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Alpha-Tocopherol in the Retinal Outer Segment of Bovine Eyes

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Summary. α-Tocopherol was identified in lipid extracts of bovine retinal outer segment (ROS) preparations. Positive identification was obtained by the thin layer chromatographic characteristics of the tocopherol form and its oxidation product α-tocopherylquinone, and by the ultraviolet spectrum of the oxidized and KBH_4-reduced form of the tocopherylquinone. In the ROS preparations used, α-tocopherol chromanol was the predominant species, the quinone form accounting for 25% or less of the total. The concentration of α-tocopherol in the ROS preparations was about 0.1 mole α-tocopherol per mole rhodopsin, or about 1 nmole/mg protein. Mitochondria from bovine retina contained about 0.4 nmole α-tocopherol per mg protein.

Histochemical work by Pearse [12, 13] demonstrated clearly that large amounts of some redox substance occur in the retinal outer segment (ROS) of vertebrate eyes. In the earlier paper, Pearse suggested that the redox compound might be ubiquinone [12]; however, Fleischer and McConnell [5] were unable to detect any ubiquinone in purified bovine ROS preparations in which Pearse also localized the redox substance histochemically [13]. The possibility occurred to us that the redox compound observed by Pearse may be α-tocopherol. This report presents the results of our experiments designed to test this hypothesis. As will be seen below, α-tocopherol does occur in the ROS preparations from cow eyes in relatively large amounts.

Methods

The ROS fragments used in this investigation were isolated by sucrose density-gradient centrifugation, under a high gravitational field, from other cell constituents of the bovine retina by the method of McConnell [8]. In one method for isolating α-tocopherol, a saponification procedure utilized 5 ml of ROS material containing about 0.7 μmole rhodopsin[1] to which was added 20 ml water, 7.5 g KOH, 7.5 g pyrogallol, and 50 ml ethanol. This mixture was refluxed 20 min, cooled, and extracted four times with 100-ml portions of petroleum ether to remove non-saponifiable lipids. In the

1 Larger amounts were used in some experiments to obtain good spectra of α-tocopherylquinone (Fig. 1).

second method, 5 ml of ROS material containing about 0.7 μmole of rhodopsin[1] was directly extracted three times with about 25 ml of 2:1 chloroform/methanol. Each extraction was allowed to proceed for approximately 15 min, after which the combined organic phases were taken up into 100-ml amounts of petroleum ether. After each of these methods, the combined petroleum ether extract was washed three times with an equal volume of water, dried over Na_2SO_4, the petroleum ether removed under vacuum and the organic residues taken up in 3 ml of n-heptane. The heptane extract, after drying over Na_2SO_4, was applied to thin layer chromatography (TLC) plates for separation of tocopherol from other lipid constituents.

TLC was carried out according to the method of Dilley and Crane [2]. A portion of the heptane extract of the ROS was applied as a streak along the origin of a TLC plate. Another sample of the extract and a sample containing pure α-tocopherol plus the extract were spotted at the origin near one edge of the plate, as controls. After development in chloroform, reducing compounds including α-tocopherol were visualized by spraying the area of the plate which included the two control spots with a mixture of ferric chloride and α-dipyridyl reagent [2]. The region of the unsprayed plate corresponding to the α-tocopherol band was scraped into ethanol to elute the tocopherol from the silica gel carrier. After sedimenting the carrier, in the clinical centrifuge, the α-tocopherol was oxidized by gold chloride to the quinone form according to the method previously published [2]. The α-tocopherylquinone was partitioned into n-heptane; the heptane phase was washed with water to remove all traces of gold chloride, then evaporated to dryness and the residue taken up in 2 ml of ethanol. The α-tocopherylquinone concentration in the ethanol was established by comparing the UV spectra of the oxidized and borohydride (KBH_4)-reduced forms of the quinone. A value of 14.3 Δ absorbance per μmole/ml was used as the extinction coefficient for the oxidized-minus-reduced form of α-tocopherylquinone at 260 nm in ethanol [2].

The advantages of measuring the quinone rather than the chromanol form of α-tocopherol are as follows. (1) The chromanol has a much lower extinction coefficient than the quinone ($E^{1\%}_{1\,cm}$ 292 nm in ETOH = 74 compared to an $E^{1\%}_{1\,cm}$ 260 nm oxid-red = 397). Thus, measuring the quinone gives a fivefold increase in sensitivity. (2) The quinone is easily reduced with KBH_4, a reductant having no significant absorbance in the UV region, whereas the common oxidants used to oxidize the chromanol to the quinone absorb UV radiation and interfere with the assay. (3) By using the oxidized-reduced difference in absorbance, one can quantitate the α-tocopherylquinone, even in the presence of other UV-absorbing materials, provided they do not also change absorbance upon KBH_4 treatment.

The method outlined above provides two qualitative criteria for identification of α-tocopherol — the chromatographic behavior of the chromanol form and the spectral characteristics of the quinone form. A third qualitative identification test was performed by chromatographing the tocopherylquinone form produced by the gold chloride oxidation of the chromanol. The identification of the quinone form on the TLC plate was accomplished by spraying, with leucomethylene blue [2], the plate containing spots of pure α-tocopherylquinone and of the oxidized ROS extract.

Assays for the rhodopsin content of the ROS preparations were carried out as previously described [10]. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall [7].

Results and Discussion

Ten different batches of ROS preparations were extracted by either the saponification or the direct solvent extraction method for estimation of

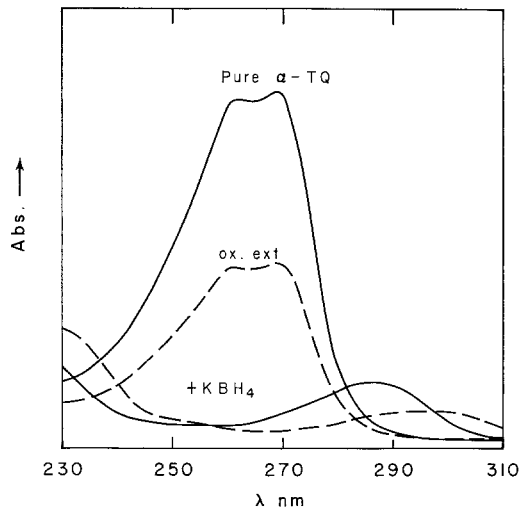

Fig. 1. Absorption spectrum of pure α-tocopherylquinone (α-TQ) and that derived from gold chloride oxidation of α-tocopherol isolated from purified bovine ROS (ROS fragments). For this spectrum only, ROS fragments containing 4.9 μmoles rhodospin were saponified as outlined in Methods. α-Tocopherol was identified on a thin layer chromatogram by the Emmerie-Engel $FeCl_3$, α−α′, dipyridyl method [2]; it was eluted from the carrier and oxidized to the quinone form by gold chloride as described in Methods. The amount of α-TQ recovered by this process was 0.20 μmole

α-tocopherol content. In all experiments, positive identification of α-tocopherol was obtained. Fig. 1 shows the UV absorption spectra of pure α-tocopherylquinone and of α-tocopherylquinone derived from gold chloride oxidation of the tocopherol isolated from ROS fragments. In both samples, the addition of KBH_4 resulted in the characteristic loss of the 260-nm absorbance. The spectra of both samples are nearly identical, providing strong evidence that the compound derived from the ROS segments is, in fact, α-tocopherylquinone.

Additional qualitative evidence for the occurrence of α-tocopherol in the ROS extract is shown in photographs of TLC plates. Fig. 2A shows that the pure α-tocopherol and the α-tocopherol in the ROS extract run with the same R_f value (columns 1 & 2). After gold chloride oxidation (column 3), the α-tocopherol spot, as expected, no longer occurs. Lighter developed spots running below the heavier spots in columns 1, 2 and 4 represent γ-tocopherol, traces of which are known to occur in the purified α-tocopherol samples obtained from commercial sources. It is apparent that the ROS extract (column 2) contains γ-tocopherol as well as α-tocopherol. Fig. 2B shows the TLC results of the gold chloride oxidation

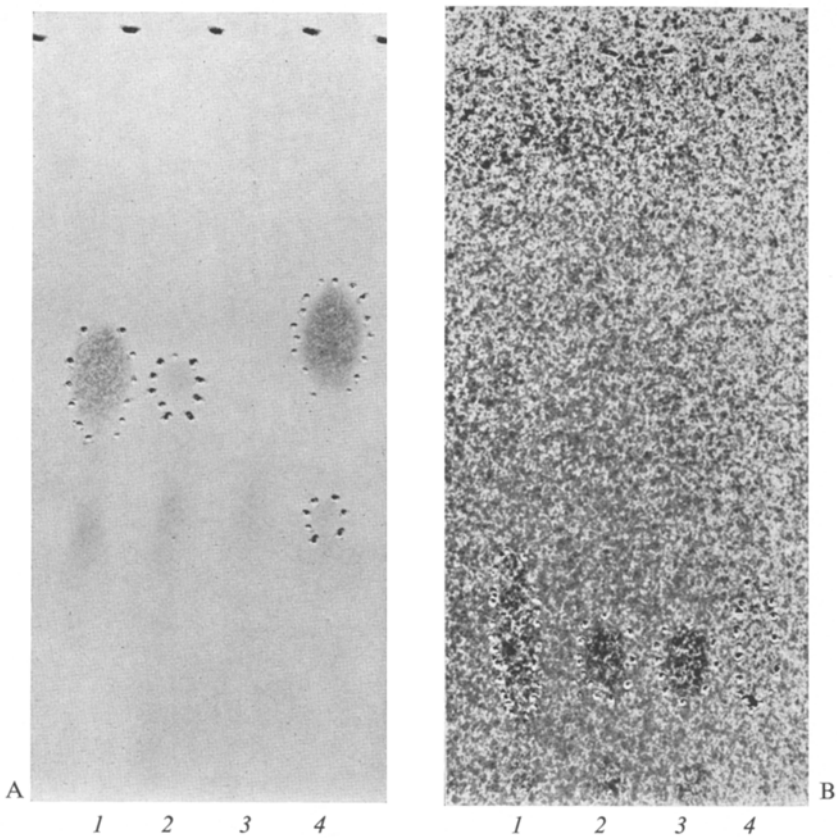

Fig. 2A and B. Chromatography of α-tocopherol and α-tocopherylquinone from ROS fragments. A. Chromatography of lipid extract after saponification as in Fig. 1. The TLC plate was sprayed with the Emmerie-Engle reagent as in Fig. 1. The reagent produces a deep-red color in the presence of compounds, such as α-tocopherol, capable of being oxidized by ferric chloride. The spots thus produced were outlined on the plate by pencil point. The origin was spotted with the following: *1* 50 λ lipid extract of ROS plus 5 λ pure α-tocopherol solution; *2* 50 λ lipid extract alone; *3* 50 λ of lipid extract after gold chloride oxidation (which converts the chromanol form to the quinone form); *4* 5 λ of pure α-tocopherol solution. B. Chromatography of sample of the lipid extract used in Fig. 2A after gold chloride oxidation. The TLC plate was sprayed with leucomethylene blue as described in Methods. The oxidized form of quinone rapidly oxidizes leucomethylene blue to the blue form, thus producing the spots outlined on the plate. The origin was spotted with the following: *1* 50 λ oxidized ROS extract plus 5 λ pure α-tocopherylquinone solution; *2* 50 λ oxidized ROS extract only; *3* 100 λ ROS extract before gold chloride oxidation; *4* 5 λ pure α-tocopherylquinone solution

product compared to pure α-tocopherylquinone. When chloroform is the solvent, the quinone form runs with a lower R_f value than the tocopherol form. It is apparent that the oxidized product in the ROS extract (column 2)

Table. *The distribution of α-tocopherol in the ROS, mitochondrial and microsomal fractions obtained from high-g sucrose density-gradient centrifugation of homogenized retinal photoreceptors of bovine eyes*

Fractions	Distribution of α-tocopherol (nmole/mg protein)		
	Exp. A	Exp. B	Exp. C
ROS	0.52	0.69	0.96
Retinal mitochondria	0.17[a]	0.38[a]	0.22[b]
Retinal microsomes	0.38[a]	0.63[a]	0.32[b]

[a] Not corrected for ubiquinone contamination on TLC plates.

[b] Corrected for ubiquinone contamination by computing the ubiquinone content from the Δ O.D. at 284 nm, the isobestic point in the oxidation-reduction difference spectrum for α-tocopherylquinone.

runs with the same R_f value as the purified α-tocopherylquinone (columns 1 & 4). The spot in column 3 indicating the presence of α-tocopherylquinone in the ROS extract which had not been oxidized with gold chloride shows either that the oxidized form of tocopherol exists naturally in the ROS material or that some of the reduced form becomes oxidized during the handling of the extract.

In such studies, the possibility must be considered that the α-tocopherol attributed to the fraction under investigation actually originates in contaminations by other subcellular particles such as mitochondria or microsomes. Whereas earlier work by McConnell [8] has shown that the technique of isolating ROS material by sucrose-density centrifugation under a high gravitational field results in very little mitochondrial contamination, to be certain, we investigated the tocopherol content in the mitochondrial and microsomal fractions obtained during isolation of the ROS. The Table shows some typical results of the distribution of α-tocopherol on a milligram protein basis in the three fractions obtained from the retina. In three different experiments (*A, B, C*), it was found that α-tocopherol is more concentrated on a milligram protein basis in the ROS fraction than in either the mitochondrial or the microsomal fraction. Both the mitochondrial and microsomal fractions contain ubiquinone (the microsomal fraction has been shown by McConnell, Ozga and Solze [9] to be contaminated by mitochondria) which, in the TLC system used in the present study, runs quite close to α-tocopherol. The presence of ubiquinone contaminating the α-tocopherylquinone formed by gold chloride oxidation of the chromanol can be detected by noting the increased Δ O.D. at 284 nm (the isobestic point of the

oxidized-minus-reduced spectrum of α-tocopherylquinone). The values of Δ O.D. at 260 and 284 nm in the oxidized-minus-reduced spectrum of ubiquinone are approximately equal [1]. Therefore, one may subtract from the observed value of Δ O.D. at 260 nm for α-tocopherylquinone contaminated by ubiquinone, the Δ O.D. value observed at α-tocopherylquinone's isobestic point − 284 nm. The corrected 260-nm Δ O.D. provides an estimate of α-tocopherylquinone content. This was done for the data of experiment C in the Table. It is seen that α-tocopherol is considerably enriched in the ROS fraction on a milligram protein basis, compared to either the mitochondrial or the microsomal fractions from the retina preparation. On this basis, one can conclude that the ROS membranes natively contain α-tocopherol, and that our observations cannot be ascribed to contamination from mitochondrial or microsomal fragments.

In calculating the amount of α-tocopherol occurring in ROS fragments, we used the criterion of percent recovery of α-tocopherol added prior to extraction, to assess the effectiveness of our overall assay procedures. A 10-fold excess of pure α-tocopherol over that contained in the ROS preparation was used in these recovery experiments. Using the chloroform : methanol solvent extraction technique, we were able to recover 77 and 84% of the added α-tocopherol in two experiments. Using these correction factors on the relative amount of α-tocopherol obtained in the ROS control sample in each experiment, we found the estimated concentration of tocopherol to be around 0.1 mole α-tocopherol per mole rhodopsin. On a protein basis, the maximum value obtained was about 1 nmole α-tocopherol per mg protein (Table).

A second control for the efficiency of our extraction and purification methods was that of extracting ubiquinone from beef-heart mitochondria (kindly supplied by Dr. Gerald Brierley). Using the saponification technique in the presence of pyrogallol, we recovered 2.1 μmoles ubiquinone per mg mitochondrial protein. This represents a 70% recovery of ubiquinone based on a value of 3 μmoles ubiquinone per mg protein found by Lester and Crane [6] in beef-heart mitochondria.

The thin layer chromatograms showed the presence of an additional unknown reducing compound which was not α-tocopherol. This substance was destroyed by the saponification technique, being found only after the direct solvent extraction procedure. The unknown compound ran ahead of α-tocopherol on the TLC plates, indicating that it was a less polar compound. The unknown compound was actually composed of three close running bands with somewhat different absorption spectra in the ultraviolet region. The bands gave positive color tests (Salkowski and Liebermann-Burchard)

for cholesterol, but their spectral characteristics were *not* identifiable with cholesterol. This unknown compound was also extracted from the retinal mitochondrial and microsomal fractions.

The high amount of α-tocopherol found in the ROS fragments (10 to 12 moles rhodopsin per mole α-tocopherol) is very similar to the amount of tocopherol occurring in higher plant chloroplasts (about 9 moles chlorophyll per mole α-tocopherol, [3]). A specific function has not been shown for tocopherol in the chloroplast system, although changes in the level of tocopherylquinone between dark- and light-treated chloroplast samples have been measured [4]. It is possible that tocopherol functions in the photoreceptor systems of the chloroplast and the eye as an anti-oxidant. Recent evidence suggests that α-tocopherol may play an important role in membrane metabolism and structure [11]. Further experiments will be necessary to elucidate the exact role of α-tocopherol in the visual apparatus.

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