*Journal of Chromatography*, 421 (1987) 71–82
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3808

# SIMULTANEOUS MEASUREMENT OF TOCOPHEROLS AND TOCOPHERYL QUINONES IN TISSUE FRACTIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH REDOX-CYCLING ELECTROCHEMICAL DETECTION

MICHAEL E. MURPHY* and JAMES P. KEHRER

*Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712-1074 (U.S.A.)*

(First received February 10th, 1987; revised manuscript received May 13th, 1987)

## SUMMARY

Tocopherols and tocopheryl quinones in lipid extracts of biological samples have primarily been measured using relatively insensitive ultraviolet detection methods. Oxidative electrochemical detection increases both the sensitivity and selectivity when measuring the tocopherols. We have developed an electrochemical detection system which sequentially reduces and oxidizes tocopheryl metabolites eluted from a reversed-phase high-performance liquid chromatography column, achieving sensitivities of about 0.05 pmol for both the tocopherols and their quinones. Using a rapid and mild extraction procedure, endogenous levels of $\alpha$- and $\gamma$-tocopherol as well as their respective quinones were measured in homogenates of chicken liver and muscle, and in dilute preparations of rat liver microsomes. The principle of the detection system could be applied to the determination of tocopheryl dihydroquinones, ubiquinols and ubiquinones with slight modifications to the mobile phase buffer and the electrode potentials of the detector.

## INTRODUCTION

Vitamin E is a biological chain-breaking antioxidant necessary for the maintenance of neuronal, muscular and reproductive tissues in a variety of animals [1,2]. In human blood cells, vitamin E is the major lipid-soluble antioxidant [1] and recent studies have demonstrated that the simultaneous analysis of $\alpha$-tocopherol ($\alpha$-TH) and its oxidation product, $\alpha$-tocopheryl quinone ($\alpha$-TQ), is useful in detailing the mechanisms of hydrogen peroxide-induced peroxidation of these cells [3].

It has been long recognized that the low concentrations of vitamin E and its oxidized metabolites in biological membranes present a severe barrier to a description of the biochemistry of vitamin E during oxidative injury. Before the

0378-4347/87/$03.50 

development of electrochemical detection (ED) techniques, the best methods of tocopherol analysis involved the separation of lipid extracts by reversed-phase high-performance liquid chromatography (HPLC) followed by UV or fluorometric detection [4]. These procedures allowed the measurement of $\alpha$-, $\beta$- and $\gamma$-tocopherols in 0.1-g tissue samples and plasma with sensitivity of about 120 pmol [4–6]. Trace quantities of $\alpha$-TQ and $\alpha$-tocopheryl dihydroquinone ($\alpha$-$TQH_2$) have also been measured in tissues using extraction procedures which included saponification followed by UV, fluorescence or $^{14}C$-labeling detection techniques [7–9]. Using extraction procedures which avoided saponification, other researchers failed to detect $\alpha$-TQ in 50 mg of rat liver [10]. However, the sensitivity of their method was limited to about 35 pmol (15 ng).

ED provides the most sensitive measurement of compounds which undergo redox reactions, although techniques to measure easily reduced compounds have not been developed as extensively as those for easily oxidized compounds [11]. The application of redox-cycling ED to the measurement of vitamin K compounds [12] suggested a similar technique would provide a more sensitive and selective method of measuring tocopheryl quinones. ED has recently been used for the measurement of $\alpha$-TH in biological samples and provided the most sensitive assay to date, with the ability to detect 0.2 pmol of the vitamin [13]. One recent paper also noted the ability of reductive ED to detect the small, endogenous levels of $\alpha$-TQ in tissue samples [11]. We have further developed this system and can simultaneously measure as little as 0.05 pmol (20 pg) of tocopherols and tocopheryl quinones in tissue extracts. This assay provides an important new tool for studying the effects of oxidative stress on vitamin E in biological membranes.

## EXPERIMENTAL

### *Chemicals*

ACS-grade potassium chloride, potassium hydrogenphosphate, dipotassium hydrogenphosphate, ethylenediaminetetraacetic acid (EDTA) disodium salt, HPLC-grade 2-propanol, acetonitrile and reagent-grade perchloric acid and acetic acid were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Sodium dodecylsulfate (SDS) (98%) and HPLC-grade heptane were from Aldrich (Milwaukee, WI, U.S.A.). Aqueous tetraethylammonium (TEA) hydroxide (20%) and $\alpha$-TH were from Sigma (St. Louis, MO, U.S.A.), $\alpha$-TQ from ICN Nutritional Biochemicals (Cleveland, OH, U.S.A.) and $\gamma$-tocopherol ($\gamma$-TH) from Eastman Kodak (Rochester, NY, U.S.A.). $\delta$-Tocopherol ($\delta$-TH) was kindly provided to us by Dr. Graham Burton, National Research Council, Ottawa, Canada; $\gamma$- and $\delta$-tocopheryl quinones ($\gamma$-TQ and $\delta$-TQ) were prepared by oxidation with iron(III) chloride as described elsewhere [7]. Prepurified nitrogen gas was from Big Three Industries (Houston, TX, U.S.A.).

### *Sample preparation*

White Leghorn strain chickens (University of Connecticut, Storrs, CT, U.S.A.) were raised from hatching with free access to Purina Starter Chow for chickens.

Blood (3 ml) was collected from six-week old chickens into a syringe containing 1 ml phosphate-buffered saline (PBS) (130 m*M* potassium chloride, 50 m*M* potassium phosphate, pH 7.4) with 0.4% EDTA. After mixing, the samples were centrifuged at 4°C at 1000 *g* for 5 min. The plasma was removed and the packed red blood cells were resuspended in 5 volumes PBS with 0.4% EDTA.

Tissue samples were obtained from four-week-old birds killed by cervical dislocation. The pectoralis major muscle was removed followed by the livers after perfusion with ice-cold, nitrogen-gassed PBS containing 0.1% EDTA. Soleus muscle was removed last. All subsequent steps were carried out at 4°C under nitrogen, using ice-cold solutions that had been pre-gassed with nitrogen. Tissues were separated from excess connective and fatty tissue, placed in 20 volumes of perfusion buffer, minced and homogenized for 30 s at medium speed with a Tekmar Tissuemizer. Aliquots of the homogenates (2.0 ml) were collected for tocopherol extraction. A known amount of $\alpha$-TQ was added as an external addition to the unused portion of some homogenate samples, which were rehomogenized for 10 s, and another 2.0-ml aliquot removed. A known amount of $\delta$-TH was added as an internal standard to other homogenate samples.

Adult, male Sprague–Dawley rats were raised from birth with free access to Purina Lab Chow. Microsomes were prepared from livers as described elsewhere [14]. Microsomal peroxidation was induced by diluting microsomal preparations to 1 mg protein per ml buffer (130 m*M* potassium chloride, 50 m*M* potassium phosphate, pH 7.4) containing 0.4 m*M* ADP, 10 $\mu M$ iron(III) chloride with or without 1 m*M* glutathione (GSH). Peroxidation was initiated by adding 0.5 m*M* ascorbate and quantitated by measuring thiobarbituric acid-reactive substances (TBARS) at 532 nm using a molar absorptivity of 156 $cm^{-1}$ $mM^{-1}$ for malondialdehyde as described elsewhere [14]. For tocopherol extraction, 2-ml aliquots of the reaction mixture were removed at time points before and after the addition of ascorbate.

The method of Burton et al. [15] was used for the extraction of tocopherols. Plasma and red blood cells (0.3 ml) were added to 2.7 ml of 30 m*M* SDS in water, while tissue homogenates and microsomal suspensions (2.0 ml) were added to 1 ml of 100 m*M* SDS in water. After mixing, 3.0 ml ethanol and 3.0 ml heptane were added sequentially with mixing. The heptane phase was removed after centrifugation of the mixture at 1000 *g* for 5 min. The heptane was evaporated under a stream of nitrogen and the extracted lipids resuspended in the HPLC mobile phase buffer (described below).

*HPLC system and electrochemical detection*

The HPLC system used included a Waters Model M-45 solvent pump and an Altex Ultrasphere-ODS 5-$\mu$m, 15 cm×4.6 mm I.D. column with a Brownlee RP-18 1.5 cm×4.6 mm I.D. guard column. The mobile phase contained 60% 2-propanol, 20% acetonitrile, 19.4% water, 0.5% TEA, and 0.1% acetic acid, pH 4.0. The pH was adjusted in a mixture of the latter three constituents by the addition of perchloric acid before the addition of the organic solvents. The buffer was filtered through 0.45-$\mu$m filters then degassed by sonication. The flow-rate was set at 0.4 ml/min and a 200-$\mu$l sample injected.

An ESA Model 5100A Coulochem electrochemical detector control module was connected to a Model 5021 conditioning cell and a Model 5010 analytical cell, which were installed in that sequence and separated from the HPLC column by an in-line carbon filter. The potential of the conditioning cell was set at +0.30 V and −0.10 V for the analysis of tocopheryl quinones and tocopherols, respectively, and was adjusted as necessary during the course of a sample run. The first analytical cell potential (D1) was set at −0.70 V and the second cell (D2) at +0.25 V for the measurement of all compounds, except as noted. Measurement of compounds by their oxidation at the second analytical cell (D2) was recorded by a Hewlett-Packard Model 3390A integrator recorder, using a peak width setting of 0.64. The output gain of the D2 cell was set between 100 and 1000 for measurement of tocopherols and between 1000 and 8000 for the measurement of tocopheryl quinones. The output response time to the recorder was set at 10 s.

*Analysis of standards*

Stock solutions of $\alpha$-, $\delta$ and $\gamma$-tocopherols and tocopheryl quinones were prepared in ethanol and their concentration determined from UV absorbance spectra [16]. After appropriate dilutions of the stock solutions with running buffer, retention times were determined and hydrodynamic voltammograms were prepared for each compound. Detector potentials were then set to maximize the measurement of the compounds, and standard curves of tocopherol and tocopheryl quinone concentrations versus peak heights were plotted. These curves were used to determine the concentrations of the compounds in tissue extracts. In addition to matching retention times, positive identification of peaks measured in tissue samples was confirmed by the addition of pure standards and by producing hydrodynamic voltammograms which matched the profiles of the authentic compounds. The tocopherol extraction efficiency of each sample was estimated by the recovery of exogenously added $\delta$-TH and $\alpha$-TQ in separate aliquots of the tissue sample, with $\alpha$-TQ levels corrected for the actual $\alpha$-TQ contents of the sample. Protein concentrations were determined using the microbiuret method with bovine serum albumin as the standard [17].

## RESULTS AND DISCUSSION

*Sensitivity and selectivity of redox-cycling ED*

Fig. 1 shows a typical chart recording of the ED output of a mixed standards solution containing 20 pmol of $\alpha$-TH, $\gamma$-TH, $\delta$-TH, $\alpha$-TQ, $\gamma$-TQ and $\delta$-TQ. The use of redox-cycling ED allowed detection of the tocopheryl quinones with at least 100-fold greater sensitivity than previously possible, and simultaneously measured tocopherols with equal sensitivity.

A recent paper noted the possibility of measuring not only tocopheryl quinones, but a whole range of biological and chemical quinones by the use of reductive ED [11]. This would be the equivalent of recording the current at the D1 cell potential of our system. The method of measuring subsequent oxidation has two significant advantages over this procedure. Firstly, the background current at the reductive electrode (D1) set at large negative voltages (−0.70 V) is high com-

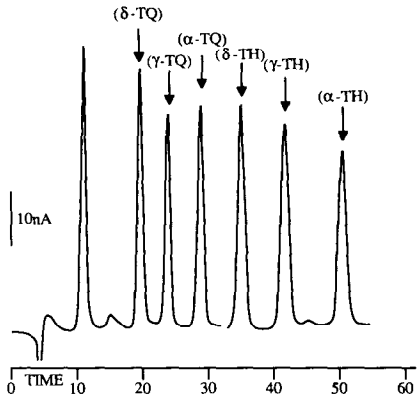


Fig. 1. HPLC profile of tocopherol standards using redox-cycling ED. The samples, dissolved in 200 μl running buffer, contained 8 ng of δ-tocopheryl quinone (δ-TQ), γ-tocopheryl quinone (γ-TQ), α-tocopheryl quinone (α-TQ), δ-tocopherol (δ-TH), γ-tocopherol (γ-TH) and α-tocopherol (α-TH). The conditioning cell potential was set at +0.30 V from 0 to 30 min, then adjusted to −0.10 V until the end of the run. The D1 cell potential was −0.70 V. Oxidation of compounds at +0.35 V was measured at the D2 cell. Output gain was 1000 and solvent flow-rate was 0.4 ml/min.

pared to the oxidative background current at +0.25 V, and so is more sensitive to the background current variations which inevitably limit the sensitivity of ED. Secondly, the appropriate choice of a subsequent oxidative cell potential adds another factor of selectivity for the assay making it possible to eliminate the measurement of virtually all interfering compounds except those redox-cycling at exactly the desired potential.

Fig. 2A shows the voltammogram of the reduction of α-, δ and γ-TQ, the oxidation of α-, δ- and γ-TH, and the oxidation of the products of quinone reductions, putatively α-, δ- and γ-tocopheryl dihydroquinone (α-, δ and γ-$TQH_2$). The voltammograms show that full oxidation of all the compounds was achieved at +0.35 V, and that full reduction of the quinones was achieved at −0.70 V. The oxidation products of α-, δ- and γ-TH, which are not the tocopheryl quinones, could not be reduced by the D1 cell set at −0.70 V (data not shown). Therefore, during the measurement of these compounds, the conditioning cell was set at −0.10 V where no appreciable oxidation occurs.

The voltammograms of α- and γ-TH produced with our system differ markedly from others in the literature. We found oxidation occurring between −0.05 and +0.15 V while other laboratories reported oxidation of the α-TH to occur between +0.20 and +0.60 V [13]. This discrepancy may be due to the high-efficiency carbon electrodes of the ESA analytical cell. The voltammograms of the standard compounds illustrate expected differences in the redox profiles of α-, δ- and γ-TH and α-, δ- and γ-TQ. In agreement with the literature [1], the trimethyl-substituted phenyl ring of α-TH allows the oxidation of the phenolic hydroxide at a lower potential than that required for δ- or γ-TH. Likewise, the reduction of tocopheryl quinones and the oxidation of the putative tocopheryl dihydroquinones demonstrate the same differences between α-, δ- and γ-forms.

In order to verify the identity of compounds from tissue sample which had

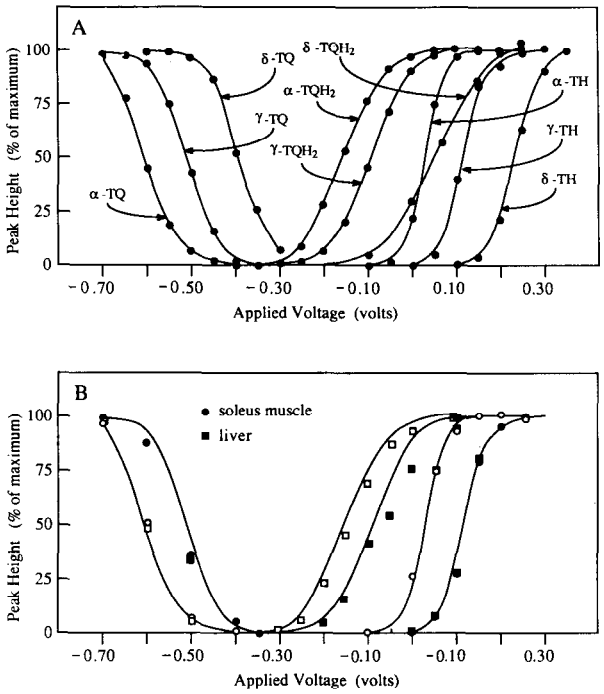


Fig. 2. Hydrodynamic voltammograms of standard tocopherol and coeluting compounds present in tissue samples from chickens. (A) Samples of standard compounds were dissolved in running buffer and peak heights recorded at various settings of the D1 cell for analysis of tocopheryl quinone reduction (with D2 cell set at +0.25 V). Replicate samples were analyzed at various settings of the D2 cell for analysis of tocopherol and dihydroquinone oxidations (with D1 cell set at −0.70 V). The conditioning cell potential was adjusted as listed for Fig. 1. (B) Similar analysis of putative tocopherol peaks in samples of soleus muscle (circles) and liver (squares). Open symbols are compounds with retention times matching $\alpha$-TQ and $\alpha$-TH isomers, closed symbols are those matching $\gamma$-isomers, and lines are reproduced from A.

retention times that matched those of standard compounds, their voltammogram profiles were determined as in Fig. 2A. Extracts of soleus muscle, liver, plasma and red blood cells each contained sufficient quantities of the four putative compounds to produce voltammogram profiles ($\geq$0.1 pmol). Peaks eluting at a given time all had similar voltammogram profiles, regardless of tissue source, and data from soleus and liver tissues are both shown in Fig. 2B. The data points in this figure, obtained from the tissue extracts, match the voltammogram profiles of pure standards, shown by lines reproduced from Fig. 2A. This verifies the identity of these compounds in these tissues. The quantities of $\alpha$-TQ and $\gamma$-TQ in pectoralis major muscle extracts were too low to verify the identity of these compounds by voltammograms, but the retention times of the putative quinones did not differ from those of standards or other tissues, and only one peak was observed in a sample to which authentic quinones were added.

Fig. 3 shows the peak height versus concentration profiles of the four standard compounds. Because peak areas were not always accurately measured by the recorder, especially in tissue samples, peak heights were measured directly from

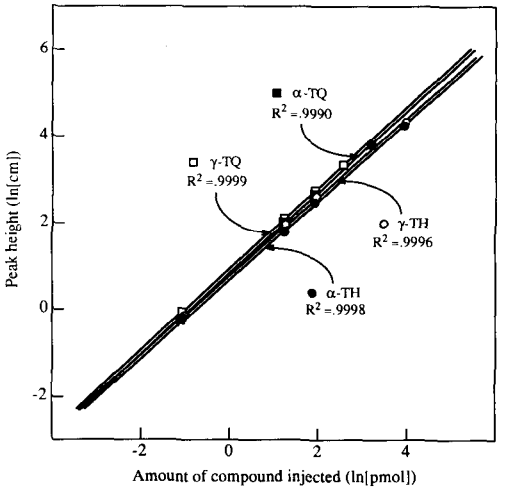


Fig. 3. Standard curves of the tocopherol compounds found endogenously in tissue. The concentrations of the four individual standards were determined by UV absorbance spectrum in ethanol. The standards were diluted in running buffer, separately and as mixtures, and 200-$\mu$l samples injected for analysis as described in Fig. 1. Peak heights were averaged from at least two recordings at appropriate output gains, then standardized to 8000 gain output heights.

the chart recording. In plotting the standard curve, a small deviation from linearity was seen when peak heights were charted directly against concentration ($r^2 = 0.996$–$0.998$). This could be attributed to a trend towards peak broadening with the higher concentrations of standards (data not shown). A log–log chart reduces this deviation and was used for the standard curve ($r^2 > 0.999$ for all four compounds). As expected of coulometric detection, equimolar quantities of the four standards demonstrated nearly identical peak areas (data not shown). Therefore, among the four standard compounds, the order of peak height-to-concentration ratios ($\gamma$-TQ > $\alpha$-TQ > $\gamma$-TH > $\alpha$-TH), as evidenced in Fig. 3, is the result of the peak broadening seen in the successive elution of these compounds during the run.

The voltammograms shown in Fig. 2 were used to select appropriate electrode potential settings for the assay of tissue extracts. Because it requires about 1 h to achieve a stable background current at the D2 cell following any change in voltage setting, we chose a potential of +0.25 V which is capable of completely oxidizing the four compounds present endogenously in tissue. The D2 cell was set to +0.35 V for analysis of samples containing internal $\delta$-TH standards. The D1 cell potential was set at −0.70 V to achieve nearly complete reduction of the tocopheryl quinones. Although $\alpha$-TQ may not be 100% reduced at this potential, the cell produced a consistent degree of reduction which would apply to $\alpha$-TQ in both standards and tissue samples. When the D1 cell was set to more negative potentials, it increased the background current at the D2 cell, perhaps by reducing residual molecular oxygen in the buffer.

*Analysis of tissue and microsomal extracts*

Fig. 4 shows a typical chromatogram for the analysis of tocopherol and tocopheryl quinone compounds in chicken soleus muscle. Other tissues showed sim-

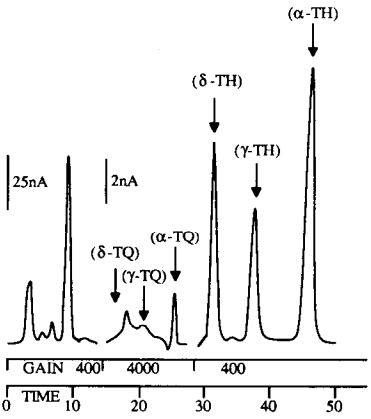


Fig. 4. HPLC profile of chicken soleus muscle. A lipid extract was prepared as described in the Experimental section, then 200 μl were injected for analysis. Several unidentified peaks eluted during this and other runs, but positive identification of putative tocopheryl quinone and tocopherol peaks were made by comparison of retention times and hydrodynamic voltammograms with those of standards. Compounds identified are those listed in Fig. 1. Different output gains were used for portions of the run and are listed below the chromatogram along with elution times in minutes. Standards of δ-tocopheryl quinone (δ-TQ) eluted at about 17 min under these conditions (shown by arrow).

ilar chromatographic patterns (data not shown). Contents of the different tocopherols varied within each tissue extract, as evidenced by the need for different gain settings during the course of the run. Table I lists the average concentration of each compound in a variety of chicken tissues measured by this technique. The tissues varied greatly in the total contents of tocopherols and in the ratio of tocopheryl quinones to tocopherols. In agreement with other reports [15], the extraction procedure was efficient at removing tocopheryl quinone and tocopherol compounds from all tissues. We obtained recoveries of 89–95% of standard additions of α-TQ and 88–99% of internal additions of δ-TH.

The technique of redox-cycling ED achieved sensitivities of about 0.05 pmol (20 pg) for the tocopherol compounds. The quantities of α- and γ-TQ we found

TABLE I

CONTENT OF TOCOPHEROLS AND TOCOPHERYL QUINONES IN CHICKEN TISSUES

Samples of chicken tissues were prepared as described in the Experimental section and 200 μl of sample injected for analysis. Concentrations of tocopherol compounds were estimated by comparison of peak heights to the standard curves. Data are expressed as pmol tocopherol compound per mg protein (mean ± S.D., $n = 4$ for all samples).

| Tissue | γ-Tocopheryl quinone | α-Tocopheryl quinone | γ-Tocopherol | α-Tocopherol |
|---|---|---|---|---|
| Plasma | 0.16 ±0.06 | 0.21±0.08 | 2.3 ±1.0 | 19 ± 4 |
| Red blood cells | 0.21 ±0.08 | 0.82±0.12 | 0.64±0.18 | 4.0± 0.8 |
| Liver | 0.085±0.026 | 1.47±1.10 | 19 ±4 | 79 ±18 |
| Pectoralis major | 0.004±0.004 | 0.07±0.08 | 8.1 ±1.2 | 35 ± 6 |
| Soleus | 0.047±0.034 | 0.49±0.26 | 25 ±8 | 99 ±28 |

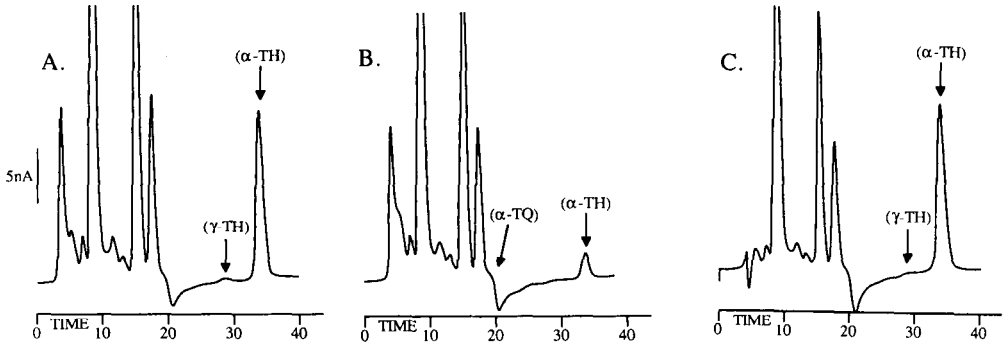


Fig. 5. HPLC profiles of rat liver microsomal extracts. Lipids were extracted from microsomal preparations undergoing oxidative attack as described in the Experimental section, and 200 $\mu$l injected for analysis. Compounds identified are $\gamma$-tocopherol ($\gamma$-TH) and $\alpha$-tocopherol ($\alpha$-TH). Samples are from microsomes (A) before the addition of ascorbate, (B) 96 min after the addition of ascorbate without glutathione in the incubation medium and (C) 120 min after the addition of ascorbate with 1 m*M* glutathione in the incubation medium. Buffer flow-rate for this analysis was 0.5 ml/min, thus accelerating the elution time profile in these runs. Standards of $\alpha$-tocopheryl quinone ($\alpha$-TQ) eluted at about 19 min under these conditions (shown by arrow).

in the tissues were considerably less than those reported by one group of investigators [9] and were less than those reported by most others who use tissue extraction procedures involving saponification [10]. In agreement with one other group [11], however, we detected very small quantities of the tocopheryl quinones in tissue even without the use of saponification. The variation in the apparent quantity of tocopheryl quinones in tissue extracted on different days was small (data not shown), indirectly suggesting that the levels measured were not produced as artifacts during sample preparation. In addition, we found no traces of oxidation of the added $\delta$-TH to $\delta$-TQ during the extraction procedures (Fig. 4). It was apparent that a greater proportion of total tocopherols were present as tocopheryl quinones in samples of plasma and red blood cells as compared to tissue homogenates, and it is possible that the high level of tocopheryl quinones in blood fractions were produced as artifacts during the extraction procedure.

One current limitation of our technique is the lack of an ideal internal standard which can indicate both the efficiency of the extraction procedures and the possible oxidation of tocopherols during sample preparation. Because $\alpha$-TQ is already oxidized, it only allows the estimation of extraction efficiency when used as an external addition. Pure standards of tocol (with a non-methylated phenyl ring) or $\delta$-tocopherol (with a single methyl group) are much better suited as oxidizable internal standards, but are slightly less oxidizable than $\alpha$-TH or $\gamma$-TH (Fig. 2A). Synthetic standards would have to have a solubility profile similar to tocopherols and oxidize in the same range of voltage potentials ($-0.10$ to $+0.25$ V) in order to be useful.

Several compounds extracted from tissue samples interfered with the accurate estimation of tocopherol contents in chicken tissues. In some samples, an unidentified compound eluted after $\gamma$-TQ and overlapped its peak. Although it was still possible to estimate the peak height of the quinone, the interfering peak

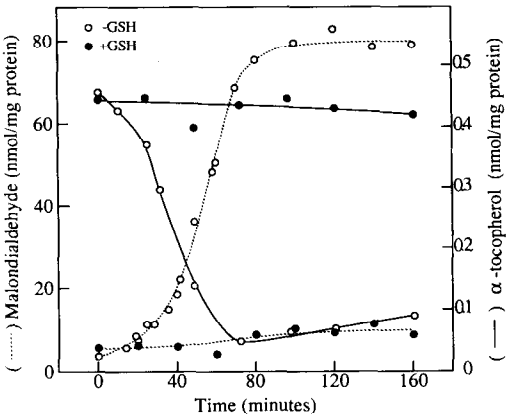


Fig. 6. Time course of $\alpha$-tocopherol ($\alpha$-TH) disappearance and malondialdehyde appearance (as measured by TBARS) in microsomal preparations. Microsomes were incubated and prepared for $\alpha$-TH and TBARS analyses as described in the Experimental section. Concentrations of $\alpha$-TH (solid lines) are given as nmol $\alpha$-TH per mg microsomal protein and concentrations of MDA equivalents are given as nmol/mg of protein. Samples were taken before and at various times after the addition of ascorbate to the incubation mixture with (closed symbols) and without (open symbols) 1 m*M* glutathione. The graph shows combined data of two separate experiments on microsomes incubated without glutathione, and one experiment on microsomes with glutathione.

might be mistaken for the quinone. As noted earlier, voltammograms provided a positive identification of peaks eluting at the same time as authentic standards. A more serious interference appeared before and after the elution of $\alpha$-TQ in samples of pectoralis major muscle. The background current dropped significantly during this period, indicating the elution of compound(s) which increased the electrical resistance of the buffer. The peak of the eluted $\alpha$-TQ was often still distinct during this interference, but the appropriate baseline current was difficult to estimate. This interference may be caused by mixtures of diacylglycerols or fatty acids (uncharged at the pH of the running buffer). This interference was the most serious limitation of the assay. A similar but smaller decrease in the background current occurs as a compound is eluted after the $\alpha$-TH peak, but does not interfere with its measurement. This compound may be cholesterol, since an injection of authentic cholesterol produces the same background deviation (data not shown).

Fig. 5A–C shows the analysis of tocopherol and tocopheryl quinone in rat liver microsomes before the initiation of peroxidation (A), after the apparent completion of peroxidation (B) and after the same length of time in the presence of 1 m*M* glutathione (C). It is evident from these profiles that $\alpha$-TH is decreased during lipid peroxidation and that this is prevented by the presence of glutathione. No tocopheryl quinone compounds were identified in any of these samples, and in contrast to chicken tissues, only trace quantities of $\gamma$-TH were found.

Fig. 6 shows the time course of the disappearance of $\alpha$-TH and the appearance of TBARS from microsomes undergoing oxidative attack, both with and without 1 m*M* glutathione in the incubation mixture. This figure shows that the microsomal content $\alpha$-TH remains constant in the presence of glutathione, a treat-

ment which others have shown to protect $\alpha$-TH from oxidative attack [18]. The technique also clearly demonstrated two surprising results for microsomes incubated in the absence of glutathione: (i) the lack of any measurable production of $\alpha$-TQ, and (ii) the maintenance of a low level of $\alpha$-TH in the microsomes even after maximal peroxidation. These results serve to illustrate the potential use for this assay in the study of the biochemistry of microsomal peroxidations.

*Potential applications of redox-cycling ED*

A wide range of applications for redox-cycling ED can be imagined. The most direct application would be towards characterizing factors involved in oxidative damage to membranes in vivo such as ionizing radiation, xenobiotics which undergo redox-cycling, and pathological conditions thought to involve oxidative stress in tissues [2,19]. The initial tocopheryl semiquinone radicals thought to form during tocopherol oxidation in vivo may be converted to tocopheryl quinone, tocopheryl dimers and trimers or a variety of bimolecular products [1,3], or may regenerate tocopherol following interactions with ascorbic acid [1] or glutathione [14,18]. Our procedure is capable of detecting the low-level tocopheryl quinones present endogenously, and can be adapted to identify the dimer and trimer forms of tocopherol. This will facilitate analyses of the physiological and environmental conditions which influence the rate of production and the proportions of these oxidation products in vivo [11] as has been attempted, for example, with the hydrogen peroxide sensitivity of erythrocytes [3]. The sensitivity of the assay allows cultured cells to be used in place of tissue samples, and also allows the study of oxidative processes in in vitro preparations such as microsomes, where the exact biochemistry can be more readily characterized [8,11].

Some reports suggest that $\alpha$-TQ may be synthesized in bacteria and perhaps mammalian cells [9]. It also has been reported that $\alpha$-TQ can be reduced to $\alpha$-$TQH_2$ by substrate-dependent mitochondrial systems [8,9] and that $\alpha$-TQ is an endogenous electron donor in biohydrogenation reactions of some bacteria [20]. The sensitivity of redox-cycling ED and its ability to simultaneously detect both quinones and dihydroquinones make it applicable to more detailed descriptions of the biochemistry of these reactions. We have prepared standards of $\alpha$-$TQH_2$ and found this compound eluted about three times faster than $\alpha$-TQ using our mobile phase buffer. Endogenous tocopheryl dihydroquinones, if present, are therefore masked in our tissue sample by multiple interfering compounds. As expected, the voltammogram profiles of $\alpha$-$TQH_2$ and $\alpha$-TQ were identical, and to measure $\alpha$-$TQH_2$ the D2 cell voltage could be set as low as 0.00 V when the quantification of tocopherols is not also desired. This change would decrease the background current at this cell as well as increase the selectivity of the assay by eliminating the measurement of compounds which oxidize at higher potentials.

Oxidative ED is a sensitive technique for the measurement of ubiquinols [21], but the redox-cycling assay could both increase the selectivity and sensitivity of the detector for ubiquinols and increase the sensitivity towards ubiquinones, which were previously best measured by UV detection. Our mobile phase buffer eluted the ubiquinones too slowly for routine analysis (10–14 h). However, other authors have described mobile phase buffers suitable for reversed-phase HPLC and ED

[11,21] which eluted ubiquinols and ubiquinones within 1 h. We have prepared a sample of $\alpha$-tocopheryl dimer and found, in our buffer system, it elutes as slowly as ubiquinones and would also be better analyzed using other mobile phase buffers. As with tocopherols, the efficient measurement of ubiquinols, ubiquinones and tocopheryl dimers would involve the choice of appropriate electrode potentials based on the voltammogram profiles of these compounds.

ACKNOWLEDGEMENTS

J.P.K. is the Gustavus Pfeiffer Centennial Fellow of Pharmacology and the recipient of Research Career Development Award HL 01435 from the National Institutes of Health. This work was supported by Biomedical Research Support Grants RR07091 and RR05849.

NOTE ADDED IN PROOF

Following submission of this manuscript, a similar method for determination of tocopherol compounds by redox-cycling ED was reported by Pascoe et al. [22] using a BAS dual-electrode LC-4B electrochemical detector.

REFERENCES

1 G.W. Burton and K.U. Ingold, Acc. Chem. Res., 19 (1986) 194.
2 L.A. Witting, in W. Pryor (Editor), Free Radicals in Biology, Vol. 4, Acadamic Press, Orlando, FL, 1980, p. 295.
3 J. Vanderpas and F. Vertongen, Blood, 66 (1985) 1272.
4 J.L. Buttriss and A.T. Diplock, Methods Enzymol., 105 (1984) 131.
5 D.B. Milne and J. Botnen, Clin. Chem., 32 (1986) 874.
6 B.J. Zaspel and A.S. Csallany, Anal. Biochem., 130 (1983) 146.
7 A.S. Csallany, H.H. Draper and S.N. Shah, Arch. Biochem. Biophys., 98 (1962) 142.
8 A. Bindoli, M. Valente and L. Cavallini, Biochem. Int., 10 (1985) 753.
9 P.E. Hughes and S.B. Tove, J. Biol. Chem., 255 (1980) 7095.
10 J.G. Bieri and T.J. Tolliver, Lipids, 16 (1981) 777.
11 M.T. Smith, D.S. Fluck, D.A. Eastmond and S.M. Rappaport, Life Chem. Rep., 3 (1985) 250.
12 Y. Haroon, C.A.W. Schubert and P.V. Hauschka, J. Chromatogr. Sci., 22 (1984) 89.
13 M.C. Castle and W.J. Cooke, Ther. Drug Monit., 7 (1985) 364.
14 G.R.M.M. Haenen and A. Bast, FEBS Letts., 159 (1983) 24.
15 G.W. Burton, A. Webb and K.U. Ingold, Lipids, 20 (1985) 29.
16 M. Kofler, P.F. Sommer, H.R. Bolliger, B. Schmidli and M. Vecchi, in R.S. Harris and I.G. Wool (Editors), Vitamins and Hormones, Vol. 20, Academic Press, New York, 1962, p. 407.
17 R.F. Itzhaki and D.M. Gill, Anal. Biochem., 9 (1964) 401.
18 S. Robey and R. Mavis, Fed. Proc., Fed. Am. Soc. Exp. Biol., 45 (1986) 1671.
19 H. Sies (Editor), Oxidative Stress, Academic Press, London, 1985.
20 P.E. Hughes and S.B. Tove, J. Biol. Chem., 255 (1980) 4447.
21 J.K. Lang, K. Gohil and L. Packer, Anal. Biochem., 157 (1986) 106.
22 G.A. Pascoe, C.T. Duda and D.J. Reed, J. Chromatogr., 414 (1987) 440.