STUDIES OF THE BIOLOGICAL FUNCTION OF VITAMIN E

I. TOCOPHEROL AND REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE–CYTOCHROME *C* REDUCTASE*

C. J. POLLARD AND J. G. BIERI

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

1. The DPNH-cytochrome *c* reductase activity was determined in heart-muscle preparations from normal and vitamin-E-deficient chicks. Although no α-tocopherol was demonstrable in the deficient preparations, the enzyme activity was not significantly different from the control preparations which contained α-tocopherol.

2. Substances which react with $FeCl_3$–bipyridyl reagent, other than α-tocopherol, were found in Florex-treated extracts of unsaponifiable matter from tissues. No unequivocal evidence was obtained for the presence of tocopherylquinone in tissues.

3. The inactivation of certain enzymes in the respiratory chain by isooctane extraction was duplicated by homogenizing the enzyme preparations with small amounts of isooctane. The inactivation, which was overcome by the addition of various lipid substances, was also reversed by simply lyophilizing or centrifuging the isooctane-treated enzyme solutions.

INTRODUCTION

Recent studies have attempted to ascertain whether α-tocopherol is an integral part of the respiratory chain[1,2]. In these reports the reactivation of isooctane-extracted enzyme preparations by tocopherol has been shown. However, in only one laboratory has specificity for tocopherol been claimed—in the aged isooctane-extracted or aged, digitonin-solubilized, mammalian cytochrome *c* reductases as prepared by DONALDSON *et al.*[3] and VASINGTON *et al.*[4]. Recently WEBER *et al.*[5] have proposed that the isoprene side chain of vitamin E and vitamin K_1 and other substances is responsible for the reactivation of isooctane-extracted succinate-cytochrome *c* reductase. These workers also postulated that the reactivation of this system by other quinones (*e.g.*, menadione) was nonspecific and could be attributed to the redox system of the quinones[6].

We have attempted to determine whether or not tocopherol acts specifically as a cofactor in the DPNH-cytochrome *c* reductase by the following lines of approach: (a) Determination of DPNH-cytochrome *c* reductase activity in normal and vitamin-

Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

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E- and vitamin-K-deficient chick hearts; (b) quantitative and qualitative analyses for tocopherol and other materials in tissues which react with ferric chloride–bipyridyl reagent; and (c) examination of the inhibitory effect of isooctane extraction on DPNH-cytochrome *c* reductase and the mode of action of its reactivation by tocopherol and other substances. In this paper it is shown that the DPNH-cytochrome *c* reductase activity in heart muscle from vitamin-E-deficient chicks is the same as that in hearts from normal chicks. It is further shown that the effect of isooctane extraction in inactivating this enzyme system can be explained by a narcosis of the enzymes by the solvent.

MATERIALS AND METHODS

Cofactors and other substances

DPNH and horse-heart cytochrome *c* were products of the Sigma Chemical Co. Crystallized bovine plasma albumin was obtained from Armour and Co. *d*-α-Tocopherol was obtained from Distillation Products Industries. Vitamin K_1 and *dl*-α-tocopherol were products of Merck and Co. Digitonin and menadione were obtained from Nutritional Biochemicals Corp. Spectral grade isooctane (2,2,4-trimethylpentane) was obtained from the Matheson, Coleman and Bell division of Matheson Company, Inc.

Enzyme preparations and assays

The DPNH-cytochrome *c* reductase activity was determined on a particulate supernatant (3,000 × *g*) of heart muscle, equivalent to fraction II of LEHMAN AND NASON[7], under the same set of conditions described by these workers. The digitonin-solubilized enzyme solutions were prepared as described by DONALDSON *et al.*[3]. Isooctane extractions and the preparation of tocopherol, vitamin K_1, and menadione suspensions in bovine albumin were done by the methods of NASON AND LEHMAN[3]. The KEILIN-HARTREE bovine heart-muscle preparation was prepared according to SLATER[9]. Protein content was determined by the method of GORNALL *et al.*[10].

Production of vitamin E-deficient and vitamin K-deficient chicks

Vitamin-E-deficient chicks were produced by feeding day-old chicks a synthetic vitamin-E-free diet (C47A) containing 4% of vitamin-E-free lard[11]. Chicks were maintained on this diet for at least 4 weeks. Two series received 15 or 30% of torula yeast added in the diet in order to accelerate the vitamin E deficiency[11]. All diets contained 0.5 p.p.m. selenium, as selenite, to prevent early death from exudative diathesis[12,13]. Normal control chicks received 100 mg *dl*-α-tocopherol acetate/kg of diet.

Vitamin-K-deficient chicks were produced by feeding day-old chicks the same diet listed above but without vitamin K. Sulfasuxidine was added at a level of 0.5%. Chicks on the vitamin-K-deficient diet received no vitamin E. Since vitamin K deficiency developed rapidly, in 12–14 days, the chicks were not yet depleted of their vitamin E stores. Control chicks received vitamin E and vitamin K (as menadione) in Tween 80–ethanol solution in the drinking water on the seventh and eleventh days.

Quantitative and qualitative analyses of tocopherol and other ferric chloride–bipyridyl reacting materials in tissues

In general, the apparent tocopherol content of tissues was determined as follows: Known amounts of tissue were added to ethanol containing pyrogallol and brought

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to boiling; 11 *N* KOH was added and the saponification continued for 15 min. An equal volume of water was added to the cooled solution and the unsaponifiable matter was extracted with hexane. After washing with water, the hexane extracts were dried over anhydrous Na_2SO_4. An aliquot of the extract was evaporated to dryness under a stream of nitrogen in a test tube, taken up in 1.0 ml benzene, and put on a 2–4-cm column of $SnCl_2$–HCl-activated Florex XXF in a Hennesy tube. The test tube was rinsed with two 0.5-ml portions of benzene. Three additional 2-ml portions of benzene were allowed to flow through the Florex column. An aliquot of the benzene effluent was evaporated, the residue dissolved in absolute ethanol, and the color produced with $FeCl_3$–bipyridyl determined at 520 mμ in a Coleman Junior Spectrophotometer after 30 sec. A water blank was used and a correction was made for the color produced by the reagents alone. For identification purposes, the Florex-treated extracts were freed of as much of the sterols as possible by repeated (three times) low-temperature freezing in methanol in an acetone dry ice bath. The extracts were then chromatographed on paper with the system of GREEN *et al.*[14], modified by using 20% benzene in isooctane (v/v) as developing solvent.

RESULTS

DPNH-cytochrome c reductase activity of heart muscle from normal and vitamin-E-deficient chicks

Preliminary experiments showed that chick heart contained a very active DPNH-cytochrome *c* reductase. (This enzyme is also present in breast muscle, but the activity is much lower.) Furthermore, it could be demonstrated that isooctane extraction of the enzyme solution led to loss of activity which could be restored by suspensions of tocopherol, vitamin K, or menadione in ethanol-albumin. Table I shows the DPNH-cytochrome *c* reductase activity of heart muscle from normal and vitamin-E-deficient chicks. Since the enzyme activities of chicks receiving the several diets were similar, the results are considered together. None of the deficient chicks appeared morbid.

TABLE I

DPNH-CYTOCHROME *c* REDUCTASE ACTIVITY OF CHICK-HEART MUSCLE PREPARATIONS

Reaction mixture contained 0.03–0.05 ml enzyme preparation (0.18–0.3 mg protein), 0.01 *M* KCN, 0.1 ml 2% aqueous cytochrome *c*, and 0.10 ml DPNH (1.15 μmoles/ml) in a total vol. of 3 ml with 0.1 *M* phosphate buffer, pH 7.5.

Normal			*Vitamin-E-deficient*		
Chick No.	*Age, weeks*	*Specific activity**	*Chick No.*	*Age, weeks*	*Specific activity**
1	6	524	1	6	620
2	6	620	2	6	524
3	7	730	3	7	529
4	7	695	4	12	485
5	7.5	580	5	12	578
6	8	705	6	12	806
	Mean with S.D. 642.3 ± 34.3		7	4**	640
			8	4**	551
				Mean with S.D. 591.6 ± 52.6	

* Specific activity = Increase in absorbance at 550 mμ/2 min/g protein.
** Chicks had encephalomalacia.

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It is apparent from Table I that there was no significant difference in the DPNH-cytochrome *c* reductase activity of heart muscle from normal or vitamin-E-deficient chicks. Also, chicks which had been depleted for 12 weeks had activities similar to chicks depleted for only 4 weeks. Extracts from either the heart muscle or the enzyme fractions from vitamin-E-deficient chicks contained no detectable α-tocopherol. However, material which reacted with $FeCl_3$-bipyridyl was present in all tissues. This material migrated with the same R_F as α-tocopherol if there was an insufficient amount of sterols removed. No unequivocal evidence for tocopherylquinone (using ascorbic acid reduction) could be found in either normal or deficient tissues.

Since no tocopherol could be found in the heart after 4 weeks on the deficient diet, it would appear doubtful that functional amounts of the vitamin could still be present after as long as 12 weeks. Two chicks receiving 12% of vitamin-E-free lard and 0.5 p.p.m. of selenium in diet C47A were killed after 15–16 weeks; gross evidence of muscular dystrophy was noted. Their heart-muscle preparations had specific activities of 520 and 444.

DPNH-cytochrome c reductase activity of heart muscle from normal and vitamin-K-deficient chicks

Table II shows the DPNH-cytochrome *c* reductase activity of muscle from 2 week-old normal and vitamin K-deficient chicks. No analyses were made for vitamin K but prolonged clotting times and deaths from internal hemorrhages were found in the deficient group in contrast to no deaths and normal clotting times in control chicks receiving vitamin K.

TABLE II

DPNH-CYTOCHROME *c* REDUCTASE ACTIVITY OF CHICK-HEART MUSCLE PREPARATIONS

Same experimental conditions as in Table I. 14–15 day old chicks*.

Normal		*Vitamin-K-deficient*	
Chick	*Specific activity***	*Chick*	*Specific activity***
A	116	A	134
B	169	B	166
C	137	C	188
D	142	D	156
E	141	E	171
	—	F	163
Mean with S.D.	141 ± 10.6	Mean with S.D.	164 ± 9.0

* 0.1 ml enzyme preparation (0.53–0.67 mg protein) used in these experiments.
** Specific activity = Increase in absorbance at 550 mμ/2 min/g protein.

It is evident from Table II that the absence of vitamin K does not cause a lowering of the DPNH-cytochrome *c* reductase activity of chick hearts. The low activity of all chicks compared with those in Table I is a reflection of the age of the chick. We have noted that there is a marked increase in the activity of this system in the chick heart between the third and fourth weeks of age.

Quantitative and qualitative analyses of tocopherol and other materials in tissues and enzyme preparations

Chromatographic analyses. It should be emphasized that quantitative data

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reported here represent the *apparent* tocopherol content of tissues and enzyme preparations. Several workers have reported finding only one spot on chromatograms of extracts from animal tissues[15,16]. However, we invariably found at least 2 spots both of which react with $FeCl_3$–bipyridyl and also with ammoniacal $AgNO_3$. In some instances all the reducing substances migrate at the same rate. Fig. 1, drawn from a chromatogram of beef-heart-muscle extract, demonstrates the behavior of reducing substances before and after sterols are removed.

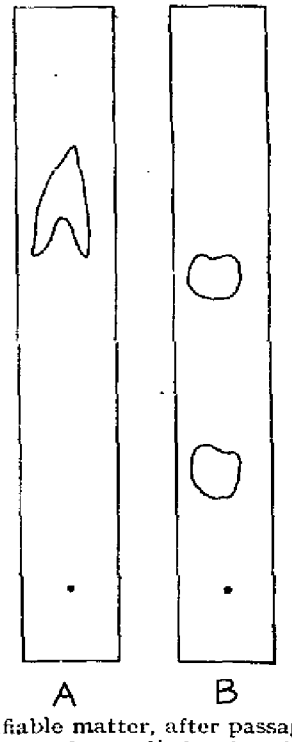

Fig. 1. Chromatograms of unsaponifiable matter, after passage through Florex XXF, from beef-heart muscle. System: $ZnCO_3$-impregnated paper[14], developed with 20% (v/v) benzene in isooctane and sprayed with $FeCl_3$–bipyridyl reagent. Solvent front at top edge of strips. A. Sample as obtained from Florex column. B. Same sample after removal of sterols.

In our hands the chromatographic system of GREEN *et al.*[14] gives varying values for the R_F of α-tocopherol depending on the amount of unsaponifiable material in the sample. Fig. 1 illustrates that unless the great bulk of the sterols are removed all of the EMMERIE-ENGEL reacting material (including added tocopherol) tends to migrate at the same rate. Such a result could be interpreted erroneously as demonstrating the presence of tocopherol in some instances where it is actually absent. This was most clearly shown by chromatographing Florex-treated extracts from vitamin-E-deficient chicks. Whereas analysis showed 9–12 μg "tocopherol" of heart muscle and the EMMERIE-ENGEL-reacting material migrated with the same R_F as α-tocopherol, upon removal of more sterols the R_F became that of the slow spot in Fig. 1B. The slow spot shown in Fig. 1B has been seen in virtually all tissues. In some instances after sterol removal 2 slow spots are seen, one immediately above the other. The R_F of the slow spot can be reduced to almost zero if sufficient amounts of

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sterols are removed. That the slow spot is not an artifact resulting from the $ZnCO_3$-paper is apparent from the finding that similar results are obtained if silicic acid-treated paper[17] and 20 % benzene in isooctane (v/v) are used as the chromatographic system.

Quantitative analyses of apparent tocopherol in tissues and enzyme preparations. BOUMAN AND SLATER[15] and DONALDSON AND NASON[18] have recently presented evidence that tocopherol may exist largely as its quinone in some tissues. The evidence rests on the increase in $FeCl_3$–bipyridyl reacting material in the unsaponifiable fraction when it is reduced before or after saponification. COWLISHAW *et al.*[16] could find no significant increase after $SnCl_2$–HCl treatment of chick-liver extracts. Tables III, IV, and V list apparent tocopherol values for beef-heart muscle, chick tissues, and various enzyme preparations.

Table III shows the tocopherol content of samples of a single beef heart at varying time intervals of storage at —20°. There is an appreciable decrease in apparent tocopherol after 25 days of storage. A greater decrease occurred in the reducible material. DJU *et al.*[19] have reported the loss of tocopherol from tissues kept deep-frozen. If tocopherol were being oxidized to the quinone during storage, some increase in reduced material would be expected, but this was not observed.

TABLE III

APPARENT TOCOPHEROL CONTENT OF BOVINE-HEART MUSCLE; ANALYSES OF ONE HEART AT VARIOUS TIME INTERVALS

Days frozen at −20°	*μg tocopherol/g muscle*		
	Unreduced	*Reduced**	*Δ*
2	40.4	70.5	30.1
		75.0**	34.6
11	31.1	55.8**	24.7
14	45.3	65.0	19.7
18***	31.2	39.3§	8.1
		40.5	9.3
25	27.4	43.0	15.6

* Unsaponifiable matter reduced with ascorbic acid–HCl or $SnCl_2$–HCl, before treatment with Florex XXF.

** $SnCl_2$H–Cl-reduced.

*** Heart muscle extracted with ethanol for 12 h in the dark in the Soxhlet apparatus.

§ Reduced before saponification.

TABLE IV

APPARENT TOCOPHEROL CONTENT OF TISSUES OF CHICKS ON TOCOPHEROL-DEFICIENT DIET*

Age	*Tissue*	*μg tocopherol/g tissue*		
		Unreduced	*Reduced*	*Δ*
1 day	Liver	67.0	117.5	49.9
1 day	Liver + yolk sac	67.2	106.5	39.3
3 days	Liver + yolk sac	47.9	77.6	29.7
13 days	Liver	5.4	12.6	7.2
28 days	Liver	4.0	7.7	3.7

* All samples extracted for 18 h in the Soxhlet apparatus with ethanol. Half of each extract was then reduced with $SnCl_2$–HCl.

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TABLE V

APPARENT TOCOPHEROL CONTENT OF ENZYME PREPARATIONS

Enzyme preparation	μg tocopherol/g protein		
	Unreduced	Reduced	Δ
Bovine heart-muscle-fraction II[7]	71	124	53
KEILIN-HARTREE bovine-heart-muscle preparation[9] *	202	243	41
KEILIN-HARTREE bovine-heart-muscle preparation[9] *	214	214	0
KEILIN-HARTREE bovine-heart-muscle preparation[9] *		191 **	
Chick-heart-muscle fraction II[7]	80	78	−2

* Extracted 12 h in the Soxhlet apparatus in the dark with ethanol.
** Reduced before saponification.

From the data in Table IV it would appear that the material which is being converted to tocopherol-like compounds upon reduction is carried over from the egg and slowly disappears with time. We have not been able to demonstrate an increase in the material in chick tissues by feeding large amounts of tocopherol.

The data in Table V demonstrate that enzyme preparations may contain appreciable amounts of apparent tocopherol. However, we have occasionally found that the color contribution described above by the slow moving material *may be as much or more* than that from tocopherol. Our values for tocopherol are much lower than those reported by DONALDSON AND NASON[18], but are of the same order as those reported by BOUMAN AND SLATER[15] for bovine heart-muscle preparations. Both of these groups of workers have noted significant increases of tocopherol upon reduction. Our Keilin-Hartree bovine-heart-muscle preparation was prepared from a heart which had been frozen for 21 days. Since Table III suggests that the material formed upon reduction decreases rapidly upon aging, this might account for the small amount in our Keilin-Hartree preparation.

Ubiquinone or Q_{275} in tissues. Attempts in our laboratory to demonstrate the presence of tocopherylquinone in tissues have been unsuccessful. It is obvious from Tables III to V that reduction of extracts of unsaponifiable matter converts some oxidized material to a reduced state. One obvious difference between reduced and unreduced Florex XXF-benzene eluates from bovine-heart-muscle unsaponifiable matter was the light yellow color of the unreduced in contrast to the reduced, which was almost colorless. This yellow material was isolated as follows: A benzene eluate from activated Florex XXF equivalent to 30 g of bovine heart muscle was evaporated to dryness under N_2 and the residue taken up in hexane. The hexane solution was placed on a 1 × 5 cm column of 2:1 silicic acid–Super Cel and 100 ml of hexane allowed to pass through the column. The yellow band at the top of the column was extruded and eluted with 10% ethanol in hexane. The ethanol–hexane solution was evaporated to dryness under nitrogen and taken up in hexane. This solution was placed on a 1 × 8 cm column of MgO–super cel (1:1) and developed with hexane. The yellow band which traveled discreetly was collected. The material had an absorption maximum at 275 mμ in absolute ethanol and gave no color with $FeCl_3$–bipyridyl. Its reduction product had a maximum at 292 mμ in absolute ethanol and reacted with the EMMERIE-ENGEL reagent. It is highly probable that the original yellow material is identical with or related to the Q_{275} of CRANE *et al.*[20] and ubiquinone

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of MORTON *et al.*[21]. Upon reduction Q_{275} may account for as much as one-fourth of the increase (after ascorbic acid–HCl treatment) in EMMERIE-ENGEL reacting material. It is possible that the slow spot seen on paper chromatograms and referred to above may be reduced Q_{275}. In view of the data presented above, we feel that further research is needed before it can be stated with certainty that other forms of α-tocopherol exist in tissues.

The effect of isooctane extraction on enzyme preparations

The reactivation of isooctane-extracted DPNH-cytochrome *c* reductase by tocopherol and other substances has been demonstrated in several laboratories[1,2,5]. DEUL, SLATER AND VELDSTRA[2] have suggested the possibility that inhibition is caused by the absorption of isooctane on the surface of the enzyme and that α-tocopherol and other fat-soluble substances act simply by dissolving the isooctane. The results given below are in accord with this hypothesis.

Preliminary experiments[22] showed that lyophilized preparations of Fraction II extracted in the dry state, with subsequent removal of isooctane *in vacuo* and reconstitution with H_2O showed similar DPNH-cytochrome *c* reductase activities as the original preparations. Experiments were then attempted wherein the enzyme was extracted in solution with isooctane, lyophilized, reconstituted with water, and the activity compared with another aliquot of the enzyme solution which was lyophilized but not extracted. In a typical experiment 3.0 ml of enzyme solution was extracted 3 times with cold isooctane (3 ml isooctane each extraction). The DPNH-cytochrome *c* reductase was measured before and after extraction. Aliquots of 2.0 ml of unextracted and extracted enzyme solution were then placed in the freeze-dry apparatus until dry. To the dried residue was added 1.8 ml of cold distilled water. Table VI shows that after removal of the isooctane essentially all of the activity was restored. That isooctane *per se* can cause an inhibition of DPNH-cytochrome *c* reductase was shown by the following experiment: 2.5 ml of chick-heart-muscle Fraction II were mixed in a Ten Broeck hand homogenizer with 0.1 ml isooctane and the enzyme activity determined. The treated and untreated enzyme solutions were then lyophilized, reconstituted with water and the enzyme activity measured again. Table VII shows that in the presence of isooctane activity is greatly diminished; removal of the solvent restored almost full activity.

TABLE VI

EFFECT OF ISOOCTANE EXTRACTION ON DPNH-CYTOCHROME *c* REDUCTASE AND THE ACTIVITY OF THE ENZYME AFTER LYOPHILIZATION AND RECONSTITUTION WITH WATER

Enzyme preparation	*Activity**				
	Before lyophilization			*After lyophilization (reconstituted)*	
	Unextracted	*Extracted*	*Extracted + vitamin K_1***	*Unextracted*	*Extracted*
Chick-heart fraction II	116	39		—	127
Chick-heart fraction II	91	19	71	51	62
Chick-heart fraction II	162	44		113	164
Solubilized rat skeletal muscle	91	35	98	73	76

* Activity = Change in absorbance × 10^3 at 550 mμ per 2 min.
** 60 μl of a solution prepared according to NASON AND LEHMAN[8] using 30 mg vitamin K_1/2 ml.

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TABLE VII

EFFECT OF HOMOGENIZING ENZYME SOLUTION WITH ISOOCTANE ON DPNH-CYTOCHROME *c* REDUCTASE ACTIVITY

*Activity**				
Control	*Homogenized with isooctane***		*After lyophilization*	
	No addition	*Plus 60 µl vitamin K_1****	*Control*	*Homogenized***
118	40	85	119	103

* Activity = $\Delta A_{550\,m\mu} \times 10^3/2$ min.
** 2.5 ml enzyme preparation homogenized with 0.1 ml isooctane.
*** See Table VI. Vitamin K_1 was added to the assay sample.

In other experiments a chick-heart-muscle Fraction II preparation was homogenized with graded amounts of isooctane in an attempt to determine the amount of residual isooctane needed to cause inhibition. Increasing amounts of isooctane from 15 to 60 µl/1.5 ml enzyme produced increasing degrees of inhibition, with the 60-µl level of the solvent almost completely inhibitory. This inhibition was partially reversed by the addition of vitamins E or K_1.

Since it appeared that lyophilization was reversing the isooctane inhibition by simply removing the solvent, another technique for accomplishing the same result was sought. Furthermore, the lyophilization procedure occasionally caused a decrease in activity of the control, unextracted samples. Centrifugation of enzyme preparations, after extraction or homogenization with isooctane, was found to restore activity[22]. In these studies the DPNH oxidase system as described by NASON *et al.*[1] was used. When preparations from rat-heart muscle were homogenized with small amounts of isooctane, the DPNH oxidase activity was reduced by 50–60 %. Centrifugation at 10,000 × *g* for 15 min in the cold resulted in a considerable restoration of activity which was further increased by a second centrifugation[22]. This effect of centrifugation was also shown with DPNH-cytochrome *c* reductase preparations.

These studies demonstrate that the inhibition produced by isooctane extraction of DPNH-cytochrome *c* reductase, and probably of other systems, is an inhibition of enzymes by the solvent rather than an extraction of an activator or cofactor. Contrary to the results of DONALDSON *et al.*[3], Table VI shows that isooctane-extracted, solubilized DPNH-cytochrome *c* reductase is reactivated by vitamin K_1. We have obtained similar results with solubilized, isooctane-extracted bovine succinate-cytochrome *c* reductase.

DISCUSSION

The experiments with vitamin-E-deficient chick hearts, although not conclusive, cast serious doubt as to a role for vitamin E in the DPNH-cytochrome *c* reductase system. It may be argued that although the tissues were depleted of α-tocopherol, some active "hidden" form of the vitamin may be present. It should be emphasized that detectable amounts of tocopherol disappeared from the tissues long before the enzyme studies were made. To our knowledge, in no other experimental vitamin deficiency does an animal continue to grow normally for two or three months after the disappearance

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of the vitamin from the body. It would seem highly improbable that functional amounts of the vitamin would still be present after this time.

There is some disagreement in the literature as to whether or not tocopherylquinone exists normally in animal tissues*. As pointed out in the present study, technical difficulties may have led workers to assume erroneously that the quinone was present in unsaponifiable extracts. We have found that substances other than the quinone are present which, when reduced by the usual procedure, give rise to a substance that reacts with the $FeCl_3$–bipyridyl reagent. This substance was not derived from α-tocopherol. In this regard, it is apparent that current quantitative analytical methods for α-tocopherol, even those employing chromatographic purification with activated earths, determine substances in addition to tocopherol. Paper chromatography followed by elution and subsequent photometric analysis appears to be the most specific method, providing the anomalous behavior of interfering substances in the presence of sterols is guarded against. Further research is needed to establish the nature of the unknown EMMERIE-ENGEL reacting materials, and also to establish whether tocopherylquinone exists normally in tissues.

In attempting to determine the biochemical role of a fat-soluble vitamin it would appear axiomatic that techniques different from those used with water-soluble metabolites may be necessary. Such new techniques as may be devised, however, must necessarily be subjected to thorough testing in order to establish their validity. Extraction of enzyme solutions with fat solvents is a procedure which has been introduced relatively recently into enzyme studies. It has been implied[1] that the extraction with isooctane of DPNH-cytochrome *c* reductase removes a fat-soluble cofactor (vitamin E). Our results, however, point to a non-specific inhibition or narcosis of the enzyme by the solvent. Such a possibility was suggested by DEUL *et al.*[2]. It may be that the solvent becomes attached to the enzyme molecule, possibly by solvation in lipid bound to the enzyme, with a resulting inhibition of the active center. Treatment of the enzyme–solvent complex with various lipid materials removes the solvent and restores activity. The reactivation by evaporation of the isooctane *in vacuo*, as shown in the present study, would be in accordance with this hypothesis. On the other hand, the fact that centrifugation also restores activity would suggest that the solvent is not firmly bound to the enzyme.

In view of the accumulated evidence from several laboratories, tocopherol can at present be said to be a specific activator for only one system of enzymes: the aged, solubilized mammalian cytochrome *c* reductases reported by DONALDSON *et al.*[3] and VASINGTON *et al.*[4]. Whether the iif-defined aging process results in a phenomenon similar to that proposed for isooctane extraction is unknown.

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*After the completion of this manuscript, BOUMAN AND SLATER[23] reported the alleged tocopheryl-quinone they had previously determined was probably entirely ubiquinone.

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FRACTIONATION OF BOVINE THYROTROPHIN AND LUTEINIZING HORMONE ON CELLULOSE ION EXCHANGE COLUMNS

PETER G. CONDLIFFE, ROBERT W. BATES AND RICHARD M. FRAPS

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. and Agricultural Research Service, Beltsville, Md. (U.S.A.)

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SUMMARY

Chromatographic studies of the behavior of TSH on DEAE-C and on CM-C have shown that crude TSH can be purified approximately 20 to 40 times with recovery of 30 to 50% of the starting activity. The potency of the most active fractions is 20 to 50 U.S.P. units/mg. LH can be separated from TSH by chromatography on DEAE-C at pH 9.5. The TSH is retained on the column while the LH passes through unadsorbed.

INTRODUCTION

Early attempts to purify pituitary thyrotrophin resulted in the preparation of fractions which were about 100 times more potent than the bovine anterior pituitary

Abbreviations: TSH, thyroid-stimulating hormone; LH, luteinizing hormone; DEAE-C, diethyl amino ethyl cellulose; CM-C, carboxymethylcellulose.

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