BBA 45799

DISTRIBUTION OF α-TOCOPHEROL IN BEEF HEART MITOCHONDRIA*

MECIA M. OLIVEIRA**, WILLIAM B. WEGLICKI***, ALVIN NASON AND P. P. NAIR

McCollum Pratt Institute, Johns Hopkins University, Baltimore, Md. 21218 (U.S.A.), Gerontology Research Center, National Institute of Child Health and Human Development, Baltimore, Md. 21224, and Biochemistry Research Division, Department of Medicine, Sinai Hospital of Baltimore, Baltimore, Md. 21215 (U.S.A.)

(Received November 27th, 1968)

SUMMARY

1. α-Tocopherol was identified and determined in purified bovine heart mitochondria by several independent procedures including thin-layer chromatography, silicic acid column chromatography, gas–liquid chromatography and spectral examination.

2. The concentration of tocopherol in purified mitochondrial preparations ranged from 0.05 to 0.44 μmole of tocopherol per g of protein, and appeared to be independent of the specific activity of cytochrome oxidase in the same samples.

3. α-Tocopherol was found to be present in all the enzymatically active lipoprotein complexes of the electron transport system of mitochondria, but apparently did not have a direct quantitative relationship with the enzymatic activities observed. The highest concentration of tocopherol per g of protein occurred in a supernatant fraction free of any electron transport chain activity.

4. The subcellular fractions of beef heart muscle were examined for vitamin E content, and the crude nuclear fraction showed a considerable amount of tocopherol per g of protein. The supernatant fraction obtained after sedimentation of the mitochondria had very little vitamin E.

5. Separation of the mitochondrial membranes by digitonin treatment showed that only the inner membranes contained tocopherol.

INTRODUCTION

The primary biological function of the tocopherols (vitamin E) is not yet known. One suggested function is that α-tocopherol participates in the mitochondrial electron transport system either as a cofactor or as a structural agent, based on reports that vitamin E is present in mitochondria and, specifically, in the terminal electron transfer chain of mitochondria (for review see refs. 1–3).

The present paper describes the characterization and quantification of α-tocopherol in bovine heart mitochondria and in various sub-mitochondrial fractions

* Contribution No. 551 of the McCollum Pratt Institute.

** On leave of absence from Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

*** Research associate of Gerontology Research Center, the National Institute of Child Health and Human Development. Present address: Cardiovascular Unit, Department of Medicine, Peter Bent Brigham Hospital, Boston, Mass., U.S.A.

including the cristae and several mitochondrial lipoprotein complexes in an attempt to evaluate a possible role of tocopherol in the terminal electron transport system.

EXPERIMENTAL PROCEDURES

Materials

Cytochrome *c* (grade III), Tris as "Trizma base" and "Trizma HCl" were obtained from Sigma Chem. Co., and standard (+)-α-tocopherol, thin-layer chromatographic "chromagram" sheets (silica gel), hexane (spectrograde), isooctane (2,2,4-trimethylpentane; spectrograde) from Distillation Products Co. Deoxycholic acid (special enzyme grade) and ammonium acetate (ultra pure) were purchased from Mann Res. Lab. Silicic acid (BioSil A, 100–200 mesh, BioRad), NADH, and NADPH were supplied by Calbiochem., and benzene (reagent grade), digitonin, α,α'-dipyridyl and OsO_4 by Fisher Scientific Co. Matheson Scientific provided succinic acid (disodium salt), and benzylamine HCl, and J. T. Baker Co. supplied reagent grade chloroform, sucrose, ethylene diamine tetra acetic acid (EDTA) and other salts and solvents. The stationary phases for gas–liquid chromatography were purchased from General Electric Co., Waterford, N.Y. (Silicone Products Division, SE 30) and from Applied Science Lab. (XE 60), State College, Pa. (U.S.A.).

Preparation of mitochondria

Fresh beef hearts were obtained from the slaughterhouse killing floor and immediately placed in ice. All subsequent operations for the preparation of mitochondria were performed at 4° according to the method of CRANE *et al.*[4]. The hearts were trimmed of fat and valvular tissue, cut into approximately one-inch cubes, and washed by suspending in cold buffer (0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris, pH 7.4). The tissue was then passed through a meat grinder, and homogenized in the above buffered sucrose (3 parts buffer and 1 part tissue) for 45 sec at low speed in the model CB-4 Waring blender, with the addition of 5–6 ml of 6 M KOH, as required, to maintain the pH at 7.4

The resultant crude muscle homogenate was centrifuged at 1600 × *g* for 12 min in a Sorvall Superspeed RC2-B centrifuge and an aliquot of the pellet, designated as crude nuclei, retained. The supernatant solution was filtered through four layers of cheesecloth and centrifuged at 40000 × *g* through the KSB-R "Szent-Gyorgyi and Blum" continuous flow system in the Sorvall Superspeed centrifuge at a flow rate of 120–150 ml/min. The centrifugation yielded a supernatant solution and a mitochondrial pellet which were subsequently assayed for tocopherol content. The latter was routinely suspended in an equal vol. of buffered sucrose, gently homogenized with a teflon pestle-smooth glass tissue homogenizer, and then treated in one of two ways depending upon the purpose for which it was intended. (a) The above mitochondrial suspension (except when used for the preparation of inner and outer membranes) was generally frozen at —15° overnight, thawed the next day, washed by suspending in the buffered sucrose, and centrifuged at 20000 × *g*; or (b) when employed for the preparation of inner and outer membranes, the mitochondrial suspension was not frozen but further processed the same day, as indicated in the appropriate experiments below.

Protein was determined by the method of LOWRY *et al.*[5] with crystalline bovine serum albumin as a standard.

Fractionation of mitochondria by deoxycholate and ammonium acetate treatment

The above bovine heart mitochondria were fractionated into component lipoprotein complexes according to the procedure of HATEFI *et al.*[6]: The thawed and washed mitochondrial pellet was first resuspended in Tris–sucrose–histidine buffer (0.05 M Tris, 0.66 M sucrose, 0.001 M histidine, pH 8.0) at a final concn. of 23 mg protein per ml. Potassium deoxycholate (10% solution, pH 9.0) was added to give a final concn. of 0.3 mg/mg protein, followed by the addition of 72 g of solid KCl per liter of suspension, which was stirred until the KCl was dissolved, and the mixture centrifuged at 78000 × *g* for 40 min (model L Spinco ultracentrifuge). The resulting crude cytochrome oxidase pellet (Table III) contained 65–85% of the cytochrome oxidase activity as compared to that of the mitochondrial suspension from which it was derived. The supernatant solution was then dialyzed against 30 vol. of 0.01 M Tris chloride (pH 8.0) for 3 h and centrifuged at 78000 × *g* for 1 h to give a pellet possessing both NADH- and succinate-cytochrome *c* reductase activities. The two enzymatic activities were separated and purified by means of increasing concentrations of ammonium acetate as indicated (Table III).

Separation of mitochondrial membranes by digitonin treatment

Outer and inner membranes of heart muscle mitochondria were separated by the digitonin method of SCHNAITMAN *et al.*[7]. Aliquots of the mitochondrial suspension at a concentration of 100 mg of protein per ml of buffer (0.25 M sucrose, 0.001 M EDTA, 0.05 M Tris, pH 7.4) were placed in an ice bath and identical aliquots of cold 2% digitonin solution in 0.25 M sucrose were added with continuous stirring to give a final concn. of 1.03 mg of digitonin per 10 mg protein. The suspension was incubated at 0° for 20 min, diluted by the addition of 3 vol. of cold 0.25 M sucrose, and centrifuged for 10 min at 9500 × *g*. The resultant pellet contained mostly inner membranes as indicated by its high cytochrome oxidase and low monoamineoxidase activity, as well as by preliminary electron microscopic examination. The 9500 × *g* supernatant solution was centrifuged for 10 min at 40000 × *g*, and the resultant supernatant solution centrifuged for 2 h at 105000 × *g* to yield a pellet possessing high monoamineoxidase activity. The electron microscopic appearance of the 105000 × *g* pellet was that of small vesicular membranous structures similar to those described as outer membranes of liver mitochondria[7].

Isolation of nuclei

A homogeneous preparation of nuclei was obtained according to the method of WIDNELL *et al.*[8].

Enzymatic assays

A. Cytochrome c reductase assays. The assay for NADH–cytochrome *c* reductase was similar to that of NASON AND VASINGTON[9]. To 0.8 ml of $5 \cdot 10^{-2}$ M phosphate buffer (pH 7.5) were added 0.1 ml of $5 \cdot 10^{-2}$ M KCN, 0.02 ml of $1.2 \cdot 10^{-3}$ M NADH, 0.04 ml of a 2% solution of oxidized cytochrome *c* in a cuvette of 1-cm light path, and the reaction initiated with 0.02 or 0.05 ml of the enzyme fraction to be assayed. The absorbance change at 550 mμ *versus* time was recorded over a 2-min period employing a Gilford Model 2000 absorbance recording photometer attached to a Beckman DU monochromator. The amount of cytochrome *c* reduced during the reaction was

calculated by applying the extinction coefficient of $2.10 \cdot 10^{-4}$ cm^2/mmole to the observed increase in absorbance at 550 mμ. Specific activity is expressed as μmoles of cytochrome *c* reduced per min per mg protein.

The assays for succinate- and NADPH–cytochrome *c* reductases were the same as that for NADH–cytochrome *c* reductase except that 0.05 ml of 0.1 M disodium succinate and 0.05 ml of $2 \cdot 10^{-3}$ M NADPH, respectively, replaced NADH as substrate.

B. Cytochrome oxidase assay. Cytochrome *c* oxidase activity was determined by recording the rate of decrease of absorbance at 550 mμ by the same procedure described above for cytochrome *c* reductase. The reaction mixture consisted of 0.9 ml phosphate buffer, $5 \cdot 10^{-2}$ M (pH 7.5), 0.02 ml reduced cytochrome *c* solution prepared with palladium asbestos in the presence of H_2 according to the method described by SMITH[11], and 0.02–0.05 ml of the enzyme solution to initiate the reaction. Specific activity is expressed as μmoles of cytochrome *c* oxidized per min per mg protein using the above indicated cytochrome *c* extinction coefficient for the observed decrease in absorbance.

C. Monoamineoxidase assay. Monoamineoxidase was assayed by a modification of the method of TABOR *et al.*[12] by following the formation for 1 min of benzaldehyde spectrophotometrically at 250 mμ and 37° in an assay system containing enzyme, 0.05 ml $2.5 \cdot 10^{-3}$ M benzylamine hydrochloride and 0.8 ml $5 \cdot 10^{-2}$ M phosphate buffer (pH 7.6) to give a final vol. of 1 ml. Specific activity is expressed as μmoles of benzaldehyde formed per min per mg protein.

Lipid extraction

Subcellular fractions were diluted with 4 vol. of absolute ethanol in 500–2000 ml round-bottom flasks and pyrogallol added to give a final concn. of 50 mg/100 ml solution. The solution was heated to its boiling point followed by the dropwise addition of 50% NaOH solution (3 ml of NaOH solution per 100 ml extract), and the mixture refluxed in the dark for 30 min. The flask and its contents were rapidly cooled to room temperature under running tap water and the non-saponifiable lipid extracted with 0.5 vol. of hexane by vigorous shaking for 10–15 sec in a separatory funnel. After separation and removal of the hexane phase, the extraction was repeated with a second 0.5 vol. of hexane. At this point, 0.2 vol. of a 5% NaCl solution was added and the solution extracted with two more 0.5 vol. of hexane. All four hexane layers were pooled, washed with 3–4 vol. of distilled water and dried over anhydrous Na_2SO_4 before being evaporated to dryness in a Büchi partial vacuum rotovaporator at 32°. The residue, designated as crude non-saponifiable lipid, was dissolved in 5–10 ml of chloroform and placed in a tared test tube. After the removal of the solvent by evaporation under a stream of N_2, the tube and its contents were weighed, and the lipid dissolved in either 1 ml of chloroform or isooctane–chloroform (1:1, by vol.) as indicated.

Determination and characterization of tocopherol

A. Thin-layer chromatography. The thin-layer plates (8 inch × 8 inch) were prepared according to the method of Skinner and Parkhurst as previously reported[13] or were commercially acquired as silica gel-coated plates ("chromagrams", Distillation Products Industry). Ascending chromatography at room temperature was

employed. Aliquots of the crude non-saponifiable lipid (in chloroform), or the non-saponifiable lipids partially purified by column chromatography as described below, were spotted 2 cm from the bottom of the thin-layer plate, the edge of which was then submerged in benzene to a depth of 3–5 mm, and the solvent allowed to run a distance of 15 cm.

The completed chromatograms were sprayed with Emmerie–Engel reagent (5% α,α'-dipyridyl in ethanol followed by 3% $FeCl_3$ in ethanol) for detection of tocopherols. The plates were then placed in a jar saturated with iodine vapors for visualization of the other lipids as well.

B. Silicic acid column chromatography. The crude non-saponifiable lipid, usually varying from 20 to 130 mg, was dissolved in 1 ml isooctane–chloroform (1:1, by vol.) and chromatographed on a silicic acid column as described below. The optimum length and diameter of the column were determined experimentally and found to conform to the following mathematical expression relating weight of lipid taken to the geometry of the column: length of column $= 7\sqrt{G}$, diameter of column $= 0.57\sqrt[4]{G}$, where $G =$ g of silicic acid determined by the relation of 10 mg non-saponifiable lipids per g of silicic acid.

Silicic acid (BioRad–BioSil A, 100–200 mesh) was activated for 30–60 min at 100° before use. Isooctane was added to the silicic acid and the mixture was poured into a glass column containing a glass wool plug for flow control, and the resultant packed silicic acid column washed with several vol. of isooctane. The lipid was added to the top of the packed silicic acid column followed by successive elution with 30-ml vol. of each of the following solvent mixtures: isooctane–chloroform (9:1, v/v), isooctane–chloroform (3:1, v/v), isooctane-chloroform (1:1, v/v), chloroform and finally chloroform–methanol (1:1, v/v).

All fractions eluted from the silicic acid column were evaporated to dryness in a Büchi partial vacuum rotovaporator at 32° and redissolved in a known amount of chloroform. An aliquot was taken for thin-layer chromatography.

C. Gas-liquid chromatography. In most cases, tocopherol was converted to its trifluoroacetate derivative prior to gas–liquid chromatography in order to afford greater resolution and sensitivity[14]. Gas chromatography was routinely carried out on U-shaped columns (6 ft × 5 mm internal diameter) containing a mixture of phases, SE 52 (methyl polysiloxane containing a small percentage of phenyl groups) and XE 60 (β-cyanoethyl methylpolysiloxane) as described by NAIR *et al.*[15]. The temperature of the column was maintained at 220° with an outlet flow rate of 100 ml/min. A modified Barber–Colman model 10 gas chromatograph equipped with a high gain electrometer–high voltage combination unit was employed in this study, and measurements were made using an Ar ionization detector containing a 20-mC ^{90}Sr source.

RESULTS

Identification and quantification of α-tocopherol in mitochondrial lipids

Thin-layer chromatography was one of the more routinely used procedures in the present study for identification of vitamin E among the non-saponifiable lipids of mitochondria. As shown in Fig. 1, thin-layer chromatography of the crude or partially purified non-saponifiable mitochondrial lipids gave an Emmerie–Engel positive spot, with the same R_F as that of standard α-tocopherol ($R_F = 0.50$ in

benzene). The method proved to be highly reproducible and afforded a rapid qualitative and semi-quantitative means for detecting tocopherol. Fig. 1B shows the same chromatogram but further developed with iodine vapors for visualization of other lipids as well.

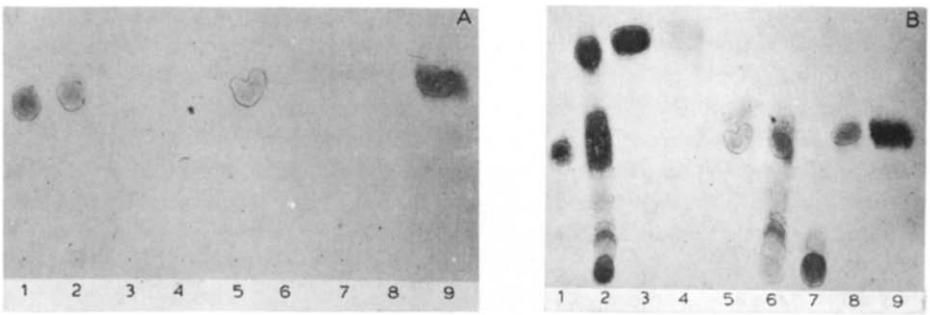


Fig. 1A. Thin-layer chromatography of authentic α-tocopherol and mitochondrial lipids developed with Emmerie–Engel reagent (solvent front not shown). 1, standard α-tocopherol, 5 μg; 2, total mitochondrial lipids; 3, isooctane–chloroform (9:1, v/v) eluate from silicic acid column; 4, isooctane–chloroform (3:1, v/v) eluate; 5, isooctane–chloroform (1:1, v/v) eluate, 50A fraction; 6, isooctane–chloroform (1:1, v/v) eluate, 50B fraction; 7, chloroform eluate; 8, reduced ubiquinone, 5 μg; 9, standard α-tocopherol, 10 μg. B. Same chromatogram but further developed with iodine vapors to show other lipids as well.

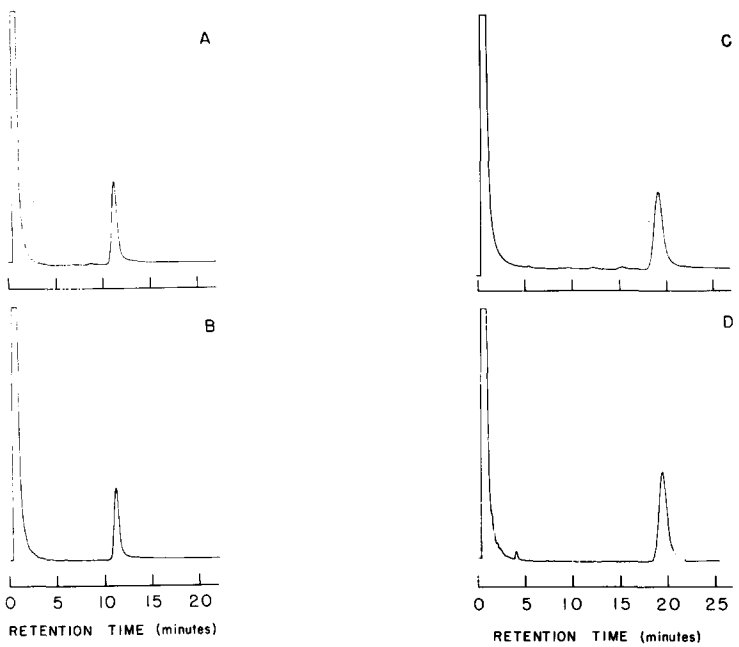


Fig. 2. Comparison of gas–liquid chromatographic tracings of authentic α-tocopherol and a purified tocopherol fraction from mitochondrial lipids. A. Standard α-tocopherol (trifluoroacetate derivative) 2 μg in 2 μl of hexane. B. Eluate from silicic acid column (1:1 (v/v) isooctane–chloroform); 50A fraction trifluoroacetate derivative, 2 μl. C. α-Tocopherol standard 4 μg in 4 μl. D. Eluate from silicic acid column (1:1 (v/v) chloroform–isooctane) 2 μl, 50A fraction. The ordinate represents the detector response $3 \cdot 10^{-9}$ A.

For quantitative separation of α-tocopherol, silicic acid column chromatography was used. Vitamin E could be obtained in most cases in a nearly pure state (90–100% pure) from the crude non-saponifiable lipid by collecting the fraction designated as 50A eluted by isooctane–chloroform (1:1, v/v) just prior to the elution of the yellow band (ubiquinone) which moved at a slower rate than tocopherol with the same solvent. Authentic α-tocopherol displayed the same elution pattern as observed with the sample obtained from mitochondrial lipids. In a typical silicic acid column an initial lipid load of 139 mg yielded a recovery of 125 mg, *i.e.*, 90% of the total. The α-tocopherol represented 0.4% of the total non-saponifiable lipid.

Quantification of tocopherol in beef heart mitochondria was done almost exclusively by gas–liquid chromatography and gave an average recovery for the vitamin of 99.7%. The method also provides additional support for the identification of vitamin E in view of identical retention times of authentic tocopherol and the biological material (previously treated by silicic acid chromatography) (Fig. 2).

Ultraviolet absorption spectra recorded on the Cary 14 and Perkin–Elmer model 220 spectrophotometers were also used to identify α-tocopherol in mitochondria. The spectrum of fraction 50A (*i.e.*, purified α-tocopherol derived from the crude non-saponifiable lipid by silicic acid column chromatography) displaying peak absorption at 292 mμ was identified with that of standard tocopherol (Fig. 3). Subsequent oxidation of the 50A fraction by $FeCl_3$ and α,α′-dipyridyl[15] transformed the ultraviolet spectrum to one now exhibiting a bicuspid peak at 262–269 mμ, characteristic of α-tocopheryl quinone (the oxidation product of tocopherol) and similar to

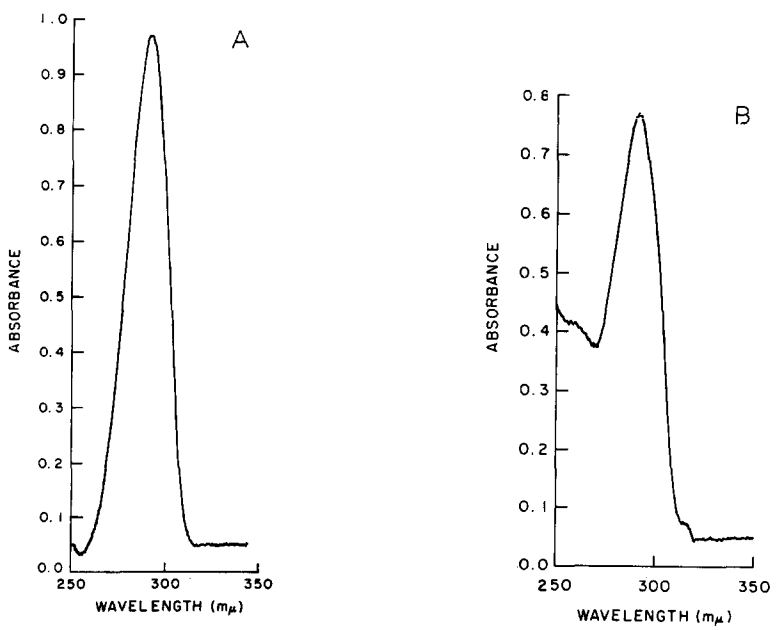


Fig. 3. Comparison of ultraviolet spectra of authentic α-tocopherol and a purified tocopherol fraction from mitochondrial lipids. A. Standard tocopherol. B. Fraction 50A (1:1 (v/v) chloroform–isooctane) eluate from silicic acid chromatography.

the spectrum of standard α-tocopherol treated in the same way (Fig. 4). A similar transformation of the ultraviolet spectrum could also be achieved by oxidation of the 50A fraction with gold chloride[16] (not shown).

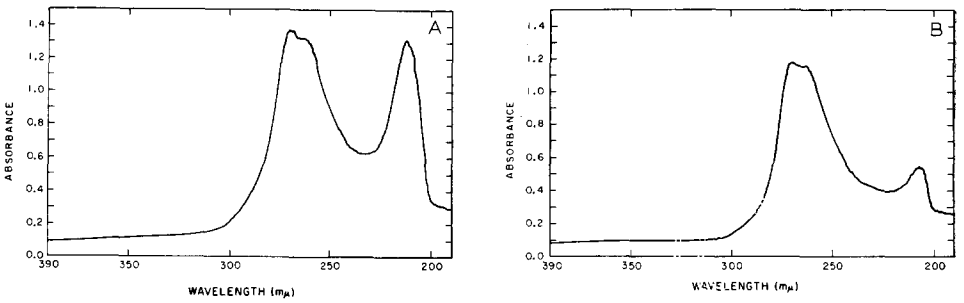


Fig. 4. Comparison of ultraviolet spectra of oxidized authentic α-tocopherol and oxidized purified tocopherol fraction from mitochondrial lipids. A. α-Tocopherol oxidized by ferric-α,α′-dipyridyl. B. Fraction 50A oxidized by ferric-α,α′-dipyridyl.

Tocopherol content of bovine heart subcellular fractions

Table I summarizes the over-all distribution of tocopherol among the fractions obtained by centrifugation of the crude heart muscle homogenates. The pellet of crude

TABLE I

TOCOPHEROL CONTENT OF BEEF HEART SUBCELLULAR FRACTIONS

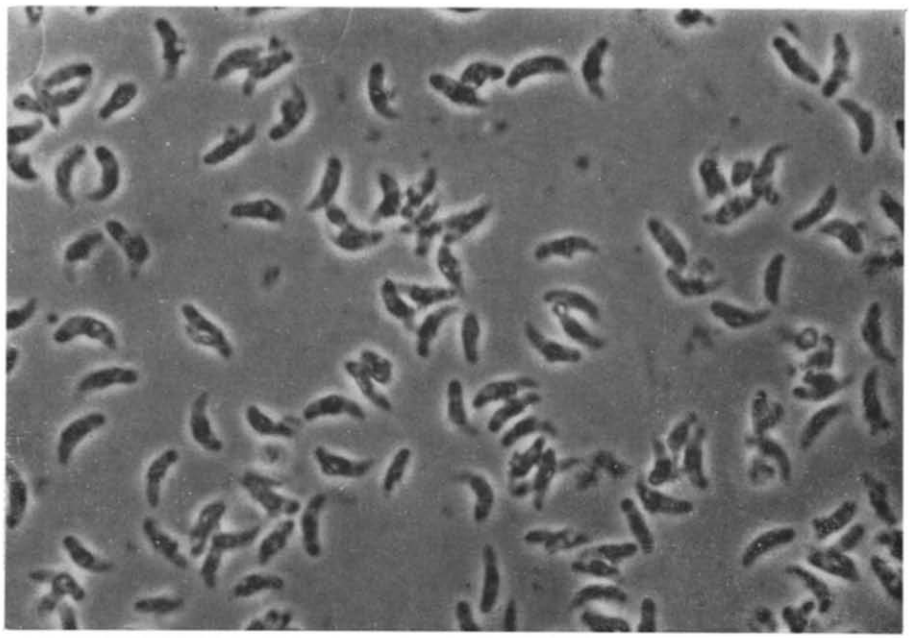
Standard conditions of assay including tocopherol determination by gas–liquid chromatography described in EXPERIMENTAL PROCEDURES.

Prep. No.	*Fraction*	*Protein (g/ aliquot)*	*Enzymatic activity**	*Mitochondrial contamination***		*Tocopherol (μg)*	*Tocopherol**** *(μg)*	*Tocopherol (μg/g protein)*
				Protein (mg)	(%)			
1	Nuclei (crude) (pellet 1600 × *g*)	53.200	0.08	425	0.8	1 142	1 065	20
	Mitochondria (pellet 20000 × *g*)	10.700	10.0	—	—	1 853	—	174
	Supernatant (40000 × *g*) (continuous flow)	41.300	0.02	1600	0.4	504	476	12
2	Nuclei (crude)	3.535	—	—	—	171.4	—	48.5
	Mitochondria	1.150	—	—	—	123.5	—	107.5
	Supernatant	1.280	—	—	—	0		0
3	Nuclei (purified)	1.080	0	0	0	65	—	60.0
	Mitochondria	2.904	0.38	—	—	164	—	56.0
4	Nuclei (crude)	3.790	0.04	83.6	2.5	126.3	123.8	36.4
	Mitochondria	1.060	1.5	—	—	30.6	—	29.8
	Supernatant	2.217	0.07	82.5		11.1	8.7	3.9

* μmoles of cytochrome *c* oxidized/min per mg protein.

** Ratio total cytochrome oxidase activity of sample/specific activity of mitochondrial cytochrome oxidase = mg mitochondrial protein in sample.

*** The values are corrected as follows: tocopherol in sample − tocopherol in contaminating mitochondria = corrected tocopherol.



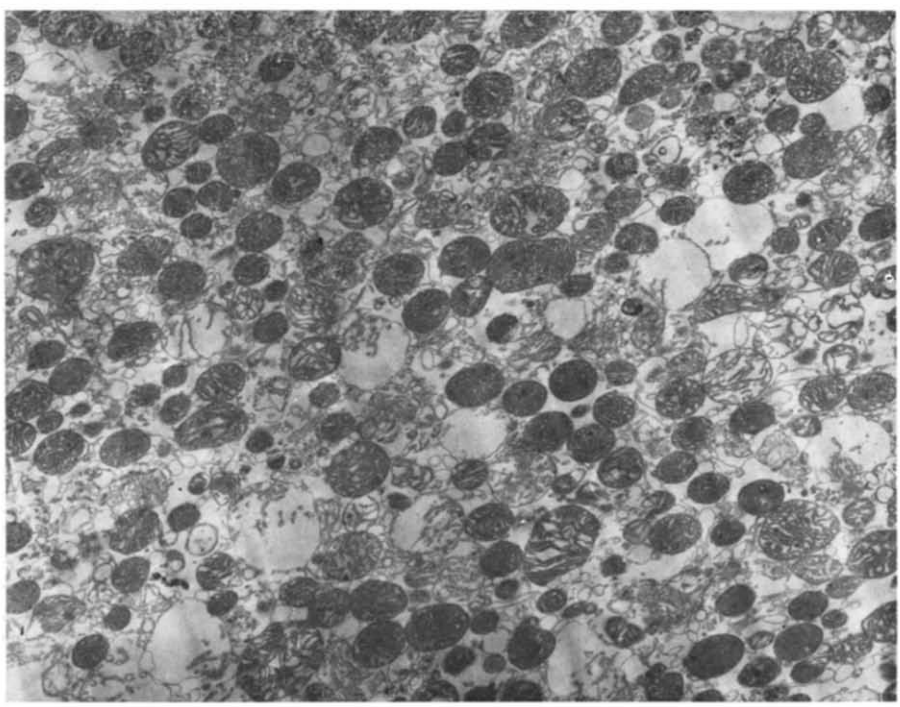


Fig. 5. Purified nuclei[8] from beef heart muscle as viewed under the light microscope, magnification approx. 1350 ×.

Fig. 6. Electron micrograph of purified beef heart mitochondria (after glutaraldehyde fixation and OsO_4 staining), magnification approx. 11500 ×.

nuclei obtained by centrifugation at 1600 × *g* for 12 min (see EXPERIMENTAL PROCEDURES) was a highly heterogeneous material consisting of myofibrils, nuclei, cellular debris and unbroken cells as indicated by microscopic examination. The tocopherol values for the nuclei are corrected for presumed contaminating mitochondria and mitochondrial fragments, based on cytochrome oxidase activity as shown in Table I. The ratio of tocopherol to protein in the nuclear fractions is appreciable, and in two experiments, one with purified nuclei[8] (Fig. 5) and one with crude nuclei, the ratio was even slightly greater than that obtained for the mitochondria from the same preparation. The concentration of tocopherol in nuclei reported in the present work is appreciably higher than that indicated in the literature[2].

The mitochondria prepared by continuous flow centrifugation at 40000 × *g* (Fig. 6) did not show microsomal contamination as assayed by NADPH–cytochrome *c* reductase activity. The supernatant solution after centrifugation at 40000 × *g* (*i.e.*, after removal of mitochondria) had the lowest tocopherol to protein ratio and was usually free of mitochondria, as assayed by cytochrome oxidase activity.

Tocopherol in bovine heart mitochondria

Table II shows the tocopherol concentration found in several preparations of beef heart mitochondria, with values ranging from 23 to 174 μg of tocopherol per g of protein. The data were collected over a period of one year, and apparently exhibited no correlation with the season in which the determinations were made (for instance, Preps. 1 and 10 were processed and assayed in summer, and Preps. 4 and 5 in winter). One explanation for the variation in tocopherol concentration in beef heart probably resides in the different diets received by the animals. Slaughterhouse officials indicated that the sacrificed animals were from different sources and had been fed in diverse ways ranging from balanced artificial diets to natural pastures. The obviously wide variety of dietary patterns could explain the almost 10-fold range of 0.05–0.44

TABLE II

TOCOPHEROL CONTENT OF BEEF HEART MITOCHONDRIA

Standard conditions of assay as described in EXPERIMENTAL PROCEDURES.

Prep. No.	*Protein (g)*	*Lipid (mg)*	*Lipid (mg/g protein)*	*Tocopherol*			*Enzymatic activity**
				Method of gas–liquid chromatographical quantitation	*Total (μg)*	*μg/g protein*	
1	4.285	98	23.2	non-derivative	479	111	—
2	7.300	139	19.0	non-derivative	850	112	0.40
3	7.960	295	37.0	TFA**-derivative	332	42	—
4	7.250	200	27.6	TFA-derivative	342	47	—
5	10.700	211	21.1	TFA-derivative	1853	174	1.0
6	1.150	36	31.4	TFA-derivative	123	107	4.0
7	1.157	13.0	11.5	TFA-derivative	116	100	1.0
8	2.904	80	27.5	TFA-derivative	164	56	1.5
9	1.060	44	41.5	TFA-derivative	30.6	30	1.5
10	1.090	28	24.8	TFA-derivative	24.3	23	3.0

* μmoles of cytochrome *c* oxidized/min per mg protein.
** TFA stands for trifluoroacetate.

TABLE III

TOCOPHEROL CONTENT OF LIPOPROTEIN COMPLEXES FROM MITOCHONDRIA TREATED WITH DEOXYCHOLATE AND AMMONIUM ACETATE

Assays and methods including tocopherol determination by gas–liquid chromatography as described in EXPERIMENTAL PROCEDURES.

Fraction	*Protein* (*g*)	*Lipid* (*mg*)	*Lipid* (*mg/g protein*)	*Total tocopherol* (*μg*)	*Tocopherol* (*μg/g protein*)	*Tocopherol* (*μg/mg lipid*)	*Enzymatic activities*		
							*Cytochrome oxidase**	*NADH–cytochrome c reductase***	*Succinate–cytochrome c reductase***
Mitochondria	—	—	—	—	—	—	0.29	0.05	0.05
Crude cytochrome oxidase fraction	5.000	78.0	15.6	142	28.4	1.8	0.09	0.01	0.00
Dialysis supernatant	9.400	77.0	8.2	341	36.3	4.4	0	0	0
16.5% ammonium acetate precipitate	0.840	17.8	21.2	3.7	4.4	0.2	0.16	0.06	0
22.8% ammonium acetate precipitate	0.610	20.0	32.8	3.4	5.6	0.2	0.04	2.80	—
26% ammonium acetate precipitate	0.150	4.8	32.0	0.5	3.3	0.1	0.04	1.28	0.24
Final supernatant	0.200	30.0	150.0	6.4	32	0.2	0	0	0

* μmoles of cytochrome *c* oxidized/min per mg protein.

** μmoles of cytochrome *c* reduced/min per mg protein

TABLE IV

TOCOPHEROL CONTENT OF SOME ELECTRON TRANSPORT FRACTIONS OF BEEF HEART MITOCHONDRIA

Assays and methods including tocopherol determination by gas–liquid chromatography as described in EXPERIMENTAL PROCEDURES.

Prep. No.	*Fraction*	*Protein*		*Tocopherol*		*Tocopherol (μg/g protein)*	*Enzymatic activities*					
							cytochrome oxidase		*NADH–cytochrome c reductase*		*Succinate–cytochrome c reductase*	
		(*g*)	(%)	(*μg*)	(%)		(*spec. act.*)*	(%)	(*spec. act.*)*	(%)	(*spec. act.*)*	(%)
1	Mitochondria	20.570	100	458.7	100	22.3	3.0	100	1.7	100	0.6	100
	Cytochrome oxidase	5.190	25	24.8	5.5	4.8	3.6	30	0	0	0	0
	Supernatant	11.000	53.5	304	66.5	27.6	0.07	2.5	0	0	0	0
2	Mitochondria	22.000	100	1122	100	51	0.38	100	—	—	—	—
	Cytochrome oxidase	9.520	43	143	12.7	15.0	0.66	75	0.21		0.02	
	Supernatant	6.675	30.4	660	59	99	0	0	0.002		0.001	
3	Mitochondria	16.468	100	494	100	30	1.5	100	0.72	100	—	—
	Cytochrome oxidase**	1.201	7.4	52.6	10.7	43.8	1.6	7.8	0	0	0	0

* μmoles of cytochrome *c* reduced (or oxidized)/min per mg protein.

** Partially purified by the procedure of HATEFI *et al.*[6].

TABLE V

DISTRIBUTION OF TOCOPHEROL IN INNER AND OUTER MITOCHONDRIAL MEMBRANES

Assays and methods including tocopherol determination by gas–liquid chromatography as described in EXPERIMENTAL PROCEDURES.

Fraction	*Protein (g)*	*Non-saponifiable lipid*		*Tocopherol content*			*Enzyme activity*	
		mg	*mg/g protein*	*Total (μg)*	*μg/g protein*	*μg/mg lipid*	*Cytochrome oxidase**	*Monoamine oxidase***
Mitochondria	4.875	9 (aliquot)	14	—	—	—	0.620	5
9500 × *g* pellet (inner membranes)	2.904	80	27	164	56.5	2.0	0.84	3
105000 × *g* pellet (outer membranes)	0.139	15	108	0	0	0	0.66	15
Last supernatant	1.702	8	4.7	0	0	0	0	0.1

* μmoles/min per mg protein.

** mμmoles benzaldehyde/min per mg protein.

μmole of tocopherol per g protein, representing an average of 0.18 μmole per g protein. The value is approximately one half the 0.4 μmole tocopherol per g protein reported by SLATER *et al.*[16] for tocopherol content of the Keilin-Hartree horse heart muscle preparations.

Table II also shows that the specific activity of cytochrome oxidase in beef heart mitochondria does not appear to be related to the absolute amount or to the concentration of tocopherol present in the same sample. Therefore, there is no obvious relationship between the concentration of this vitamin (at least within the range found) and the ability of the enzyme preparation to catalyze the transfer of electrons from reduced cytochrome *c* to molecular oxygen.

Tocopherol in fractions of the electron transport system of mitochondria

The method of HATEFI *et al.*[6] was utilized for the fractionation of mitochondria into their component lipoprotein complexes, all of which had tocopherol (Table III). The fraction possessing NADH-cytochrome *c* reductase activity (22.8% ammonium acetate precipitate) had less tocopherol per g of protein than the dialysis supernatant fraction and the final supernatant fraction both of which lacked enzymatic activities.

The crude cytochrome oxidase complex which was the enzymatic fraction with the largest protein content also exhibited an appreciable content of tocopherol averaging about 10% of that of the total mitochondria (Table IV). When cytochrome oxidase was purified one step further[6] (Prep. 3 in Table IV), there was an increase in the ratio of α-tocopherol to protein. The supernatant fractions as seen in Table III (*viz.*, dialysis supernatant and final supernatant) and Table IV (*viz.*, supernatants of Preps. 1 and 2) contained the highest percentage of the original mitochondrial protein and lipid including 60% of the total tocopherol of the starting material, and were devoid of electron transport enzymatic activities. These fractions were designated as supernatant preparations because they represented the soluble material remaining after the cytochrome oxidase and NADH and succinate-cytochrome *c* complexes had been precipitated.

Tocopherol distribution in inner and outer membranes of mitochondria

The recent methods available for separation of membranes in rat liver mitochondria[7,17,18] were successfully adapted for beef heart mitochondria. In spite of the different viewpoints on enzyme markers for these membranes[19], several laboratories[17,18] have reported findings in good agreement with those of SCHNAITMAN *et al.*[7].

Table V summarizes the results of tocopherol distribution in inner and outer membranes of mitochondria. Tocopherol was confined entirely to the inner membranes (or cristae) of mitochondria: vitamin E could not be detected in the outer membranes. These findings, as well as the low level, if not absence, of tocopherol in the supernatant solution (Table V) support in some respects the proposal of the different origins of the two membrane systems of mitochondria, the outer ones being related in origin, and perhaps in properties, to the endoplasmic reticulum (see discussion of biogenesis of mitochondria by LEHNINGER[20]). The relatively high specific activity of cytochrome oxidase observed in the present experiments in the outer membranes could be simply due to a slight contamination by the inner membranes in view of the small protein content of the outer membranes. The presence of some monoamine-oxidase in the inner membranes might be ascribed to its incomplete removal by the procedure employed.

DISCUSSION

The experiments described in this paper clearly demonstrate the presence of tocopherol in purified beef heart mitochondria. The several independent methods applied in the present work for the determination of vitamin E represent an improvement over previous reports for a number of reasons. First, the use of a silicic acid column employing chloroform–isooctane mixtures as solvents resulted in the isolation of tocopherol in a relatively pure state from other lipids. Moreover, the silicic acid columns are easier to prepare than either the floridin or alumina columns classically employed in vitamin E studies. In a recent review on the silicic acid column chromatography of tocopherol, it was stated that the separation of tocopherol from other contaminants, using a Skellysolve F–ethyl ether mixture as a solvent, was not satisfactory[21]. The effective resolution and recovery of vitamin E from a lipid mixture was attained in the present experiments by use of chloroform–isooctane mixtures as solvents. Secondly, quantification of tocopherol by gas–liquid chromatography eliminates the possibly erroneous values found with the colorimetric procedure of Emmerie–Engel due to the presence of reducing substances other than vitamin E. On the other hand, the gas–liquid chromatographic method is more time consuming, but the accuracy obtained is compensatory. Finally, thin-layer chromatography also proved to be very useful for qualitative or semi-quantitative analyses, being a reproducible and rapid technique. It was used routinely to monitor the fractions eluted from silicic acid columns.

BOUMAN AND SLATER[22] were the first to claim the presence of vitamin E in active respiratory systems. Since then, several laboratories have reported the occurrence of tocopherol in Keilin–Hartree preparations, mitochondria and some enzymes of the electron transport system[2], thus implying a possible role for tocopherol in the electron transport chain. The findings of NASON AND LEHMAN[23] and DONALDSON *et al.*[24] on the action of tocopherol in restoring the enzymatic activity of the NADH– and succinate–cytochrome *c* reductases partially inactivated by aging or isooctane extraction, together with the observation of the reversal of the inhibitory effect of digitonin upon the same system by tocopherol[25], have led to the postulation of possible vitamin E participation in the mitochondrial electron transport system.

In the present studies, it seems that tocopherol, although undoubtedly found in bovine heart mitochondria, does not appear to have a direct quantitative relationship with the enzymatic activities observed, such as cytochrome oxidase and NADH– and succinate–cytochrome *c* reductases. It may be possible that the amount of vitamin E in the normal cell is in excess of a critical concentration below which deficiencies and correlations with observed enzymatic activities would occur. This question might be answered utilizing tissues from laboratory animals on controlled diets.

Fractionation of the electron transport chain of mitochondria into its component lipoprotein complexes by the method of HATEFI *et al.*[6] involves disruption of mitochondria with potassium deoxycholate and further precipitation of the proteins with KCl and ammonium acetate. The final distribution of the mitochondrial lipid may be questioned in such a rigorous procedure, particularly since deoxycholic acid is known to act on the lipid–protein bonds thereby allowing for a possible redistribution of lipids in the various lipoprotein fractions. Nevertheless, the enzymatic systems thus obtained, when reunited into one complex are able to catalyze the

transport of electrons from NADH to molecular oxygen[26], proving that its functional properties are retained.

Another explanation for the observed ubiquity of tocopherol in all fractions (with or without enzymatic activity) obtained by treatment of mitochondria with deoxycholate is that tocopherol is possibly involved as a structural component of the mitochondrial cristae, perhaps as part of a lipoprotein that functions in the physical organization of the electron transport system. This viewpoint is supported by the present findings that tocopherol apparently occurs only in the inner mitochondrial membrane fraction (the cristae), namely in a structure where both the enzymes of electron transport system and the bulk of the mitochondrial structural proteins are located.

Of some interest is the observation that appreciable levels of tocopherol are present in the nuclei of beef heart muscle. In at least two experiments, the purified nuclei possessed a tocopherol to protein ratio greater than that observed for the corresponding mitochondria. Perhaps in line with this observation, tocopherol also serves in some role in nucleic acid metabolism, as originally suggested by Dinning and associates (see review by VASINGTON *et al.*[2]), in addition to its mitochondrial functions.

ACKNOWLEDGEMENTS

This investigation was supported in part by research grants from the National Institutes of Health, U. S. Public Health Service (AM-5923, AM-02131, and General Research Support Grant 5SO-1FR-05478-07).

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