots), freshly prepared and dried as in method IV were taken for the reactions with the reductants. Accompanying each reaction with a reductant was a "control" with 10% ascorbic acid to estimate the amount of **POH** in the reaction (see **II.8.2**). A gentle stream of nitrogen was blown into the reaction mixture for 1, 10, 20 and 40 min and the mixture then extracted with 10% isopropanol in hexane (0.5 ml), dried (Na_2SO_4), filtered and an aliquot (25 µl) chromatographed by NPLC (solvent D).

II.8.3.2.1. Reactions of POH and PQ (150 µM) with ascorbic acid (3 mM). (See also Fig. 45, p. 181).

Table 40. HPLC analysis of the reaction of **POH** and **PQ** with ascorbic acid (expressed as a % of the original **POH** or **PQ** concentration).

Time	Reaction products of **POH**			Reaction products of **PQ**.		
(min.)	**PH**	**PQ**	**POH**	**PH**	**PQ**	**PQH_2**
1	77.0	-	15.0	0.0	100.0	0.0
10	91.0	-	3.6	0.0	100.0	0.0
20	100.0	-	0.0	0.0	100.0	0.0
40	100.0	-	0.0	0.0	100.0	0.0

II.8.3.2.2. Reactions of POH (150 µM) and PQ (150 µM) with sodium ascorbate (3 mM).

There was no reaction with either **POH** or **PQ**.

II.8.3.2.3. Reactions of POH (150 μM) and PQ (150 μM) with sodium dithionite (3 mM).

The sodium dithionite solution was freshly made immediately before the reaction.
Results are recorded in Table **41** and Fig. **46**, p. 182.

Table 41. HPLC analysis of the reaction of **POH** and **PQ** with sodium dithionite (expressed as a % of the original **POH** and **PQ** concentration).

Time (min.)	Reaction products of **POH**			Reaction products of **PQ**.		
	PH	**POH**	**PQH_2**	**PH**	**PQ**	**PQH_2**
			I + II			
1	75	12	0	0	45	30
10	75	3	0	0	12	56
20	85	0	0	0	15	61
40	68	2 *	0	0	5	67

* most likely the **PQ** formed by oxidation of **PH** or **POH**.

II.8.3.2.4. Reactions of POH (150 μM) and PQ (150 μM) with dithiothreitol (3 mM).

Results are recorded in Table **42** and Fig. **47**, p. 183.

Table 42. HPLC analysis of the reaction of **POH** and **PQ** with dithiothreitol (expressed as a % of the original **POH** or **PQ** concentration).

Time (min.)	Reaction products of **POH**			Reaction products of **PQ**.		
	PH	**POH**	**PQH_2**	**PH**	**PQ**	**PQH_2**
1	21	82	0	0	65	0
10	8	90	0	0	70	19
20	9	68	0	0	22	61
40	12	83	0	0	15	69

II.8.3.2.5. Reactions of POH (150 μM) and PQ (150 μM) with NADH (2.4 mM) and FAD (3 mM).

The freshly made **POH** was mixed with NADH then a gentle stream of nitrogen was blown into the mixture for 5 sec and immediately the FAD solution was added (still under nitrogen) and again nitrogen was blown into the mixture and the remainder of the

reaction was as above. The NADH and FAD solutions were made up in 0.05 M phosphate buffer pH 7 immediately before the reactions.

Results are recorded in Table **43** and Fig. **49**, p. 184.

Table.43. HPLC analysis of the reaction of **POH** and **PQ** with NADH plus FAD (expressed as a % of the original **POH** and **PQ** concentration).

Time	Reaction products of **POH**			Reaction products of **PQ**.		
(min.)	**PH**	**POH**	**PQH_2**	**PH**	**PQ**	**PQH_2**
1	0	64	0	0	65	0
10	0	1	73	0	5	42
20	0	0	51	0	7	28
40	0	1	28	0	4	33

II.8.3.3. Experiments using hexane/ethyl acetate as the extracting solvent.

There was a solubility problem in extracting the **PQH_2** from the reaction mixture as can be observed by the inconsistency of the recovery of the reactions where **PQH_2** was formed. For this reason the experiments were repeated and the reactions products were extracted by hexane-ethyl acetate (9:1) or (8:2). In these experiments a gentle stream of nitrogen was blown into the tube throughout the reactions.

II.8.3.3.1 Reactions of POH (150 μM) with sodium dithionite (3 mM), extracted with hexane/ethyl acetate.

Table.44. HPLC analysis of the reactions of **POH** with sodium dithionite (expressed as a % of the original **POH** concentration).(See Fig. **50**, p. 185).

Time	Extraction with hexane/ethyl acetate (8:2)			Extraction with hexane/ethyl acetate (9:1)		
(min)	**PH**	**PQH_2**	**POH**	**PH**	**PQH_2**	**POH**
1	100	0	0	12	31	0
10	100	0	0	50	58	0
20	100	0	0	54	42	0
40	100	0	0	27	69	0

II.8.3.3.2. Reactions of POH (150 μM) with dithiothreitol (3 mM), extracted with hexane-ethyl acetate.

Table 45. HPLC analysis of the reaction of **POH** with dithiothreitol (expressed as a % of the original **POH** concentration). (See Fig. **51**, p. 186).

Time (min)	Extraction with hexane-ethyl acetate (8:2)			Extraction with hexane-ethyl acetate (9:1)		
	PH	**PQH_2**	**POH**	**PH**	**PQH_2**	**POH**
1	12	26	77	10	0	75
10	11	26	90	10	0	90
20	21	0	77	25	0	75
40	32	21	58	30	0	75

II.8.3.3.3. Reactions of POH (150 μM) with NADH (2.4 mM) plus FAD (3 mM), extracted with hexane/ethyl acetate (8:2).

Table 46. HPLC analysis of the reaction of **POH** with NADH plus FAD (extracted with hexane/ethyl acetate (8:2), expressed as a % of the original **POH** concentration). (See Fig. **52**, p. 186).

Time (min)	**PH** (mole %)	**PQH_2** (mole %)	**POH** (mole %)
1	0	14	86
10	6	77	0
20	10	50	0
40	8	62	5

II.8.3.3.4. Reactions of POH (150 μM) with potassium iodide (1 M, pH 3), extracted with hexane-ethyl acetate.

Table 47. Products of reaction of **POH** with potassium iodide (1M, pH3) (extracted with hexane/ethyl acetate (9:1), expressed as a % of the original **POH** concentration). (See Fig. **48**, p. 184).

Time (min)	**PH** (mole %)	**PQH_2** (mole %)	**POH** (mole %)
1	77	0	23
10	78	0	28
20	100	0	0
40	89	0	17*

*This is most likely the **PQ** formed due to the oxidation of **POH** by I_2.

Reactions with potassium iodide solution 1M (pH 9.25) did not show any reduction, neither did the reaction with potassium chloride (1M, pH3). **PQ** (the same concentration as **POH**) was not reduced by KI (1 M, pH 3).

Another series of experiments was performed using **PQ** in hexane (heterogenous solution) with the same protocol as in **II.6.3.2.**, i.e. **PQ** (20 mM) and the reductants (400 mM), except for NADH (40 mM)/FAD (200 mM) and NADH (320 mM)/FAD (400 mM) (Table **48**, p. 116).

Table 48. Reduction of **PQ** (0.02M) by several reductants in heterogeneous solution.

Time	ascorbic acid (0.4M)		sodium ascorbate (0.4 M)		sodium dithionite (0.4 M)		dithiothreitol (0.4 M)		NADH (0.04M) plus FAD (0.2M) in water		NADH (0.04M) plus FAD(0.2M) in buffer		NADH (0.32M) plus FAD (0.4M) in buffer	
(min)	PQ	PQH_2	PQ	PQH_2	PQ	PQH_2	PQ	PQH_2	PQ	PQH_2	PQ	PQH_2	PQ	PQH_2
1	96	4	86	14	30	70	59	41	79	21	55	45	4	97
10	88	12	61	39	30	70	7	93	47	53	13	87	3	97
20	93	7	38	62	3	97	5	95	81	19	4	96	5	95
40	80	20	24	76	2	98	4	96	88	12	8	92	5	95

Concentration of **PQ** and PQH_2 are expressed as % of the original **PQ**.

II.8.4. Preparation and reactions of 8a-hydroxytocopherone with several reductants.

II.8.4.1. UV analysis.

The rate of decomposition of **TOH** was investigated by UV analysis as in **II.8.1.**

A solution of **TH** (22 mg; 0.05 mmol) in acetonitrile (10 ml) was cooled in an ice bath and added rapidly to a stirred, chilled solution of N-bromosuccinimide (NBS, 36 mg; 0.2 mmol) in a mixture of 0.1M phosphate (KH_2PO_4, 10 ml) and acetonitrile (10 ml). The cool solution was stirred for 1 minute then hexane (HPLC, 20 ml) added and the mixture shaken vigorously. The hexane layer was removed and washed with ice cold water (4 x 10 ml) and dried rapidly (Na_2SO_4). The whole process was accomplished in 6 min. Prior the UV analysis, the the freshly prepared **TOH** solution was diluted with hexane (15-fold) and the data of this observation were used to determined the rate of decomposition of **TOH** (Fig. **54**, p. 189).

II.8.4.2. HPLC experiments.

II.8.4.2.1. Estimation of the concentration of TOH.

To check the yield of the **TOH** formed in the preparation, the freshly made **TOH** as in **II.8.4.1.** was reacted with ascorbic acid (10%) as soon as possible for 10,20, and 30 minutes. Results are recorded in Table **49**.

Table 49. HPLC analysis of the reaction of **TOH** with ascorbic acid (10%).

Time (min.)	**TH** (mM)	**TQ** (mM)	Total yield (mM)	% yield
10	0.67	0.11	0.78	77
20	0.75	0.09	0.84	82
30	0.74	0.07	0.81	79

II.8.4.2.2. Reaction of TOH (0.8 mM) with sodium dithionite (32 mM).

The control reaction with vitamin C (10%) gave 0.79 μmol/ml yield.

Table 50. HPLC analysis of the reaction of **TOH** (0.8 mM) with sodium dithionite (32 mM).

Time (mins)	**TH** (% mole)	**TQ** (%mole)
10	76	16
20	85	19
30	79	19
45	79	18

The control with **TQ** solution (0.8 mM) did not give **TH** or TQH_2.

II.8.4.2.3. Attempted preparation of TOH (0.02M).

I. **TH** (22 mg, 0.05 mmol) in acetonitrile (2 ml) was added to a stirred chilled solution of NBS (36 mg, 0.2 mmol) in a mixture of acetonitrile (1 ml) and 0.1M KH_2PO_4 (1 ml). The mixture was stirred for 30 seconds (the colour turned yellow instantaneously). Hexane (2 ml) was added while still stirring vigorously and the hexane phase was removed and washed with ice cold water (3 x 2 ml). An aliquot of the hexane was dried (Na_2SO_4), diluted (10 x) and an aliquot (5 µl) chromatographed by NPLC, solvent C. A further aliquot (100 µl) of the hexane phase was reacted with ascorbic acid (200 mM, 100 µl) to estimate the yield of the **TOH** produced. The concentration of **TOH** was 0.008 M (30% of theoretical) while that of **TQ** was 0.012 M.

II. **TH** (22 mg, 0.05 mmol) in acetonitrile (5 ml) was added to a stirred chilled solution of NBS (36 mg, 0.2 mmol) in a mixture of acetonitrile (2.5 ml) and 0.1M KH_2PO_4 (2.5 ml). The remainder of the preparation was the same as **II.8.4.2.3.** protocol I except that the **TOH** was extracted with 5 ml hexane instead of 2 ml. The concentration of **TOH** was 0.007 M (70% of theoretical) while that of **TQ** was 0.0023 M.

After several other attempts, it was clear that the best yield of **TOH** which could be obtained was 0.005 M or 5 mM. Therefore all the reactions were performed using 5 mM **TOH** and **TQ**, while the concentration of the reductants was 100 mM. Thus the

molar ratio of **TOH**/reductant was 1:20, as in the reaction of **POH** and all the reactions were performed in a long, narrow tube under nitrogen.

II.8.4.2.4. Reaction of TOH (5 mM) and TQ (5 mM) with NADH (80 mM) plus FAD (100 mM).

TH (11 mg, 0.026 mmol) in acetonitrile (5 ml) was added to a chilled and stirred NBS solution (18 mg, 0.1 mmol) in acetonitrile (5 ml) and 0.1M phosphate solution (5 ml). The mixture was stirred in an ice-bath for 30 sec, then extracted with hexane (5 ml), washed with ice cold bidistilled water (2 x 3 ml), dried (Na_2SO_4), then immediately taken for reactions with the various reductants. Freshly made **TOH** (0.5 μmol) in hexane (100 μl) was added to NADH (8 μmol) in 0.05M phosphate buffer (pH 7, 100 μl) in a long, narrow tube and a gentle stream of nitrogen was blown into it. FAD (10 μmol) in 0.05M phosphate buffer (pH 7, 100 μl) was added (still under nitrogen) and again nitrogen was blown to the bottom of the tube until a black colour was developed. Then the reaction mixture was stirred under nitrogen for 1, 10, 20 and 40 minutes and was extracted with hexane-isopropanol (9:1, 1 ml). The organic layer was dried over anhydrous Na_2SO_4 (0.5 g) in a pasteur pipette and an aliquot (10 μl) chromatographed by NPLC solvent C. Each af the above reacton times was determined as a separate reaction since the reaction mixture was heterogeneous.

Table.51. Products of reactions of **TOH** (5 mM) and **TQ** (5 mM) with NADH (80 mM) plus FAD (100 mM). (Fig. **69**, p. 198).

Time (min)	Products of reduction of **TOH**			Products of reduction of **TQ**		
	TH (mole %)	**TQH_2** (mole %)	**TOH** (mole %)	**TH** (mole %)	**TQH_2** (mole %)	**TQ** (mole %)
1	0	1	100	0	10	97
10	0	8	93	0	11	98
20	0	11	90	0	40	61
40	0	31	72	0	74	21

II.9.4.2.5. Reduction of TOH (5 mM) and TQ (5 mM) by sodium dithionite (100 mM).

Results are recorded in Table **52** and Fig. **68**, p. 197.

Table.52. HPLC analysis of the reactions of **TOH** (5 mM) and **TQ** (5 mM) with sodium dithionite (in 20 minute reaction of **TOH**, the residual **TQ** was reduced).

Time (min)	Products of reduction of TOH			Products of reduction of TQ		
	TH (mole %)	**TQH_2** (mole %)	**TOH** (mole %)	**TH** (mole %)	**TQH_2** (mole %)	**TQ** (mole %)
1	11	1	90	0	3	97
10	56	8	28	0	18	77
20	59	36	0	0	40	48
40	41	9	50	0	28	71

II.9.4.2.6. Reduction of TOH (5 mM) by ascorbic acid (100 mM).

Results are recorded in Table 53 and Fig. **67**, p. 196.

Table.53. HPLC analysis of the reactions of **TOH** (5 mM) with ascorbic acid (100 mM).

Time (min)	**TH** (mol %)	**TQH_2** (mol %)	**TOH** (mol %)
1	9	0	90
10	30	0	68
20	53	0	49
40	41	0	63

There was no reduction of **TOH** by sodium ascorbate and dithiothreitol and no reduction of **TQ** by ascorbic acid, sodium ascorbate and dithiothreitol.

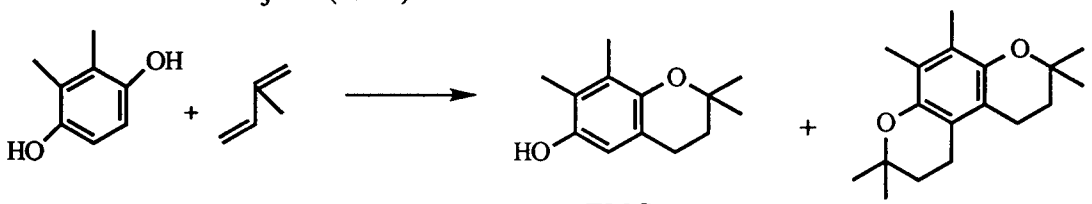
III. CHAPTER THREE.

RESULTS AND DISCUSSION

III. 1 SYNTHESIS.

III.1.1 PREPARATION OF 5-HYDROXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OH).

A two step reaction was involved in the preparation of 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**, p.41-43), including preparation of 2,2,7,8-tetramethyl-6-chromanol (γ-T model compound, **TMC**) (scheme **5**), followed by hydroxymethylation of **TMC** with paraformaldehyde and boric acid as catalyst according to Nakamura and Kijima (1972).



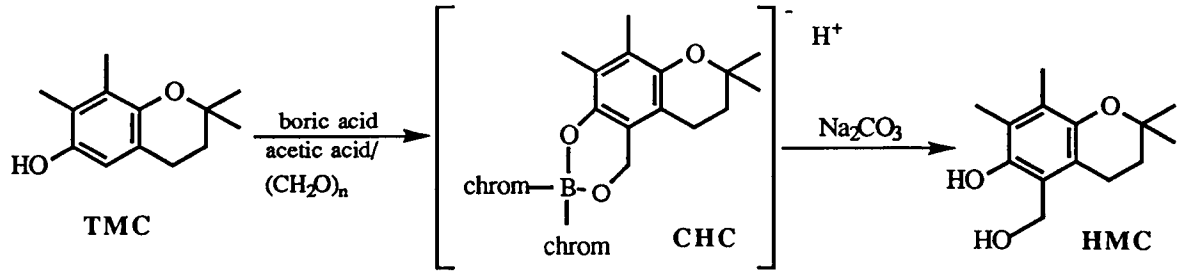
TMC

minor product

bichroman of **TMC**
major product

Scheme **5**. Formation of **TMC**.

Addition of boric acid formed a chroman complex (**CHC**), which was then broken up by mixing with Na_2CO_3 (scheme **6**).



Scheme 6. Formation of **PMOH**

III.1.2. PREPARATION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ).

When **PQ** was prepared by oxidising **PH** with silver nitrate in absolute ethanol and heating to 85°C for 8 min, the yield was only 44% compared to 64% when **PH** was stirred at room temperature in methanol for 5 min. In the first method, the reaction took place at a higher temperature and for a longer time and the reaction was stopped when the colour of the solution was orange-yellow indicating that some **PR** has also formed together with **PQ** (see scheme 7: oxidation of **PH**, p. 124). It was very difficult to separate the two compounds on TLC as they always overlap. Only after using solvent a (light petroleum 60-80°C/chloroform = 9:1) and solvent b (light petroleum 60-80°C/chloroform/ethyl acetate = 4:1:5 with a composition solvent a/b (1:1) was a good separation achieved, even though quite substantial amount of **PQ** was lost in purification. In the second method care was taken that **PR** was not formed and the reaction was stopped when the solution was bright yellow and before red colour appeared. The above solvent was used on purification by TLC. Crystallisation of **PQ** was achieved only from a very concentrated solution in light-petroleum (60-80°C) when the solvent removed under a gentle stream of nitrogen.

III.1.3. PREPARATION OF 2,2,7,8-TETRAMETHYLCHROMAN-5,6-DIONE (PR).

Formation of **PR** may occur by oxidation of **PH** as outlined in scheme **8** (p. 130), it may also formed from **PQ** (scheme **9**, p. 140) and from **PM-OEt**.

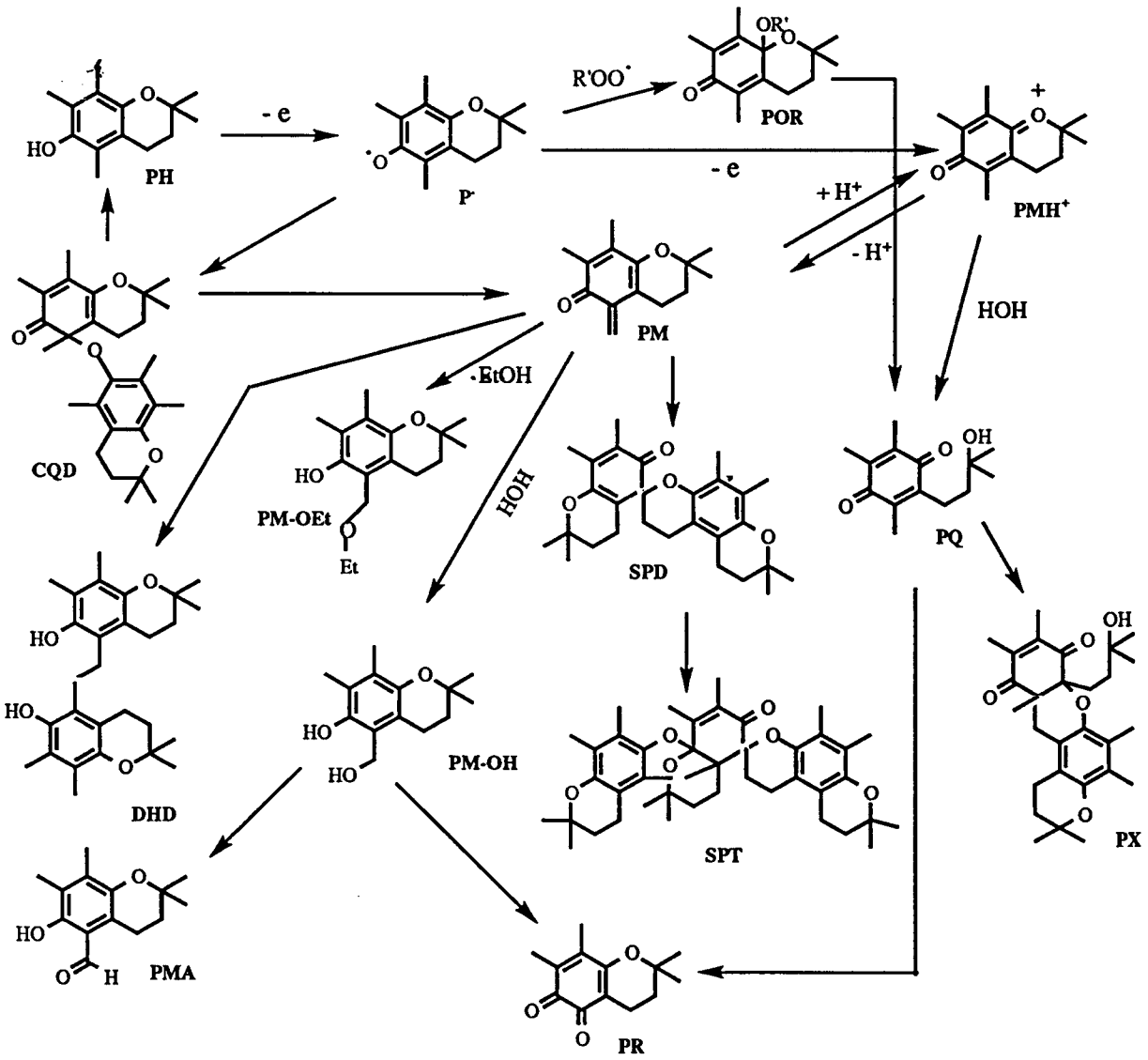
III.1.4. PREPARATION OF 1,4-DIHYDROXY-2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYLBENZENE (PQH2).

It was very difficult to obtain pure $\mathbf{PQH_2}$ and also its spectra as this compound was very unstable. The IR spectrum could be done only on freshly prepared $\mathbf{PQH_2}$. The KBr disk was prepared under nitrogen and the spectrum run immediately. The colourless crystals of freshly made $\mathbf{PQH_2}$ need only 3 h in the refrigerator under nitrogen to acquire a pinkish colour. The $\mathbf{PQH_2}$ turned yellow as soon as it was dissolved in deuterated acetone and a satisfactory ^{1}H NMR spectrum could not be obtained.

III.2. OXIDATION EXPERIMENTS

III.2.1. OXIDATION OF 2,2,5,7,8-PENTAMETHYL-6-CHROMANOL IN CHLOROFORM WITH *TERT*-BUTYLHYDROPEROXIDE.

The first product of oxidation of **PH** is the free radical (**P•**, scheme 7), which may react with peroxyl radicals to form 8a-peroxy-2,2,5,7,8-pentamethyl-6-chromanone radical (**POR**, scheme 7). Two molecules of **P•** are believed to dimerise to chromane quinone dimer (**CQD**, scheme 7) which may disproportionate into one molecule of the quinone methide (**PM**) and one molecule of **PH** (scheme 7).

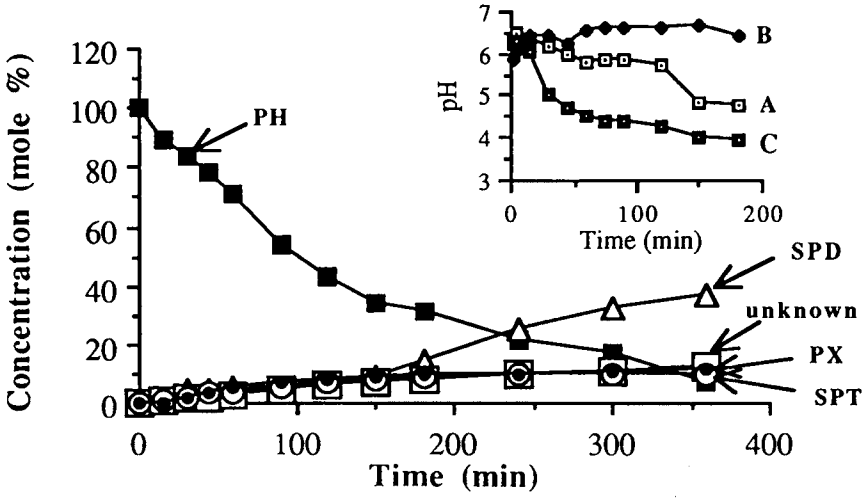


Scheme 7.

The quinone methide is unstable and may dimerise into **SPD** , trimerise into the **SPT**, (scheme 7) or react further to form **PM-OEt, PMA, PM-OH** and **PR** (scheme 7). The presence of a protic material such as an alcohol or water may induce an equilibrium between the quinone methide and its protonated form, the phenoxylium ion (**PMH$^+$**, scheme 7). The more acidic is the protic material, the more the equilibrium is shifted toward the phenoxylium ion. Water reacts rapidly with the phenoxylium ion to form **PQ** (John et al., 1939) and subsequently 1H-2,3-dihydro-3,3,5,6,9,10,11a(R)heptamethyl-7a(S)-3-hydroxy-3-methylbutyl)-pyrano-[2,3-a]xanthe-ne-8(7aH),11(11aH)dione (**PX**, scheme 7) (Suarna, et al., 1988). The phenoxylium ion may also react with alcohols (Goodhue and Risley, 1965) to form 8a-alkoxy-2,2,5,7,8-pentamethyl-6-chromanones. Thus depending on the presence or absence of a protic material, the reaction pathway will proceed through the neutral quinone methide or its cationic form.

Reaction of **PH** with *t*-butyl hydroperoxide in chloroform saturated with water yielded **SPD, SPT** and **PX** as the major products and also **PQ, PR, PMA** and the dihydroxy dimer (**DHD**) as the minor products (Suarna, 1992). When the pH was observed in a duplicate reaction (Fig.3, insert, line **A**, p. 125) it fell very slowly from a starting value of 6.2 to a value of 4.9 after 180 min.

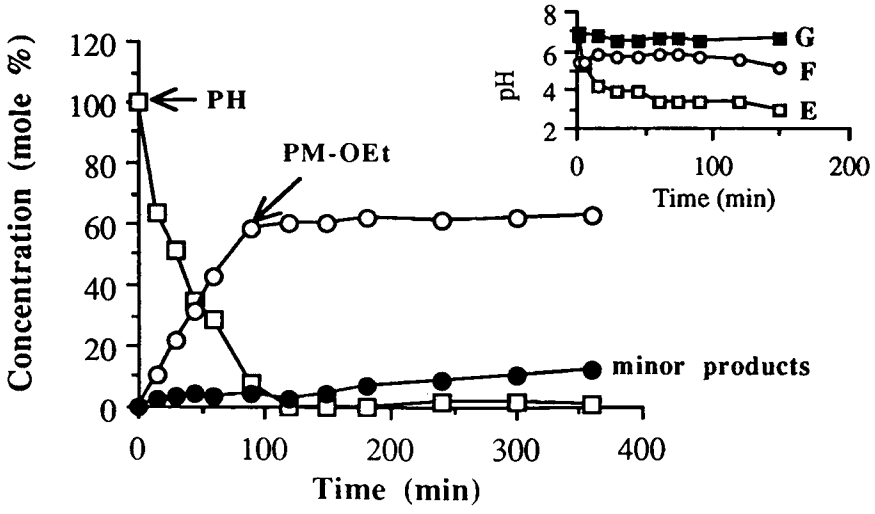
Fig. 3. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and **PH** in chloroform saturated with water at 60°C (data for graph of products printed by kind permission of Dr. C. Suarna).



However when **PH** by itself was heated in chloroform saturated with water the pH remained constant (Fig.3, insert, line **B**). When *t*-butyl hydroperoxide was heated in chloroform saturated with water, the pH fell more rapidly than as in the reaction with **PH** (Fig.3, insert, line **C**, p. 125). The reason that the pH of the decomposing *t*-butyl hydroperoxide is less than that of the reaction between **PH** and *t*-butyl hydroperoxide is unknown. This reaction also shows that when only a small amount of water is present in a heterogenous system, the reaction proceeds slowly and predominantly via the quinone methide since the majority of products, eg **SPD, SPT, PR, PA, DHD** and the greatest molar percentage is derived from the quinone methide. In the absence of acidity (pH drops only from 6.2 to 4.9 in a 3h period), the quinone methide <-> phenoxylium ion equilibrium lies towards the quinone methide which may then dimerise to **SPD** and trimerise to **SPT**. The dimerisation and trimerisation of the quinone methide dominate over its reaction with water because molecules of the quinone methide are more likely to encounter each other in the chloroform phase than to encounter water in the heterogenous water phase.

Reaction in purified chloroform plus 20% ethanol gave **PM-OEt** as the major product, and **PMA, PQ** and **PR** as the minor products (Suarna & Southwell-Keely, 1989).

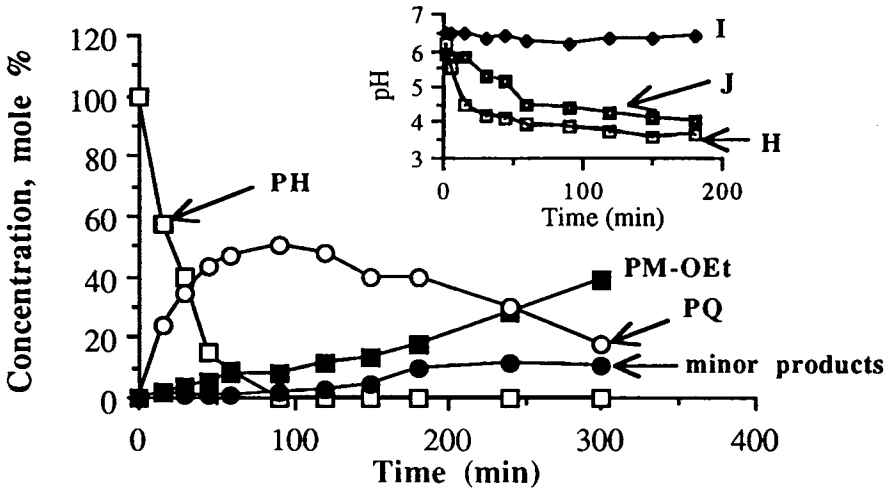
Fig. 4. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and **PH** in chloroform containing 20% ethanol at 60°C (data for graph of products printed by kind permission of Dr. C. Suarna).



The pH showed a sharp decrease from 6.71 to 2.95 in 180 min. (Fig **4**, line **E**), while the pH of *t*-butyl hydroperoxide when heated in chloroform plus 20% ethanol slightly decreased (Fig. **4**, line **F**, p. 126), the pH of the solvent itself (chloroform containing 20% ethanol) when heated at 60°C remained constant. In the presence of acid the quinone methide <-> phenoxylium ion equilibrium should shift towards the phenoxylium ion, but because the proportion of ethanol was much greater than water, the quinone methide reacted with ethanol first to form **PM-OEt**.

In the presence of water and ethanol in homogeneous solution (chloroform plus 18.5% ethanol and 1.5% water), the major products were **PQ**, **PM-OEt** and **PX** (Suarna and Southwell-Keely, 1992), there was a rapid pH decrease during the course of reaction, 6.42 to 2.84 in 150 min (Fig.**5**, insert, line **H**) and the pH of *t*-butyl hydroperoxide also gradually decreased (Fig.**5**, insert, line **J**), while when **PH** by itself was heated in chloroform containing 18.5% ethanol and 1.5% water at 60°C, the pH remained constant (Fig. **5**, insert, line **I**). In this reaction the solvent was completely homogenous and contained two competing nucleophiles - ethanol and water.

Fig. 5. Formation of oxidation products in the reaction between *t*-butyl hydroperoxide and **PH** in chloroform containing 18.5% ethanol and 1.5% water at 60°C (data for graph of products printed by kind permission of Dr. C. Suarna).



This oxidation was much faster than that of oxidation in chloroform saturated with water and the most rapidly formed product was **PQ** which rose to a peak and then declined (Suarna and Southwell-Keely, 1992). **PM-OEt** was formed much more slowly than **PQ**

but continued to increase throughout the reaction and was the major product after 5 h (Suarna and Southwell-Keely, 1992). When water was present in a homogeneous system, the reaction proceeded predominantly via the phenoxylium ion to form **PQ** very rapidly. **PM-OEt** representing the quinone methide pathway, is formed much more slowly. The fact that **PM-OEt** continues to build up after **PH** has disappeared suggests that it is predominantly a secondary rather than a primary product of this reaction. The fall in the concentration of **PQ** is matched by a corresponding rise in the concentration of **PM-OEt** suggesting that **PM-OEt** is formed from **PQ**. The progressive fall of pH (Fig.5, line **H**) suggests that the increase in acidity shifts the **PM** <--> **PMH**$^+$ equilibrium towards the **PMH**$^+$ which reacts with water to form **PQ**. The pH continues to drop until it has reached 3.8 which is sufficiently low to promote the recyclisation of **PQ** (a tertiary alcohol) into the **PM**. The **PM** can then react with ethanol to form **PM-OEt**.

As a consequence of the above reactions one might predict that, if **TH** is oxidised beyond the level of the tocopheryl radical in aqueous membrane systems, such as occur in all living organisms, the major oxidation product is likely to be the quinone (**TQ**) rather than one of the many products of the quinone methide (**PM-OEt**, scheme 7). There have been many reports of the isolation of **TQ** from tissues of animals (Morton and Philips, 1959; Csallany et al., 1962; Gallo-Torres et al., 1971; Hughes et al., 1980; Bieri et al., 1981; Vatassery, 1987; Murphy et al., 1989). **TQ** has been by far the most widely reported metabolite of **TH** although there have been a few instances of the isolation of the spirodimer and the spirotrimer (Draper et al., 1967).

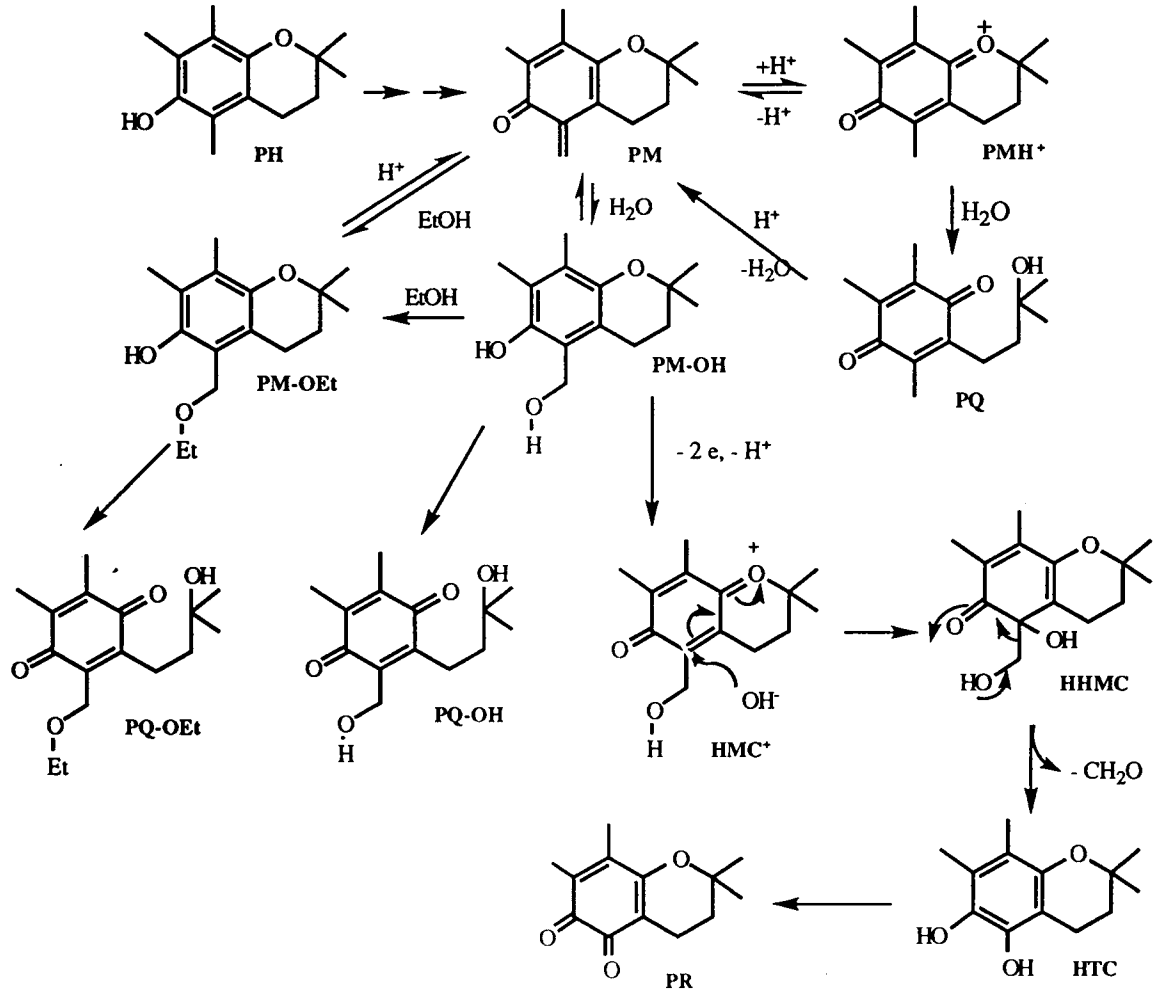
III.2.2. OXIDATION WITH SILVER NITRATE AND REACTIONS IN ACIDS.

There has been much discussion in the literature as to whether α- or γ-tocopherol is the better antioxidant. Much of the early work suggested that γ-tocopherol was a better antioxidant than α-tocopherol (Olcott & Emerson, 1937, Hove & Hove, 1944, Lea & Ward, 1959, Skinner & Parkhurst, 1970) whereas more recent work has

suggested the reverse (Kunkel, 1950, Grams & Eskins, 1972, Burton & Ingold, 1981, Niki et al., 1986). It has been proposed that antioxidant activity may be best assessed in an initiated autoxidation system and that, in such a system, α-tocopherol is better than γ-tocopherol (Burton & Ingold, 1986). Using an uninitiated autoxidation system Suarna and Southwell-Keely (1991) found that 2,2,7,8-tetramethyl-6-chromanol (**TMC**), the model compound of γ-tocopherol, was a far superior antioxidant to **PH**, the model compound of a-tocopherol. Thus their results agree with those in the early literature. They also suggested that the superior performance of **TMC** over **PH** in the uninitiated system may be due to oxidation products of **TMC** rather than **TMC** itself (Suarna and Southwell-Keely, 1991). Therefore, to shed somelight on this hypothesis, **TMC** was treated with silver nitrate under the same condition as **PH** (see p.144). Thus 2,2,7,8-tetramethylchroman-5,6-dione (**PR**), which is a major oxidation product of **TMC**, has antioxidant activity and may extend the activity of **TMC** whereas 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**PQ**), which is the major oxidation product of **PH**, has no antioxidant activity in bulk phase (Suarna and Southwell-Keely, 1991). However, Lindsey et al. (1985) observed that in nonbiological system **TH** and γ-tocopherol (γ-T) had similar capacities in inhibiting the peroxidation of arachidonic acid and that **TQ** also functioned as an antioxidant but γ-tocopherylquinone showed a crossover effect by functioning as an antioxidant at low concentration and a prooxidant at high concentration. While in biological system, **TH**, γ-T and **TQ** had similar antioxidant capacities in inhibiting the lipid peroxidation in smooth muscle cells, but γ-tocopherylquinone was highly cytotoxic for cells. **PR** in addition to being a major oxidation product of **TMC**, can also be formed by overoxidation of **PH** (John et al., 1939).

Oxidation of PH with $AgNO_3$ in absolute ethanol at 60°C. Treatment of **PH** with silver nitrate at 60°C in absolute ethanol for 2 h gave **PR** (40%) and **PM-OEt** (30%) as the major products (Suarna et al., 1991a). Formation of both these compounds is believed to occur through an intermediate quinone methide (**PM**) (Scheme 8, p. 130). Addition of

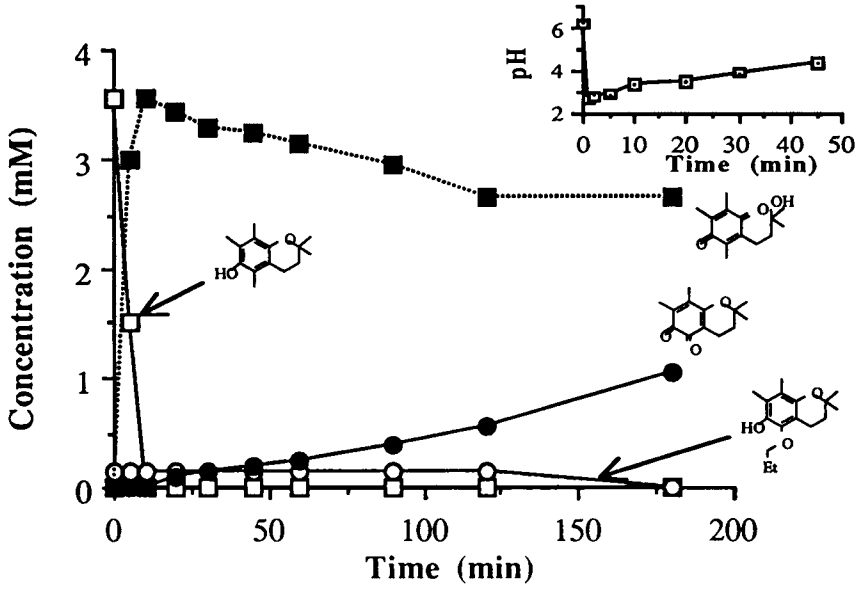
ethanol to **PM** would form **PM-OEt** while addition of water to **PM** followed successively by oxidation, further addition of water, deformylation and further oxidation would form **PR** (Suarna et al., 1991a). Although this reaction was performed in absolute ethanol, there was sufficient water (0.5%) in the solvent to account for formation of compound **PR** by this pathway. Nonetheless, there was an overwhelming excess of ethanol in the reaction, and this led to the thought that compound **PM-OEt** might also be an intermediate in the formation of **PR**. The earliest report of **PR**, which did not characterise its structure, indicated that it could be formed from **PH** and also from **PQ** by silver nitrate oxidation (John et al., 1939). Although **PQ** was not a significant product in the silver nitrate oxidation of **PH** (Suarna et al., 1991a), it was decided to follow the rate of oxidation of compounds **PM-OEt**, 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**PM-OH**) and **PQ** in turn with silver nitrate to determine the most probable pathway to **PR**.



Scheme 8. Formation of **PR** from **PH**.

Fig.6 (p. 131) shows that **PH** was oxidised completely to **PQ** in 10 min after which the concentration of **PQ** fell gradually but it still was the major product (70 mole % after 180 min). **PR** did not appear until 10 min and then rose gradually to 28% at 180 min. **PM-OEt** rose rapidly to 5% within 5 min and then fell very slowly to zero at 180 min.

FIG. 6. HPLC analysis of the oxidation of 2,2,5,7,8-pentamethyl-6-chromanol (**PH**) with silver nitrate in absolute ethanol at 60°C.

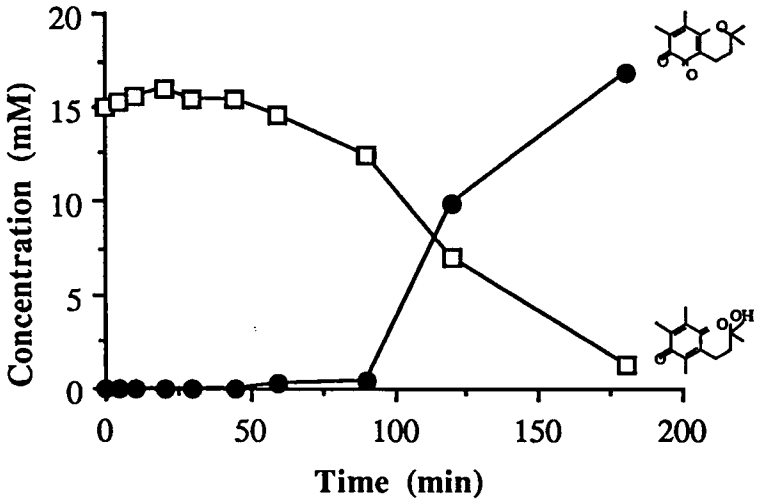


The product distribution in this silver nitrate oxidation of **PH** differed a great deal from that observed previously (Suarna et al., 1991a). The major product by far in the present reaction was **PQ** which was only observed in very small amounts by Suarna and co-workers (Suarna et al., 1991a). By contrast **PM-OEt** which had been one of the major products in the previous reaction (Suarna et al., 1991a) was only a minor product in the present reaction. The reason for this product reversal may be the ratio of silver nitrate to **PH** which was 3.5:1 in the previous reaction by Suarna and co-workers (Suarna et al., 1991a) and was 10:1 in this reaction. Also the amount of water in the reactions was different, the molar ratio of water/**PH** in the present reaction (61:1) was much greater than in the previous study (3:1). It has been shown that a relatively low concentration of water in the presence of a large excess of ethanol favours the formation of **PQ** rather than **PM-OEt** (Suarna et al., 1992). Although this reaction was performed

in absolute ethanol, there was sufficient water (0.5%) in the solvent to account for formation of **PR** (scheme 8).

Oxidation of PQ with $AgNO_3$ in absolute ethanol at 78°C. The fact that **PR** did not appear until **PH** had disappeared completely suggests that **PR** was a secondary product. Also **PR** began to form after **PQ** had reached a maximum and was beginning to decline. This suggests that **PR** was being formed from **PQ**. In order to test this theory, **PQ** was heated at 60°C and, subsequently, at 78°C in ethanol in the presence of silver nitrate, but was found to be stable and unchanged after several hours. The original report of the formation of **PR** indicated that it could be formed from **PQ** as well as from **PH** (John et al., 1939). The lack of reactivity of **PQ** in the present reaction was in conflict with the original report until it was realized that the original authors had used a much more concentrated solution of **PQ** (13 mg/ml) than that had been used in this reaction (1 mg/ml). On changing to a more concentrated solution (5 mg/ml) and heating at 78°C, the results shown in Fig.7 were observed. The reaction showed a lag period of almost one hour before any change occurred, after which **PQ** was converted fairly rapidly into **PR**. The reason for this lag period is unknown.

FIG.7. HPLC analysis of oxidation of **PQ** with silver nitrate in boiling absolute ethanol.



As **PQ** at a concentration of 1 mg/ml was stable when heated directly with silver nitrate at 60°C, but decomposed steadily at 60°C after it was formed in the **PH** oxidation (Fig. 6),

it appeared that another product of **PH** oxidation might be promoting the decomposition of **PQ**. A study of pH change during **PH** oxidation revealed an initial, rapid drop to pH 2.7, followed by a much slower rise (insert Fig. **6**, p. 131).

A pH of 2.7 corresponds closely to that which would have been obtained if **PH** had dissociated completely. However, as **PH** is a very weak acid, these results suggest that silver nitrate reacts with **PH** to liberate a proton as follows:

$$Ag^+ + ArOH \text{ ----------> } Ag + ArOH^{+\bullet} \text{ ----------> } ArO^\bullet + H^+ \qquad [1]$$

This pH drop was definitely due to reaction between silver nitrate and **PH**, because a solution of silver nitrate in ethanol had a pH of 6.5.

*Reaction of **PQ** in absolute ethanol containing 0.03M H_2SO_4 at 60°C.* In order to determine the effect of acid on the course of oxidation of **PH** with silver nitrate, **PQ** was heated at 60°C under nitrogen in the presence of 0.03M sulfuric acid and in the absence of silver nitrate.

FIG. 8. HPLC analysis of the reaction of **PQ** in absolute ethanol containing 0.03M sulfuric acid at 60°C.

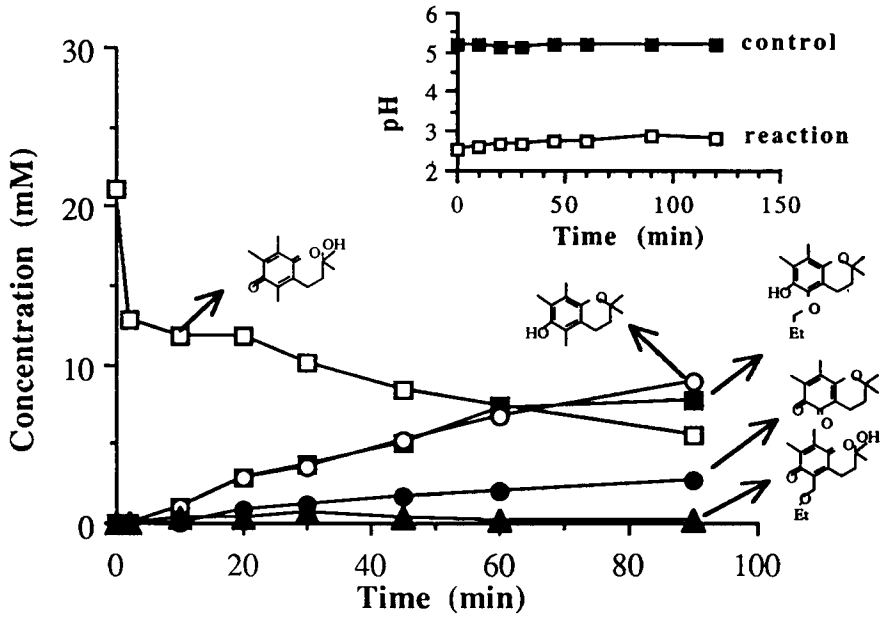


Fig. **8** shows that the concentration of **PQ** fell by 40% within 2 min and then by a further 30% in the ensuing 88 min. Compound **PQ** was converted into **PM-OEt**, **PH**, 2-(3-

hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (**PQ-OEt**) and **PR**.

Reaction of **PQ** with 0.001M sulfuric acid in absolute ethanol was also performed.

FIG. 9. HPLC analysis of the reaction of **PQ** with 0.001M sulfuric acid in absolute ethanol at 60°C.

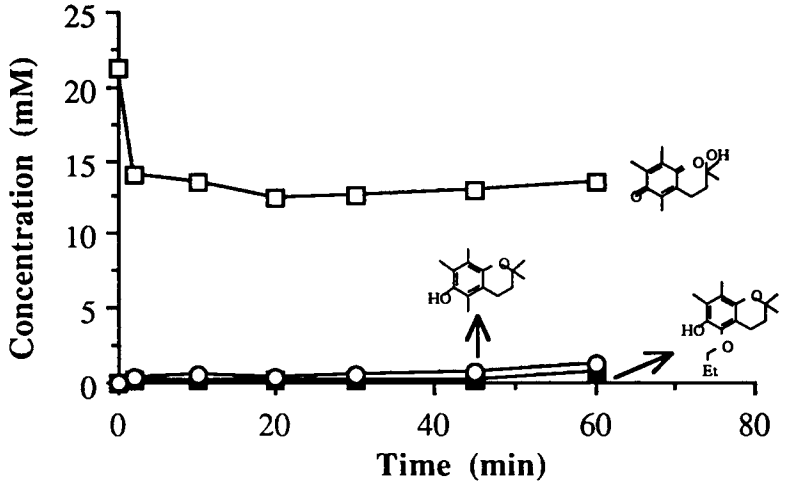
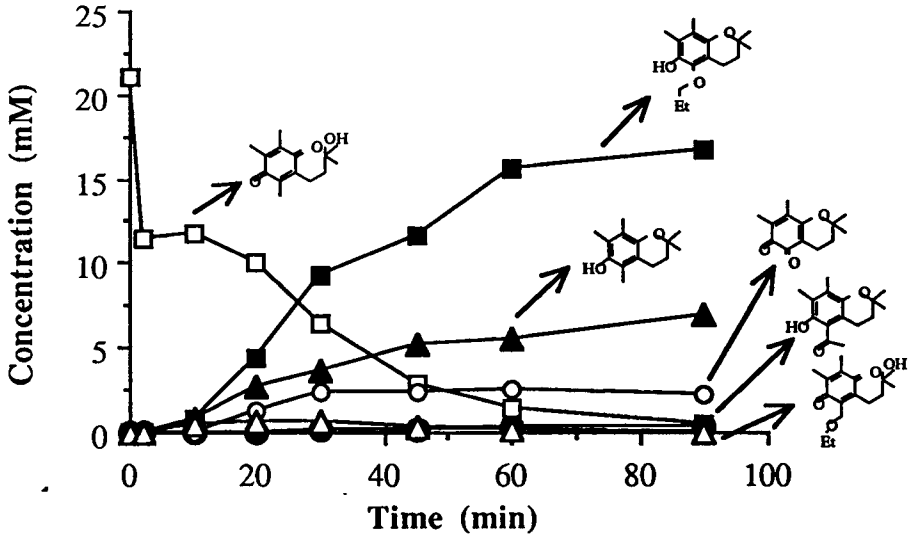


Fig. **9** shows that the concentration of **PQ** also fell by 40% within 2 min and then remained practically constant in the ensuing 88 min. There was no **PR** and only a small amount of **PH** and **PM-OEt** formed in this reaction.

Reaction of PQ in absolute ethanol containing 0.03M HCl. When the reaction of **PQ** with 0.03M sulfuric acid was repeated using 0.03 M hydrochloric instead of sulfuric acid, the rate was faster and the same products were formed (Fig. **10**, p. 135).

The reaction of **PQ** in 0.03M HCl was much faster than that in 0.03M sulfuric acid. **PQ** declined steadily to 26% at 90 min in the reaction with 0.03 M sulfuric acid, while in hydrochloric acid it dropped to 2% at 90 min. The major products in both reactions were **PM-OEt** and **PH** which reached 37% and 42% respectively in the reaction with 0.03M sulfuric acid, while in the reaction with hydrochloric acid the amounts of **PM-OEt** and **PH** were 79% and 33% respectively.

FIG. 10. HPLC analysis of the reaction of **PQ** in absolute ethanol containing 0.03M HCl at 60°C.

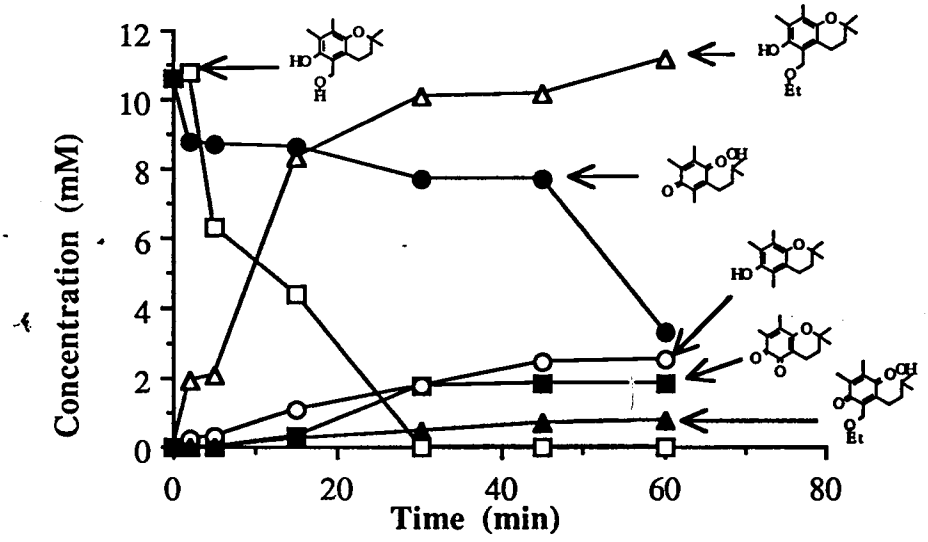


There was a big increase of **PM-OEt** in the hydrochloric acid reaction. **PR** formed in the 0.03 M sulfuric acid reaction was 13% at 90 min, while in hydrochloric acid it reached its maximum (10%) at 30 min. **PQ-OEt** reached its maximum (3%) at 30 min in the 0.03M sulfuric acid reaction and at 20 min in the hydrochloric acid reaction.

Reaction of PQ plus PM-OH in absolute ethanol containing 0.001M sulfuric acid. One may conclude that, in the presence of acid, **PQ** is readily dehydrated to **PM** which can then add ethanol to form **PM-OEt** (Scheme 8, p. 130). Alternatively, **PM** can add water to form **PM-OH** (Suarna et al., 1988)) which may react with ethanol in acid solution to form **PM-OEt**. Formation of **PR** and particularly **PH** in this reaction is more difficult to explain. **PR** is believed to be formed by the oxidative deformylation of **PM-OH** (Suarna et al., 1991a) (scheme 8). However, as the reaction of **PQ** with sulfuric acid was performed under nitrogen, the only oxidant present was **PQ** itself. A possible explanation is that **PQ** was dehydrated by acid to **PM** which then added water to form **PM-OH**. **PM-OH** is a phenol (reducing agent) which may be able to react with **PQ** (oxidizing agent) to form **PR** (oxidation product) and the hydroquinone of **PQ** (**PQH_2**; reduction product). In acid solution **PQH_2**

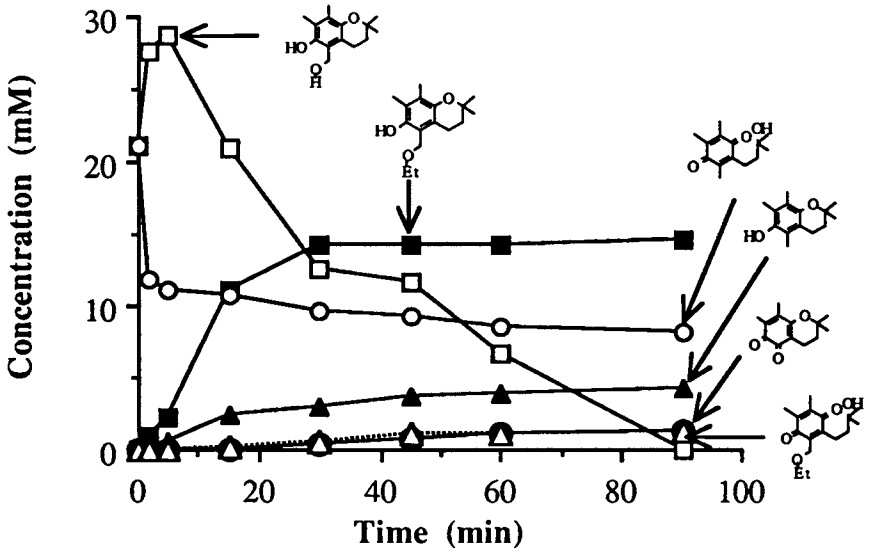
would cyclise to **PH** (John et al., 1939). To test this theory, **PQ** was reacted with **PM-OH** at 60°C in the presence of 0.001M sulfuric acid (to simulate the approximate pH of the **PH** oxidation)(Fig.6).

FIG. 11. Products of the reaction of **PQ** (10.5mg) and **PM-OH** (10.5 mg) in absolute ethanol containing 0.001M sulfuric acid at 60°C.



At these concentrations of **PQ** and **PM-OH** (10.5 mg of each starting material), **PM-OH** increased 2% at 2 min then decreased rapidly to nil at 30 min, while the **PQ** decreased progessively to 30% in the ensuing 60 min. However, in a more concentrated reaction (25 mg of **PQ** and **PM-OH**, Fig. 12, p. 137), the **PM-OH** showed a substantial increase (36 %) from 2 to 5 min, then rapidly decreased and disappeared at 90 min, while the **PQ** also decreased. **PR** starts forming at 15 min in the more dilute reaction and at 30 min in the more concentrated reaction, reaching similar concentration in both reactions. **PH** formed at about the same rate in both reactions and so did **PQ-OEt**. **PM-OEt** formed much faster in the dilute reaction than in the more concentrated reaction.

FIG. 12. Products of the reaction of **PQ** (25 mg) and **PM-OH** (25 mg) in absolute ethanol containing 0.001M sulfuric acid at 60°C.



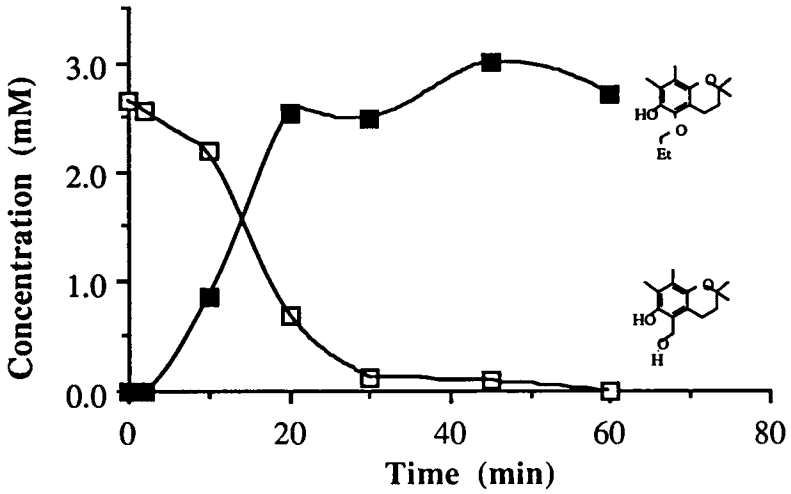
In the reaction between **PQ** and **PM-OH**, compound **PM-OEt** was the major product while **PR**, **PQ-OEt** and **PH** were formed in smaller amounts. Although, initially, there were equal amounts of **PQ** and **PM-OH** in the reaction mixture, it appears that the first step involves the effective isomerisation of **PQ** into more **PM-OH** and thus the concentration of **PM-OH** rises as that of **PQ** falls.

$$\mathbf{PQ} \xrightarrow{H^+,\ -H_2O,\ +H_2O} \mathbf{PM\text{-}OH} \qquad [2]$$

A number of reactions then follow. **PM-OH** reacts very readily with ethanol in the presence of acid to form **PM-OEt** (Eq. 3, Fig. **13**, p. 138) and the reaction does not require **PQ**.

$$\mathbf{PM\text{-}OH} + EtOH \longrightarrow \mathbf{PM\text{-}OEt} \qquad [3]$$

FIG. 13. Products of the reaction of **PM-OH** (10.5 mg) in absolute ethanol containing 0.001M sulfuric acid at 60°C.



PQ reacts with **PM-OH** in the presence of acid (but not in the absence) to form **PR** and **PH**. The conversion of **PM-OH** into **PR** is a 4 electron oxidation and therefore the production of one mole of **PR** would require 2 additional moles of **PQ** and lead to the formation of 2 moles of **PH** (eqn [4] and [5])

2 x **PQ** + **PM-OH** + H_2O ----------->2 x $\mathbf{PQH_2}$ + **PR** + HCHO [4]

2 x $\mathbf{PQH_2}$ ----------> 2 x **PH** + 2 H_2O [5]

In summary, equations [2], [4], [5] give

3 x **PQ** ----------> **PR** + 2 x **PH** + H_2O + HCHO [6]

It is possible to conceive of an additional pathway from **PQ** to **PR** which does not involve the intermediacy of **PM-OH** (scheme **9**, p. 140). Thus enolisation of **PQ** followed by the addition of water would yield 1,4-dihydroxy-2,3-dimethyl-5-(3-hydroxy-3-methylbutyl)-6-hydroxymethylbenzene, the hydroquinone of **PQ-OH** ($\mathbf{HQH_2}$)

PQ + H_2O ----------> $\mathbf{HQH_2}$ [7]

A second molecule of PQ could then oxidize $\mathbf{HQH_2}$ to **PQ-OH** and be itself reduced to $\mathbf{PQH_2}$

PQ + $\mathbf{HQH_2}$ ----------> **PQ-OH** + $\mathbf{PQH_2}$ [8]

Addition of water to **PQ-OH** would form 4,6-dihydroxy-2,3-dimethyl-5-(3-hydroxy-3-methylbutyl)-6-hydroxymethylcyclohexa-2,4-dienone (**HCD**, scheme **9**) which could deformylate to 1,2,4-trihydroxy-5,6-dimethyl-3-(3-hydroxy-3-methylbutyl)benzene (**THB**, scheme **9**, p. 140).

PQ-OH + H_2O ----------> **HCD** [9]

HCD ----------> **THB** + HCHO [10]

As discussed previously (Suarna et al.,1991a), the deformylation is a typical retro-aldol type of reaction whose driving force is the establishment of aromaticity in **THB**.

Cyclisation of **THB** would produce 2,2,7,8-tetramethyl-5,6-dihydroxychroman (**CC**) which would be oxidized by a third molecule of **PQ** to form **PR**.

THB ----------> **CC** + H_2O [11]

PQ + **CC** ----------> **PR** + PQH_2 [12]

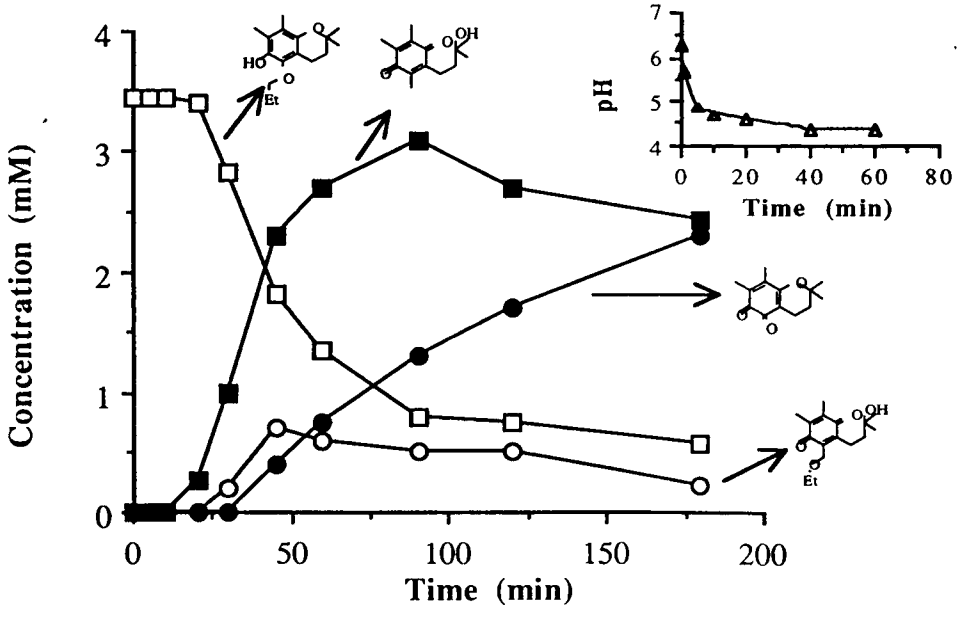
Cyclisation of PQH_2 in acid conditions would yield **PH**

2 x PQH_2 ----------> 2 x **PH** + $2H_2O$ [13]

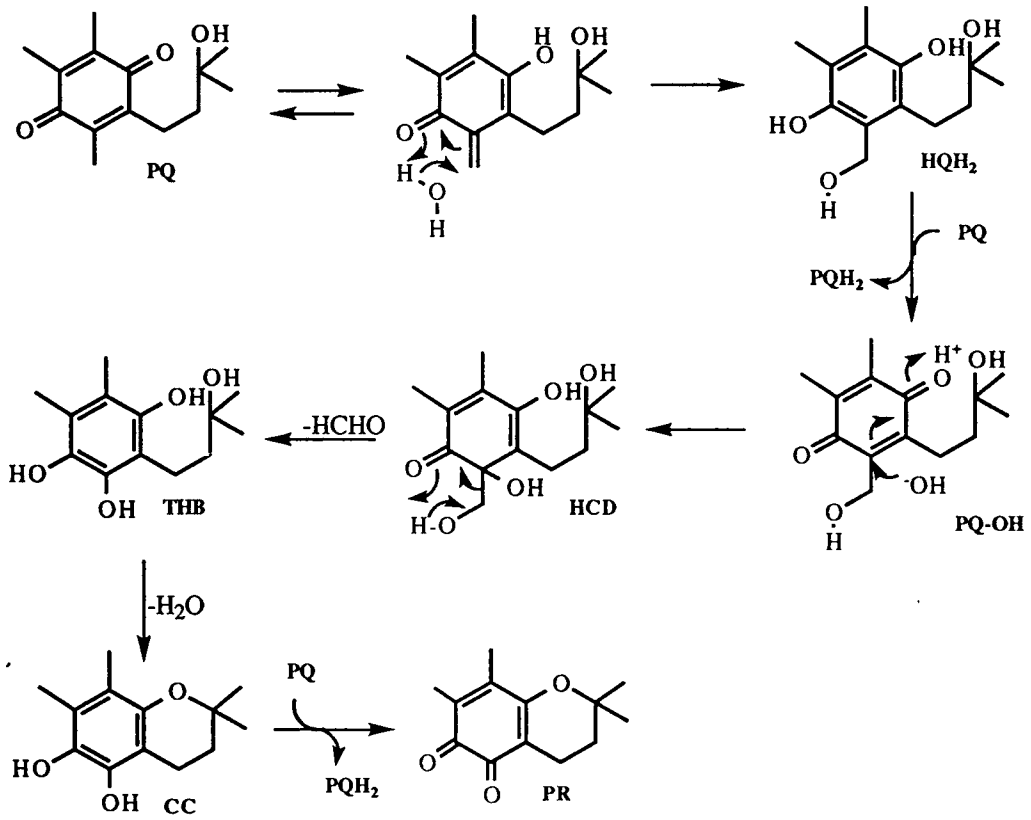
The sum of equations [7]-[13] is equation [6].

*Oxidation of **PM-OEt** with Ag NO_3 in absolute ethanol at 60°C.*

FIG.14. Products of oxidation of **PM-OEt** with silver nitrate in absolute ethanol at 60°C.



In order to determine whether **PM-OEt** could be converted into **PR**, it was oxidised with silver nitrate. Fig.**14** (p. 139) shows that oxidation of **PM-OEt** although relatively rapid, is much slower than **PH** and appears to go through a 20 min lag period before commencing. During the lag period the pH dropped from 6.2 to 4.2 (Fig.**14**, insert) so that acid appears to play a role in this reaction also. **PR** was a major product of the reaction (44 mole % in 180 min) but it did not appear until after 30 minutes and was, therefore, a secondary product. The first-formed product was again **PQ** which reached a maximum at 90 min and then began to decline. Thus it appeared that **PM-OEt** was being converted *via* the quinone methide (**PM**) into **PQ** and then into **PR** as before (see scheme **9**, p. 140). Although **PR** formed more slowly than **PQ**, it reached a higher concentration at 240 min and formed more rapidly than in the oxidation of **PH**.

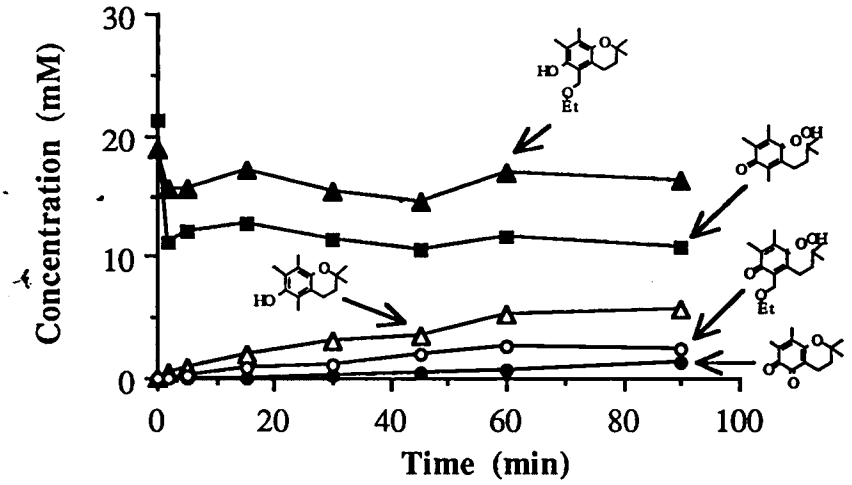


Scheme 9. Formation of **PR** from **PQ**.

*Reaction of **PQ** plus **PM-OEt** in absolute ethanol containing 0.001M H_2SO_4 at 60°C.* To check the interaction between **PQ** and **PM-OEt**, these two compounds were reacted in absolute ethanol containing 0.001M sulfuric acid in the same manner as the

reaction between **PQ** and **PM-OH**. The reaction was slower than the **PM-OH** reaction. The concentration of **PQ** dropped by 50% and that of **PM-OEt** by 25% within 2 min but then remained constant throughout the reaction. **PH**, **PM-OEt** and **PR** were formed to a much lesser extent than in the reaction of **PQ** and **PM-OH**.

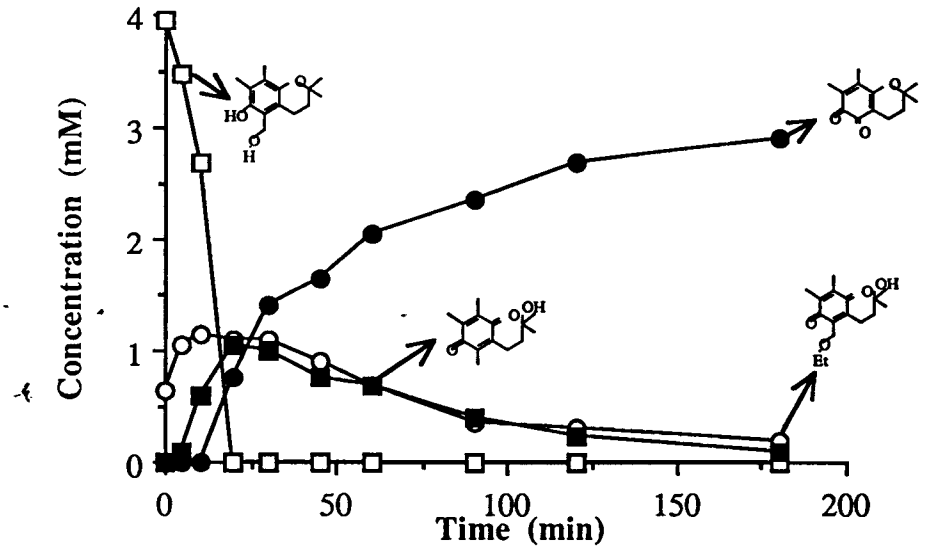
FIG. 15. HPLC analysis of the reaction of **PQ** and **PM-OEt** in absolute ethanol containing 0.001M sulfuric acid at 60°C.



Oxidation of PM-OH with $AgNO_3$ in absolute ethanol at 60°C. **PM-OH**, which was believed to be a key intermediate in the formation of **PR** from **PH** (Suarna et al., 1991a) was oxidised completely by silver nitrate within 20 min (Fig. **16**). Although **PR** was the major product of the reaction (90% after 180 min), and was formed faster in this reaction than in any other reaction, it was formed predominantly after **PM-OH** had disappeared. Thus it must be concluded that **PR** was, at least in part, a secondary product of this reaction as well. **PM-OEt** and **PQ-OH** were formed more rapidly than **PR** early in the reaction and subsequently declined. Thus, it would appear that **PM-OH** undergoes 3 separate reactions simultaneously, oxidative deformylation to **PR**, oxidation to **PQ-OH** and reaction with ethanol to form **PM-OEt**. Again it appeared that **PM-OEt** and **PQ-OH** were being converted into **PR**. A possible pathway from **PM-OEt** to **PR** would be protonation of the ethoxy oxygen of **PM-OEt** followed by loss of ethanol to form the quinone methide (**PM**). Addition of water to **PM** would form **PM-OH**, oxidative deformylation of which would yield **PR**. The pathway by which **PQ-OH** is converted

into **PR** is not clear but may involve acid-catalysed cyclisation to the enol of 5-formyl-2,2,7,8-tetramethyl-6-chromanol followed by oxidative loss of formic acid to form **PR**.

FIG.16. Products of oxidation of **PM-OH** with silver nitrate in absolute ethanol at 60°C.



Oxidation of **PH** leads progressively to the quinone methide (**PM**) and quinone methide cation (**PMH**$^+$). **PM** may add water to form **PM-OH** or ethanol to form **PM-OEt**. Further oxidation of **PM-OH** (scheme 8, p. 130) and **PM-OEt** form 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-3-hydroxymethyl-1,4-benzoquinone (**PQ-OH**) and 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-3-ethoxymethyl-1,4-benzoquinone (**PQ-OEt**) respectively. Oxidation of **PM-OH** may form the phenoxylium ion (**HMC**$^+$) which with the addition of OH^- (from water) could then form 2,2,7,8-tetramethyl-5,6-dihydroxychroman (**HHMC**). With the loss of formaldehyde **HHMC** is converted to 5-hydroxy-2,2,7,8-tetramethyl-6-chromanol (**HTC**). **HTC** contains the very labile catechol structure and would be oxidised rapidly to **PR**. **PMH**$^+$ may add water to form **PQ** which then by adding proton and losing water forms **PM** and eventually **PR** through the **PM-OH** (Suarna, et al., 1991a).

From the reactions above it can be concluded that **PR** can be formed from **PH**, **PM-OEt**, **PM-OH** and **PQ** and is formed most rapidly from **PM-OH** suggesting that **PM-OH** is the immediate precursor of **PR**. In all oxidations except that of **PQ**, **PR** was

a secondary product. Conversion of **PH** into **PR** may be explained as in scheme **8** (see p. 130).

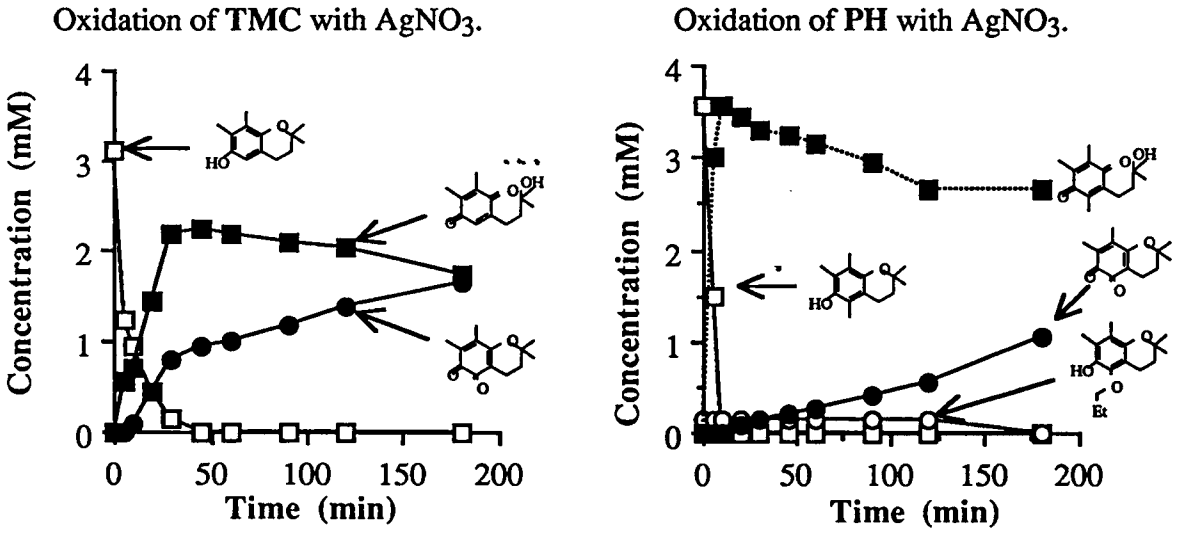
Reaction of silver ion with **PH** produces silver metal and the radical cation of **PH** (eqn 1). Dissociation of the radical cation liberates the chromanoxyl radical of **PH** and a proton which causes an immediate drop in pH (eqn. 1). Two molecules of the chromanoxyl radicals disproportionate to give one molecule of the unstable intermediate quinone methide (**PM**) and one molecule of **PH**. **PM** can react immediately with ethanol to form **PM-OEt**. Alternatively, **PM** may react with a proton to form the cation of quinone methide (PMH^+) which can then react with water to form **PQ** and regenerate a proton. **PQ** is unstable in the present of acid and undergoes dehydration and recyclization into **PM** which then in turn undergoes hydration to **PM-OH** and oxidative deformylation to **PR**. Although silver nitrate is the oxidant for the deformylation of **PM-OH**, it is interesting to note that **PQ** is also capable of bringing about the deformylation of **PM-OH** with the concomitant formation of **PH** in mild acid conditions. It is also possible that **PQ** may be converted into **PR** without the intermediacy of **PM-OH** as shown in scheme **9** (p. 140).

Acid also promotes the loss of ethanol from **PM-OEt** and its conversion into **PM**. Surprisingly, in this reaction **PM** does not proceed directly to **PM-OH** and **PR** but rather to **PQ**. Then as the acid increases further **PQ** is recyclised to **PM** and then on to **PM-OH** and **PR**. Thus the oxidation of **PH** is under the control of the equilibria **PM-OEt<->PM<->PMH+<->PQ** (Scheme 7, p. 124). In the absence of water the major product is **PM-OEt** (Suarna et al., 1992). In the presence of water, the equilibria shift to the right and a mixture of **PM-OEt** and **PQ** will be formed with **PQ** dominating as the concentration of water rises. Then as the acid increases, the equilibria shift to the left and to the key intermediate **PM** which undergoes hydration and further oxidation

It is worth noting that the spirodimer and spirotrimer of **PH** were not observed in any of the above reactions. This confirms previous observations that **PM** shows a much greater tendency to react with nucleophiles such as water and alcohols than it does to polymerise (Suarna et al., 1992).

Oxidation of TMC with $AgNO_3$ in absolute ethanol at 60 °C. **TMC** was consumed more slowly than **PH**, which supports the data of Burton and Ingold (1986) on their relative rates of reaction (**TMC** was oxidised completely after 30 min, **PH** after 10 min). The major products of **TMC** oxidation were 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (**TMCQ**) and 2,2,7,8-tetramethylchroman-5,6-dione (**PR**)(Fig. **17**). **TMCQ** was formed much faster then **PR** and reached a maximum at 30 min after which it slowly declined. Formation of **PR** only began after 10 min suggesting that it was a secondary product. However **PR** continued to increase throughout the reaction and after 180 min almost the same as **TMCQ**. The rate of formation of **PR** in this reaction was much faster than from **PH**. The fact that **PR** has antioxidant activity may explain the greater antioxidant activity of **TMC** than **PH** in the uninitiated autoxidation system.

FIG. 17. Products of the oxidation of **TMC** and **PH** with silver nitrate in absolute ethanol at 60°C.



Isolation of 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (**TMCQ**). The ^1H NMR spectrum of **TMCQ** was notably different from that of **PQ** in that the ring methyl groups of **PQ** appear as singlets while those of **TMCQ** appeared to be quartets. In order to confirm this observation NOE experiments were performed and appear in Fig. **18**, p. 146.

(1) The normal spectrum.

(2) Irradiation of the methyl signal at 2.01 ppm changed the methyl signal at 2.02 ppm from quartet into a singlet which proved there was a 5 bond coupling between the methyl groups at C_5 and C_6.

(3) Irradiation of the 2'-methylene signal at 1.63 ppm simplified the 1'-methylene signal at 2.51 ppm.

(4) Irradiation of the 1'-methylene signal at 2.51 ppm changed the olefinic proton signal at 6.52 ppm from triplet to singlet and simplified the 2'-methylene signal at 1.63. This demonstrates the 4 bond coupling between the olefinic and the 1'-methylene protons.

(5) Irradiation of olefinic proton signal at 6.52 ppm made the 1'-methylene signal at 2.51 simpler.

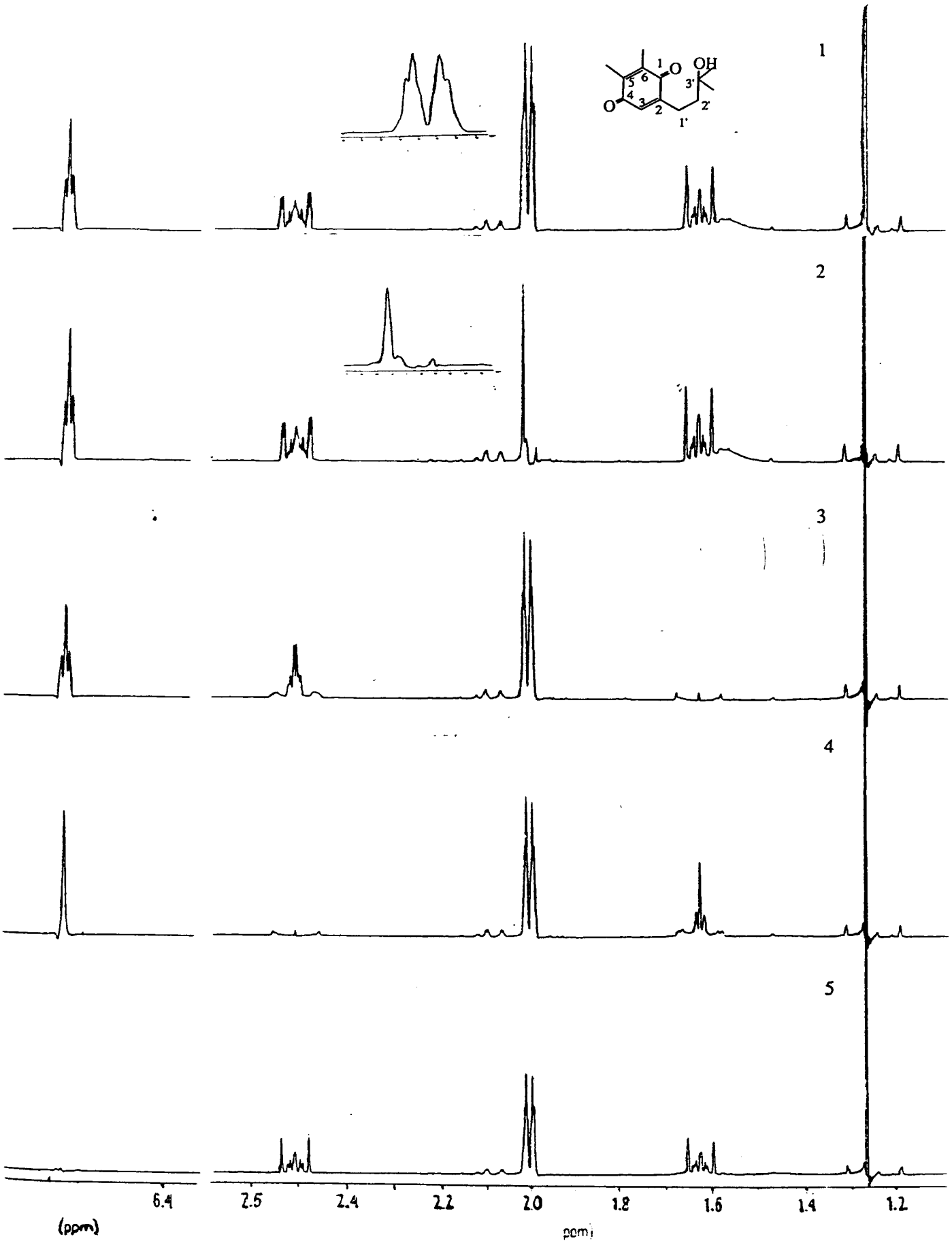


Fig. 18. NOE spectra of 2-(3-hydroxy-3-methylbutyl)-5,6-dimethyl-1,4-benzoquinone (TMCQ).

III.3. REDUCTION EXPERIMENTS.

REDUCTION OF 2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ) AND α-TOCOHEYRLQUINONE (TQ) BY VARIOUS REDUCTANTS

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.

Reduction of PQ by various reductants.

III.3.1. Experiments using UV spectrophorometer.

The UV measurement of the reactions between **PQ** and ascorbic acid (1-, 2- and 4-fold molar ratio), sodium ascorbate (1- and 2-fold molar ratio), glutathione (2- and 10-fold molar ratio) and NADH (1- and 10-fold molar ratio) in buffer phosphate pH 7/methanol (1:1) did not show a visible reduction of **PQ** into PQH_2. The reactions with sodium dithionite (10- and 25- fold molar ratio to **PQ**) showed a visible reduction of **PQ** to PQH_2 starting at 5 min and slowly decreasing at 20 to 60 min (Fig. **19**, p.148), while sodium dithionite 1.5-fold (by molar ratio to **PQ**) did not show any PQH_2 peak at all. The reactions with dithiothreitol (5-fold molar ratio to **PQ**) showed a rapid reduction of **PQ** to PQH_2 at 1 min and was quite stable until 60 min (Fig. **20**, p.149).

It is very difficult to quantitate the reaction products by UV measurement because at the time **PQ** and PQH_2 both were presence in the solution, the peaks overlapped, thus the quantitation was performed by HPLC.

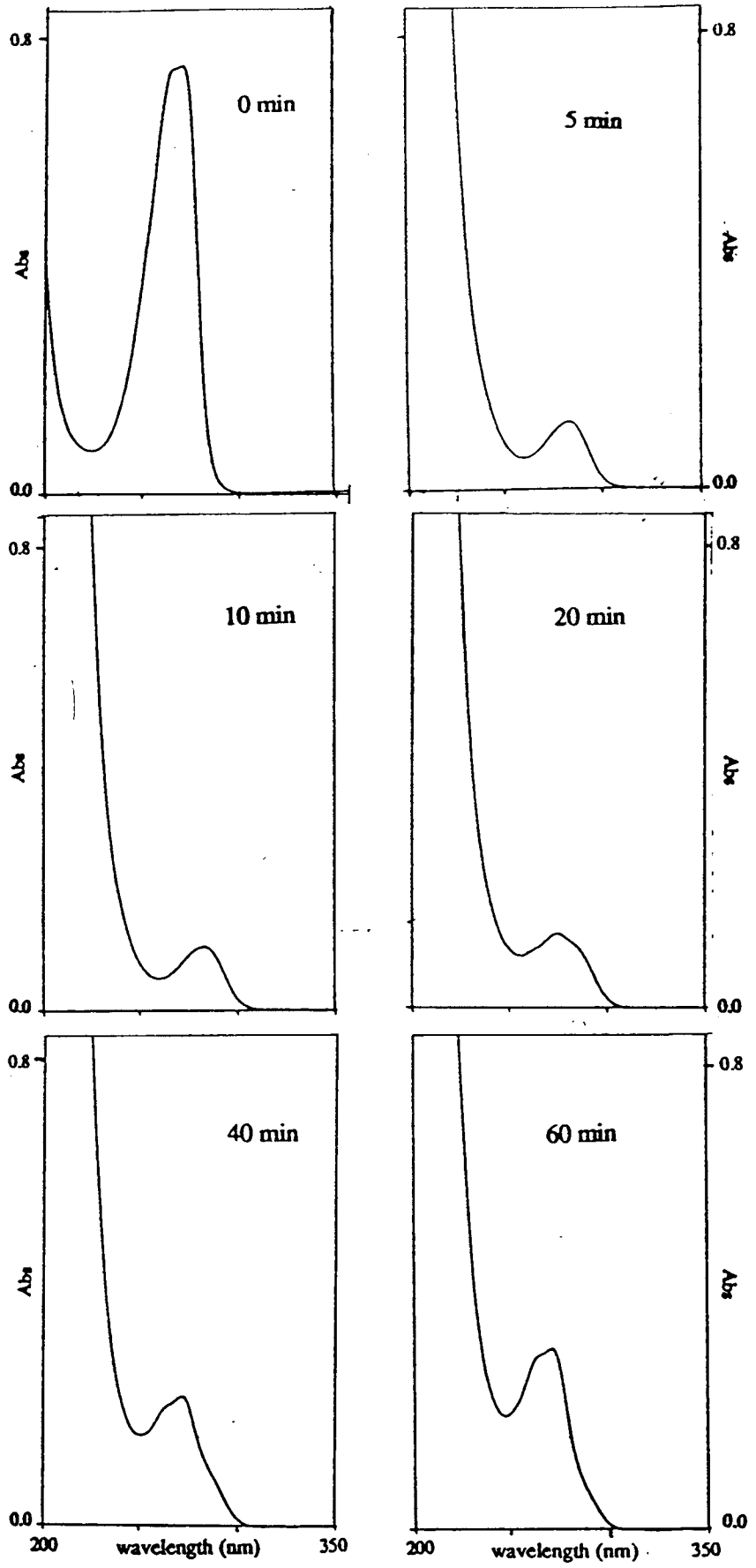


Fig. 19. Reduction PQ (10 mM) by sodium dithionite (250 mM). UV spectra are shown at various times after the beginning of the reaction.

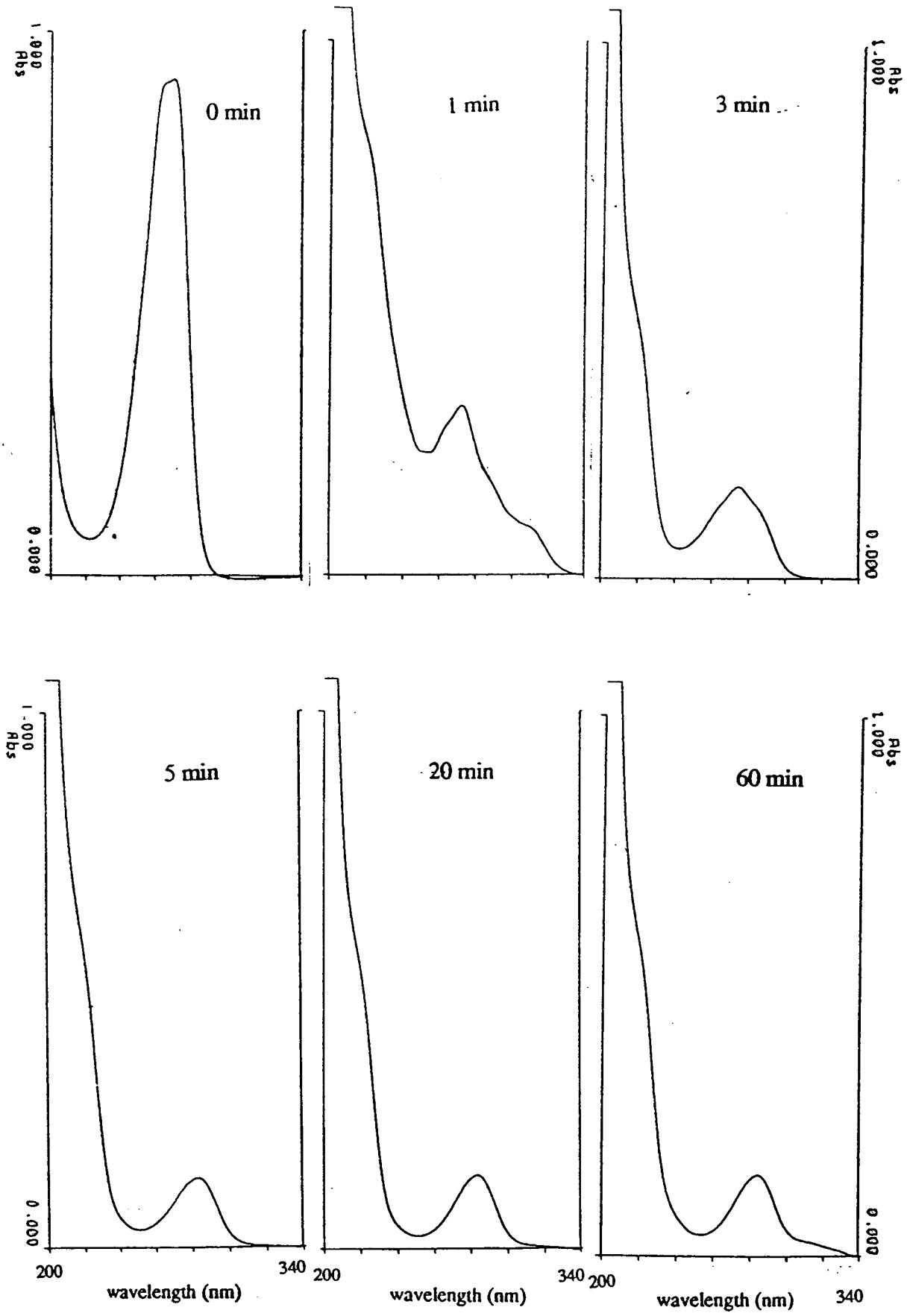
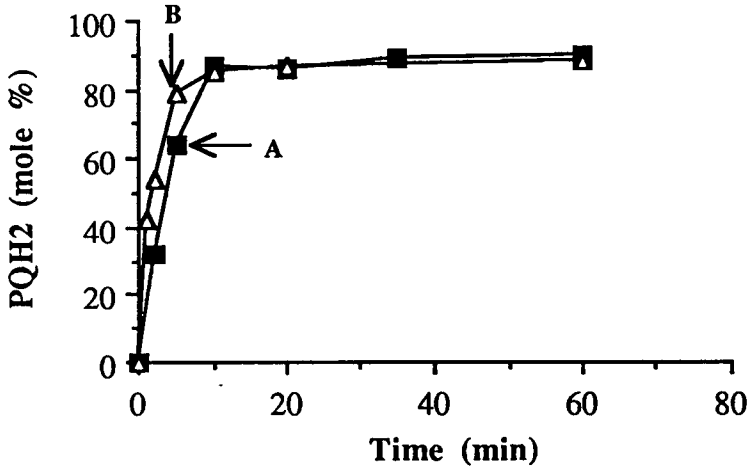


Fig. 20. Reduction of **PQ** (10 mM) by dithiothreitol (50 mM). UV spectra are shown at various times after the beginning of the reaction.

III.3.2. Reduction of PQ in air, analysed by NPLC.

III.3.2.1. Reduction of PQ (10 mM) by dithiothreitol (50 and 200 mM).

Fig. 21. 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 (see p. 154).

III.3.2.2. Reduction of PQ (10 mM) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).

Fig. 22. HPLC analysis of the reduction of **PQ** (10 mM) by sodium dithionite (200 mM) and by sodium ascorbate (200 mM).

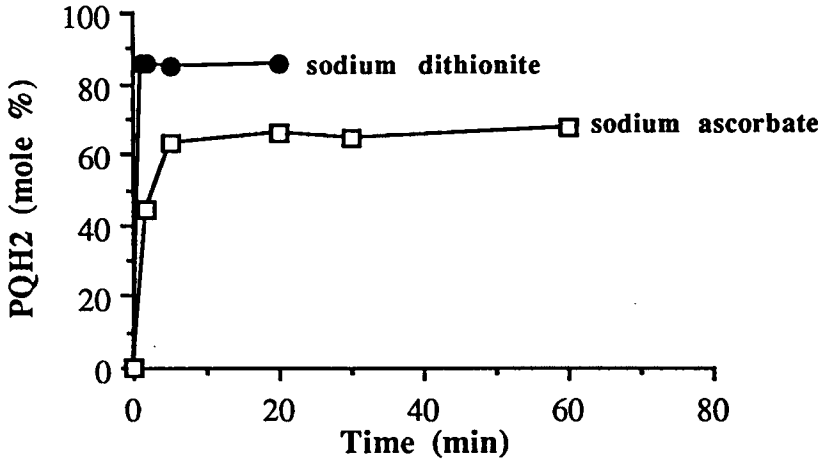


Fig. 22 shows that like **DTT**, sodium dithionite and sodium ascorbate were still capable to reduce **PQ** into PQH_2 in air and sodium dithionite reduced faster than sodium ascorbate (see p. 154).

III.3.2.3. Reduction of PQ (7 mM) by various concentration of NADH plus FAD.

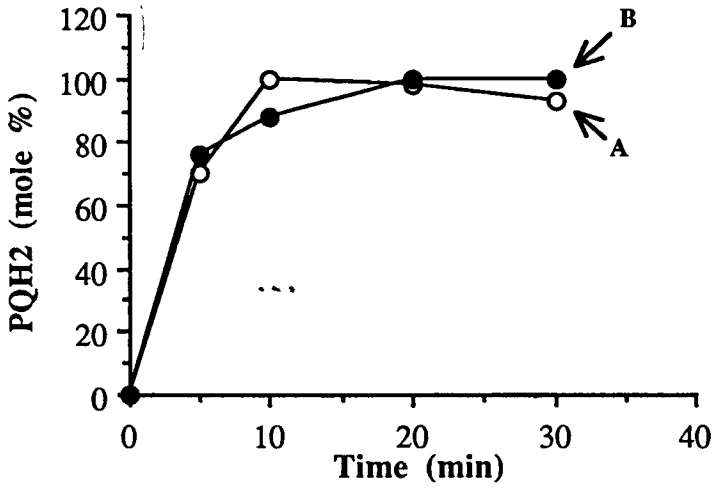
Reduction of **PQ** (7 mM) by:

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 are presented in Fig. 23.

Fig. 23. 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)) (see p. 154).

III.3.3. Reduction of PQ performed under a gentle stream of nitrogen.

III.3.3.1. Reduction of PQ (10 mM) by dithiothreitol (20 and 100 mM).

Fig. 24. Formation of PQH_2 (mole %) from the reduction of **PQ** (10 mM) by dithiothreitol (**A** = 20 mM and **B** = 100 mM).

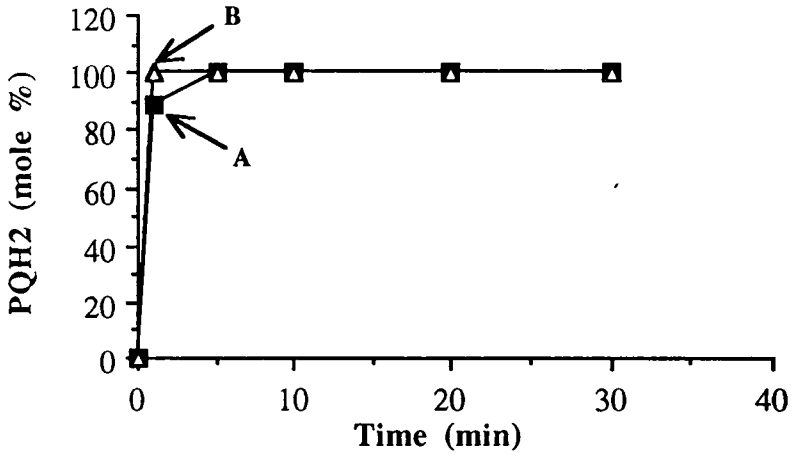


Fig. **24** shows that under N_2, even with lower concentrations (2 mM and 40 mM), **DTT** reduced **PQ** to PQH_2 faster than in the reactions in air (see p. 155).

III.3.3.2. Reduction of PQ (10 mM) by sodium dithionite (20 and 100 mM).

Fig. 25. Formation of PQH_2 (mole %) from the reduction of **PQ** (20 mM) by sodium dithionite (**A** = 20 mM and **B** = 100 mM).

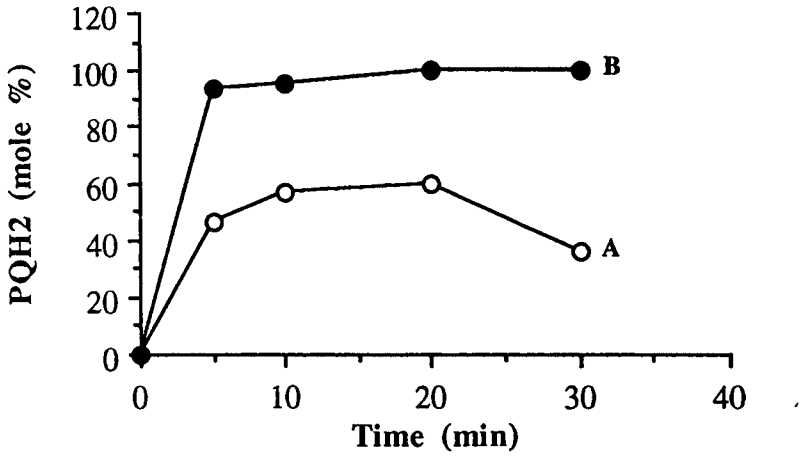


Fig. **25** shows the rate of reduction of sodium dithionite in different concentration (see p. 155).

III.3.3.3. Reduction of PQ (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

Fig. 26. Formation of PQH_2 (mole %) from the reduction of **PQ** (10 mM) by sodium ascorbate (100 mM) and by ascorbic acid (100 mM).

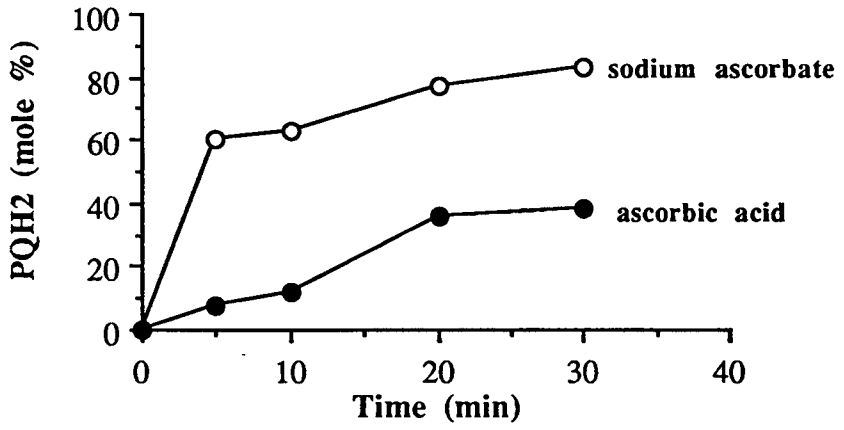


Fig. **26** shows in the reaction under N_2 ascorbic acid was capable to reduce **PQ** to PQH_2 and the reduction of sodium ascorbate was faster than the one in air (see p. 154).

III.3.3.4. 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. 27. 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).

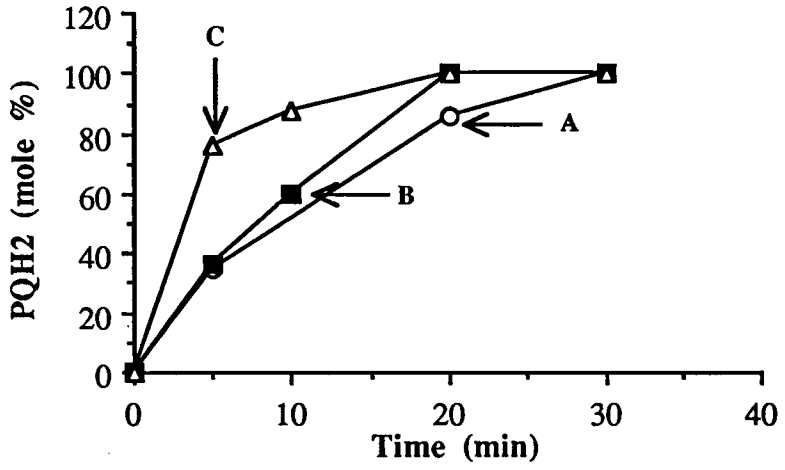


Fig. **27** shows the rate of reduction of NADH plus FAD in several concentration (see p. 154).

III.3.3.5. Reduction of PQ (40 mM) by glutathione (1.6 M).

Glutathione at concentrations up to 1.6 M did not reduce **PQ**.

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.**21**, line **A**, p. 150). Even when the ratio of dithiothreitol to **PQ** had been increased to 20:1 it did not make much difference.(Fig.**21**, line **B**, p. 150). When the reduction was performed under nitrogen, with the smallest ratio of 2:1, dithiothreitol gave a total reduction at 5 min (Fig.**24**, line **A**, p. 152), while a 10-fold molar ratio gave a total reduction at 1 min (Fig.**24**, line **B**, p. 152).

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.**22**, p. 150). 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.**25**, line **A**, p.152). 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.**25**, line **B**, p.152).

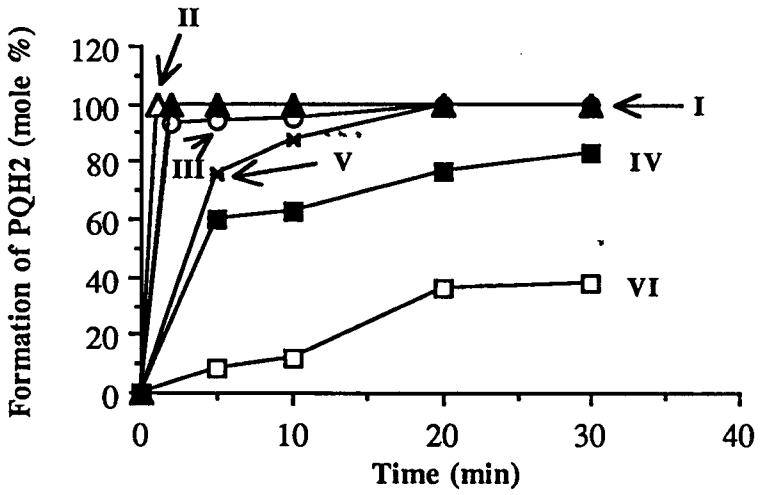
A 20:1 molar ratio of sodium ascorbate in air gave 44% reduction at 2 min and then gradually progressed to 68% at 60 min (Fig.**22**, p.150). However a 10:1 molar ratio of sodium ascorbate under nitrogen gave a slightly faster reaction which reached 84% reduction in 30 min (Fig. **26**, p.153).

NADH plus FAD gave total reduction (100%) at 30 min even in air (Fig.**23**, 151). While reduction under nitrogen was much faster (Fig.**27**, p. 153),. 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. **23**, line **B** and Fig. **27**, line **C**, p.151 and 153).

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.**28**, p. 155.

Fig. 28. 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 %).

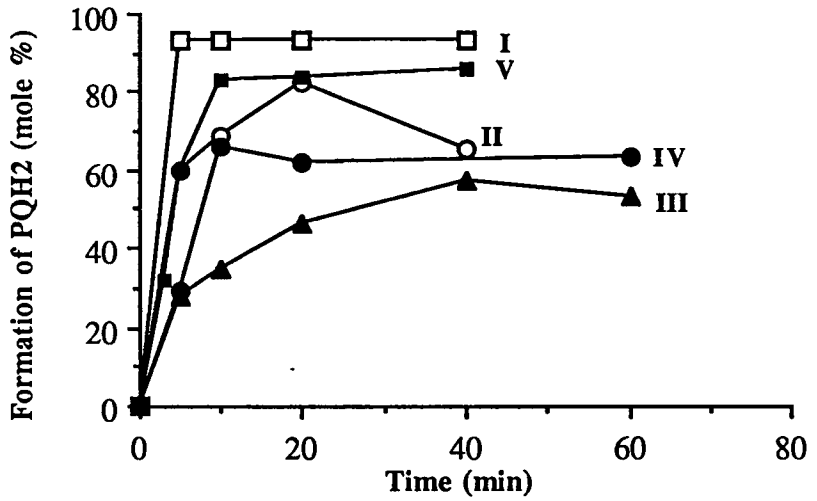


Legends : **I**: Dithiothreitol (100 mM); **II**: NADH (56 mM) plus FAD (70 mM);**III**: sodium dithionite (100 mM): **IV**: sodium ascorbate (100 mM); **V**: NADH (14 mM) plus FAD (70 mM); **VI**: ascorbic acid (100 mM).

III.3.4 Reductions of α-tocopherylquinone (TQ).

III.3.4.1. Reduction of TQ by several reductants.

Fig. 29. Formation of TQH_2 (mole %) from the reduction of **TQ** by several reductants.



Legends: **I**: sodium dithionite (200 mM) in homogeneous solution; **II**: sodium dithionite (100 mM) in homogeneous solution; **III**: NADH (28 mM) plus FAD (70 mM) in homogeneous solution; **IV**: NADH (40 mM) plus FAD (100 mM) in heterogeneous solution; **V**. NADH (80 mM) plus FAD (100 mM) in heterogeneous solution.

Fig. **29** shows that in the reduction of **TQ**, the reactivity of the reductants decrease in the order of sodium dithionite (200 mM) > NADH (80 mM) plus FAD (100 mM)/heterogeneous solution > sodium dithionite (100mM) > NADH(40 mM) plus FAD (100 mM)/heterogeneous solution > NADH (28 mM) plus FAD (70 mM)/homogeneous solution.

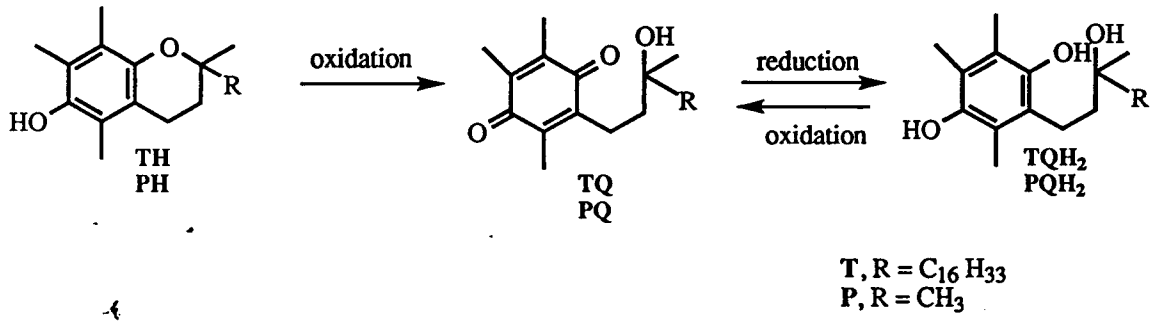
It shoud be noted that in the reactions of **PQ** with NADH plus FAD, the amounts of the reactants were the same both in homogeneous and heterogeneous reactions, however their concentrations vary because of the homogeneous or heterogeneous nature of the reactions.

III.3.4.2. Reduction of TQ (20 mM) by DTT (200 mM).

DTT gave only slight reduction which was evident at 5 min (8%) and 20 min (12%) but not at 30 min. 400 mM Dithiothreitol gave 76% reduction of **TQ** to TQH_2 at 45 min but only when a small quantity of methanol was added during the process.

Several solvents were used in the trial to get the reduction of the **TQ** to TQH_2, such as acetonitrile, acetone and glycerol, but it did not improve the situation. Also a suspending agent, xanthan (0.1% in buffer), was tried without effect.

Sodium ascorbate and ascorbic acid at the same concentration as the other reductants, 100 and 200 mM, did not give any result.



Scheme 10.

III.3.5. Reduction of ubiquinone-10 (UQ-10, 20 mM)) by NADH (80 mM) plus FAD (100 mM).

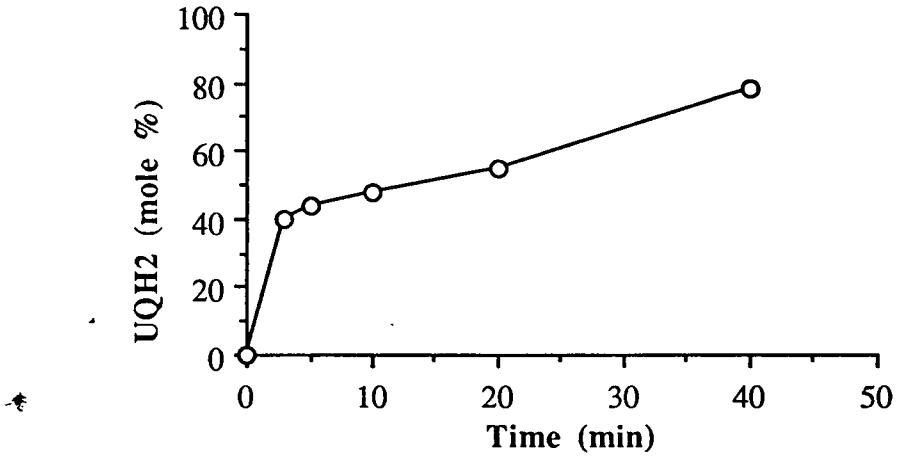
In view of the outstanding antioxidant activity of ubiquinol-10 (UQH_2)(Mellors and Tappel, 1966, Frei et al., 1990, Stocker et al., 1991), and its proposed mode of action, it seemed that there may be an additional explanation for the excellent antioxidant activity of **TH**. Thus, if **TH** is readily oxidised in various tissues to **TQ**, it is quite likely that there may one or more enzymes present in living cells that could reduce **TQ** to its hydroquinone TQH_2 (scheme **10**) which could be expected to be as good an antioxidant as UQH_2. For example, TQH_2 would also be expected to reduce T•, thereby inhibiting potential prooxidant activity of **TH**.

In order to compare the rate of reduction of ubiquinone-10 (**UQ**) and **TQ** by NADH plus FAD, **UQ** was reduced by NADH plus FAD and a mixture of **UQ** and **TQ** was reduced by the same reductant as well. The **UQ** was dissolved in hexane, while the NADH and FAD were dissolved in phosphate buffer.

Fig. **30** (p. 158) showed that reduction of **UQ** by NADH (160 mM) plus FAD (200 mM) was slower than that of **TQ** at the same concentration (Fig. **29**, line **V**, p.

156). However, when **TQ** and **UQ** were reduced together with the same concentration of NADH plus FAD, **UQ** was reduced faster than **TQ** (Fig. **31**, p. 158).

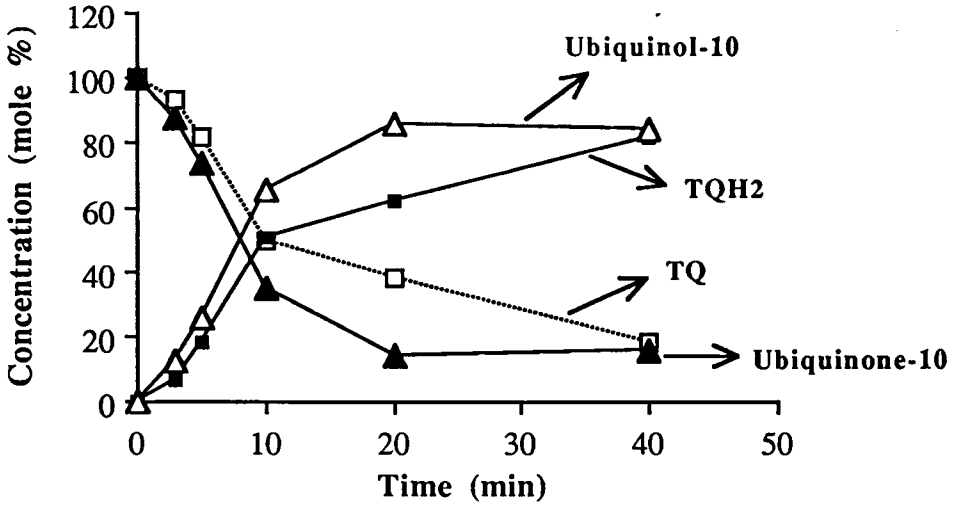
Fig.30. Formation of ubiquinol-10 (UQH_2) (mole %) from the reduction of **UQ** (20 mM) by NADH (80 mM) plus FAD (100 mM).



III.3.6. Reduction of TQ (20 mM) and UQ (20 mM) by NADH (80 mM) plus FAD (100 mM).

This experiment was performed in heterogeneous solution.

Fig.31. HPLC analysis of the reduction of **TQ** (20 mM) and **UQ** (20 mM) by NADH (80 mM) plus FAD (100 mM)



It can be concluded that the mode of action of **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 $\mathbf{PQH_2}$ by blood, the *in vivo* reduction of **TQ** to $\mathbf{TQH_2}$ after ingestion and also by a mixed population of leucocytes suggest that **TH** may be able to function in a completely different manner as as antioxidant *in vivo*. If, for some reason, $\mathbf{T}^{\bullet}$ 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 $\mathbf{TQH_2}$. Additional lipid peroxyl radicals could then reoxidise the $\mathbf{TQH_2}$ 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.

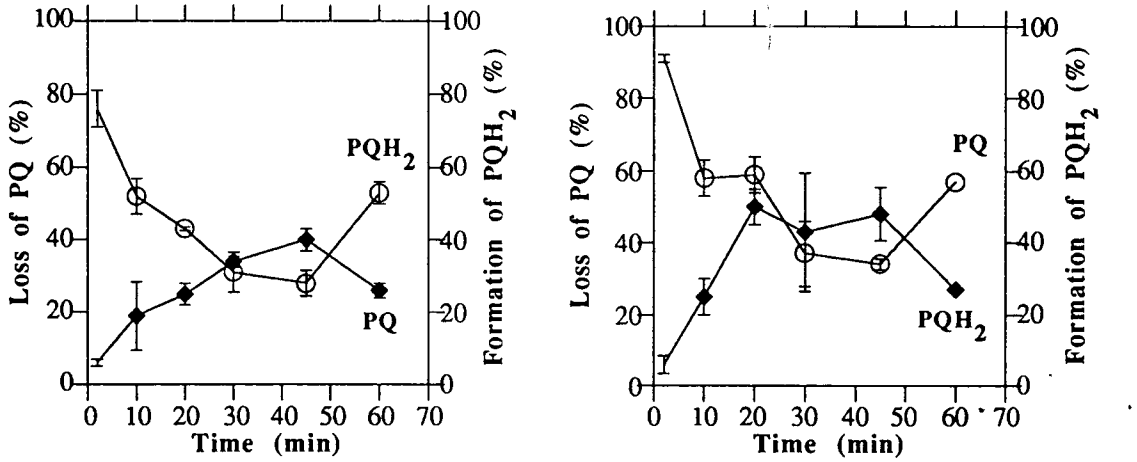
III.4. BIOLOGICAL EXPERIMENTS.

III.4.1. Reduction of PQ by blood.

Studies have shown that mitochondria, chloroplasts and microbial plasma membranes possess enzymes capable of the reduction of **TQ** to TQH_2 (Crane, 1977, Hughes and Tove, 1980, Bindoli et al., 1985). Fig. **32** shows that fresh and 2 day old blood are capable of reducing **PQ** to PQH_2 rapidly at 37°C and that spontaneous reoxidation begins to occur after 45 min.

Fig. 32. Reduction of **PQ** by fresh and 2 day old blood (calculated as % of **PQ** added).

Reduction of **PQ** by fresh blood. Reduction of **PQ** by 2 day old blood.



There was no evidence of the formation of **PH** in this reaction. Thus, if **TH** is oxidised in vivo to **TQ**, it seem most unlikely that **TQ** could be reduced back to **TH** by one of the natural reductants.

III.4.2. Reduction of TQ by blood.

III.4.2.1. Recovery of TQH_2 from bovine serum albumin.

In contrast with the reduction of **PQ** to PQH_2 by blood, incubation of **TQ** in blood failed to produce TQH_2, which was thought due to the difficulty of extracting TQH_2 from blood. Thus, a recovery experiment of TQH_2 from bovine serum albumin at 37°C (for several time points) was performed which showed recovery between 42-82%.

III.4.2.2. Recovery of TQ from whole blood and packed cells.

Several experiments for the recovery of **TQ** from blood were performed as well, which showed that **TQ** could be recovered from blood in a relatively good yield (80-99% by NPLC analysis, Table **54**, p. 161) and that the age of the blood (fresh and one day old blood) had no influence on the recovery. Similarly a combination of hexane/isopropanol extracting solvent and RPLC analysis gave good recoveries **TQ**. Hexane/isopropanol proved a much better extractant than hexane itself. Using NPLC, solvent E (hexane/isopropanol 95:5) the recovery was between 80-99% and using RPLC system, solvent J (methanol/Milli-Q water 98:2) and hexane as the extracting solvent showed 65-93% recovery (Table **54**, p. 161). When **TQ** was added to a suspension of packed cells in PBS recoveries of 77-88% were achieved showing that **TQ** is not bound strongly to the cells.

Table 54. Recovery of **TQ** from whole blood and packed cells as % of added **TQ**.

Incubation time (min)	Whole blood analysed by NPLC (solvent E)		Fresh blood analysed by RPLC (solvent J)			
	Type of blood		Extracting solvent			Replace plasma with PBS (packed cells)
	Fresh	1 day	hex/isopr 19:1	hexane	hex/isopr 9:1	
0	111	96				
2			102	62	112	87
10	86	86	93	64	120	77
20	99	86	112	63	124	81
30	99	88	107	51	124	
45	88	81		69	124	88
60	86	79	88	83	148	83

Each time point was performed as a separate experiment (hex/isopr = hexane/isopropanol).

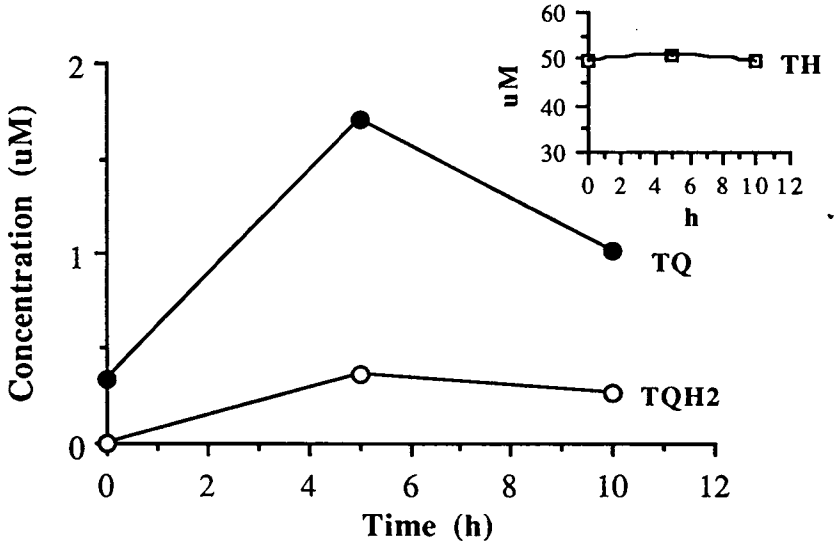
It was concluded that recovery of TQH_2 and **TQ** from blood was not the problem but that the much greater hydrophobicity of **TQ** than **PQ** may have inhibited **TQ** from making contact with its reducing enzymes in this experimental system. This argument is in line with the observation of other workers (Stocker and Suarna, 1993), that ubiquinone-1, but

not **UQ**, is rapidly reduced to the hydroquinone upon addition to whole blood. In an attempt to ensure its dispersion, **TQ** was added to blood in a number of solvents including hexane, methanol, acetone and also incorporated in liposomes. In none of these systems was **TQ** reduced.

III.4.2.3. Reduction of TQ *in vivo*.

However, ingestion of **TQ** led to a substantial rise (from 0.1 to 1.8 μM in 5 h) in its plasma concentration and also to the appearance of TQH_2 (0.3 μM after 5h). The formation TQH_2 was confirmed by its correct retention time and because its concentration in the hexane extract fell to 50% of its initial value within 20 min due to rapid aerial oxidation. TQH_2 was never noted in normal blood while the concentration of **TQ** in normal blood is similar to that reported by others (Murphy et al., 1992). Thus **TQ** is capable of being reduced by the body at a site as yet unknown.

Fig. 33. Plasma concentrations of **TQ**, TQH_2 and **TH** following ingestion of **TQ**

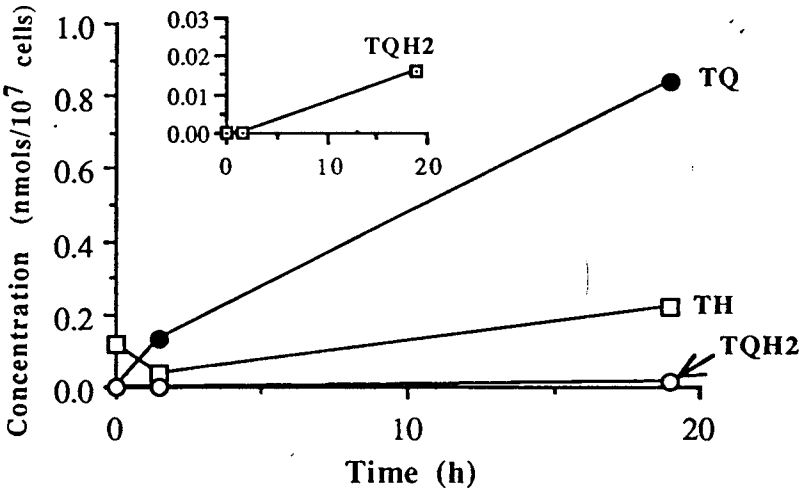


III.4.3. Reduction of TQ by human mononuclear cells.

Evidence of the relatively facile reduction of **TQ** by living systems came from an experiment in which a mixed population of lymphocytes and monocytes was able to convert **TQ** into TQH_2. (Fig. **34**). This was a relatively slow reaction and required cellular uptake of **TQ**. Another interesting feature of this reaction was that the

concentration of **TH** increased from 0.11 to 0.22 nmol/10^7 cells during the 19 h of the reaction (Fig. 34). As these results were obtained from a single experiment, it is not known whether this increase in **TH** is significant. However it may imply that **TQ** is being reduced to **TH** by the leukocytes. Reduction of **TQ** to **TH** can occur readily *in vitro* in the presence of acid but has not been reported in biological solutions of neutral pH.

Fig. 34. Uptake and reduction of **TQ** to TQH_2 by a mixed population of lymphocytes and monocytes.



III.4.4. Optimisation of extraction of endogenous TH and TQ from blood.

It was observed that the volume of plasma extracted with a certain amount of methanol and hexane played an important role in the extraction of endogenous **TH** and **TQ** from plasma. Table **55**, protocol 1 (p. 164) showed that extraction of 100 ml plasma using methanol (500 ml) followed by hexane (2 ml) gave an optimum yield of **TH** with minimum oxidation of **TH** to **TQ**. The best combination of solvents to extract **TH** and **TQ** from blood are methanol/hexane (0.5:2) ml and methanol/hexane/isopropanol 95:5 (0.5:2) ml) and also ethanol/hexane (0.5:2)ml (Table **55**, protocol 2, p. 164).

Table 55. Extraction of **TH** and **TQ** from plasma using different solvents and methods.

Protocol 1			Protocol 2				
Amount of plasma (µl)	TH (µM)	TQ (µM)	Age/temp. of plasma	Name/amount (ml)/temp. of solvent to which plasma was added	How/time the plasma and solvent were mixed	TH (µM)	TQ (µM)
400	5.4	0.7	fresh/r.t.	ACN/0.5/r.t.	flicked, 5 sec	13.0	ud
300	13.6	2.0	fresh/r.t.	MeOH/1/r.t.	vortexed, 2 sec	39.0	ud
200	24.6	2.5	fresh/r.t.	MeOH/0.4/r.t	flicked, 60 sec	40.7	ud
100	53.2	0.7	fresh/4°C	MeOH/0.5/4°C	shaken, 5 sec	40.0	ud
50	46.4	ud	7 day/r.t	MeOH/0.5/r.t.	shaken, 5 sec	44.4	5.0
			10 day/r.t.	MeOH/0.5/r.t.	shaken, 5 sec	47.3	0.8
			10 day/r.t.	MeOH/0.5/r.t.	flicked, 20 sec	43.1	0.3
			fresh/r.t.	IprOH/0.5/r.t.	shaken, 5 sec	41.4	ud
			10 day/r.t.	EtOH/0.5/r.t.	shaken, 5 sec	45.2	0.5
			4 day/r.t.	hexane/2/r.t.	vortexed, 3 sec, then centrifuged 3 min	ud	ud
			fresh/r.t.	MeOH/0.5/r.t	shaken 5 sec, then extrd. with dichloromethane	58.1	ud
			7 day/r.t.	MeOH/0.5/r.t	shaken 5 sec, then extrd. with hexane	52.1	0.9
			7 day/r.t.	MeOH/0.5/r.t	shaken 5 sec, then extrd. with hexane/isoprOH 95:5	50.9	0.8

Abbreviation in Table **55**: r.t. = room temperature, temp. = temperature, ud = undetectable, extrd = extracted, ACN = acetonitrile, MeOH = methanol, EtOH = ethanol, IprOH = isopropanol.

III.4.5. Analysis of blood using the SMAD data acquisition interface.

SMAD data acquisition was used because of its sensitivity and accuracy. Using this interface, some experiments were performed to find the detector wavelength that could give the optimum reading for both the **TH** and **TQ**, because these compounds absorb at different wavelenghts (297 nm and 268 nm respectively) and with different extinction coefficient (4000 and 20,000 respectively). However, the results were not conclusive. Analysis of **TH** did not give significant difference (around 40 mM) and analysis of **TQ**, eventhough it gave the highest yield at 280 nm, it was probably an artifac, because the level of **TQ** in plasma was not that high as was found by others (Howell and Wang, 1982) and also in other present experiments (Table **62**, p. 169 and Table **63**, p. 173). Also 280 nm suppose to give better reading for **TH** because it is nearer to the maximum wavelength of **TH** (297 nm) than that of **TQ** (268 nm).

Table 56. Analysis of plasma using SMAD data acquisition at 280, 275 and 270 nm.

Wavelength (nm)	Conc. of **TH** (μM)	Conc. of **TQ** (μM)
280	42.3 ± 2.4	4.5 ± 1.2
275	38.3 ± 3.3	1.6 ± 1.2
270	33.0 ± 5.2	0.5 ± 0.9

Because the amount of **TQ** in plasma was very small and fluctuated from undetectable to 5 mM (Table **55**, p. 164), several other methods of analysis using the SMAD interface were performed, by varying the extracting solvents (Table **57** and **58**, p.166), adding surface active agents to enhance the extraction of **TH** and **TQ** (Table **59**, p.167), adding papain and proteinase K (Table **60** and **61**, p. 168).

Table 57. Analysis of **TH** and **TQ** in plasma by several protocols using SMAD data acquisition interface.

Protocol	Conc of **TH** (μM)	Conc of **TQ** (μM)
A	34	1.1
B	46	0.2
C	40	0.6
D	42	0.4

Protocol A, B, C and D differ in the method of mixing plasma with ethanol and in the method of extraction.

Protocol A: plasma was added to ethanol, the mixture extracted with solvent C (hexane/isopropanol = 98:2), then vortexed (20 sec) and centrifuged before collection of hexane layer (see p. 98).

Protocol B: plasma was added to ethanol, the mixture extracted with solvent C, then vortexed (4 sec) and the mixture was allowed to settle (3 min) before collection of hexane layer (see p. 99).

Protocol C: ethanol was added to plasma then the mixture extracted with solvent C, vortexed (4 sec) and allowed to settle (1 min) before collection of hexane phase (see p. 99).

Protocol D: ethanol was added to plasma, the method of extraction was as in protocol C, but performed twice (p. 99).

These experiments did not give significant results.

Table 58. Analysis of **TH** and **TQ** in plasma by different extracting solvents and stages of extraction.

Type of plasma	Extracting solvent	Stage of extraction	Conc. of **TH** (μM)	Conc. of **TQ** (μM)
7 day old	solvent C	(i) + (ii) + (iii)	50	undetected
fresh	solvent C	(i) + (ii) + (iii)	45	0.1
fresh	solvent C	(i) + (ii)	38	0.3
fresh	solvent C	(i)	52	0.3
fresh	solvent E	(i) + (ii) + (iii)	43	0.4
fresh	hexane	(i)	44	0.5

Solvent C: hexane/isopropanol = 98:2, solvent E: hexane/isopropanol = 95:5.

Stage (i): extraction with solvent C (1 ml), vortexed (3 sec), more solvent C (1 ml) was added and vortexed (4 sec). The mixture was allowed to settle for 1 min, then an aliquot

of the hexane layer (1.7 ml) dried under a gentle stream of nitrogen (see p. 99, protocol E).

Stage (ii): to the residue of (i) was added solvent C (2 x 1 ml) as in (i) and an aliquot of hexane layer (1.8 ml) combined with the dried residue of (i) and the solvent removed under a gentle stream of nitrogen (see p. 100, protocol E).

Stage (iii): the aqueous plasma residue of (ii) was treated as in (ii), except an aliquot (2 ml) was collected instead of 1.8 ml.

These experiments gave similar yields for the analysis of **TH** and **TQ** in plasma.

Table 59. Analysis of **TH** and **TQ** in plasma with and without surface active agent.

Surface active agent	Stage of extraction	Conc. of **TH**(μM)	Conc. of **TQ** (μM)
None	(i)	49 ± 6	0.4 ± 0.07
None	(i) + (ii)	41 ± 1	0.7 ± 0.3
1% sod.laurylsulphate	(i) + (ii) + (iii)	43	0.8
1% tween	(i) + (ii) + (iii)	43	1.3

It was felt that if **TQ** were strongly bound to proteins, either denaturation of the protein by a surface active agent such as sodium lauryl sulfate or hydrolysis of the protein by enzymes should improve the extraction of **TQ**. However, sodium lauryl sulfate and also papain or proteinse K did not really improve the extraction of **TH** and **TQ** from blood (see also Table **60** and **61**, p.168).

Table 60. Analysis of **TH** and **TQ** in blood fractions using papain stock solution or proteinase K

Type of blood	Type of enzyme added	Amount of enzyme added	Time of incubation	**TH** (μM)	**TQ** (μM)
plasma	-			36 ±1.5	0.8±0.5
w. blood	-			18±3.1	2.6±0.8
plasma	papain	2 mg/20 ml	35 min	42	1.3
plasma	papain	1.5 mg/100 ml	15 min	41	0.4
plasma	papain	1.5 mg/100 ml	30 min	42	0.4
packed cell	papain	1.5 mg/100 ml	30 min	4	1.3
packed cell	papain	4.5 mg/300 ml	15 min	6	2.7
plasma	proteinase K	50 mg/50 ml	15 min	40±0.6	0.3±0.1
plasma	proteinase K	50 mg/50 ml	30 min	38±2.2	0.6±0.3
w. blood	proteinase K	50 mg/50 ml	15 min	16±0.6	3±0.1
w. blood	proteinase K	50 mg/50 ml	30 min	16±0.9	3±0.5

w. blood = whole blood

Table 61. Analysis of **TH** and **TQ** in plasma and packed cells using papain stock solution (40 ml, 8 mg) for varying times.

Time (min)	Plasma		Packed cells	
	TH (μM)	**TQ** (μM)	**TH** (μM)	**TQ** (μM)
15	45.3	1.8		
30	44.8	0.5	10.1	1.8
45	46.2	0.5	16.5	1.9
60	48.5	0.4	14.2	2.3
90	47.1	0.5	11.4	1.9

III.4.6. Analysis of blood fractions for TH and TQ.

These experiments showed that, of blood fractions, plasma contained the highest concentration of **TH** and the lowest concentration of **TQ**. By contrast,. packed cells, red cells and white cells contained a much lower concentration of **TH** and a much higher

concentration of **TQ**. This may mean that **TH** is the most important form of the vitamin in plasma while **TQ** has a more important role to play in cells.

Table 62. Analysis of blood fractions for **TH** and **TQ**.

Blood fraction	**TH** (μM) (n = 4)	**TQ** (μM) (n = 4)
Whole blood	23.0 ± 1.7	2.3 ± 0.7
Plasma	49.8 ± 0.3	0.2 ± 0.04
Packed cells	4.3 ± 0.5	1.6 ± 0.2
White cells	6.5 ± 0.7	1.6 ± 0.3
Red cells	5.0 ± 0.2	1.4 ± 0.2

Values of **TH** in red cells agrees with the ones found by other methods: TLC followed by GLC (5.5 μM ± 0.37 μM) (Bieri et al., 1970), spectrophotometric method based upon the Emmerie-Engel tocopherol assay (2.91 mg/ml = 6.7 μM)(Kayden et al., 1973), spectrofluorometric (3.71 μM) (Taylor et al., 1976), RPLC with spectrophotometric detection (4.24 ± 0.21 μM) (Bieri et al., 1979), (5.05 ± 1.72 μM) (Cuesta Sanz et al., 1986), and (4.06 ± 0.68 μM, with normal range: 3.04 - 5.22 μM)) (Sierra et al., 1992), RPLC with fluorometric detection (3.2 - 8.8 μM) (Lehman and Martin, 1983),

Values of **TH** in plasma found by spectrophotometric method based upon the Emmerie-Engel tocopherol assay (13.8 mg/ml = 32.1 μM)(Kayden et al., 1973), NPLC with fluorometric detection (16.3 - 48.4 μM) (Jansson et al., 1980), RPLC with fluorometric detection (10.2 - 41.9 μM)(Lehman and Martin, 1983), with UV detection (44.2 μM)(Bieri et al., 1979), (27.5 ± 5.2 μM) (Cuesta Sanz and Castro Santa-Cruz, 1986) and (33.7 ± 8.19 μM, with normal range: 22.2 - 52.1 μM)(Sierra et al., 1992).

The ratio of **TH** content in red cells/plasma agrees to Sierra (1986) report (0.1 %), however, other reports showed a bigger ratio (0.2 %)(Bieri et.al., 1970 and 1979, Kayden et al., 1973, Lehmann and Martin, 1983, Cuesta Sanz et al., 1986).

According to Howell and Wang (1982), small quantities of **TQ** could be detected in human plasma along with **TH**, but a quantitatible level of **TQ** (16 mM) was found only

in the patient with the highest level of **TH** (250 μM), while Murphy et al. (1992) observed the content of **TH** and **TQ** in human plasma were 21.0 ± 2.5 μM and 0.36 ± 0.17 μM respectively.

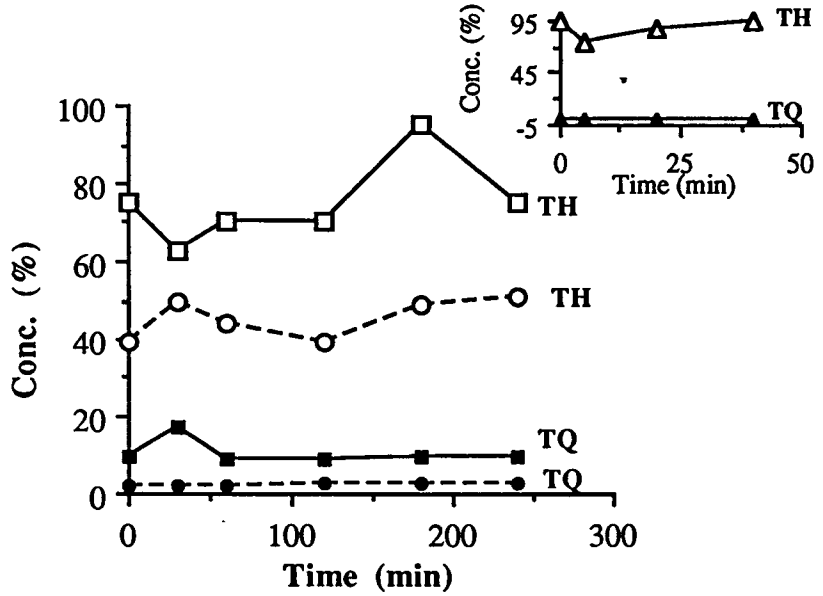
Bieri et al. (1981) mentioned that methods of tocopherol separation by HPLC coupled with ultraviolet detection for simultaneous quantitation of **TH** and **TQ** lack sufficient sensitivity for small biological samples. However, our experiment on the analysis of **TH** and **TQ** showed similar results as of the above mentioned workers.

III.4.7. Reaction of TH and PH with haemoglobin (Hb) solution at room temperature and 37°C.

To simulate the reaction that might take place between the haemoglobin in the blood and **TH**, **TH** and also **PH** were incubated with Hb and BSA (as a control) solution at room temperature and at 37C.

III.4.7.1. Reaction of TH with Hb or BSA at room temperature.

Fig. 36. Recovery of **TH** from BSA (insert) and Hb solution at room temperature (calculated as % of added **TH**).



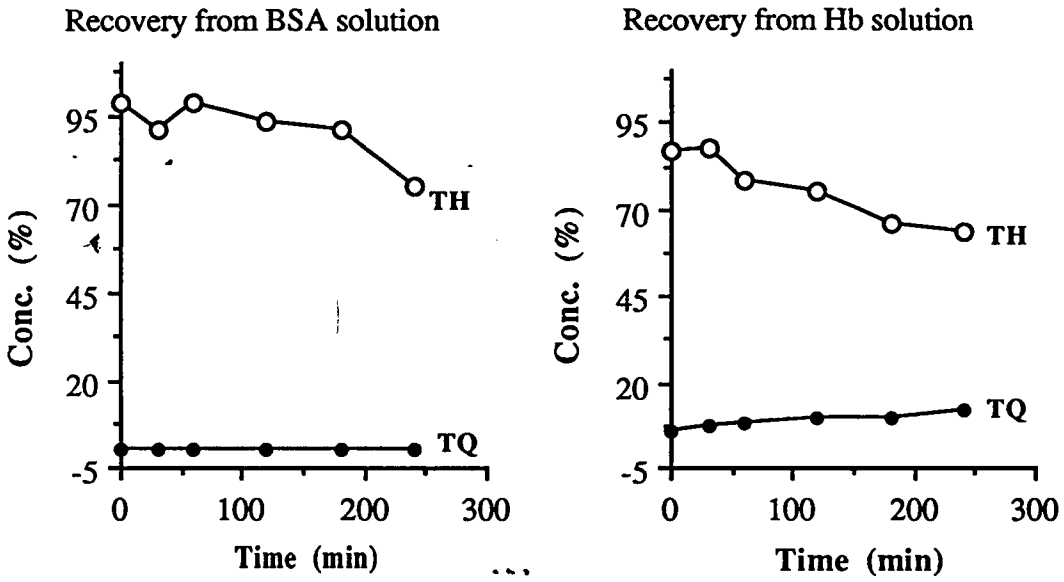
——— recovery from 12% Hb ---------- recovery from 15 % Hb

In the experiment using Hb, 12% Hb gave higher yield than 15% Hb both for **TH** and **TQ** recovery, most probably due to the binding of **TH** and **TQ** in haemoglobin.

In the Hb reaction there was a slight increase in **TQ** (~ 6%) during the 4 h reaction which was, however, much less than the fall in **TH** during the same period (~ 20%). This implies also that the fall in **TH** was not simply due to oxidation but to binding with the protein.

III.4.7.2. Reaction of TH with Hb or BSA at 37°C

Fig. 37. Recovery of **TH** from BSA and Hb solution at 37°C.



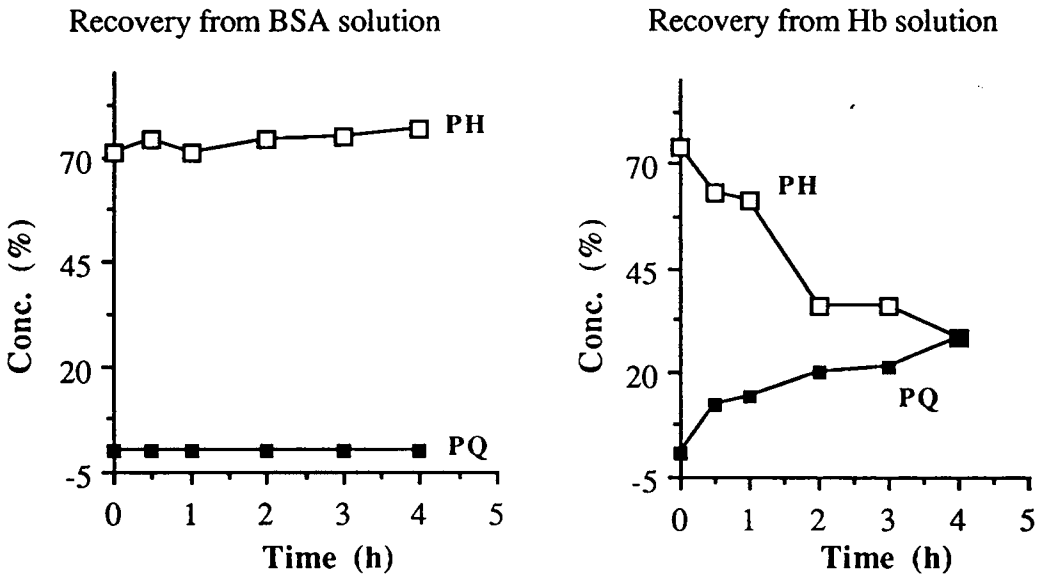
The recovery of **TH** from both proteins decreased progessively during the 4 h reaction. In the BSA reaction the concentration of **TQ** did not rise, suggesting that the drop in recovery of **TH** was not due to formation of **TQ** but rather stronger binding, perhaps covalent bonding with the BSA. Also in the recovery experiments at 37°C, the reaction mixtures were diluted with bidistilled water prior to extraction and the results showed that diluting the reaction mixture with water gave more consistent results, which may also point to the bonding with protein in which was stronger in a more concentrated mixture.

III.4.7.3. Reaction of PH with Hb or BSA solution at 37°C.

Fig. 38 (p. 172) showes that the recovery of **PH** from BSA did not decrese at all during the 4 h reaction and neither did the concentration of **PQ** rise suggesting that **PH** did not progressively bind or react with BSA in the same way as did **TH**. However, the

reaction of **PH** with Hb was a remarkable contrast with that of **TH**. The recovery of **PH** decreased from 74% at the start of the incubation and 30% after 4 h. In the same period the concentration of **PQ** had risen from 0 to 27%. Thus it appeared that Hb had catalysed the oxidation of **PH** to **PQ**.

Fig. 38. Recovery of **PH** from BSA and Hb solution at 37°C (calculated as % of added **PH**).



However, the loss of **PH** was much greater (44%) than the formation of **PQ** (27%) suggesting the **PH** may bind in some way with the Hb. Thus Hb is capable of oxidising **PH** to a much greater extent than **TH** which suggests that in some way "the tail" of **TH** has substantial inhibiting effect on the oxidation of the chromanol nucleus.

III.4.8. Reaction of blood with carbon monoxide.

It was expected that the **TH** would be oxidised to some extent into **TQ** by haemoglobin. The blood was treated with carbon monoxide to block the iron of the haemoglobin so that it could not react with oxygen and cause any oxidation. Table **63** (p. 173) showed that the **TH** and **TQ** content of whole blood, packed cells and plasma of the treated blood were almost the same as those of untreated blood. These results demonstrated that there was very little if any oxidation of **TH** to **TQ** by Hb. There it is

likely that the higher **TQ** levels observed in the whole blood and the cells are real and not the result of oxidation during extraction.

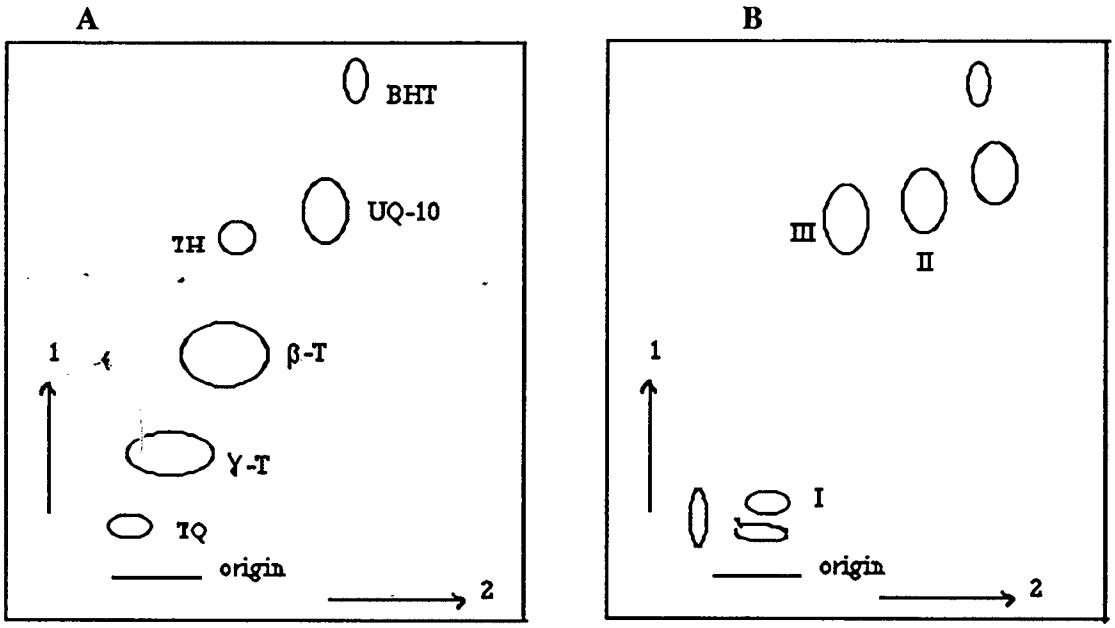
The analysis of red blood cells for **TH** required a slightly larger volume than for plasma because red cells contain about one-fifth as much of the vitamin as does plasma (Bieri et al., 1970, Kayden.et al., 1973).

Table 63. Concentration of **TH** and **TQ** (μM) in blood fractions.

	TH	**TQ**
A. Untreated blood analysed by NPLC		
1. Whole blood	17.45	1.28
2. Packed cells	3.54	0.90
3. Plasma	38.62	0.22
B. Treated blood by analysed NPLC		
1. Whole blood	15.22	1.03
2. Packed cells	3.67	0.91
3. Plasma	33.80	0.16
C. Untreated blood analysed by RPLC		
1. Whole blood	15.68	1.55
2. Packed cells	3.92	1.03
3. Plasma	35.26	0.28

III.4.9. ANALYSIS OF HEART MUSCLE AND LIVER FOR α-TOCOPHEROL AND α-TOCOPHERYLQUINONE BY THIN LAYER CHROMATOGRAPHY.

Fig.39. TLC (two dimensional) analysis of heart muscle for **TH** and **TQ**.

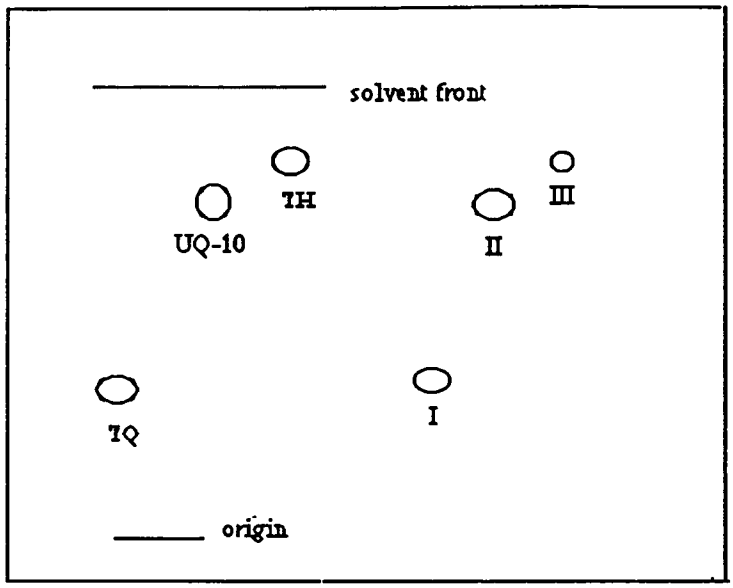


A. standard BHT, **TH**, β-T, γ-T, **TQ** and **UQ-10** (**TQ** and **UQ-10** were yellow, the other spots were all colourless).

B. lamb's heart extract. (I and II were yellow, III was faintly visible, became obvious only after 1 day).

Spots I, II and III and also standard **TH**, **TQ** and **UQ-10** were scraped off the TLC, dissolved in a little hexane and run on another TLC plate using hexane-ethanol 9:1 as the developing solvent.

Fig. 40. TLC (two dimensional) analysis of spots I, II and III from the lamb's heart extract.



Rf of standards: **TH**, 0.86; **UQ-10**, 0.77; **TQ**, 0.33.

Rf of sample: I, 0.33; II, 0.77; III, 0.86.

Qualitative analysis by two dimensional TLC (Fig. **38**, p. 176 and Fig. **39**, p. 177) showed that lamb's heart muscle contained **TH**, **TQ** and **UQ-10**.

III.4.10. Analysis of dog's heart muscle and liver for TH and TQ.

Table 63. Analysis of dog's heart muscle and liver for **TH** and **TQ**.

	Heart muscle	Liver
TH (nmol/g)	15.4	49.5
TQ (nmol/g)	0.2	0.8

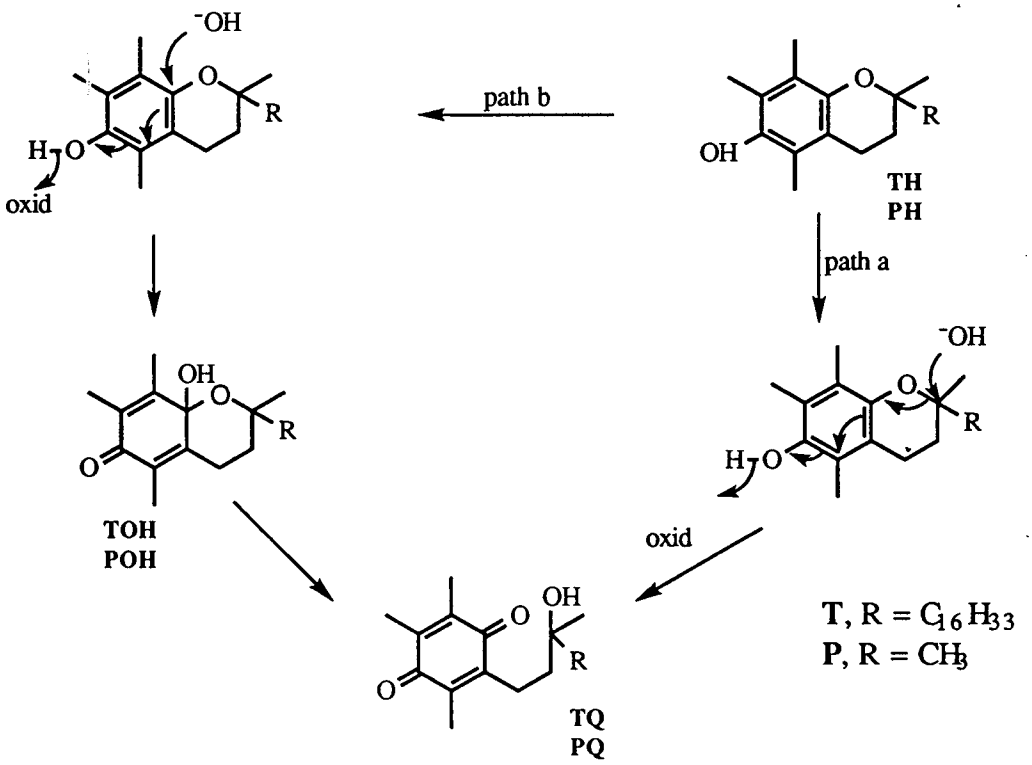
To compare the amount of **TH** and **TQ** in animals heart and liver, a fresh dog's heart and liver were used for this experiment. Hughes and Tove (1980) reported that **TQ** found in the rat's heart and liver was 22 nmol/g heart and 124 nmol/g liver, measured by fluorescence, spectrophotometric and radioactive measurements. Bieri and Tolliver (1981) found the content of **TH** and **TQ** in rat liver analysed by TLC followed by RPLC with

UV detection (280 nm) was 26.4 nmol/g and 3.6 nmol/g liver respectively. While Howell and Wang (1982) using RPLC with UV detection (275 nm) observed the content of **TH** in rat's heart was 54 ± 12 nmol/g heart and that of **TQ** was below reliable level of quantitation (2.9 nmol/g heart). Murphy and Kehrer (1987) using RPLC with oxidative electrochemical detection found that the **TH** and **TQ** content in chicken liver was 79 ± 18 pmol/mg tissue and 1.47 ± 1.10 pmol/g tissue respectively. Compare with others observations our results was contradictory, however, it may due to the difference in the method of analysis and to that the **TH** and **TQ** in animals tisues were different depends on the species of the animals and the individual as well.

III.5. EXPERIMENTS WITH 8a-HYDROXYTOCOPHERONE (TOH) AND ITS ANALOGUE, 8a-HYDROXY-2,2,5,7,8-PENTAMETHYL-6-CHROMANONE (POH).

III.5.1. Experiments with 8a-hydroxy-2,2,5,7,8-pentamethyl-6-chromanone (POH).

Durckheimer and Cohen (1964) discussed two possible pathways - a and b (Scheme **11**) of formation of **TQ** from **TH**. They concluded from UV spectral evidence that pathway b operated and that **TOH** was the labile, immediate precursor of **TQ**.



Scheme 11.

It seemed possible that another pathway may operate in which **TH** might be recycled and its activity thereby preserved. Thus **TOH**, formed in the oxidationof **TH**, may be reduced to **TH** or to TQH_2 before the stage of **TQ** had been reached.

It was decided to investigate the ease of reduction of **TOH** and **POH** by several reduction of **TOH** and **POH** by several biological and non-biological reductants.

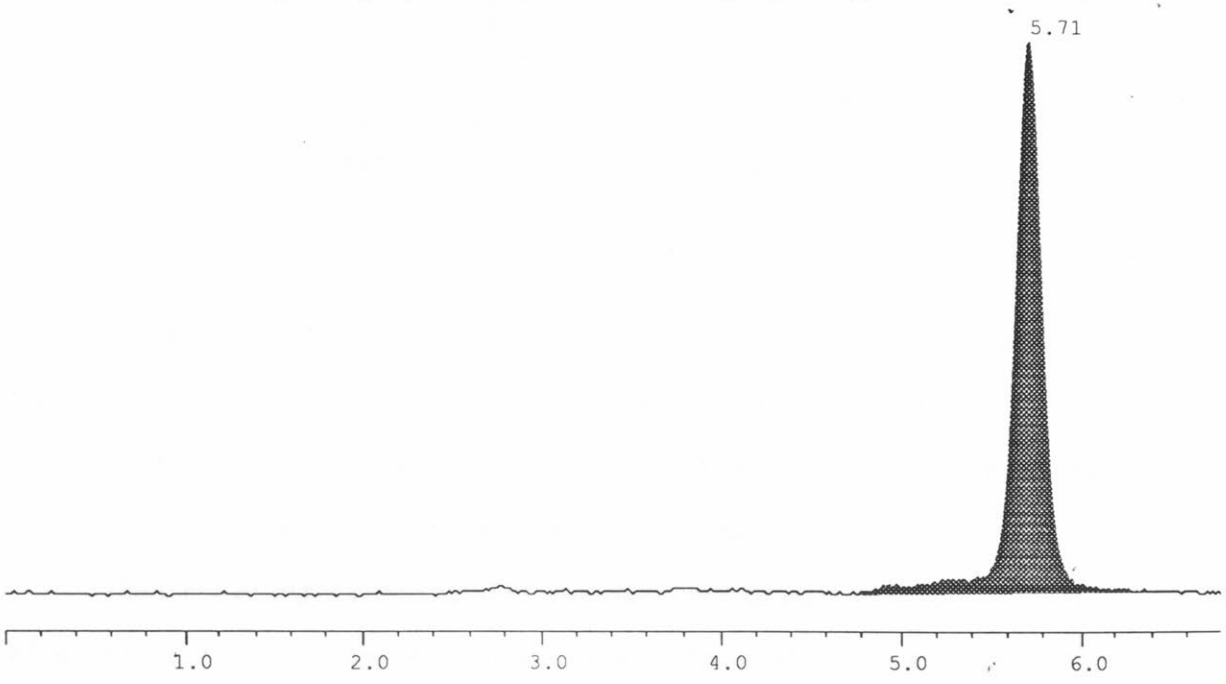
Therefore several attempts had been performed to make 8a-hydroxy-2,2,5,7,8-pentamethyl-6-chromanone (**POH**) in high concentration using the Durckheimer method (Durckheimer and Cohen, 1964), by varying the amount of hexane as the extracting solvent. Hexane was used in order to make it comparable to the later 8a-hydroxytocopherone experiments. The highest concentration that could be achieved was 0.15 mM (30% of theoritical).

POH (λ 238 nm) is unstable, decomposes spontaneously into **PQ** (λ_{max} 260, 268 nm) and its rate of decomposition may be determined by UV analysis. Thus within 50 min in dried solvent **POH** had decomposed completely into **PQ**.(Fig.**54**, p. 189).

The reaction between **POH** with the reductants were performed under nitrogen, as reactions in air gave good results only in the reaction with ascorbic acid, while with other reductants, the results were very poor to none.

When **POH** was chromatograph by NPLC and RPLC, it rearranged immediately to **PQ** on the column and was not observed at all (Fig. **41**, p. 178). This effect had been noticed earlier with **TOH** by Liebler et al. (1989).

Fig.41. NPLC chromatogram (solvent D, hexane/isopropanol = 96:4) of **POH** immediatedly after preparation (Retention time (RT) of **PQ** was 5.7 minutes)



The only possible evidence of **POH** on the chromatogram was a slight shoulder which eluted just before the **PQ** and which disappeared when the **POH** was reacted with ascorbic acid, dithionite and NADH plus FAD. Since the identity of the shoulder was unknown and very small, it was ignored for purposes of quantitation.

The fact that **POH** rearranges to **PQ** immediately upon chromatography means that it cannot be analysed directly but suggests that it may be able to be analysed indirectly as **PQ**. However this would not allow differentiation between **PQ** which was already present in the **POH** preparation and **PQ** that had been formed from **POH** during chromatography.

Fig.42. Chromatogram of the reaction between **POH** and ascorbic acid (10%) for 20 min, analysed by NPLC, solvent C. RT of **PH** is 3.6 and **PQ** 5.8 minutes.

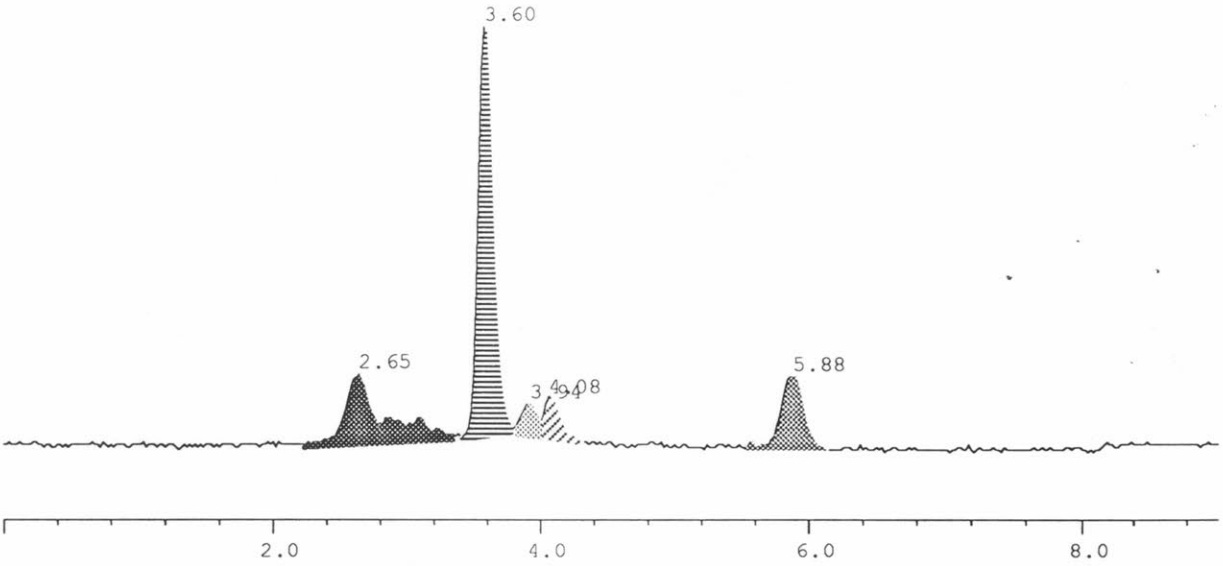


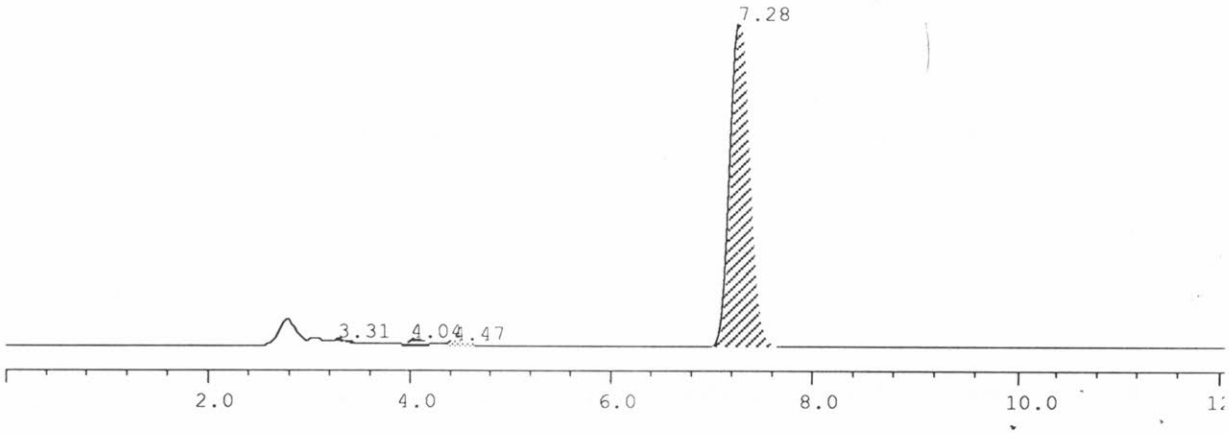
Fig. **42** (p. 179), shows a chromatogram of a 20 min reaction between **POH** and ascorbic acid. The majority of the **POH** has been reduced to **PH** (peak at 3.6 min). However, the peak at 5.88 min shows that the **POH** preparation contains a small amount of **PQ**. The identity of the other small peaks is unknown.

The fact that **PQ** is not reduced by ascorbic acid acid under these condition (Fig. **45**, p. 181) allows us to estimate the initial concentration of **POH** by its convertion into **PH**. In all of the reactions of **POH**, with various reductants, a control reaction with

ascorbic acid was performed to estimate the initial concentration of **POH** and **PQ**. Unreacted **POH** at various times during the reaction was estimated by subtracting the initial **PQ** concentration from that of the **PQ** at any subsequent time.

Fig. **43** (p. 180) shows a chromatogram of a 10 min reaction between **POH** and dithiothreitol (DTT). The peak at 3.6 min shows that only a small amount amount of **PH** has been formed while the majority of **POH** remains unreacted and rearranges to **PQ** as soon as it is chromatographed.

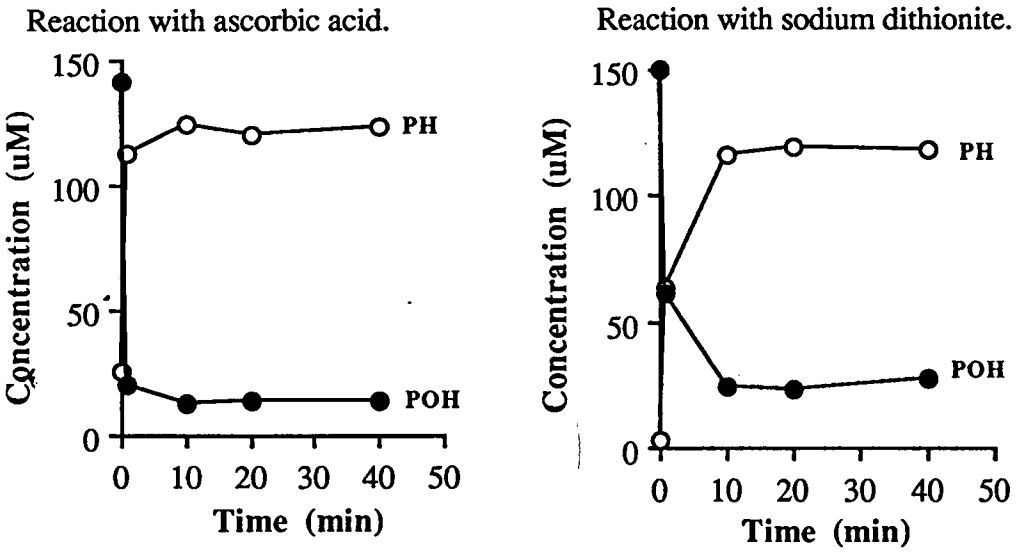
Fig.43. Chromatogram of the reaction between **POH** (0.15 mM) and dithiothreitol (3 mM) in 10 minutes.



III.5.2. HPLC analysis of the reaction of POH (0.15 mM) with various reductants.

III.5.2.1. Reactions in air.

Fig. 44. HPLC analysis of the reactions of **POH** (0.15 mM) with ascorbic acid (3 mM) and with sodium dithionite (3 mM) in air.



III.5.2.2. Reactions under nitrogen.

Fig. 45. HPLC analysis of the reaction of **POH** and **PQ** (0.15 mM) with ascorbic acid (3 mM).

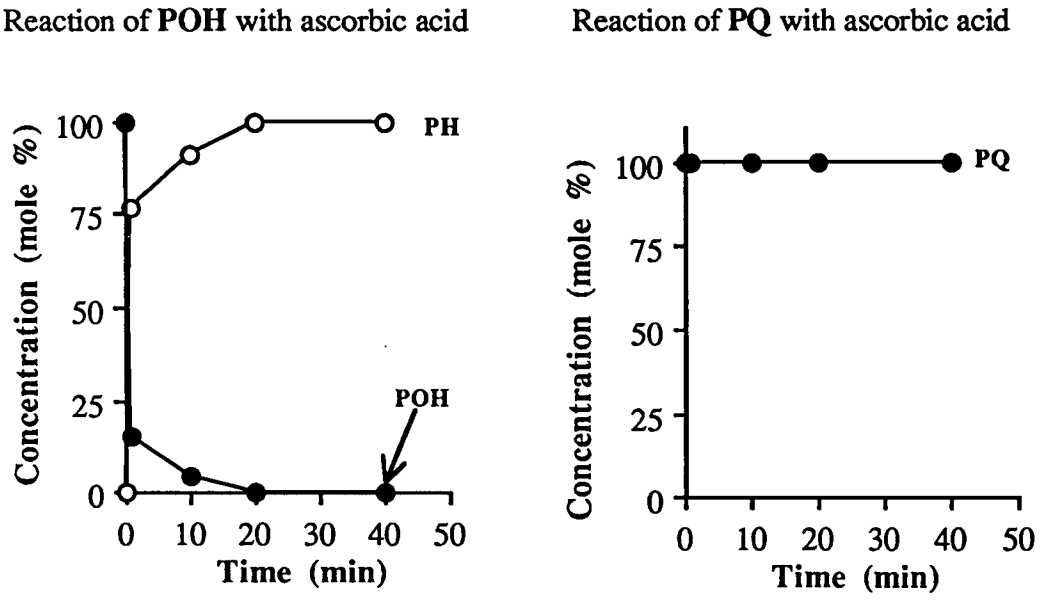


Fig. **45** (p. 181) compares the reactions of **POH** and **PQ** with ascorbic acid, **POH** is completely reduced to **PH** in 20 min, while there is no reaction between **PQ** and ascorbic acid.

Fig. 46. HPLC analysis of the reaction of **POH** (0.15 mM) or **PQ** (0.15 mM) with sodium dithionite (3 mM).

Reaction of **POH** with sodium dithionite. Reaction of **PQ** with sodium dithionite.

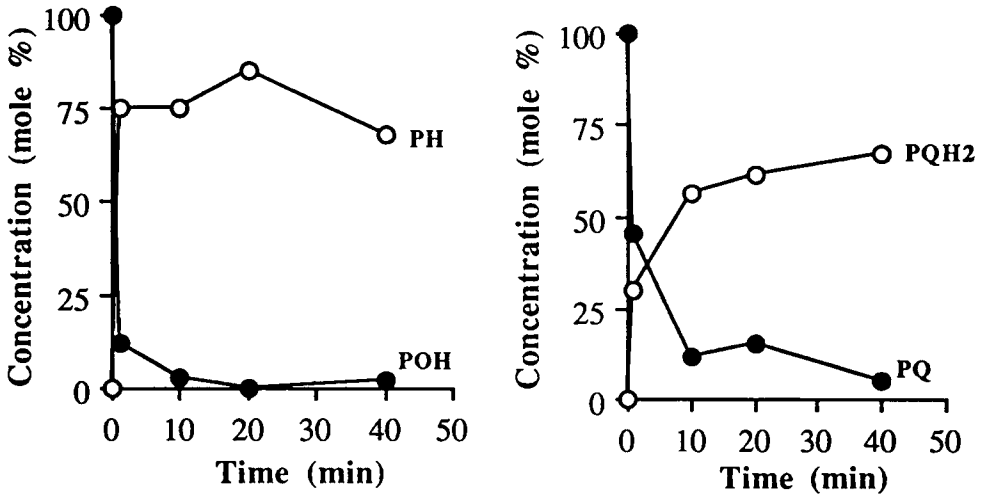


Fig. **46** (p. 182) compares the reactions of **POH** and **PQ** with sodium dithionite, **POH** is almost completely reduced to **PH** within 20 min and no PQH_2 formed while **PQ** is reduced more slowly (70% in 40 min) to PQH_2 and no **PH** is formed.

Fig. 47. HPLC analysis of the reaction of **POH** (0.15 mM) or **PQ** (0.15 mM) with DTT (3 mM).

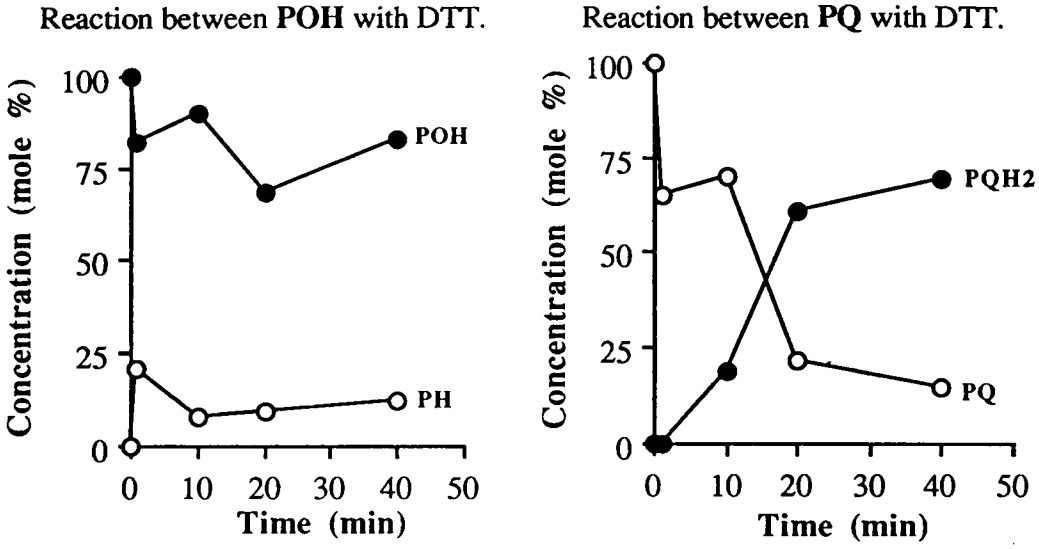


Fig. **47** compares the reactions of **POH** and **PQ** with DTT. DTT brings about a small (about 20%) reduction of **POH** to **PH** which occurs rapidly and then comes to a halt, while $\mathbf{PQH_2}$ is not formed. By contrast DTT brings about a 70% reduction of **PQ** to $\mathbf{PQH_2}$ in 40 min and **PH** is not formed,

Fig. **48** (p. 184) shows that **POH** is reduced readily to **PH** by KI in acid solution. In fact KI in acid appeares to be as good a reducing agent for **POH** as ascorbic acid and sodium dithionite. $\mathbf{PQH_2}$ is not formed in this reaction. KI in neutral solution however has no effect on **POH** and neither does 0.1M KCl in pH 3 acid. However, KI in acid pH 3 has no effect on **PQ**.

Fig. 48. HPLC analysis of the reaction of **POH** with KI (0.1M, pH 3), extracted with hexane/ethyl acetate 9:1.

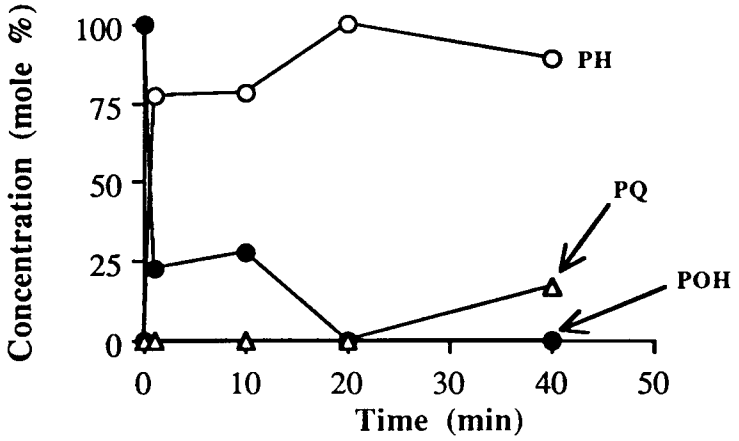
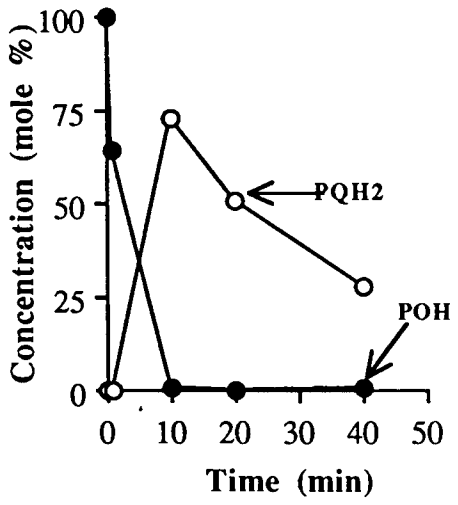


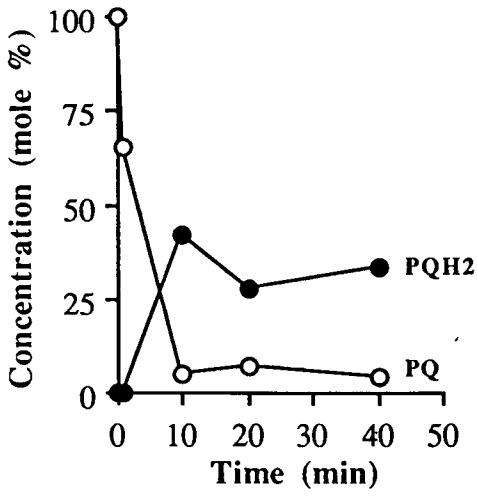
Fig. **49** (p. 184), compares the reduction of **POH** and **PQ** with NADH plus FAD. **POH** is converted to 75% of the theoritical PQH_2 within 10 min and the concentration of PQH_2 then begins to fall presumably due to reoxidation of the PQH_2. **PH** is not formed. By contrast **PQ** is reduced to the PQH_2 and to the extent of about 40% in 10 min and the PQH_2 then begins to reoxidise. No **PH** is formed. It thus appears that NADH plus FAD can reduce **POH** equally as well, if not better than **PQ**.

Fig. 49. HPLC analysis of the reaction of **POH** (0.15 mM) or **PQ** (0.15 mM) with NADH (2.4 mM) plus FAD (3 mM).

Reaction between **POH** with NADH plus FAD



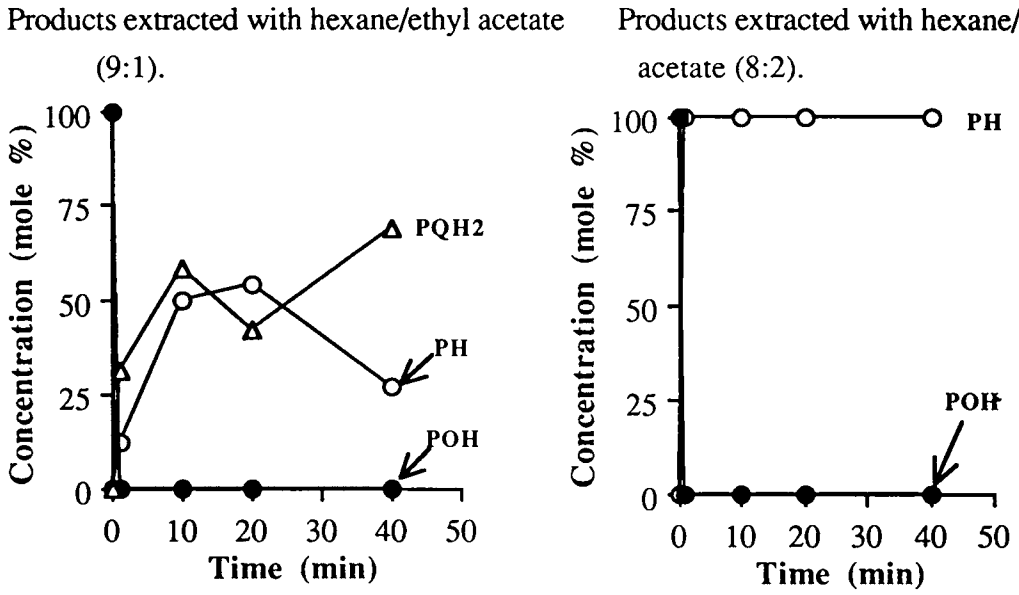
Reaction between **PQ** with NADH plus FAD.



III.5.2.3. Reaction of POH with various reductants. Influence of extracting solvent.

Failure to observe PQH_2 in the **POH** reductions, apart from the NADH plus FAD reaction, led to the thought that it was not being extracted effectively from the solution. Fig. **50** compares two extracting solvents of different polarity on the dithionite reduction of **POH**. In the hexane/ethyl acetate (9:1) reaction PQH_2 and **PH** were observed for the first time whereas PQH_2 had not been observed previously with this solvent. By contrast in the hexane/ethyl acetate (8:2) extraction only **PH** was observed as a product.

Fig. 50. HPLC analysis of the reaction between **POH** (0.15 mM) and sodium dithionite (3 mM), the products extractedwith hexane/ethyl acetate (9:1) or (8:2).



These very different results cannot be explained by the extracting solvent since the more polar solvent hexane/ethyl acetate (8:2) would have been expected to extract more PQH_2. The reason for the differences may lie in the speed with which sodium dithionite is used after dissolving it in solution. Thus dithionite in solution reacts very rapidly with oxygen, thereby reducing its effective concentration.

Fig. 51. HPLC analysis of the reaction between **POH** (0.15 mM) and DTT (3 mM), the products extracted with hexane/ethyl acetate (9:1) or (8:2).

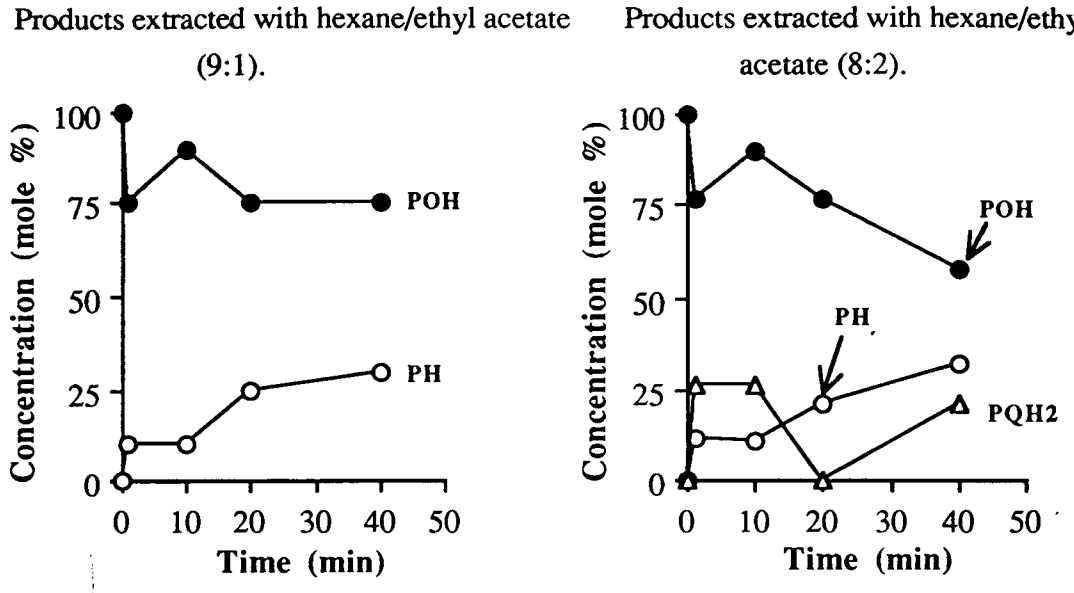


Fig. **51** compares the two extracting solvents on the reaction between **POH** and DTT. With the less polar solvent (hexane/ethyl acetate (9:1)), only **PH** is observed as a product, while, with the more polar solvent, some $\mathbf{PQH_2}$ was also observed. In this case it appears that the more polar solvent does manage to extract $\mathbf{PQH_2}$.

Fig. 52. HPLC analysis of the reaction of **POH** (0.15 mM) with NADH (2.4 mM) plus FAD (3 mM), extracted with hexane/ethyl acetate (8:2).

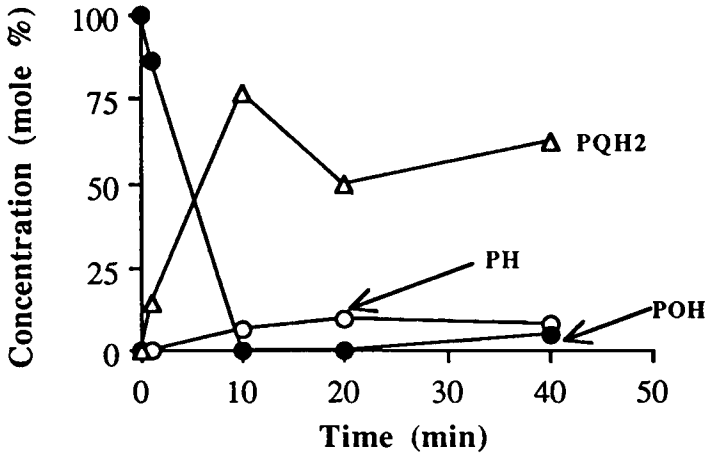


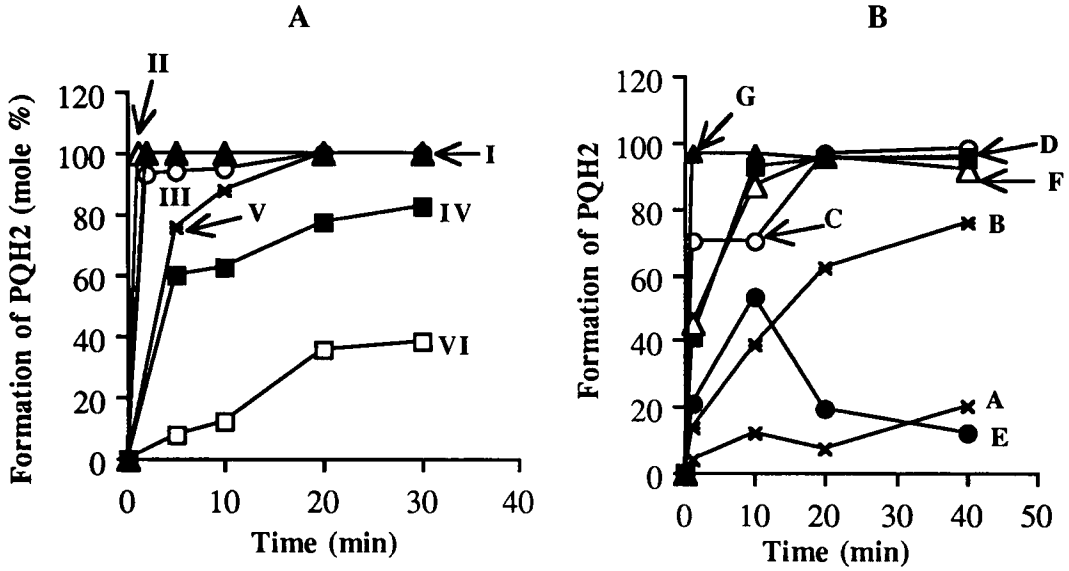
Fig. **52** (p. 188) shows the effect of the more polar extracting solvent (hexane/ethyl acetae (8:2)) on the reduction of **POH** by NADH plus FAD. The reaction

is similar to that shown in Fig. **47** except that a small amount of **PH** also appears in this reaction.

Fig. 53. Formation of PQH_2 from the reduction of **PQ** in homogeneous and heterogeneous solution.

A. Formation of PQH_2 from the reduction of **PQ** (10 mM, except in NADH/FAD reaction, the concentration of **PQ** was 7 mM) by several reductants (100 mM) in homogeneous solution. Presented as formation of PQH_2 (mole %). **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).

B. Formation of PQH_2 from the reduction **PQ** (20 mM) by several reductants in heterogenous solution (hexane/water or hexane/phosphate buffer pH 7 for the reaction with NADH plus FAD). **A** = ascorbic acid (400 mM), **B** = sodium ascorbate (400 mM), **C** = sodium dithionite (400 mM), **D** = DTT (400 mM), **E** = NADH (40 mM) plus FAD (200 mM) in water, **F** = NADH (20 mM) plus FAD (100 mM) in phosphate buffer pH 7, **G** = NADH (160 mM) plus FAD (200 mM) in phosphate buffer pH 7).



It is obvious that the reduction of **PQ** to PQH_2 by NADH plus FAD is much faster in a pH 7 buffer solution than in water (Fig. **53B** line **E** and **F**).

DTT reduced **PQ** more rapidly in homogeneous solution (100% in 2 min) than in heterogeneous solution (93% in 10 min). Reduction of **PQ** by NADH (56 mM) plus FAD(70 mM) in homogeneous solution was very similar to that by NADH 160 (mM)

plus FAD (200 mM) in heterogeneous solutin. Reduction by sodium dithionite in a homogeneous system reached 93% at 2 min and was completed at 20 min, while in a heterogeneous system it led to 70% reduction at 1 min and 98% at 40 min Ascorbic acid in a homogeneous system reached 38% reduction at 30 min and only 20% at 40 min in a heterogeneous solution. At the beginning of the reaction sodium ascorbate also showed a much slower reduction in a heterogeneous system (39% at 10 min) than in a homogeneous system (63% at 10 min).

It is interesting to note that the reduction of **PQ** by the above mentioned reductants in heterogeneous system was slower than that in homogeneous system, especially at the beginning of the reaction, eventhough the concentration in the heterogeneous system was much higher than that in the homogeneous system.

III.9.2. EXPERIMENTS OF 8a-HYDROXYTOCOPHERONE (TOH).

The rate of decomposition of **POH** and **TOH** was determined by UV analysis and recorded in Fig. **54**, p. 189. Fig. **54** shows that **POH** decomposes to **PQ** much more rapidly than **TOH** decomposes to **TQ** (see p. 178).

Because the chromatograms of **TOH** analysed by NPLC (solvent C) showed quite a big shoulder on the **TQ** peak which was impossible to quantitate (Fig. **63**, p. 194), several attempts were made to separate these peaks, using different wavelengths for the analysis (250 nm, 270 nm, 280 nm and 290 nm) which did not give satisfactory results.

Then some experiments using different HPLC solvents were performed as follows:

I. Normal phase using solvent system A, which showed tailing of the **TH** peak (Fig. **56**, p. 191). **TOH** also appeared as a broad tailing peak before the **TQ** peak (from 3.4 - 5 min) (Fig. **55**, p. 191).

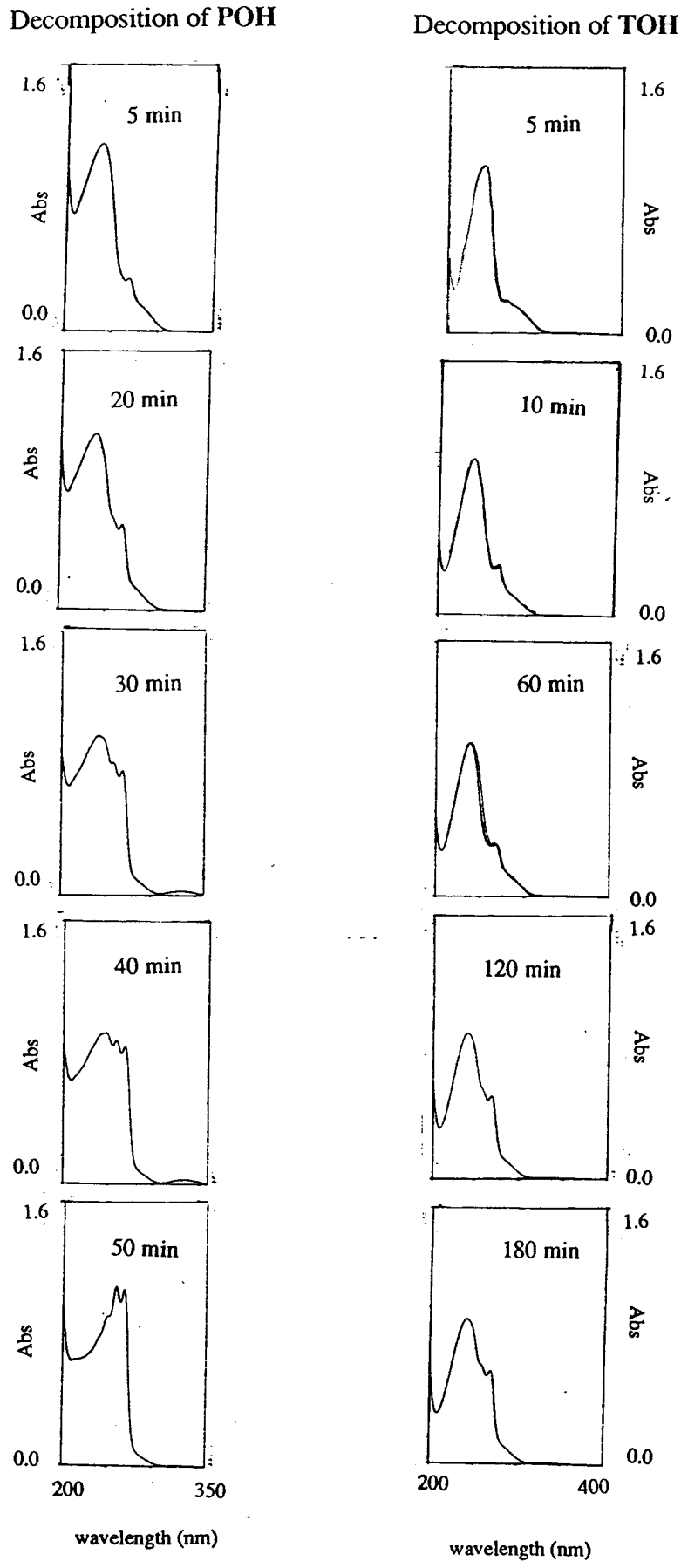


Fig. 54. Rate of decomposition of **POH** and **TOH** determined by UV spectrometry.

II. Cyano column (Millipore Waters, 8mm x 10 cm) with solvent system:

1. hexane/isopropanol (98:2) (solvent system C), showed **TH** and **TQ** peaks were very closed together and **TQ** was eluted first (Fig. **57**, p. 192).
2. hexane/isopropanol (99:1), **TH** and **TQ** were eluted at the same time and appeared as one peak (not shown).
3. hexane/isopropanol (99.25:0.75); **TH** and **TQ** peaks appeared very close together and **TH** eluted first.(Fig. **58**, p. 192).
4. hexane-/isopropanol (99.5:0.5) showed satisfactory resolution of **TH** and **TQ** but failed to give good resolution for the $\mathbf{TQH_2}$ which also important to detect (Fig. **59**, p. 192 and Fig. **60**, p. 193). The $\mathbf{TQH_2}$ peak also showed some unchanged **TQ** as an impurity.

Thus, depending on the polarity of the eluting solvent the cyano column behaved as a reverse phase (Fig. **57**) or a normal phase (Fig. **59**) column.

III. Reverse phase liquid chromatography (solvent J : methanol/milliQ water (98:2)). It does not show **TOH** as a tailing peak or a shoulder, but as a **TQ** peak (Fig. **62**, p. 193). It also gave a good resolution between the **TH** and **TQ** peak (Fig. **61**, p. 193). The disadvantages of this system were that **TQ** and **TH** have long retention times (11.5 and 17.1 min respectively), which also explains why all **TOH** was converted into **TQ** on the column (no shoulder peak of **TOH** was observed). Also the protocol was more complicated than as in NPLC system which was not good for the unstable compounds such as **TOH** and $\mathbf{TQH_2}$.

Fig. 55. Chromatogram of **TOH** on silica column with hexane/ethyl acetate (4:1) as the HPLC solvent.(measured at 280 nm). It showed a broad tail before **TQ** peak and a small shoulder at 3.97, near the **TH** peak.

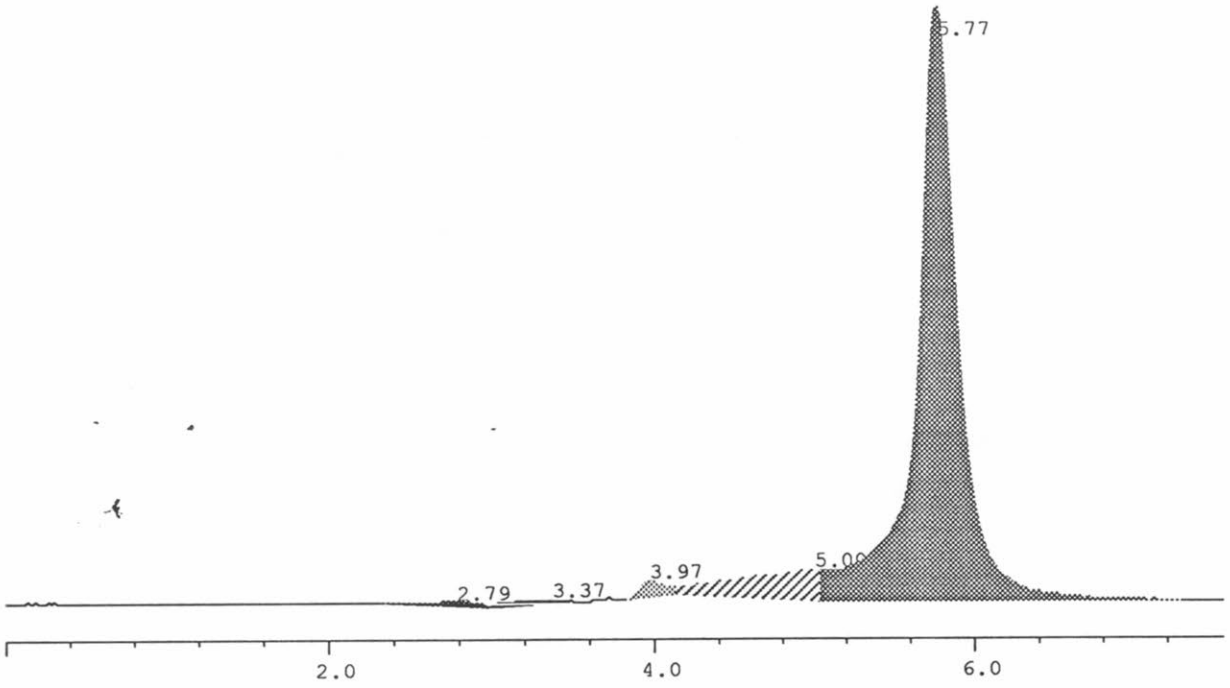


Fig.56. Chromatogram of **TH** and **TQ** standards in silica column with hexane/ethyl acetate (4:1)(solvent A) as the mobile phase (measured at 280 nm). RT of **TQ** was 5.6 and **TH** 3.5 minutes and tailing.

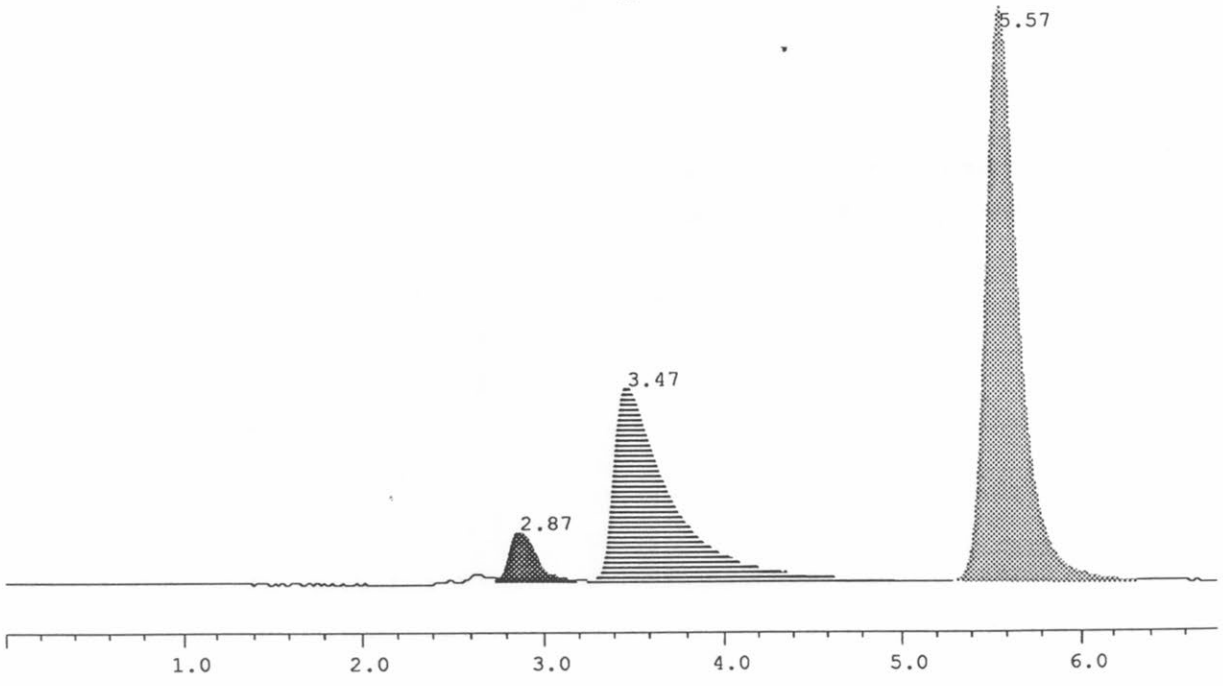


Fig. 57. Chromatogram of **TH** and **TQ** standards on the cyano column with hexane/isopropanol (98:2)(solvent system C) as the mobile phase (measured at 280 nm). RT of **TQ** was 2.1 and **TH** 2.4 minutes.

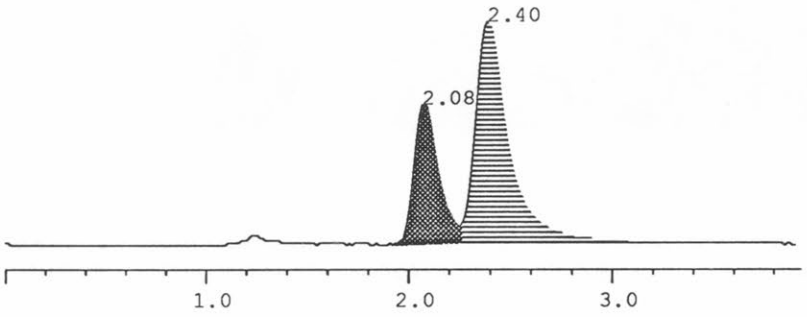


Fig. 58. The chromatogram of **TH** and **TQ** standards on the cyano column with hexane/isopropanol (99.25:0.75) as the mobile phase. RT of **TH** was 3.6 and **TQ** 3.9 minutes.

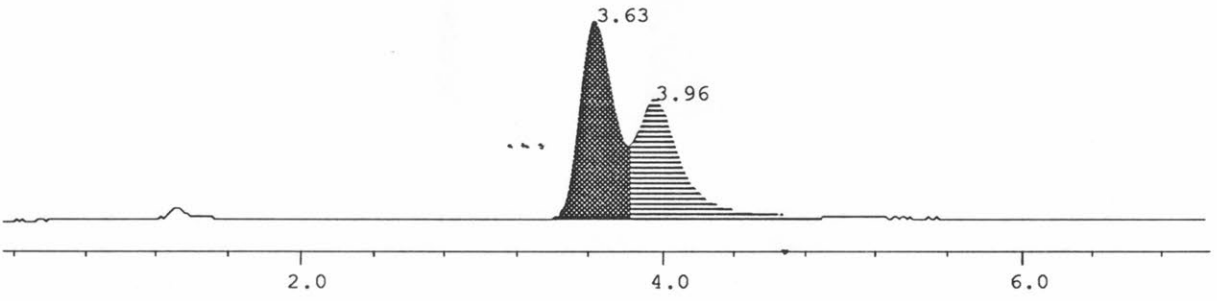


Fig. 59. Chromatogram of **TH** and **TQ** standards on the cyano column with hexane-isopropanol (99.5:0.5) as the mobile phase. RT of **TH** was 4.7 and **TQ** 5.9 minutes.

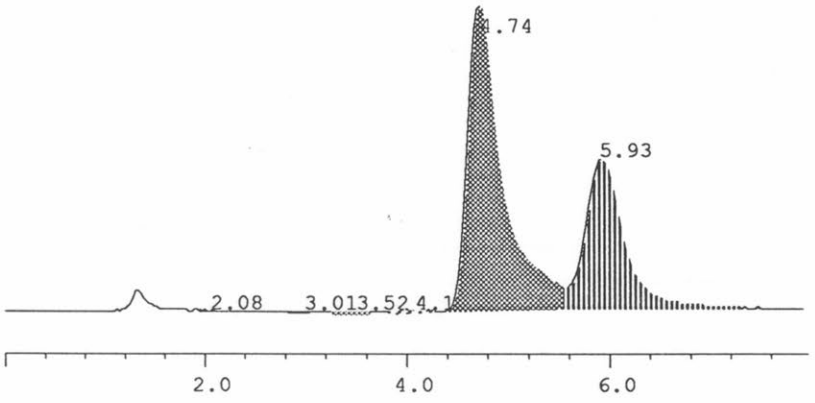


Fig. 60. Chromatogram of **TQH_2** on the cyano column with hexane-isopropanol (99.5:0.5)

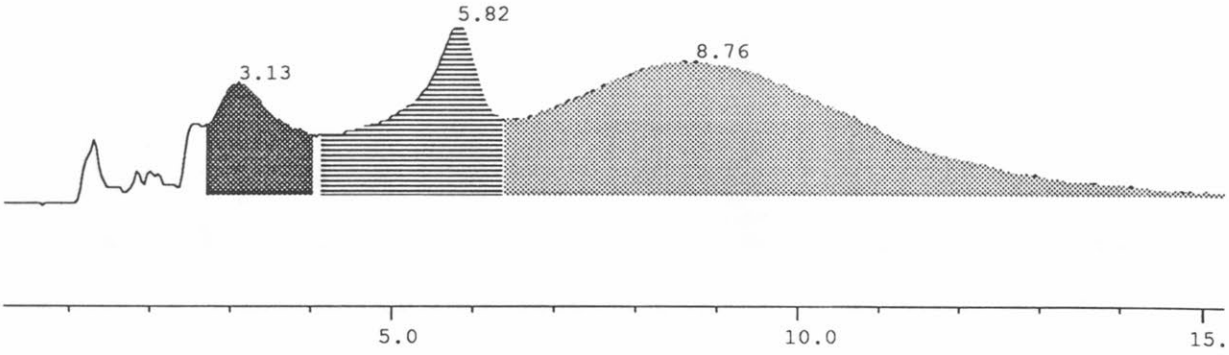


Fig. 61. Chromatogram of standard **TH** and **TQ** analysed by RPLC (with methanol/milliQ water (98:2)(solvent J).(RT of **TH** was 17.1 min and **TQ** 11.5 min.

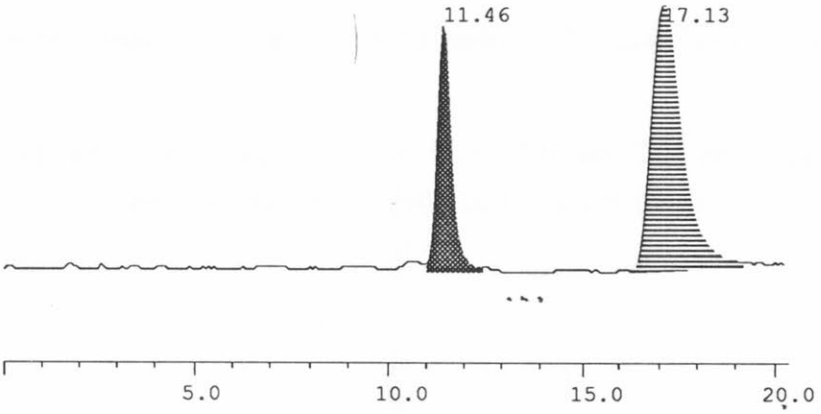


Fig. 62. Chromatogram of **TOH** analysed by RPLC (with methanol/milliQ water (98:2)(solvent J).(RT of **TQ** was shifted to 13.4 min)

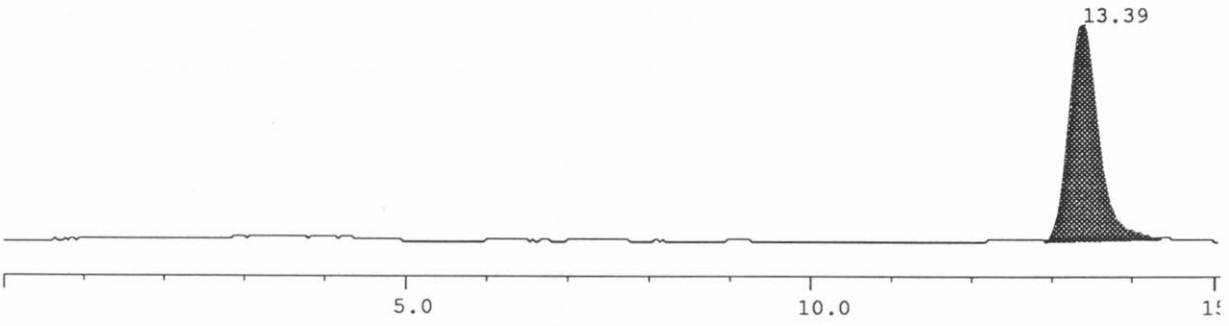
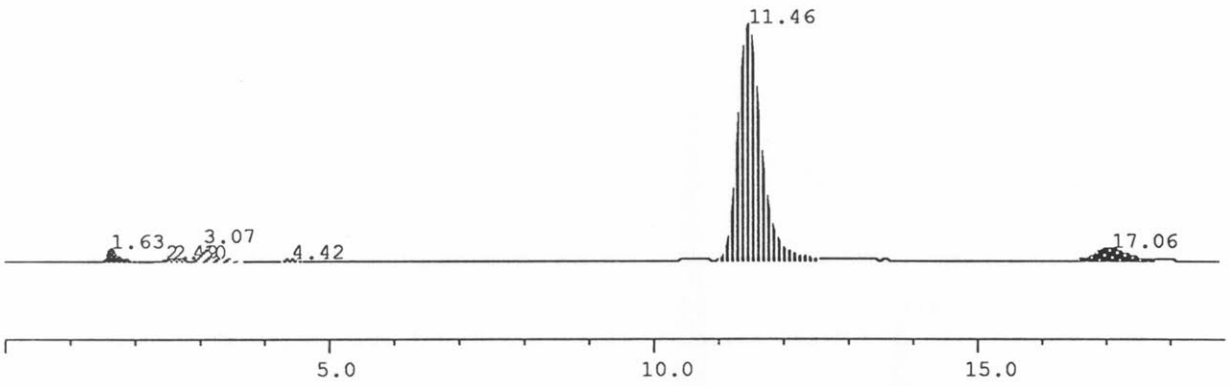


Fig. 63. Chromatogram of the reaction between **TOH** and ascorbic acid (10%) for 40 min, analysed by RPLC, solvent (RT of **TQ** is 11.5 min and **TH** 17.1 min).



Because of these disadvantages, all the experiments with **TOH** were performed on a normal phase system with solvent system C (hexane/isopropanol (98:2)) and the quantitation only based on the formation of **TQ** and **TQH_2**, and the reformation of **TH**.

Fig. 64. Chromatogram of standard **TH** and **TQ** analysed by NPLC (solvent C: hexane/isopropanol (98:2))(RT of **TH** was 3.9 and **TQ** 5.1 min).

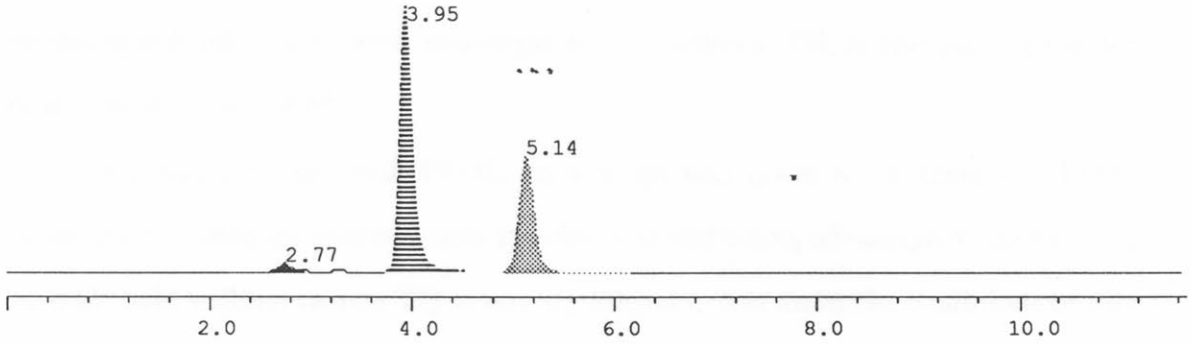


Fig. 65. Chromatogram of **TOH** immediately after preparation analysed by NPLC (solvent C: hexane/isopropanol (98:2))(RT of **TQ** was 5.1 min).

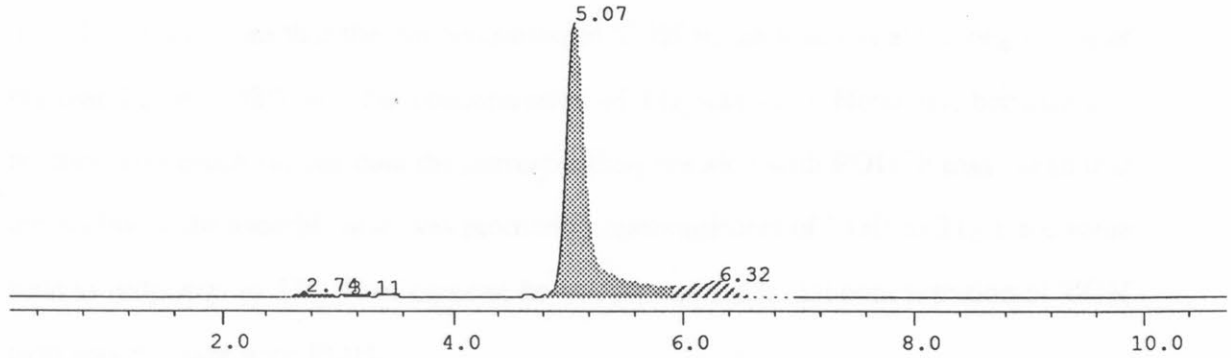
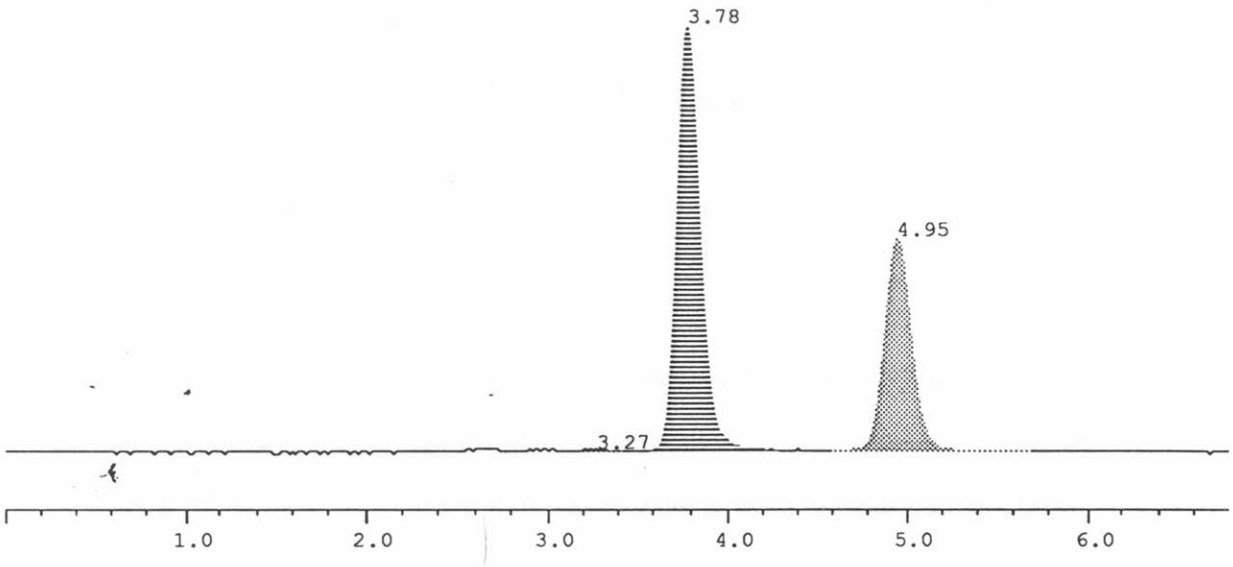


Fig. 66. Chromatogram of the reaction between **TOH** and ascorbic acid (10%) for 25 minutes (RT of **TH** was 3.7 and **TQ** 4.9 minutes).

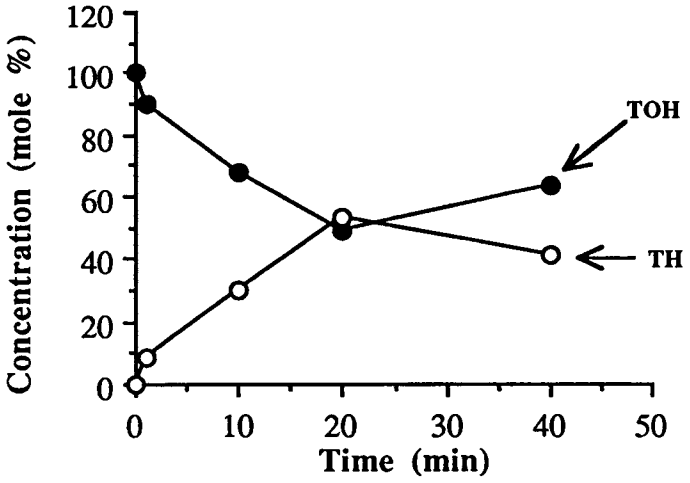


TOH and also its analogue, **POH** decomposed to **TQ** and **PQ** respectively on silica columns and were thus too unstable to analyze directly by HPLC. Both were measured indirectly by ascorbic acid-dependent reduction to **TH**, as also used by Liebler et al.(Liebler et al., 1989).

As was the case with **POH**, an attempt was made to estimate the **TOH** concentration using the reaction with ascorbic acid and taking advantage of the fact that ascorbic acid will not reduce **TQ** to any significant extent under the conditions of this reaction.

Fig. **67** (p. 196) shows that a maximum yield of **TH** (53%) was achieved in 20 min. This may mean that the concentration of **TOH** in the solution at the beginning of the reaction was 53% and the concentration of **TQ** was 47%. However, because this reaction was much slower than the corresponding reaction with **POH**, it may mean that the acidity of the ascorbic acid was promoting rearrangement of **TOH** to **TQ** at the same time as reduction to **TH**. Thus one can be less sure of the initial concentration of **TOH** than was the case with **POH**.

Fig. 67. HPLC analysis of the reaction of **TOH** (5 mM) with ascorbic acid (100 mM).

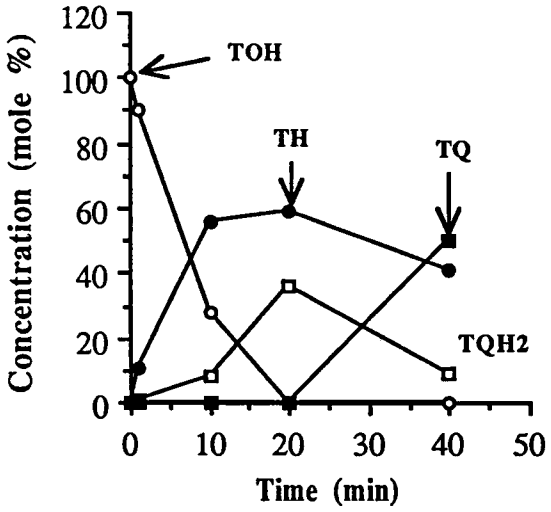


Therefore the reaction of **TOH** with various reductants assume that the initial concentration of **TOH** was the same in every case and the yields of products are reported as percentages of the **TH** which was originally used to generate the **TOH.**

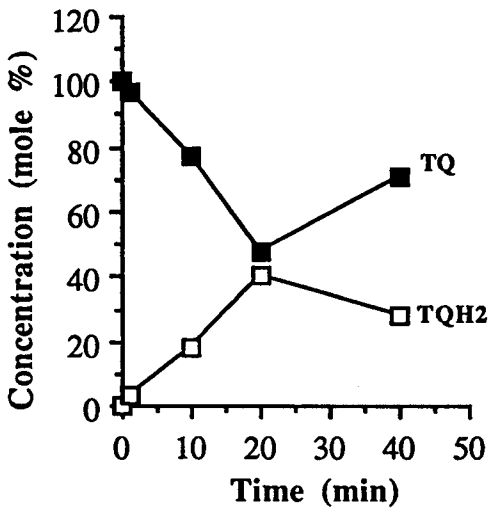
Treatment with sodium dithionite (stochiometry 1:20) resulted in 56% reduction to **TH** at 10 min and 59% at 20 min. **TOH** was also reduced to **TQH2** which reached the maximum (36%) at 20 min and decreased to 9% at 40 min, while at the same time **TH** also decreased from 59% to 41% and **TQ** formed (50%) (Fig. **68**, p. 196).

Fig. **68**. HPLC analysis of the reaction of **TOH** (5 mM) and **TQ** (5 mM) with sodium dithionite (100 mM).

Reaction of **TOH** with sod. dithionite.



Reaction of **TQ** with sod. dithionite.



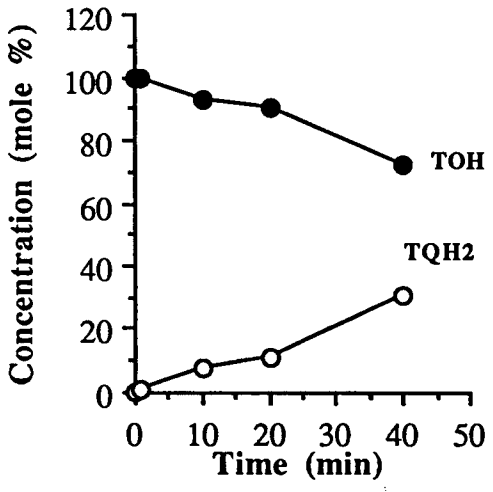
It appeared that reduction of **TOH** is better with sodium dithionite than with ascorbic acid (at the same concentration), because it reached maximum **TH**

concentration faster (at 10 min) than did ascorbic acid (20 min) and it also formed TQH_2 as well (at 20 min the combined concentrations of **TH** and TQH_2 accounted for 96% of the initial **TOH**). Sodium dithionite is very unstable and in solution it decomposes to thiosulfate and bisulfite and acquires an acid reaction which explains its ability to convert **TOH** into **TH** (which requires acid reaction) and TQH_2 as well. The instability of sodium dithionite suggests why the reduction of **PQ** or **TQ** in dilute solution (0.15 mM **PQ** or 5 mM **TQ**) gave a low yield of PQH_2 or TQH_2. Sodium dithionite reacts rapidly with and is destroyed by the oxygen in the air. In dilute solution, the concentration of dithionite may be reduced to such a level that it cannot protect PQH_2 or TQH_2 against reoxidation to **PQ** and **TQ** respectively.

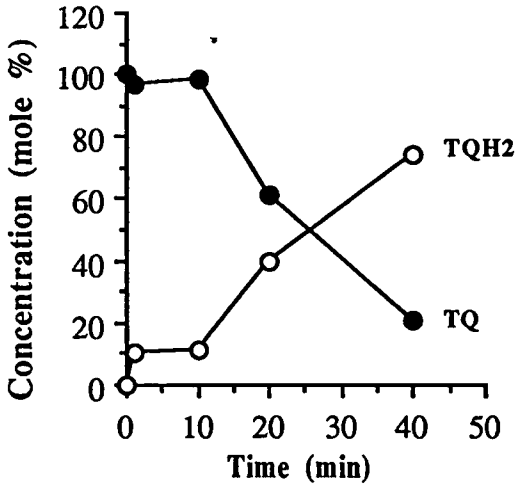
NADH plus FAD reduced both **TOH** and **TQ** to TQH_2, the higher yield (75%) being obtained in the **TQ** reaction.

Fig. 69. HPLC analysis of the reaction of **TOH** (5 mM) and **TQ** (5 mM) with NADH (80 mM) plus FAD (100 mM).

Reaction of **TOH** with NADH plus FAD



Reaction of **TQ** with NADH plus FAD

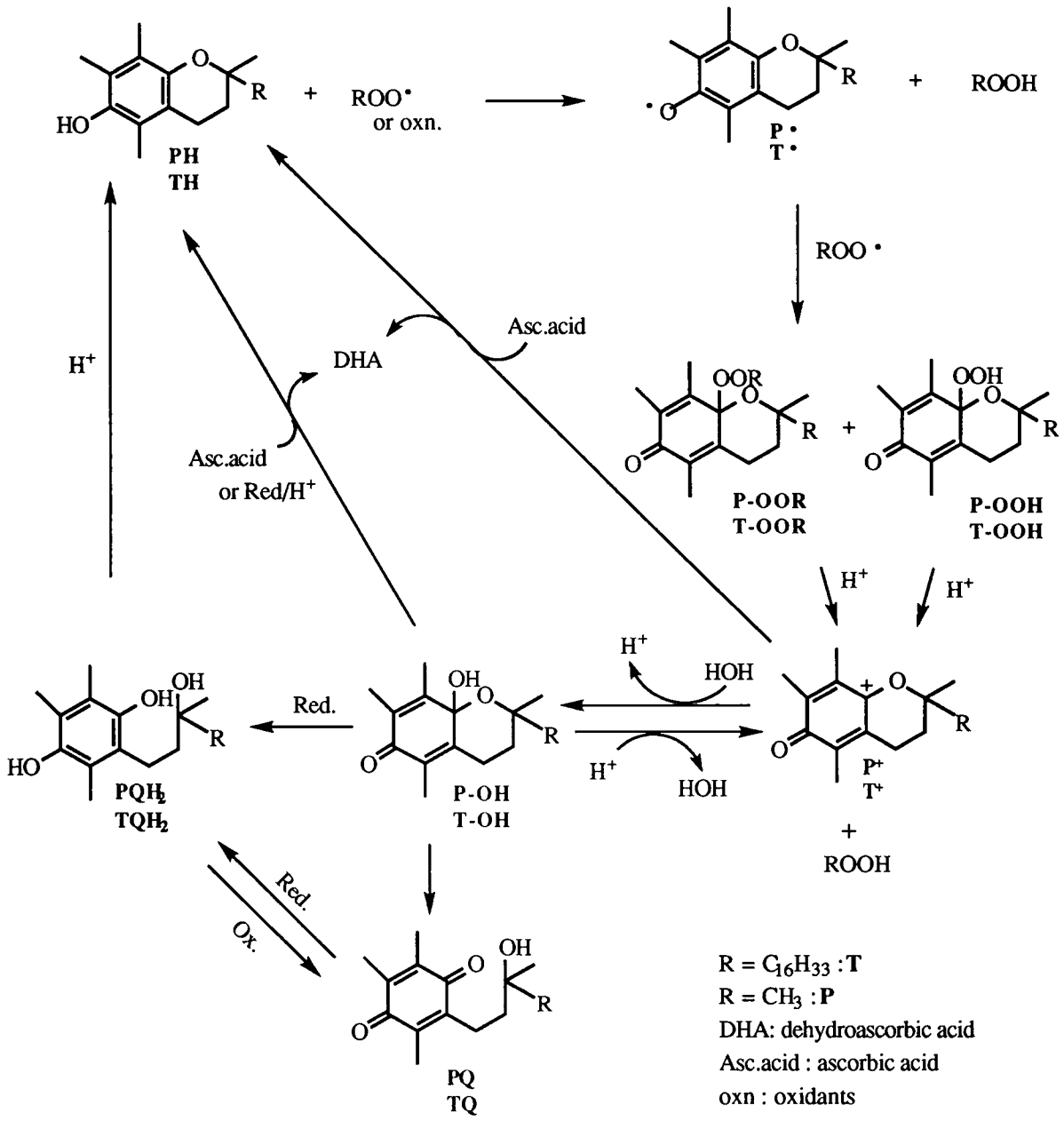


On the other hand, the treatment of its analogue, **POH** with ascorbic acid resulted in a much faster regeneration of the **POH** to **PH**. It was completed at 20 minutes without decreasing at 40 minutes and no rearrangement to **PQ**, while with sodium dithionite the maximum was the same as in **TOH**, which was 20 minutes

followed by a decrease at 40 minutes, and more over, there was no **PQH_2** formation could be observed. This may be pointed to the concentration dependent, as it was impossible to make a higher concentration of **POH** in our system (the concentration of **TOH** was 5 mM compared to 0.15 mM of **POH**).

As the control, reactions of **TQ** and its analogue, **PQ** with the same reductants and the same conditions as in **TOH** and its analogue, **POH** was also performed. The control reactions showed that neither **TQ** nor **PQ** can be regenerated into **TH** or **PH**.

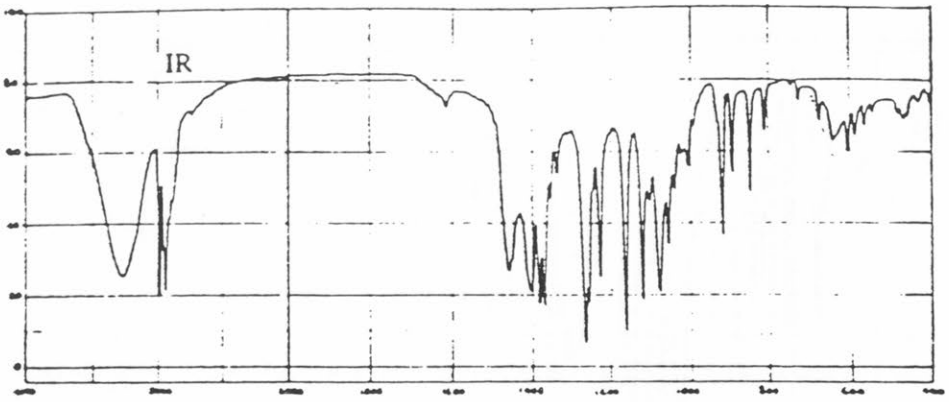
TH reacts with peroxyl radical by hydrogen atom transfer (scheme **12**, p. 199) to form a hydroperoxide and **TH** radical (**T•**), a resonance-stabilized phenoxyl radical which does not easily participate in radical propagation reactions. It is believed that **T•** is reduced back into **TH** by ascorbic acid thus creating at **TH** <----> **T•** redox cycle and explaining how a very small amount of **TH** can protect a relatively large number of phospholipid molecules. Some of the **T•** which do not complete redox cycle with ascorbic acid can undergo further reaction with peroxy radicals and may form 8a-(alkyldioxy)tocopherones. Acid-catalyzed loss of the 8a-(alkyldioxy) moiety yields T^+ which then is reduced by ascorbic acid to **TH** and also can possibly reversibly hydrolyze to **TOH** which then rearranges to **TQ**. Thus, 8a-(alkyldioxy)tocopherone, 8a-hydroperoxytocopherone and **TOH** are reduced by ascorbic acid to **TH**, whereas hydrolysis and rearrangement convert them to **TQ** (Liebler & Burr, 1992) (see scheme **12**, p. 199).

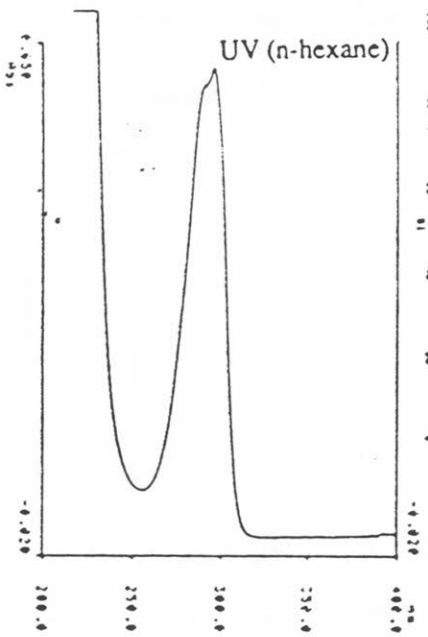


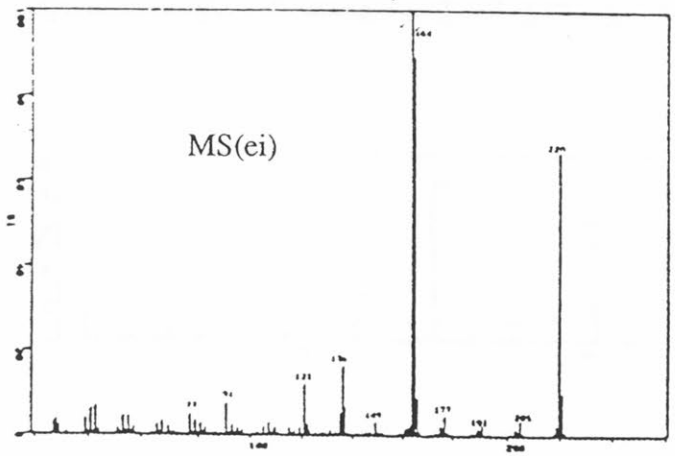
Scheme 12

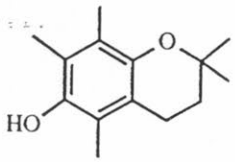
Present results have shown that **POH** and **TOH** can be readily reduced by a variety of reducing agents into **PH** and **TH**, if acid is present. In the absence of acid, for example sodium ascorbate and potassium iodide in neutral solution, reduction to **PH** and **TH** does not occur. Instead **POH** and **TOH** rearrange to **PQ** and **TQ** respectively. It is very interesting to note however that NADH plus FAD at neutral pH is capable of reducing both **POH** and **TOH** to PQH_2 and TQH_2 repectively.

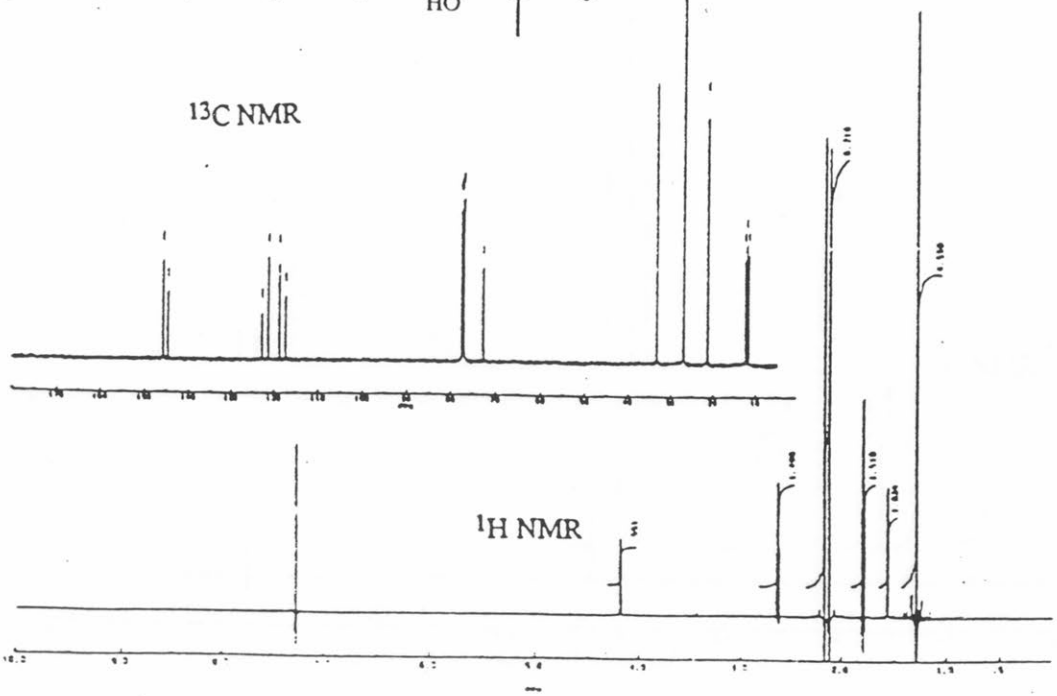
APPENDIX



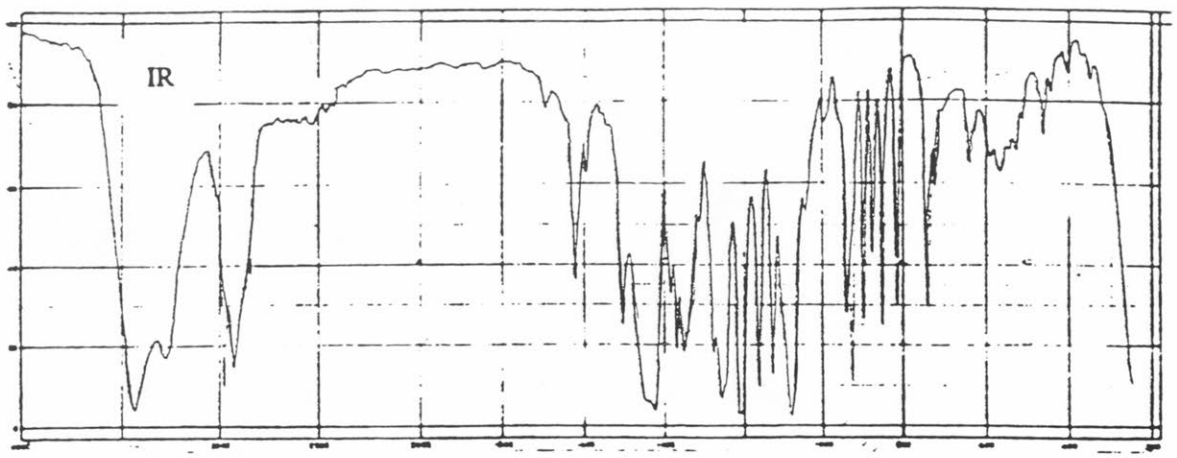


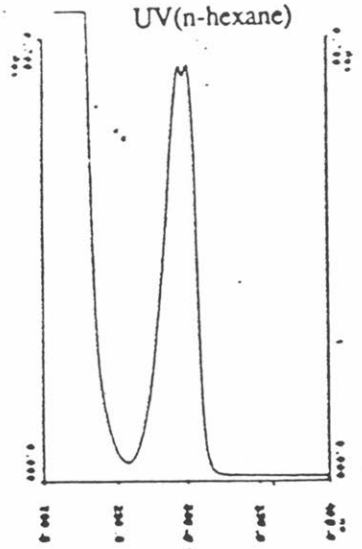


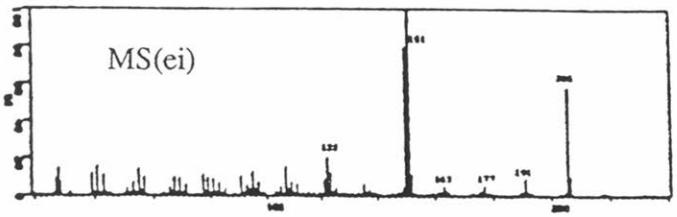


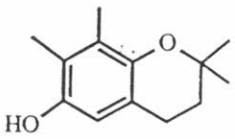


2,2,5,7,8-PENTAMETHYL-6-CHROMANOL (PH)

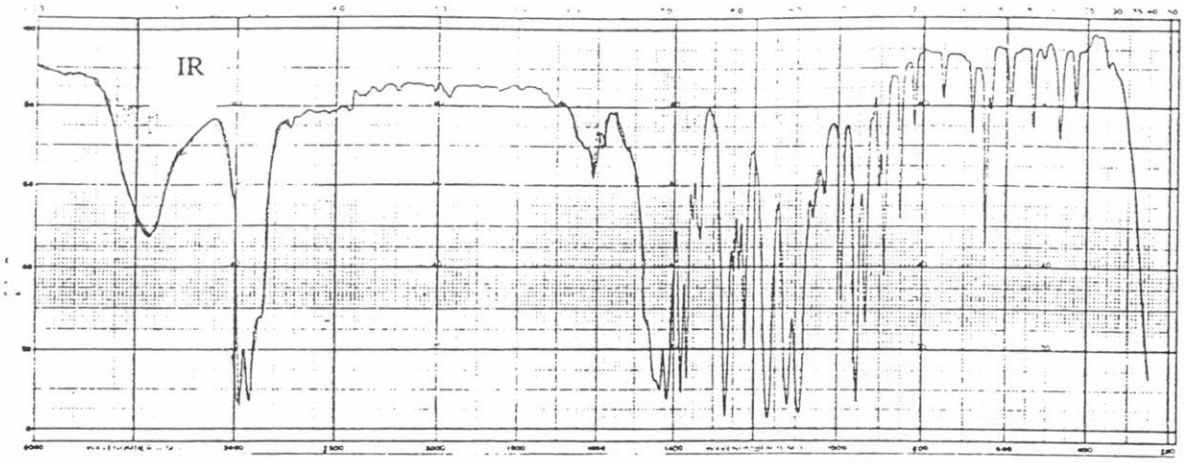


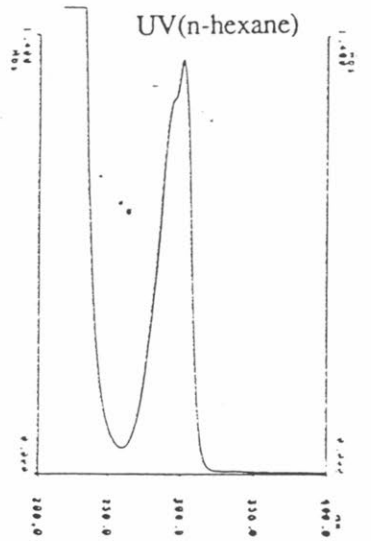


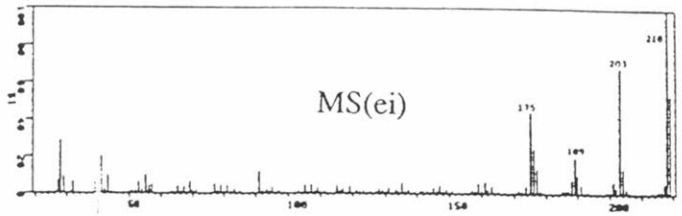


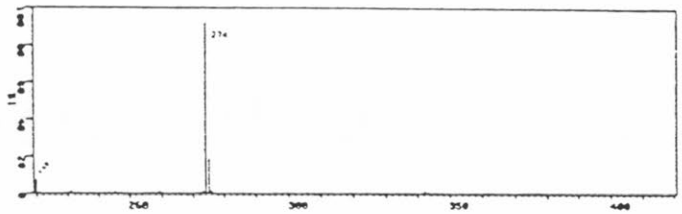


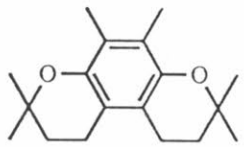
2,2,7,8-TETRAMETHYL-6-CHROMANOL (TMC)



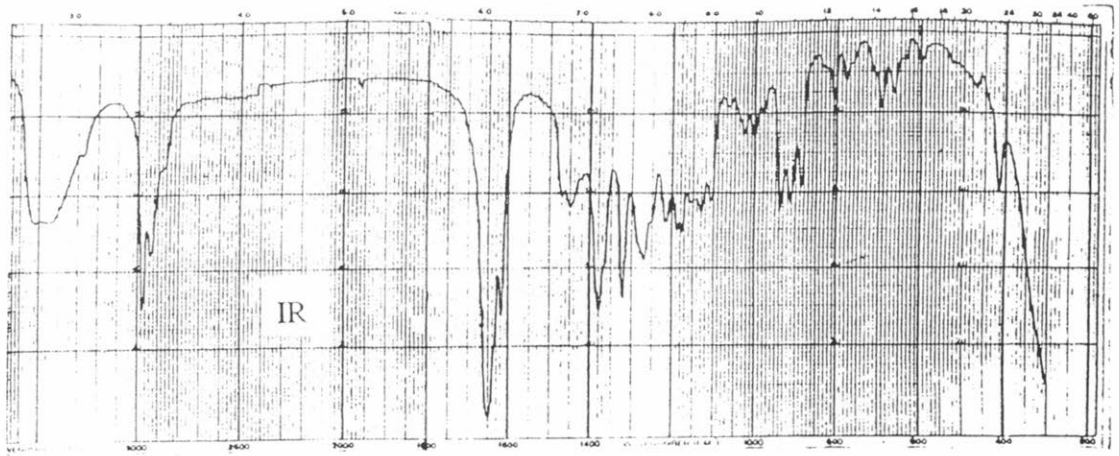


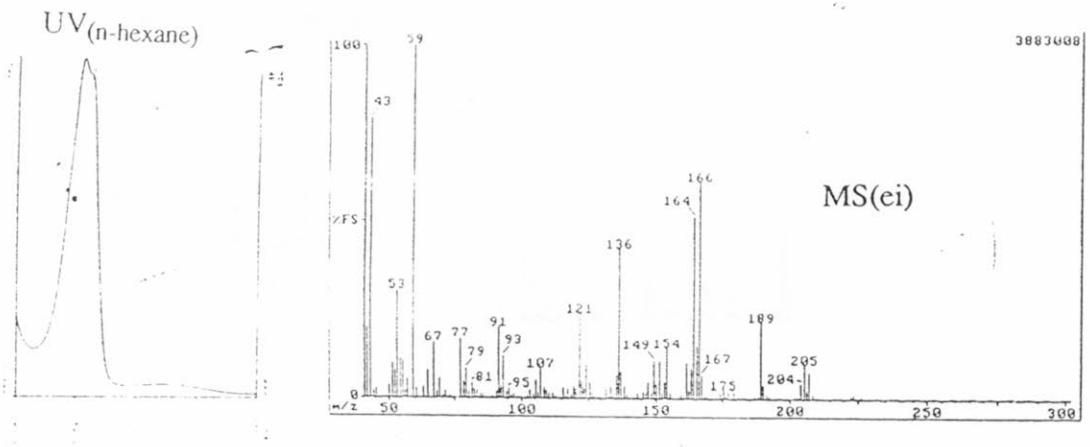


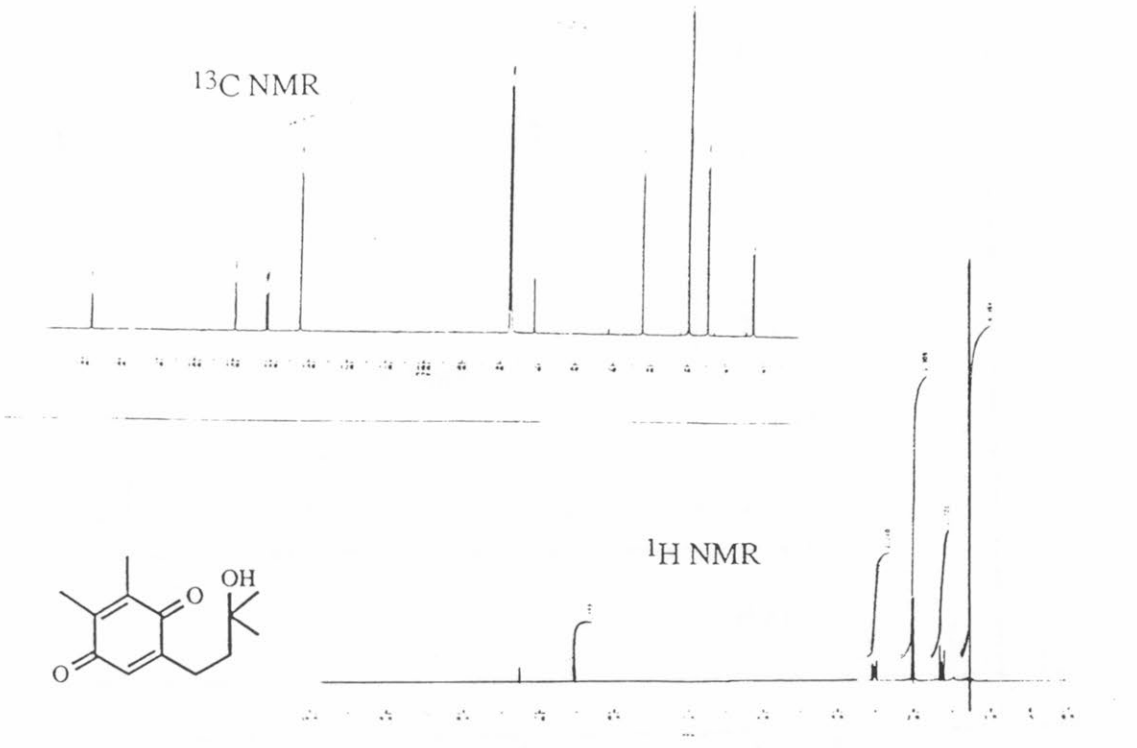




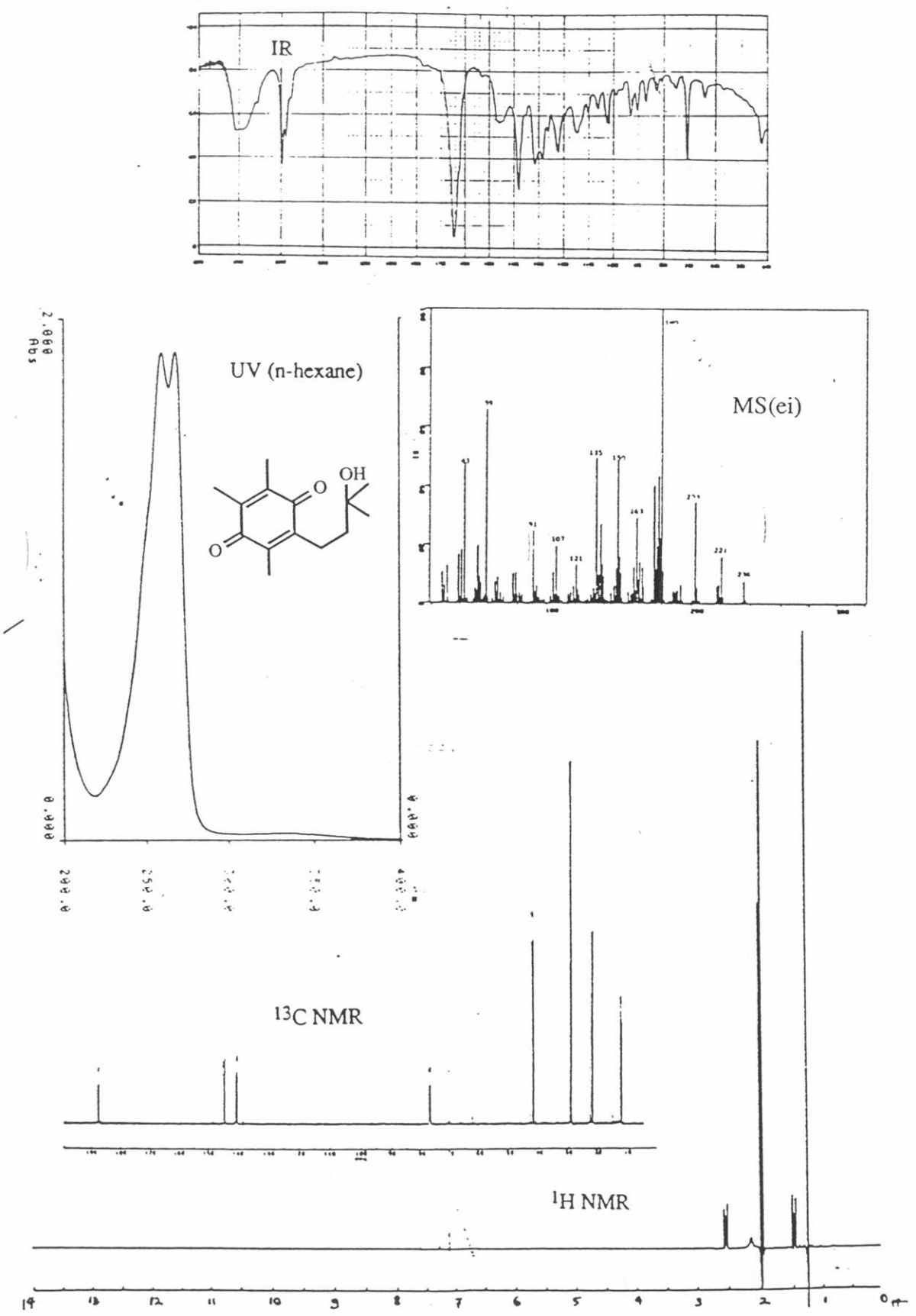
BICHROMAN OF TMC



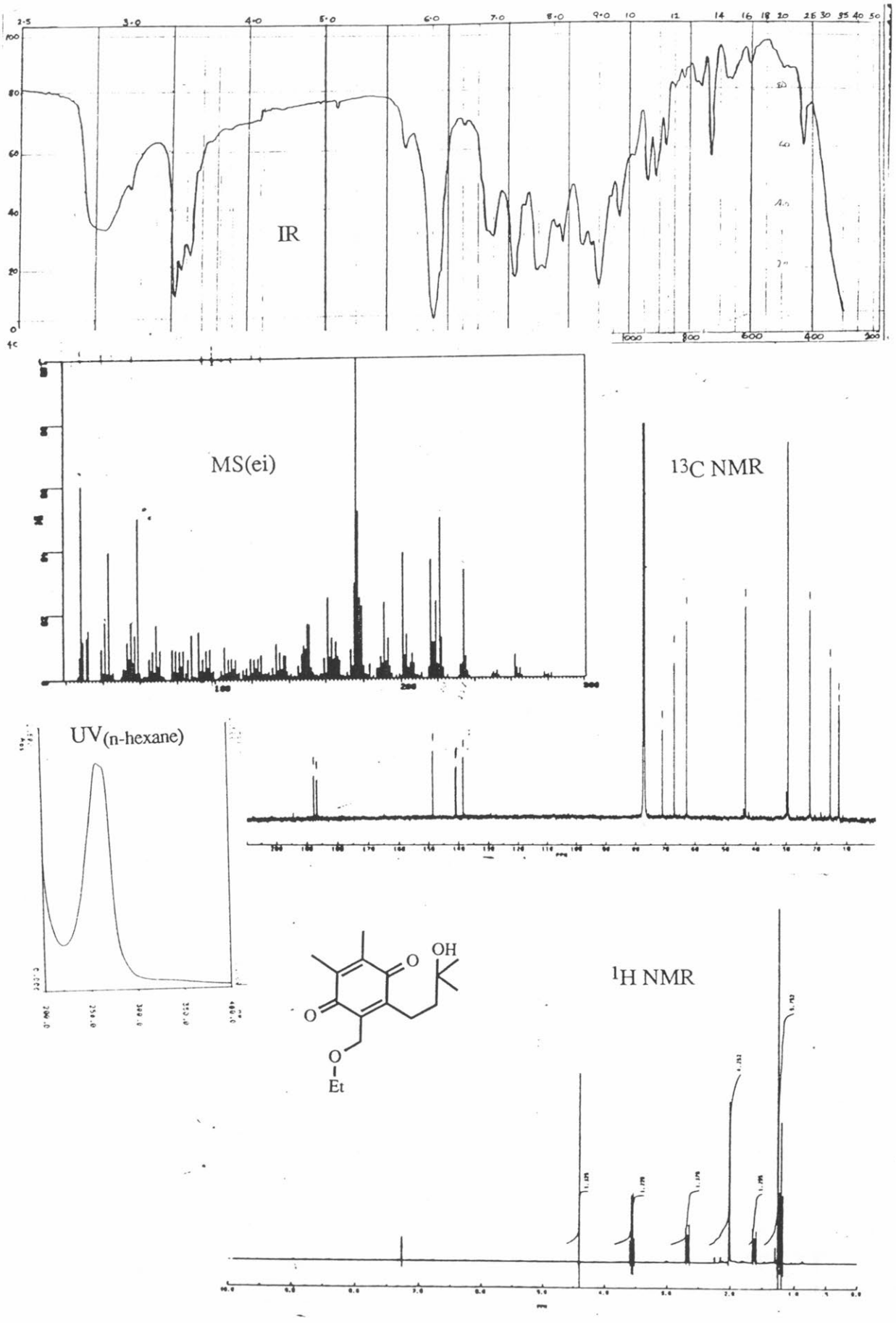




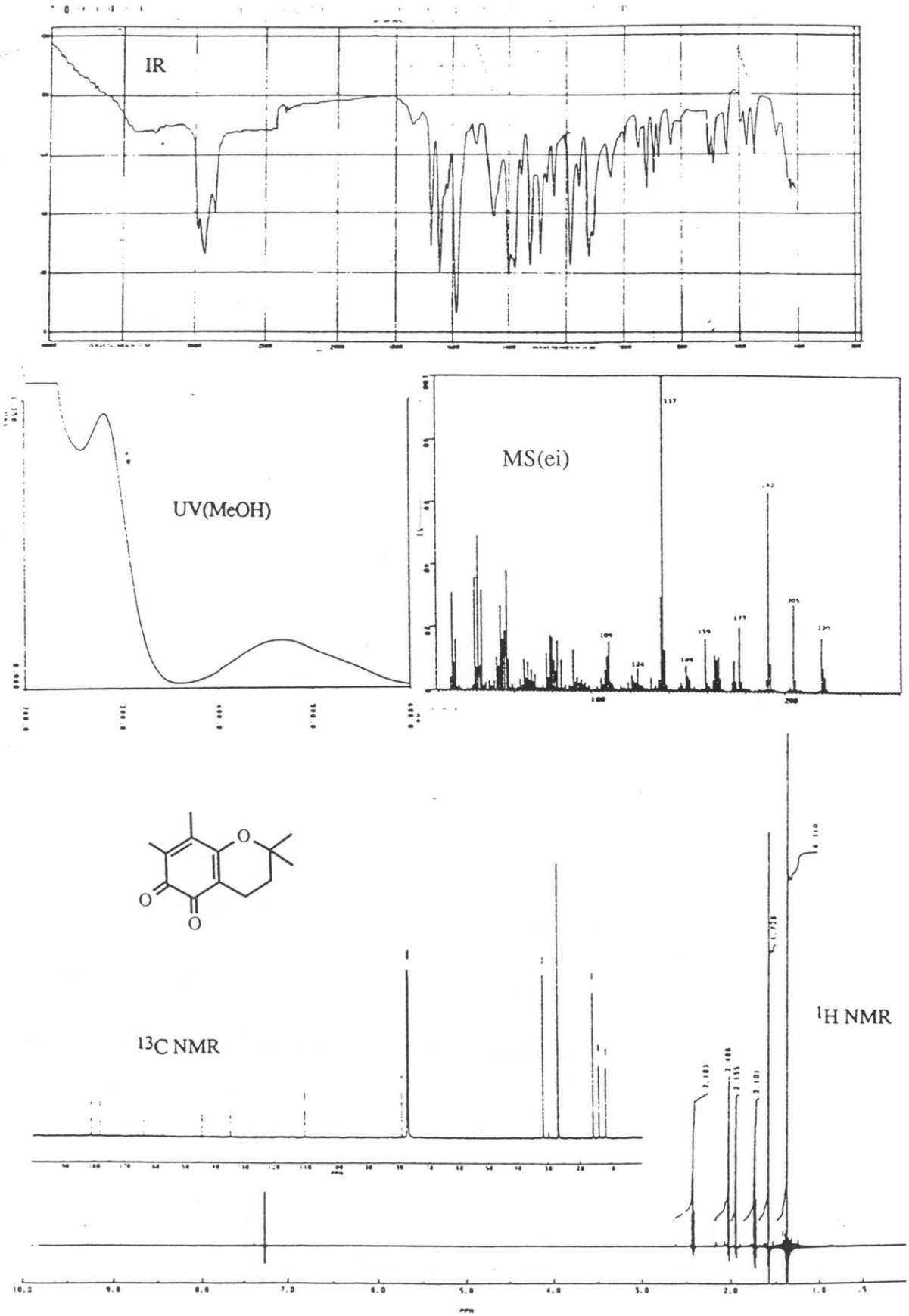
2-(3-HYDROXY-3-METHYLBUTYL-5,6-DIMETHYL-1,4-BENZOQUINONE (TMCQ)



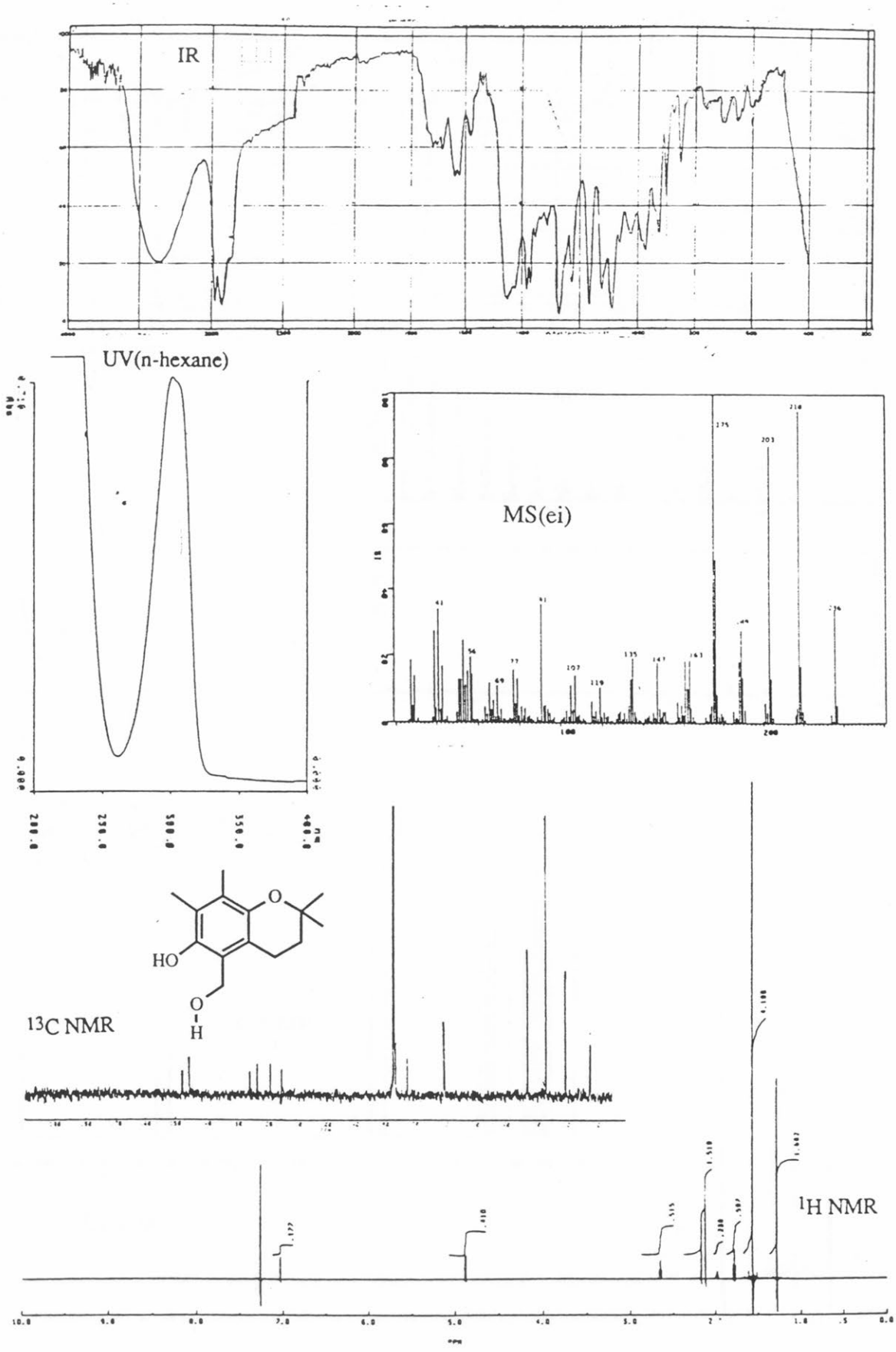
2-(3-HYDROXY-3-METHYLBUTYL)-3,5,6-TRIMETHYL-1,4-BENZOQUINONE (PQ)



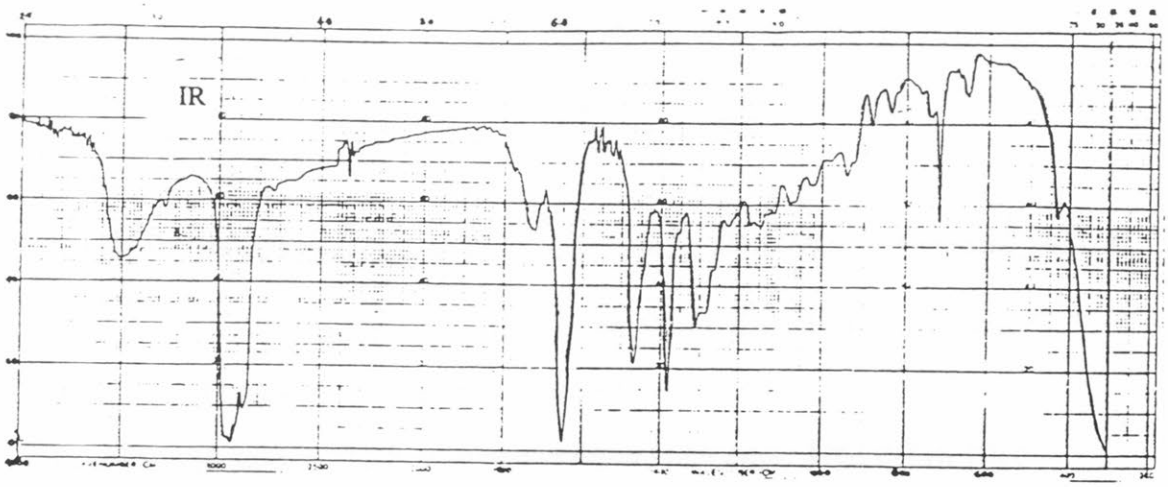
2-(3-HYDROXY-3-METHYLBUTYL)-3-ETHOXYMETHYL-5,6-DIMETHYL-1,4-BENZOQUINONE (PQ-Et)

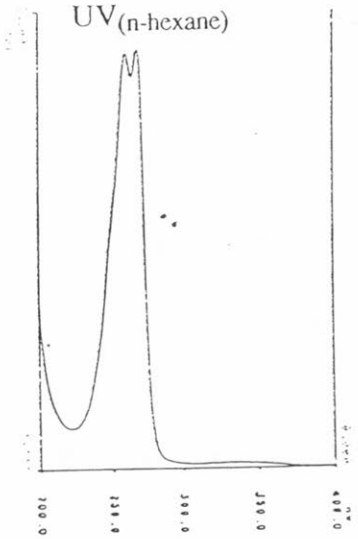


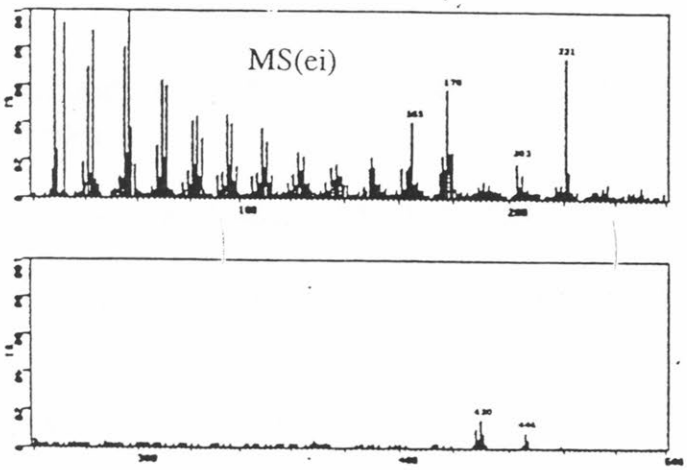
2,2,7,8-TETRAMETHYLCHROMAN-5,6-DIONE (PR)

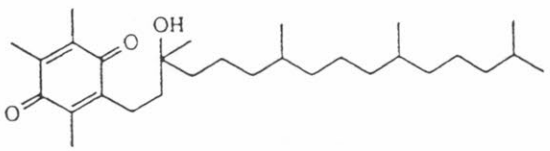


5-HYDROXYMETHYL-2,2,7,8-TETRAMETHYL-6-CHROMANOL (PM-OH).









α-TOCOPHERYLQUINONE (TQ)

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