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† The following abbreviations are used: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide respectively; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide respectively.

‡ Recent experiments have clearly indicated that thyroxine does inhibit the transhydrogenase reaction. It has also been possible to show that the naphthoquinone SN5949 does not exert its inhibitory effect by a direct action upon the transhydrogenase enzyme.

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*INTERRELATIONSHIP BETWEEN VITAMIN E AND LIPIDE COFACTOR IN THE CYTOCHROME C REDUCTASE SYSTEM**

BY KENNETH O. DONALDSON† AND ALVIN NASON

MCCOLLUM-PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND

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Although the importance of lipides in the oxidation of reduced diphosphopyridine nucleotide (DPNH) and succinate via the cytochrome system has been well established,[1-8] their mode of action has remained somewhat obscure. It has already been demonstrated that added vitamin E (the tocopherols) increases the activity of the diphosphopyridine nucleotide (DNP)- and succinate-cytochrome c reductases from rat skeletal muscle and bovine heart muscle; and that the almost complete loss of enzymatic activity resulting from iso-octane extraction can be completely restored by the addition of tocopherol or, even more effectively, by the lipide residue obtained from vacuum distillation of the iso-octane after extraction. It has also been shown that the added tocopherol acts in a catalytic fashion in the sequence of electron transport just before the cytochrome c position and that there is a competitive inhibition of antimycin A with tocopherol.[6-8]

The present paper reports new experiments demonstrating that the crude lipide residue obtained by iso-octane extraction of the cytochrome c reductases from rat

skeletal muscle and bovine heart muscle can be shown to contain vitamin E, most of it as a presumed tocopheryl quinone, in amounts too small, however, to account for enzyme reactivation. The active compound of the crude lipide residue, designated as the "lipide cofactor," has been purified and tentatively identified as a mixed triglyceride. Further evidence is presented which suggests that added lipide cofactor may be acting indirectly by releasing endogenous vitamin E of the preparation to the "active sites" of the enzyme.

Methods and Materials.—The particulate cytochrome c reductases from rat skeletal muscle and bovine heart muscle were prepared and assayed as already described.[7] Tocopherol,[9] other lipides, and the lipide cofactor were used in the enzymatic reaction mixtures as the usual bovine serum albumin suspensions.[8] Iso-octane extraction of the enzymes was performed as previously indicated.[8] Tocopherol was determined colorimetrically by a modification of the iron chloride–bipyridyl method of Emmerie and Engel[10] and confirmed to be α-tocopherol by the paper-chromatographic procedure of Green *et al.*[11] The conversion of tocopheryl quinone to tocopherol by means of an HCl–ascorbic acid reduction procedure has recently been described by Harrison *et al.*[12] This procedure was used in the present work to convert a naturally occurring substance, presumably tocopheryl quinone, to α-tocopherol in the nonsaponifiable fraction of crude lipide obtained from the above enzyme preparations.

Results and Discussion.—Tocopherol is now found in the nonsaponifiable fraction of the concentrated crude residue obtained by iso-octane extraction of large quantities of rat muscle enzyme (Table 1). Most of it is present presumably as the

TABLE 1

TOCOPHEROL CONTENT OF PARTICULATE ENZYME PREPARATIONS FROM RAT SKELETAL MUSCLE AND BOVINE HEART MUSCLE

(μM Tocopherol/Gm Protein)

	Iso-octane Extracted		Remaining*		
Preparation	Before Reduction	After HCl–Ascorbic Acid Reduction	Before Reduction	After HCl–Ascorbic Acid Reduction	Total†
Rat skeletal muscle enzyme:					
1	0.18	0.42	0.82	4.08	4.5
2	0.08	0.16	0.75	4.94	5.1
3	0.18	0.90	1.55	4.30	5.2
Bovine heart muscle enzyme:					
1	1.33	1.60	4.44	8.40	10.0
2	1.88	1.65	5.68	9.65	11.3
3	1.55	1.57	3.70	8.43	11.0

* The tocopherol remaining in the enzyme preparations after iso-octane extraction was determined in the non-saponifiable fraction of the total lipide (see Nason and Lehman, *J. Biol. Chem.*, **222**, 511, 1956) by the procedures already indicated under "Methods and Materials."

† Calculated by summation of the values for "Iso-octane Extracted" and "Remaining" tocopherol after HCl–ascorbic acid reduction.

tocopheryl quinone which can be reduced to tocopherol by the HCl–ascorbic acid procedure, as already demonstrated by Bouman and Slater[13] for a Keilin-Hartree heart muscle preparation. Calculations of the data of Table 1 show that only 3–17 per cent of the total tocopherol of the rat skeletal muscle enzyme is removed by iso-octane extraction. This occurs with a concomitant loss of approximately 90 per cent of the cytochrome c reductase activity. As much as 85 per cent of the total tocopherol in the enzyme appears to be present in the presumed tocopheryl quinone

form. Iso-octane extraction removes only 10–20 per cent of the tocopherol form. The ratio of tocopherol to presumed quinone present in the corresponding bovine heart muscle enzyme is larger when compared to that of the rat skeletal muscle system. In most preparations virtually all the vitamin E removed from the bovine heart muscle enzyme by iso-octane extraction is in the tocopherol form and represents only 15 per cent of the total vitamin E in the enzyme preparation. Occasionally, however, a bovine heart muscle preparation was obtained which was similar to that of rat muscle with respect to the ratio of tocopherol to the presumed quinone. Of the vitamin E remaining (85 per cent) in the iso-octane-extracted bovine heart muscle enzyme, about half is present as tocopherol and the other half as the presumed tocopheryl quinone. The bovine heart muscle system contains twice as much vitamin E as the rat skeletal muscle enzyme.

Previous inability to demonstrate the presence of tocopherol in the crude lipide residue obtained by iso-octane extraction of the rat skeletal muscle enzyme can be attributed to a combination of circumstances: (1) most of the vitamin E is present in the presumed tocopheryl quinone form and could be detected only by converting it to tocopherol through the recently described HCl–ascorbic acid reduction method,[12] and (2) relatively small quantities of enzyme (15–50 ml.) were formerly used for iso-octane extraction. However, by using large quantities of rat muscle enzyme (100–500 ml.), it is now possible to measure tocopherol in the lipide residue obtained by iso-octane extraction. Thus in a typical experiment the quantity of crude lipide residue obtained by iso-octane extraction of particulate rat skeletal muscle enzyme which restored 50 per cent of the activity of the extracted cytochrome c reductase contained only 0.03 μg. of tocopherol even after HCl–ascorbic acid reduction. It has already been established[8] that 45–50 μg. of d-α-tocopherol are necessary for 50 per cent reactivation under the same conditions. Therefore, enzymatic reactivation cannot be ascribed to the tocopherol content of the residue. Instead, reactivation is due to the presence of a lipide cofactor in the residue.

The lipide cofactor of the crude residue obtained by iso-octane extraction and responsible for restoration of cytochrome c reductase activity has been purified 200-fold from bovine heart muscle homogenate. The purified lipide is about 20 times as active as tocopherol, mole for mole, and does not show the presence of tocopherol even after HCl–ascorbic acid treatment. Alkaline hydrolysis completely destroys the activity of the lipide cofactor, whereas it leaves that of d-α-tocopherol unchanged. By means of infrared analysis, characterization of the chemical derivatives, and chromatographic and countercurrent distribution techniques the lipide cofactor has been tentatively identified as a mixed triglyceride with stearate, palmitate, and oleate components. Of the more than one hundred different substances tested for restoration of activity,[8] including steroids, mono-, di-, and triglycerides and other esters, phospholipides, fat-soluble vitamins, and antioxidants, approximately ten of these showed significant restoration of activity. These included milk, butter, oleomargarine, synthetic corn oil, triolein, ethyl stearate, n-butyl stearate, and certain synthetic glycerides.

Two facts are of interest: (1) iso-octane extraction removes only about 10 per cent of the total tocopherol of the particulate cytochrome c reductase system, with a concomitant loss of 90 per cent of enzymatic activity; and (2) structurally dissimilar molecules such as the tocopherols and certain triglycerides and long-chain

fatty acid esters are effective in restoring activity of the iso-octane-extracted enzyme. Apparently the small quantity of tocopherol in the crude lipide residue cannot account for enzymatic restoration. However, it is possible that before iso-octane extraction it was originally effective at the "active enzyme sites." The possibility also exists that addition of the lipide cofactor or of certain other effective esters replenishes the "active centers" of the extracted enzyme by releasing some of the remaining tocopherol, perhaps from a "bound" form. This would be tantamount to adding tocopherol to the system. The activity of these effective esters could thus be attributed directly to tocopherol. A partial test of this hypothesis would be an examination of the effects of various lipides in potentiating the removal of tocopherol from the enzyme by iso-octane extraction. Table 2 shows

TABLE 2*

EFFECT OF VARIOUS LIPIDES ON THE SUBSEQUENT RELEASE OF TOCOPHEROL BY ISO-OCTANE EXTRACTION OF BOVINE HEART ENZYME

SUBSTANCES	EFFECT IN RESTORATION OF ISO-OCTANE-EXTRACTED ENZYME	μM OF TOCOPHEROL EXTRACTED	
		Before Reduction	After HCl–Ascorbic Acid Reduction
EtOH-albumin (control)	Inactive	0.07	0.33
Glycerol dipalmitate	Inactive	0.09	0.36
Tristearin	Inactive	0.09	0.35
Purified lipide cofactor (from bovine heart muscle)	Active	0.16	0.86
Oleomargarine	Active	0.27	1.09
n-Butylstearate	Active	0.14	0.65

* 1.5 gm. of each substance were homogenized with 20 ml. of ethanol–bovine serum albumin in a Ten Broeck homogenizer and added to 100 ml. each of particulate bovine heart enzyme in two separate portions. After each addition, the enzyme was extracted twice with an equal volume of cold iso-octane in the usual manner (see Nason and Lehman, *J. Biol. Chem.*, **222**, 511, 1956). The combined iso-octane extracts were evaporated *in vacuo*, the lipide residue taken up in absolute ethanol, and tocopherol determined in the nonsaponifiable faction as indicated.

that the addition of the lipide cofactor or of other indicated active substances to the bovine heart muscle enzyme results in a two- to threefold release of tocopherol and presumed tocopheryl quinone, as compared with the control, upon subsequent extraction with iso-octane. Inactive compounds such as glycerol-dipalmitate and tristearin are ineffective in potentiating the removal of tocopherol by subsequent iso-octane extraction. Glycerol monostearate (not shown), which is inactive, was also ineffective in this respect. It is also worthy of note that treatment of the enzyme with lipide cofactor followed by iso-octane extraction yields a lower cytochrome c reductase activity as compared with the control. The addition of lipide cofactor and α-tocopherol, separately or in various combinations, in most cases did not restore enzymatic activity. Morrison *et al.*,[14] using iso-octane-extracted succinate cytochrome c reductase from pig heart have recently confirmed the reactivating effect of *d*-α-tocopherol and the lipide residue derived from iso-octane extraction. Reactivation was also effected by a hemin-lipide complex isolated from heart muscle. It is not unlikely that the hemin-lipide might be acting like the other active compounds in potentiating the mobility of endogenous tocopherol as suggested above.

The present experiments offer further support for the function of vitamin E as a cofactor in the cytochrome c reductase system.[6, 8] Moreover, they provide a reasonable explanation for the fact that such structurally unlike molecules as the tocopherols and certain triglycerides and long-chain fatty acid esters can restore enzyme activity. The new findings that some vitamin E is removed by iso-octane

extraction, while most of it still remains in the enzyme, and that only those lipides which are effective in reactivation of cytochrome c reductase also potentiate the mobility or extractability of endogenous tocopherol point to a primary role for the vitamin. The additional observation that a gradual removal of tocopherol from the enzyme system results in a gradual loss of activity lends further strength to this suggestion. It is also of interest that tocopherol has been reported to be the only fat-soluble vitamin present in a Keilin-Hartree heart muscle preparation.[13] Nevertheless, the possibility of a more direct role of the lipide cofactor has not been excluded.

Thus far, it has not been possible to demonstrate that vitamin E functions directly as an electron carrier in the cytochrome c reductase system, although it has already been shown to function in a catalytic manner.[8] However, the possibility has not been eliminated that undetectable quantities of vitamin E bound to the "active enzyme sites" undergo oxidation-reduction changes. Chemical studies demonstrating reversible oxidation reactions of the vitamin tend to favor an electron-transport role in the enzyme system. Alternately, the possibility still exists that vitamin E may be functioning indirectly as a binding or cementing substance for other components of the cytochrome c reductase system. Work is now in progress in this laboratory to elucidate further the mechanism of action of vitamin E.

Summary.—By using large quantities of particulate cytochrome c reductase prepared from rat skeletal muscle and bovine heart muscle, it has been possible to show that the crude lipide residue obtained by iso-octane-extraction of the enzyme contains vitamin E, most of it as a presumed tocopheryl quinone. Iso-octane removes only 10–20 per cent of the total vitamin E, with a concomitant loss of 90 per cent of the cytochrome c reductase activity. The amount of vitamin E extracted, however, cannot account for enzyme reactivation by added crude lipide residue. Instead, restoration is attributed to a lipide cofactor tentatively identified as a mixed triglyceride with stearate palmitate and oleate components. It is possible, however, that the small quantity of vitamin E removed by iso-octane extraction may originally have been effective at the "active enzyme sites." The fact that the lipide cofactor and other active lipides potentiate the extractability of endogenous vitamin E is interpreted to suggest that they are acting by releasing the vitamin to the "active sites" of the enzyme. Whether the vitamin is functioning directly in electron transfer or indirectly as a binding substance remains to be established.

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† Postdoctoral fellow of the McCollum-Pratt Institute.

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*MEIOTROPHIC MUTANTS OF PASTEURELLA PESTIS AND THEIR USE IN THE ELUCIDATION OF NUTRITIONAL REQUIREMENTS**

BY ELLIS ENGLESBERG AND LAURA INGRAHAM

BIOLOGICAL LABORATORY, LONG ISLAND BIOLOGICAL ASSOCIATION, COLD SPRING HARBOR, NEW YORK

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During the course of characterizing the natural breaks in the biosynthesis of amino acids by *Pasteurella pestis* and of isolating mutants requiring fewer growth factors than the prototroph (meiotrophic mutants[1]), it became apparent that this procedure of meiotrophic mutant isolation and characterization was of general value in the elucidation of the amino acid requirements of other fastidious heterotrophic[2] micro-organisms.

Pasteurella pestis, strain A1122B1, avirulent, and various meiotrophic mutants were employed in this study. Stock cultures were maintained on casein hydrolyzate mineral glucose agar. The complete synthetic medium employed had the following composition: glucose, 0.2 per cent; $MgSO_4(7H_2O)$, 0.05 per cent; $CaCl_2$, 10^{-3} per cent; $FeCl_3(6H_2O)$, 2.5×10^{-4} per cent; Na_2SO_3, 0.1 per cent; Na_2HPO_4-KH_2PO_4 (pH 7), $M/20$; agar (Bacto), 1.5 per cent; and the following amino acids in amounts to yield a 0.004 per cent solution: *l*-cystine (C), *dl*-methionine (M), *dl*-valine (V), *dl*-isoleucine (I), and *dl*-phenylalanine (P). The *l* forms of the amino acids can be substituted for the *dl*. The medium was prepared by autoclaving together agar, Na_2SO_3, and distilled water, and the other ingredients were added subsequent to autoclaving as sterile concentrated solutions. In some early experiments a smaller concentration of sulfite (0.05 per cent) was employed, and in some cases thiosulfate was used in place of cystine. Although 0.05 per cent sulfite was subsequently found to be inadequate to fully neutralize the inhibitory effect of agar on *P. pestis*,[3] it gave a medium highly sensitive to the detection of isoleucine and valine inhibitions. Agar autoclaved at acid pH was employed initially as another means of preventing the inhibitory effect of the agar on the growth of *P. pestis*.

Mutation rates to meiotrophy were determined by a modification of the Luria and Delbrück[4] procedure and were calculated by the median method of Lea and Coulson.[5]

Pasteurella pestis A1122B1 has been previously shown to require cysteine, phenylalanine, methionine, valine, and isoleucine for growth at 30° C. in a mineral glu-