Reduction of Cytochrome *c* by Tocopherols in the Presence of Unsaturated Fatty Acids*

Thomas C. Detwiler and Alvin Nason

ABSTRACT: Addition of cytochrome *c* to a suspension of α-tocopherol and unsaturated fatty acid results in reduction of the cytochrome and a decrease in turbidity of the suspended lipids. The reaction is specific for long-chain unsaturated free acids and tocopherols, and has a pH optimum of 8. Kinetic and stoichiometric evidence indicates that fatty acids act as catalysts and that tocopherol is a two-electron donor in the oxidation–reduction reaction with cytochrome *c*.

Although evidence for reversal of the reaction has not been obtained, the reaction attains an apparent equilibrium, permitting calculation of oxidation–reduction potentials (E_0', pH 8) of +0.20 for α-tocopherol, +0.22 for β- and γ-tocopherols, and +0.24 for δ-tocopherol.

In the course of investigations of lipid function in electron-transfer enzymes we observed that when cytochrome *c* was added to a suspension of α-tocopherol and oleic acid, the cytochrome was rapidly reduced while the suspended lipids apparently became soluble. Although many biological compounds are known to reduce cytochrome *c* nonenzymatically, this reaction was of particular interest because of several known biological interrelationships of the three classes of compounds involved.

First, all three compounds occur as components of mitochondria of mammalian tissue. Mitochondrial membranes are composed of 35–40% lipid (largely phospholipids containing esters of unsaturated fatty acids) (Lehninger, 1964a) and also contain much of the cell's cytochromes (Lehninger, 1964b) and tocopherol (Lehninger, 1964b; Vasington *et al.*, 1960; Crane *et al.*, 1959; Slater *et al.*, 1961; Hatefi *et al.*, 1961). Secondly, peroxidation of unsaturated lipids is known to be catalyzed by heme proteins, including cytochromes, and inhibited by antioxidants, including tocopherols (Tappel, 1961), processes that have been demonstrated *in vitro* and that are probably involved in many nutritional aspects of tocopherol (Vasington *et al.*, 1960; Tappel, 1961, 1962). Finally, the possibility that tocopherols might function as a component of the terminal respiratory system, either as an electron carrier or structural agent, has often been discussed (Vasington *et al.*, 1960; Boyer, 1960), although there is no unequivocal or direct evidence for such a role.

In view of the above biological relationships, the reaction of oleic acid, the tocopherols, and cytochrome *c* was studied in an attempt to determine the nature of the chemical and physical changes involved and to evaluate its possible biological significance. The results reported here indicate that tocopherol is the electron donor for the reduction of cytochrome *c* and that unsaturated fatty acids apparently act as catalysts.

Experimental Section

The *d*-tocopherols and their derivatives and the vitamin A used in these studies were kindly provided by Distillation Products Industries, Division Eastman Kodak, Rochester, N. Y. *l*-α-Tocopherol was a gift from F. Hoffman-La Roche and Co., Basle. Coenzyme Q compounds were provided by Dr. Karl Folkers, Stanford Research Institute, Menlo Park, Calif., and by Dr. David Hendlin, Merck and Co., Rahway, N. J. Oleic, linoleic, linolenic, palmitic, lauric, and caprylic acids (all A grade), Δ^{10}-undecenoic acid (C grade), and oleic acid-1-^{14}C were obtained from Calbiochem, Los Angeles, Calif. Oleic alcohol, methyl oleate, elaidic acid, palmitoleic acid, crotonic acid, glyceryl monooleate, glyceryl dioleate, and Δ^2-decenoic acid were purchased from K and K Laboratories, Jamaica, N. Y. Stearic acid, butyric acid, β-carotene, vitamin D_2, and bovine serum albumin were obtained from Mann Research Laboratories, N. Y., and butylated hydroxytoluene was a gift from Dr. Klaus Schwarz, Veteran's Administration Hospital, Long Beach, Calif.

Cytochrome *c* (type II, 65–70% pure, and type III, 95–100% pure) was purchased from Sigma Chemical Co., St. Louis, Mo. As obtained these cytochrome preparations were about 30% reduced and were usually converted to either a completely oxidized or completely reduced form with either potassium ferricyanide or ascorbic acid, respectively. Ferri- and ferrocyanide was removed by passing the aqueous solution through

* From the Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, New York, and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland. *Received July 1, 1966.* Contribution No. 483 of the McCollum-Pratt Institute. This investigation was supported in part by Research Grant AM 5923 from the National Institutes of Health, U. S. Public Health Service.

a column of Dowex 1-acetate and ascorbic acid was removed on a column of G25 Sephadex or by dialysis. The concentration of cytochrome *c* in solution was calculated from A_{550} using the extinction coefficient (reduced − oxidized) of 2.12×10^4 cm²/mmole (Van Gelder and Slater, 1962).

In some experiments, lipids were used as suspensions prepared by first dissolving the lipid in absolute ethanol and then homogenizing with 0.2% aqueous bovine serum albumin in a ground-glass tissue grinder to give a stable suspension of lipid with 15% ethanol–0.17% albumin. Suspensions of unsaturated lipids were used within 2 or 3 hr after preparation to reduce the possibility of autoxidative destruction. In other experiments lipids were used as ethanol solutions prepared just prior to use.

Precoated plates for thin layer chromatography were obtained from Brinkman Instrument Co., Westbury, Long Island. Autoradiograms were made with Kodak no-screen medical X-ray film. Absorption spectra and kinetic studies were recorded with either a Cary 14 or a Beckman DK-2 spectrophotometer.

Results

Measurement of the Reaction. Changes occurring on addition of cytochrome *c* to an albumin suspension of oleic acid and α-tocopherol may be readily observed with a recording spectrophotometer, as shown in Figure 1. The decrease in turbidity of the lipid suspension was measured by the decrease in light scattering at 600 mμ, where the extinction coefficient of both oxidized and reduced cytochrome *c* is small, whereas reduction of cytochrome *c* was measured at 550 mμ, the α-peak absorption maximum of reduced cytochrome *c*. Since turbidity also causes light scattering at 550 mμ, a correction was made by subtracting A_{600} from A_{550}. The reaction is considerably faster in Tris than in phosphate buffer and turbidity decreases to essentially zero sufficiently fast to permit A_{550} to be used without correction after the first minute. If the concentration of lipids relative to cytochrome is decreased, turbidity is even less important and it is possible to obtain an increase in A_{550} that is linear with time and can therefore be used as a quantitative rate assay.

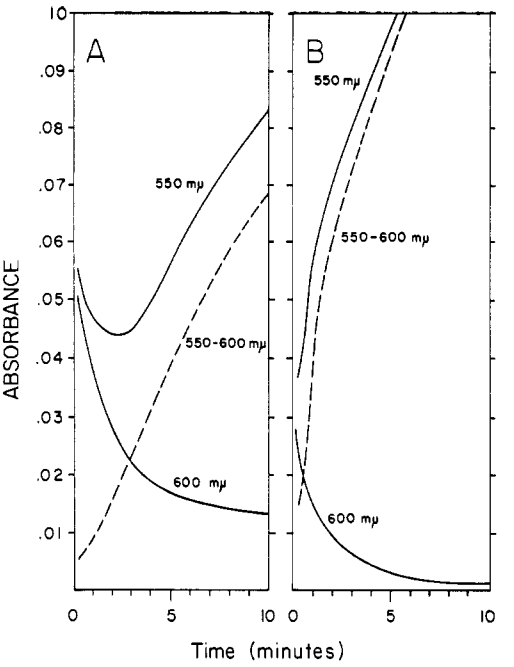

FIGURE 1: Spectrophotometric recording of the reactions of α-tocopherol, oleic acid, and cytochrome *c*. The reaction cuvets contained 60 μM oleic acid, 10 μM α-tocopherol, and 10 μM oxidized cytochrome *c*. The reference cuvet contained 10 μM oxidized cytochrome *c*. Reactions were started by adding cytochrome *c* to the lipids, which were used as albumin suspensions; (A) in 70 mM potassium phosphate, pH 8.0; (B) in 70 mM Tris–chloride, pH 8.0.

TABLE 1: Qualitative Method of Measuring Reaction of α-Tocopherol, Oleic Acid, and Cytochrome *c*.[a]

Time, *t* (min)	Wavelength (mμ)	Absorbance (reaction cuvet − ref cuvet)
0	600	0.630
0.1 ml of cytochrome *c* added to reference and reaction cuvets		
5	550	0.854
	600	0.072
Turbidity change	$A_{600}(t = 5\text{ min}) - A_{600}(t = 0) = -0.558$	
Cytochrome reduction	$A_{550}(t = 5\text{ min}) - A_{600}(t = 5\text{ min}) = 0.782$	

[a] Absorbance was measured in 1.0-ml cuvets (1.0-cm light path) with a Beckman DU spectrophotometer. The buffer was 0.1 M potassium phosphate, pH 8.0. Lipids were suspended with albumin as described under Experimental Section at a concentration of 5 mg/ml. Cytochrome *c* was added as an aqueous solution of 20 mg/ml. The reference cuvet contained 0.8 ml of buffer and 0.06 ml of albumin solution. The reaction cuvet contained 0.8 ml of buffer, 0.03 ml of α-tocopherol, and 0.03 ml of oleic acid.

In earlier experiments, prior to development of the assay shown in Figure 1, the reaction was observed by measuring A_{550} and A_{600} at a fixed time after adding cytochrome *c* to a lipid suspension. Decrease in turbidity was the difference between A_{600} before adding cytochrome *c* and A_{600} after a given time interval, usually either 3 or 5 min, following the addition of cytochrome *c*. Reduction of cytochrome *c* was measured as A_{550} after the given time minus A_{600} at the same time with unreacted cytochrome *c* as the blank. Since A_{550}

and A_{600} had to be measured at essentially the same stage of the reaction in order for the correction to be valid, it was necessary to wait until the reaction had neared completion so that absorbance changes were small during the time required to make readings at the two wavelengths. Therefore, these measurements do not strictly correspond to rate measurement and the assays are at best only semiquantitative. An example of this assay is shown in Table I. The absorbance changes observed are known to represent actual reduction of cytochrome *c* because the reduced minus oxidized difference spectrum of tocopherol–oleic acid reduced cytochrome is identical with that of dithionite reduced cytochrome.

Specificity of the Reaction. The specificity of the reaction was studied by substituting various lipids for either oleic acid (Tables II and III) or α-tocopherol (Tables IV and V). (It is important to note that in Tables II and IV the results are not strictly quantitative.) Table II shows the results of substituting various fatty acids and fatty acid derivatives for oleic acid using the qualitative assay as described in Table I. Only long-chain unsaturated free acids were reactive. The long-chain unsaturated alcohol or esters, saturated acids, or short-chain unsaturated acids were not reactive. A later experiment, using a quantitative assay to determine the relative effectiveness of reactive compounds (Table III) indicates that three mono unsaturated fatty acids were approximately equally effective, but a diunsaturated fatty acid (linoleic) was slightly less effective and a triunsaturated acid (linolenic) was considerably less effective, requiring a concentration about three times greater than the monounsaturated acid for an equal rate.

TABLE II: Specificity for Fatty Acids in Reaction with α-Tocopherol and Cytochrome *c*.[a]

Fatty Acid or Derivative	Turbidity Change A_{600} (t = 5 min) $-A_{600}$ (t = 0) ($\times 10^3$)	Cytochrome Reduction A_{550} (t = 5 min) $-A_{600}$ (t = 5 min) ($\times 10^3$)
None (control)	−35	25
Oleic acid ($C_{18:1}$)	−365	675
Oleic alcohol	−50	40
Methyl oleate	−50	40
Glyceryl monooleate	−50	60
Glyceryl dioleate	−65	40
Elaidic acid ($C_{18:1}$)	−420	320
Linoleic acid ($C_{18:2}$)	−690	760
Stearic acid (C_{18})	−55	65
Palmitic acid (C_{16})	−45	−40
Palmitoleic acid ($C_{16:1}$)	−285	420
Lauric acid (C_{12})	−75	−55
Δ^{10}-Undecenoic acid ($_{11:1}$)	−35	80
Δ^{2}-Decenoic acid ($C_{10:1}$)	−40	85
Caprylic acid (C_8)	−60	110
Crotonic acid ($C_{4:1}$)	−40	65
Butyric acid (C_4)	−45	65

[a] α-Tocopherol (0.03 ml) (5 mg/ml) and 0.03 ml of test lipid (10 mg/ml) were added as albumin suspensions to 0.8 ml of 0.1 M potassium phosphate, pH 8.0. At t = 0, 0.1 ml of cytochrome *c* (20 mg/ml) was added. The rate of reaction was measured as described in Table I.

TABLE III: Rate of Reduction of Cytochrome *c* by Tocopherol and Various Fatty Acids.[a]

Fatty Acid	Concentration (μM) 25	50	75	100	150
	$\Delta A_{550} \times 10^3$/min				
None	0	1			
Oleic acid ($C_{18:1}$)	55	87			
Elaidic acid ($C_{18:1}$)	60	99			
Palmitoleic acid ($C_{16:1}$)	30	98			
Linoleic acid ($C_{18:2}$)	25	73			
Linolenic acid ($C_{18:3}$)	6	14	37	79	95

[a] The reaction mixture contained 50 μM α-tocopherol, 50 μM oxidized cytochrome *c*, and fatty acid in 100 mM Tris–chloride, pH 8.0. Lipids were added as albumin suspensions. Absorbance at 550 mμ was measured in a Beckman DU spectrophotometer against 50 μM oxidized cytochrome *c*. Readings were made at 30-sec intervals beginning 15 sec after adding cytochrome *c* to start the reaction.

Table IV shows the results of substituting various lipids for α-tocopherol. Only β-, γ-, and δ-tocopherols were reactive; tocopherol esters, tocopherylquinone and hydroquinone, coenzyme Q compounds, and other vitamins were not effective. The lack of reactivity of coenzyme Q_4 chromanol is especially interesting, since tocopherol is also a chromanol. A comparison of reaction rates using a quantitative assay (Table V) indicates that *d*-α- and *l*-α-tocopherols are equally effective; β-, and γ-tocopherols are about one half as effective and δ-tocopherol only about 20% as effective as α-tocopherol. It is interesting that these are approximately the relative biological potencies of these tocopherols (Mattill, 1954).

Effect of pH. The reaction has a pronounced dependence on pH with an optimum at about pH 8. Initial results using the qualitative assay (Figure 2) indicate that both reduction of cytochrome *c* and decrease in

TABLE IV: Specificity for Tocopherols in Reaction with Oleic Acid and Cytochrome *c*.[a]

Lipid	Turbidity Change A_{600} (t = 5 min) − A_{600} (t = 0) (×10³)	Cytochrome *c* Reduction A_{550} (t = 5 min) − A_{600} (t = 5 min) (×10³)
None (Control)	−20	15
α-Tocopherol	−605	850
β-Tocopherol	−565	580
γ-Tocopherol	−665	800
δ-Tocopherol	−375	205
α-Tocopherylhydroquinone	−145	40
α-Tocopherylquinone	−150	30
α-Tocopheryl acetate	−195	70
α-Tocopheryl phosphate	−115	5
α-Tocopheryl succinate	−75	25
Tocophersolan[b]	0	30
Coenzyme Q_{10}	−105	60
Coenzyme Q_4	−50	−90
Coenzyme Q_4 chromanol	−130	−35
Vitamin K_1	−100	−10
Vitamin A alcohol	−45	35
Vitamin A acetate	−60	−25
β-Carotene	−55	120
Vitamin D_2	−55	−160

[a] Oleic acid (0.03 ml) (10 mg/ml), 0.03 ml of test lipid (5 mg/ml) as albumin suspensions, and 0.1 ml of cytochrome *c* (20 mg/ml) were used as in Table II. [b] Distillation Products Industries' trade name for α-tocopheryl polyethylene glycol 1000 succinate, a water-soluble tocopherol derivative.

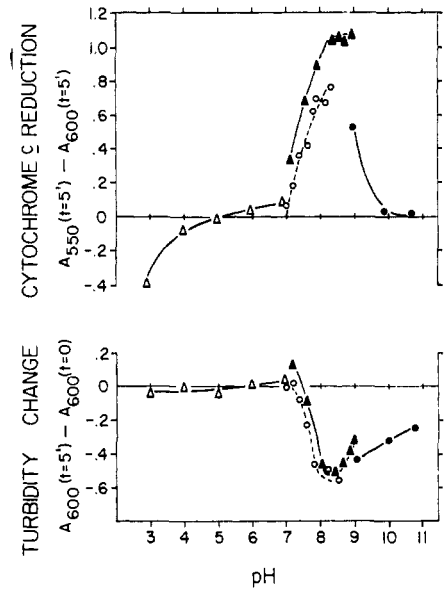

FIGURE 2: The effect of pH on the reaction of α-tocopherol, oleic acid, and cytochrome *c*. The reaction mixture consisted of 0.03 ml of an albumin suspension of tocopherol, 5 mg/ml; 0.03 ml; of an albumin suspension of oleic acid, 5 mg/ml; 0.8 ml of 0.1 M buffer; and 0.1 ml of cytochrome *c*, 20 mg/ml. The reaction was measured as described in Table I. △——△, citric acid–sodium phosphate buffer; ○----○, potassium phosphate buffer; ▲——▲, Tris–chloride buffer; ●——●, sodium carbonate–sodium bicarbonate buffer.

TABLE V: Rate of Reduction of Cytochrome *c* by Oleic Acid and Various Tocopherols.[a]

Tocopherol	Concentration (μM) 12.5	25	50	100
	$\Delta A_{550} \times 10^3$/min			
None	0	0		
d-α-Tocopherol	50	90	125	
l-α-Tocopherol	50	85		
d-β-Tocopherol	20	49	75	
d-γ-Tocopherol	23	48	98	
d-δ-Tocopherol	8	17	35	65

[a] The reaction mixture contained 10 μM oleic acid, 50μM oxidized cytochrome *c*, and tocopherol in 0.1 M Tris–chloride, pH 8.0. Lipids were added as albumin suspensions. Absorbance at 550 mμ was measured in a Beckman DU spectrophotometer against 50 μM oxidized cytochrome *c*. Readings were made at 30-sec intervals beginning 15 sec after adding cytochrome *c* to start the reaction.

turbidity are dependent on pH and have the same optimum. Later measurements of initial rate showed a sharper optimum at pH 8.0 for cytochrome reduction but did not permit accurate measurement of the very rapid and slight turbidity changes.

Possible Involvement of Peroxidation and Autoxidation. Since heme proteins, including cytochrome *c*, are known to catalyze the peroxidation of unsaturated fatty acids (Tappel, 1961, 1962), experiments were carried out to determine if this was related to the reaction reported here. Table VI shows the lack of effect of removing oxygen or adding inhibitors of autoxidation and heme-catalyzed peroxidation, indicating that the reaction is not dependent on either autoxidation or heme-catalyzed peroxidation. This conclusion is supported by the specificity studies showing that (a) esters of unsaturated fatty acids, which are also subject to autoxidation, were not reactive, and (b) polyunsaturated fatty acids, which

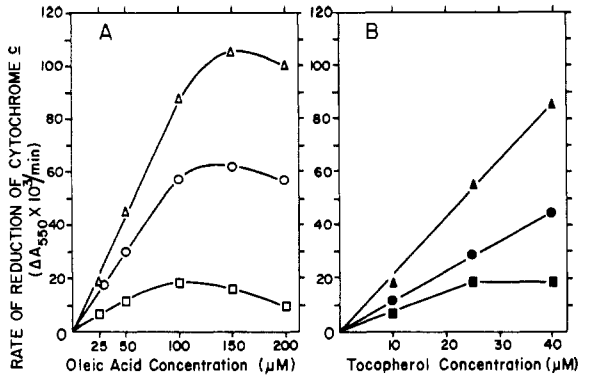

FIGURE 3: Relationship between concentrations of α-tocopherol and oleic acid and initial rate of reduction of cytochrome *c*. The reactions were started by adding oxidized cytochrome *c* to lipids suspended with albumin. A_{550} was recorded and rates were measured from the initial linear part of the recording. The reaction mixtures contained 30 μM cytochrome *c* plus the indicated amounts of tocopherol and oleic acid in 70 mM Tris-chloride, pH 8.0. (A) □—□, 10 μM tocopherol; ○——○, 25 μM tocopherol; △——△, 40 μM tocopherol; (B) ■——■, 25 μM oleic acid; ●——●, 50 μM oleic acid; ▲——▲, 100 μM oleic acid.

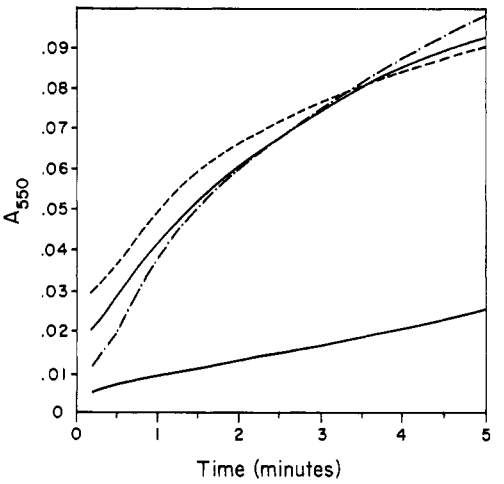

FIGURE 4: Reduction of cytochrome *c* by α-tocopherol and oleic acid with one or two rate-limiting reactants. Reduction of cytochrome *c* was observed by recording absorbance at 550 mμ after adding oxidized cytochrome *c* to an albumin suspension of oleic acid and tocopherol. For each curve the reference cuvet contained the same concentration of oxidized cytochrome *c* as the sample. For the lower, solid line, oleic acid was rate limiting with 10 μM oleic acid, 40 μM oxidized cytochrome *c*, and 40 μM α-tocopherol. The upper curves are: — — — — (cytochrome *c* limiting), 60 μM oleic acid, 15 μM α-tocopherol, and 5 μM cytochrome *c*; —— (tocopherol limiting), 60 μM oleic acid, 5 μM α-tocopherol, and 20 μM cytochrome *c*; —·—·—·— (tocopherol and cytochrome *c* equally limiting), 60 μM oleic acid, 10 μM α-tocopherol, and 10 μM cytochrome *c*.

TABLE VI: Effect of Removal of Oxygen and Addition of Antioxidants on Cytochrome *c* Reduction by α-Tocopherol and Oleic Acid.[a]

Conditions of Reaction	Turbidity Change	Cytochrome *c* Reduction
	A_{600} (t = 5 min) − A_{600} (t = 0) (×10³)	A_{550} (t = 5 min) − A_{600} (t = 5 min) (×10³)
Control (as in Table I)	−580	575
−Oxygen	−745	480
+Butylated hydroxytoluene	−595	540
+KCN	−524	554

[a] Reactions were measured by the method in Table I. A Thunberg cuvet, with cytochrome *c* in the side arm, was used for the oxygen-free reaction. Oxygen was removed by repeatedly evacuating and flushing with nitrogen. Butylated hydroxytoluene was included in the suspension of oleic acid to give a final concentration in the reaction of 10^{-3} M, a concentration found by Tappel (1961) to inhibit heme-catalyzed lipid peroxidation. Potassium cyanide, which inhibits heme-catalyzed peroxidation but not autoxidation (Tappel, 1961) was included in the reaction mixture, where indicated, at a final concentration of 5×10^{-3} M.

would be more susceptible to oxidation than the monounsaturated fatty acids, were less reactive.

Kinetics of the Reaction. For information about the general kinetics of the reaction and to determine the best assay conditions, initial rates of reduction of cytochrome *c* were determined with various concentrations of oleic acid and α-tocopherol (Figure 3). As shown in Figure 3A, the reaction rate is proportional to oleic acid concentration over a certain range, above which the rate decreases. The optimal concentration of oleic acid is higher at higher tocopherol concentrations with maximum rates apparently occurring within a range of relative concentrations of reactants, namely ratios of oleic acid:tocopherol of from 4:1 to 10:1. When the same data are plotted as rate against tocopherol concentration (Figure 3B), the rate is proportional to tocopherol concentration except at the lowest concentration of oleic acid. The rate is no longer proportional to tocopherol below a ratio oleic acid:tocopherol of about 1:1. The concentration of cytochrome *c* was not included as a variable in these experiments because of the difficulty of finding an assay that was reliable over a significantly wide range of

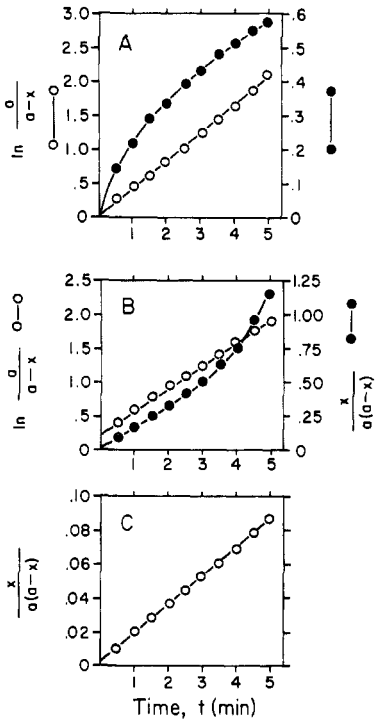

FIGURE 5: Graphs of data from Figure 4 with first- and second-order rate equations. The amount of reduced cytochrome *c* at time, *t*, was calculated from A_{550} in Figure 4. One mole of cytochrome *c* reduced was assumed to correspond to either 1 or 2 moles of tocopherol oxidized. The first- and second-order rate equations are: $\ln a/a - x = k_1 t$, and $x/a(a - x) = k_2 t$, where a is the concentration (micromolar) of reactant at $t = 0$, and x = concentration (micromolar) of product at t (Moore, 1955). (A) Data from Figure 4 where tocopherol is rate limiting with calculation based on a one-electron oxidation, O——O, or on a two-electron oxidation, ●——●. (B) Data from Figure 4 where cytochrome *c* is rate limiting. (C) Data from Figure 4 where tocopherol and cytochrome *c* are equally limiting when calculations are based on a one-electron oxidation.

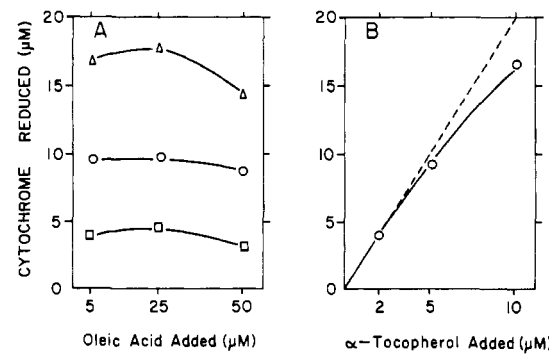

FIGURE 6: Relationship between concentrations of oleic acid and tocopherol and the extent of reduction of cytochrome *c*. The reaction mixtures consisted of 50 μM oxidized cytochrome *c* plus ethanol solutions of α-tocopherol and oleic acid in 50 mM Tris-chloride, pH 8.0. The tubes were repeatedly evacuated and flushed with nitrogen and then left in the dark at room temperature overnight. Reduction of cytochrome was determined by measuring A_{550} against blanks of 50 μM oxidized cytochrome *c*. (A) □——□, 2 μM α-tocopherol; O——O, 5 μM α-tocopherol; Δ——Δ, 10 μM α-tocopherol. (B) Each point represents the average of the three concentrations of oleic acid used in part A. The broken line represents the results expected with 2 moles of cytochrome reduced/mole of tocopherol added.

cytochrome *c* concentrations and lipid:cytochrome ratios.

To determine the approximate kinetic order of the reaction, reduction of cytochrome *c* was recorded under conditions where individual reactants were rate limiting (Figure 4). When oleic acid is rate limiting, reduction is linear with time. The evidence shown in Figure 3 that rate is proportional to oleic acid concentration indicates that oleic acid is not being consumed as the reaction proceeds but is probably acting as a catalyst.

When either tocopherol or cytochrome *c* is rate limiting, or when tocopherol and cytochrome *c* are in equal concentration but still limiting, more nearly exponential curves are obtained. These curves are analyzed by graphing the integrated forms of simple rate equations (Figure 5). The concentration of reduced cytochrome *c* at any time was calculated from A_{550} at that time. The concentration of oxidized tocopherol was calculated from the amount of cytochrome *c* reduced on the basis of either 1 or 2 moles of cytochrome *c* reduced/mole of tocopherol oxidized, that is, assuming either a one- or two-electron oxidation.

In Figure 5A, the data for the situation where tocopherol is rate limiting is plotted with calculations based on a one- or two-electron oxidation. A good fit of a first-order rate equation is obtained when calculations are based on a one-electron oxidation.

In Figure 5B, the data for the case where cytochrome *c* is rate limiting is plotted with both a first- and second-order rate equation. Neither equation gives an ideal fit; the line for the first-order equation is straight but does not go through the origin and the line for the second-order equation deviates from linearity after about 3 min. The failure to go through the origin may be due to the fact that when cytochrome *c* is limiting, the decrease in turbidity is rather slow and not complete. Therefore in these experiments, where turbidity has been ignored, some positive error in A_{550} may occur, resulting in an upward displacement of the line in Figure 5B. The deviation from linearity of the second-order plot might also be explained since it occurs when the kinetics may be complicated by a reverse reaction, or by the existence of a reaction sequence in which the second step would not be appreciable until the concentration of an intermediate

accumulated. The existence of two monomers of cytochrome *c* with different reaction rates, as observed by Greenwood and Palmer (1965), would also result in deviations from simple kinetics. Thus, this experiment is inconclusive, and the kinetics with respect to cytochrome are examined further in Figure 5C.

An hypothesis that the reaction was first order with respect to cytochrome *c* and to tocopherol with a one-electron oxidation of tocopherol was tested by recording reduction of cytochrome *c* when cytochrome and tocopherol were present in equal concentrations. The plot of this curve for a second-order rate equation (Figure 5C) seems to verify this hypothesis. However, because of the complications discussed for the reaction with cytochrome limiting, this must be a tentative conclusion.

Stoichiometry of the Reaction. Additional information about the reaction was obtained by measuring the amount of cytochrome *c* reduced at completion of the reaction with various amounts of oleic acid and tocopherol in the reaction mixture. Figure 6 shows that the amount of cytochrome *c* reduced at completion is not dependent on oleic acid concentration but is dependent on tocopherol concentration. At lower concentrations of tocopherol, 2 moles of cytochrome *c* were reduced/mole of tocopherol added, but at higher concentrations of tocopherol the ratio is slightly less (Figure 6B). It was found that the relationship of 2 moles of cytochrome reduced/mole of tocopherol added was observed only when cytochrome *c* was present in a considerably greater concentration than tocopherol, suggesting that the reaction did not go to completion but that an equilibrium was reached. A separate experiment, where tocopherol was measured directly in an extract of the reaction mixture (Table VII), confirmed that 2 moles of cytochrome are reduced/mole of tocopherol oxidized.

The lack of a stoichiometric relationship between oleic acid added and cytochrome reduced confirms the kinetic finding that oleic acid acts as a catalyst. The stoichiometric relationship between tocopherol and cytochrome *c* reduced, as well as the results in Table VII, confirm the kinetic finding that tocopherol is consumed during the reaction, and must, therefore, be the electron donor. The apparent disagreement between the kinetic evidence for a one-electron oxidation of tocopherol and the stoichiometric demonstration of a two-electron oxidation is probably a reflection of the fact that reduction of cytochrome *c* is a one-electron reaction. It can be assumed that the reaction consists of either two sequential steps with an intermediate one-electron oxidation product of tocopherol that reduces a second cytochrome *c*, or the simultaneous transfer of two electrons from tocopherol to two cytochromes. If the first mechanism exists, it would account for our observations of (a) kinetics that are first order with respect to tocopherol with a one-electron oxidation, (b) somewhat more complicated kinetics with respect to cytochrome *c* since it will react with two different reductants at possibly different rates, and (c) a stoichiometric relationship indicative of a two-electron oxidation of tocopherol.

TABLE VII: Measurement of Tocopherol and Cytochrome *c* at Equilibrium.[a]

Cytochrome *c* (μM)		α-Tocopherol Remaining after 4 hr (μM)	
Added to Reaction	Reduced in 4 hr	Measured	Calcd (1 tocopherol oxidixed/2 cytochromes reduced)
0	0	27	30
30	17	18	22
60	36	12	12
90	46	8	7

[a] The concentrations of the constituents in the reaction mixtures were as follows: 30 μM α-tocopherol, 100 μM oleic acid, either 30, 60, or 90 μM oxidized cytochrome *c*, and 100 mM Tris–chloride, pH 8.0. Aliquots were diluted 1:10 for measurement of absorbance at 550 mμ immediately after adding cytochrome *c* to start the reaction and after the reaction had proceeded for 4 hr under anaerobic conditions (in nitrogen) in the dark at room temperature. Cytochrome *c* reduced was calculated from the change in absorbance at 550 mμ. Duplicate aliquots were also taken after 4 hr for extraction of tocopherol by the method of Duggan (1959). The extracts were evaporated *in vacuo* and the residue was dissolved in 1.0 ml of ethanol for measurement of tocopherol by ultraviolet absorbance. To avoid errors due to irrelevant absorbance or to possible interferring absorbance by the tocopherol oxidation product, spectra were recorded from 250 to 320 mμ. A line drawn between the 280- and 310-mμ points of the spectrum was used as a base line for measuring absorbance of tocopherol at 292 mμ. The reaction extracts were compared to standard tocopherol solutions treated in a similar way.

Reversibility of the Reaction and Calculation of an Oxidation–Reduction Potential of Tocopherol. The kinetic and stoichiometric data indicate a reduction of cytochrome *c* by tocopherol by a reaction that does not go to completion but instead attains an equilibrium. It is implicit in the existence of an equilibrium that the reaction is reversible but our attempts to demonstrate this directly have been only partially successful. When reduced cytochrome *c* was added to a reaction mixture that had reached equilibrium, there was a slow oxidation of the cytochrome. The rate of oxidation was only about 5% the initial rate of reduction but addition of more oleic acid (to twice the original amount) increased the rate of oxidation to about 50% the initial rate of reduction. However, when extracts of the reaction mixtures were analyzed for tocopherol, as in Table VII, it was found that there had been no regeneration of tocopherol associated

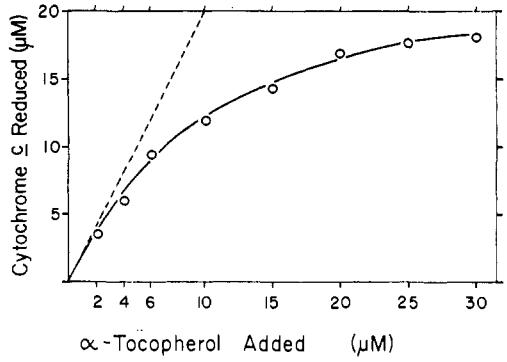

FIGURE 7: Extent of reduction of cytochrome *c* at various concentrations of α-tocopherol. The reaction mixtures consisted of 20 μM oleic acid and 20 μM oxidized cytochrome *c* and α-tocopherol in 50 mM Tris–chloride, pH 8.0. Lipids were added as ethanol solutions. The tubes were repeatedly evacuated and flushed with nitrogen and left at room temperature in the dark for 4 hr. Reduction of cytochrome *c* was determined by measuring A_{550} against blanks with 20 μM oxidized cytochrome *c*.

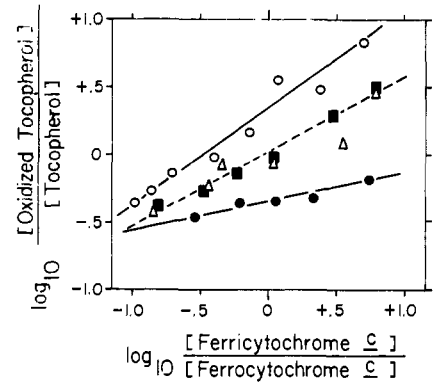

FIGURE 8: Graphs for calculation of oxidation-reduction potentials by the method described in the text. The data for α-tocopherol are from Figure 7 and for the other tocopherols from identical experiments. O——O, α-tocopherol; Δ----Δ, β-tocopherol; ■----■, γ-tocopherol (the points for β- and γ-tocopherols were considered together for placing the broken line); ●——●, δ-tocopherol.

with the oxidation of cytochrome. While this may indicate that we have not found the proper conditions for observing the reversal, it is quite possible that the reaction is not as simple as some of the data suggest and that it does not attain a "true" equilibrium. Nevertheless, the apparent existence of a stable equilibrium seemed to justify the application of thermodynamic relationships to the reaction for calculation of an oxidation–reduction potential of tocopherol.

Accordingly, the extent of reduction of cytochrome *c* at equilibrium was determined over a wide range of tocopherol concentrations (Figure 7). The concentration of reduced cytochrome *c* was calculated from A_{550} and the concentration of oxidized tocopherol was calculated on the basis of 1 mole of tocopherol oxidized/2 moles of cytochrome *c* reduced (*cf.* Table VII). Thus the concentrations of product for both tocopherol and cytochrome *c* were calculated and from the known amounts of reactants present, the ratio [oxidized]:[reduced] could be calculated for cytochrome *c* and tocopherol at equilibrium for each point in Figure 7. The logarithms of these ratios are plotted in Figure 8 for calculations based on the equation (Neilands and Stumpf, 1958)

$$E_h = E_0' + \frac{0.06}{n} \log \frac{[\text{oxidized}]}{[\text{reduced}]} \qquad (1)$$

where E_h is the oxidation–reduction potential of the system, E_0' is the potential of the half-reduced system at a stated pH, and n is the mole equivalent of electrons involved in the reaction. When [oxidized]:[reduced] = 1, log [oxidized]:[reduced] = 0, and E_h equals E_0'. Using a value for E_0' (pH 8) for cytochrome *c* of +0.22 (Rodkey and Ball, 1950), when log [ferricytochrome *c*]:[ferrocytochrome *c*] = 0, E_h is +0.22 and, from Figure 8, log [oxidized tocopherol]:[tocopherol] equals +0.33. Therefore, in eq 1

$$+0.22 = E_0' + \frac{0.06}{1}(0.33) \qquad (2)$$

and for tocopherol, E_0' (pH 8) = +0.20.[1]

Experiments identical with the one described in Figure 7 were done with β-, γ-, and δ-tocopherols and the data are also plotted in Figure 8. The potentials are higher than for α-tocopherol, the values being +0.22 for β- and γ-tocopherols and +0.24 for δ-tocopherol. These potentials should be interpreted only with recognition of the facts that (a) the reaction is not clearly reversible, (b) the mechanism of the reaction and the product of tocopherol oxidation are not known, and (c) equilibrium might be influenced by binding of tocopherol or its product by the cytochrome.

Other methods have previously been used to measure the oxidation–reduction potentials of tocopherols. Golumbic and Mattill (1940) using potentiometric indicators in 95% ethanol at 75° found an "apparent potential," defined as the potential at which 30% of the tocopherol is oxidized in 30 min for the oxidation of tocopherol to tocopherylquinone to be between the potentials of toluquinone and *p*-xyloquinone,

[1] These calculations assume that the reaction proceeds in two sequential one-electron steps, only one of which determines the equilibrium and thus the oxidation–reduction potential, so $n = 1$. If there is actually a single two-electron step, $n = 2$ and the potentials are +0.21, +0.22, and +0.23 for α-, β- and γ-, and δ-tocopherols. It is also assumed that the lipids do not change the potential of cytochrome *c*.

about +0.63 at pH 0 or +0.21 at pH 8. Since the over-all reaction was not reversible, they assumed that the first step was reversible and rate limiting, while the second step was not reversible. There does not appear to be a similar situation with the reaction we have studied because it would not lead to the apparent equilibrium we observe.

Polarographic measurements, when calculated for pH 8, indicate potentials of +0.29 for α-tocopherol with higher values for β- and γ-tocopherols (Smith *et al.*, 1942), and +0.27, +0.34, +0.35, and +0.41 for α-, β-, γ-, and δ-tocopherols (Baltes, 1956). It is not possible to make direct comparisons of our results with those obtained by polarographic measurements because of differences in buffers and solvents, uncertainties in pH corrections, and the fundamental differences in the nature of the process. It is significant, however, that with both procedures the relative values of the potentials are $\alpha < \beta \approx \gamma < \delta$.

Preliminary Investigations of Reaction Products. Reaction products have not been identified or studied in detail, but some preliminary studies have yielded significant information. Complete reaction mixtures and controls with one or more reactants omitted, or with oxidized cytochrome *c* replaced with reduced cytochrome *c*, were extracted with ethanol–ether (3:1) or chloroform–methanol (2:1) and ultraviolet absorbance spectra of the extracts were recorded. All controls had a tocopherol absorbance spectrum but the extract of the complete reaction mixture had no absorbance maximum above 220 mμ. Thin layer chromatography of the extracts on silica gel with either benzene or chloroform using 60% sulfuric acid or 10% ammonium molybdate–10% sulfuric acid for detection (Skinner *et al.*, 1964), also showed the absence of tocopherol. Both a faster and a slower spot were observed in place of tocopherol, but small amounts of the same spots were also present in the controls. They appear to arise, at least in part, during extraction and concentration of the extracts and may not be related to the reaction product.

Since previous results suggested that oleic acid acted only as a catalyst, it was anticipated that a negative proof would be needed with regard to an oleic acid reaction product, that is it would be necessary to demonstrate that there was no change in oleic acid. Therefore, a very sensitive means of detecting products was essential. Oleic acid-1-^{14}C was used and autoradiograms were made of thin layer chromatographs of extracts of reaction mixtures. There were equal amounts of tocopherol and oleic acid and an excess of cytochrome *c* in these experiments so that 2 moles of cytochrome were reduced/mole of oleic acid present. Therefore, any product of oleic acid formed in any stoichiometric relationship to cytochrome *c* reduction should be readily located. Reactions were allowed to proceed for 2 hr and the reaction mixtures were then extracted twice with five volumes of ethanol–ether (3:1). The extracts were concentrated and chromatographed on activated silica gel thin layer plates with hexane–ether–acetic acid (70:30:2), which gave an R_F for oleic acid of 0.52, and autoradiograms were made of the plates. Controls consisted of mixtures with either tocopherol or cytochrome *c* (or both) omitted and with oxidized cytochrome *c* replaced by reduced cytochrome *c*. With a 1 day exposure of the film, almost maximal darkening was obtained for oleic acid and all samples appeared identical with the control of oleic acid alone. With a 6-day exposure, two small faster moving spots were observed with those samples that contained both oleic acid and tocopherol; these spots appeared slightly darker with the complete reaction mixture. The spots for the complete reaction mixture were eluted with absolute ethanol and aliquots were dried on planchets for counting with a gas-flow counter. The two small spots contained 3 and 1% of the total activity eluted so they can not be considered as significant reaction products, confirming the previous conclusions that oleic acid is not changed during the reaction. About 15% of the radioactivity added to the complete reaction mixture was not recovered in the extract. This loss could represent oleic acid that was bound tightly to the cytochrome or was in some other way not extracted, but it is probably due to loss during the manipulations required to extract, concentrate, and dry the extracts.

The decrease in turbidity following addition of cytochrome to a suspension of tocopherol and oleic acid suggests the formation of a lipid–cytochrome complex. The state of this presumed complex has not been studied, but it has been determined that the reduced cytochrome is capable of serving as an enzyme substrate in that it can be oxidized by mammalian cytochrome oxidase.

Discussion

Previous Work on Oxidation of Tocopherol. The existence of oxidation–reduction reactions involving tocopherol was recognized in early work on the chemistry of tocopherols (Karrer, 1939) and has been studied extensively; the literature has been reviewed by Vasington *et al.* (1960), Boyer (1960), and more recently by Green and McHale (1965). A monovalent oxidation, yielding an unstable free radical, was observed by Michaelis and Wollman (1950) by irradiating a solution of α-tocopherol at liquid air temperature. There are many bivalent oxidation reactions of tocopherol, leading to 9-substituted tocopherones (Harrison *et al.*, 1956; Durckheimer and Cohen, 1964; Goodhue and Risley, 1965), α-tocopherylquinone (Karrer and Geiger, 1940), or to tocopherol dimers (Csallany and Draper, 1963) (although Durckheimer and Cohen (1962) believe that dimers result from monovalent oxidation). Various products of further oxidation have also been isolated (Vasington *et al.*, 1960; Boyer, 1960; Green and McHale, 1965).

We are aware of only one previous report of an oxidation–reduction involving tocopherol and cytochrome *c*. Harrison *et al.* (1956), in a report concerning tocopherol oxidation products, mentioned briefly

that if tocopherol and cytochrome *c* were mixed in 0.1 M phosphate, pH 7.4, and extracted immediately with isooctane, 51% of the tocopherol was oxidized. Their reaction differs from the one we have observed in that we are unable to obtain appreciable oxidation of tocopherol in the absence of unsaturated fatty acids, and they found 4.8 moles of tocopherol oxidized/mole cytochrome *c* present, whereas we always find 0.5 mole or less of tocopherol oxidized/mole of cytochrome present.

Possible Mechanism of Tocopherol Reduction of Cytochrome c. The kinetic, stoichiometric, and chromatographic experiments make it clear that the fatty acids in the reaction reported here function as catalysts. The main evidence available for considering a possible mechanism is the observation of the dependence on pH with an optimum at pH 8. Theoretical considerations of the effect of pH on the components of the reaction indicate that the phenolic hydroxyl of tocopherol would dissociate at alkaline pH, but we estimate the pK of the substituted phenol to be near pH 10 or 11 (Brown *et al.*, 1955). Therefore, at pH 8 tocopherol would be less than 1% dissociated, which is probably not significant to the pH effect. The carboxyl group of oleic acid has a pK in water of about 5 (Jukes and Schmidt, 1935), so that as pH is increased it dissociates, making the compound increasingly soluble in water. We have observed that if oleic acid and tocopherol are both added to Tris buffers from pH 7 to 9, they form a turbid suspension at pH 7.5 and below but a nearly clear solution at pH 8 and above. It is possible that this solubilizing effect is at least partly the cause of increased reactivity as pH is increased, but it would not account for decreased reactivity above pH 8 so it may not fully explain catalysis by fatty acids.

Another possible mechanism of the catalysis would be the formation of a complex of cytochrome and fatty acid to permit a hydrophobic association of cytochrome and tocopherol. Oleic acid has been shown to form salts with lysine and arginine and with basic proteins (Jukes and Schmidt, 1935), and acidic phospholipids are known to complex with cytochrome *c* (Das *et al.*, 1962; Reich and Wainnio, 1961), apparently by electrostatic attraction of the basic groups of lysine and arginine followed by complexing of additional lipid through hydrophobic bonding. Thus, formation of a cytochrome–oleic acid–tocopherol complex is not unreasonable. Such a mechanism would be consistent with the observation that only free acids are reactive. However, it would seem unusual that there is such a high degree of specificity for unsaturated acids; this could reflect specific requirements for permitting tocopherol to be accessible to the heme group, which is considered to be protected in a "crevice" of the protein (Theorell and Akesson, 1941a). Since the complex would depend on the fatty acid ionized, it would also be consistent with increased reactivity with increased pH, but again there is no explanation for decreased reactivity above pH 8. It should be noted that if catalysis is through the formation of a cytochrome–oleic acid complex, it must be an unstable complex since the reaction proceeds with an oleic acid:cytochrome ratio as low as 1:10 (*cf.* Figure 6), indicating that each oleic acid would be available to more than one cytochrome.

Decreased reactivity above pH 8 might be due to changes in cytochrome *c*, which is titrated in this region. On the basis of oxidation–reduction potential changes, a pK of 7.8 (Rodkey and Ball, 1950) has been identified for cytochrome *c* and on the basis of spectral changes pK values of 9.35 and 12.76 have been observed (Theorell and Akesson, 1941b). The groups responsible for these pK values are apparently associated with the active center of the cytochrome and could be responsible for a pH effect in this pH range. Increasing pH would also decrease the cationic nature of cytochrome *c*, a strongly basic protein containing 19 lysine and 2 arginine moieties/molecule (Margoliash *et al.*, 1961) and having an isoelectric point of about pH 10 (Theorell and Akesson, 1941c). This could decrease formation of a complex with anionic oleic acid, but the titration of cytochrome *c* indicates that few of these groups would be titrated below pH 9 (Theorell and Akesson, 1941d). Greenwood and Palmer (1965) have shown that pH also affects the tertiary structure of cytochrome *c*. At alkaline pH it exists as two monomers, one rapidly reduced by ascorbate and tetrachlorohydroquinone and the other slowly reduced. The percentage of the total cytochrome existing in the slowly reactive form increases with pH above 8, but at pH 9 it is only about 45% so that this phenomenon alone could not account for almost complete lack of reaction at pH 9. It is, of course, possible that the changes in tertiary structure observed by Greenwood and Palmer (1965) could have a greater effect with the lipid reductant used by us. It is interesting that they noted slower reduction in phosphate than in Tris, as we also observe (Figure 1).

Until the oxidation product of tocopherol is identified, it is not possible to discuss mechanisms, or likely intermediates, of the actual electron-transfer reaction. It should be noted, however, that if the product were tocopherylquinone, which has a much stronger ultraviolet absorbance than tocopherol and is extracted by the procedure used, it would have been readily detected in extracts of the reaction mixture unless it was destroyed or so tightly bound to the protein that it could not be extracted.

Significance of the Reaction. The most obvious significance of the reduction of cytochrome *c* by tocopherol is related to current research into the function of tocopherol. One line of research concerns the possible participation of additional electron carriers in the terminal electron-transfer chain and the possibility that quinones and chromanols may function in coupling oxidation to phosphorylation (Vasington *et al.*, 1960; Slater *et al.*, 1961; Boyer, 1960; Green and McHale, 1965; Scott, 1965). These are especially attractive proposals because tocopherol is known to exist in either a reduced form or several oxidized forms, and because tocopherol is localized in the

particulate mitochondrial preparations that also contain the electron-transfer chain. Additional experimental support stems from the observation that terminal electron-transfer enzymes could be inactivated by extraction with isooctane and subsequently reactivated by addition of tocopherol, as well as certain other substances, and the finding that NADH[2]–cytochrome *c* reductase preparations lost activity with aging and that the activity could be restored with tocopherol. However, considerable work on these phenomena has failed to demonstrate an enzyme cofactor or electron carrier role of tocopherol (see Vasington *et al.*, 1960, for a critical review of this work). Although tocopherylquinone has been identified as a metabolite of tocopherol (Csallany *et al.*, 1962), Slater *et al.* (1961) found tocopherol, but not tocopherylquinone, to be present when all other components of the Keilin–Hartree heart muscle respiratory chain were oxidized. Thus, the possibility of a tocopherylquinone system functioning directly in electron transfer seems remote. Our observation that tocopherol can participate in an oxidation–reduction reaction with cytochrome *c* (in which tocopherylquinone is apparently not a product, although it is not entirely ruled out), suggests that there does exist a reaction mechanism by which tocopherol could participate directly in an electron-transfer sequence or possibly in coupled phosphorylation. This reaction should permit more detailed study of the type of reaction that might be expected of tocopherol in the electron-transfer chain, and the calculation of the oxidation–reduction potential permits an estimate of the region of the cytochrome chain where the reaction would most likely occur. Identification of the tocopherol oxidation product should permit a more direct and thorough investigation of the possible participation of an oxidation–reduction reaction of tocopherol in electron-transfer enzymes.

Tocopherol is also thought to function, at least partly, as an antioxidant (Tappel, 1961, 1962). It has been demonstrated that tocopherols inhibit the heme-catalyzed oxidation of unsaturated fatty acids (Tappel, 1961, 1962), and at an early stage of the present investigation it seemed reasonable that the reaction we were observing might be related to this phenomenon. However, we soon demonstrated by (a) the observation that removal of oxygen and addition of inhibitors of heme-catalyzed oxidations had no effect on the reaction, and (b) by the findings that only free acids are reactive and polyunsaturated acids are less reactive than monounsaturated acids, that there is no apparent relationship between fatty acid oxidation and cytochrome *c* reduction. In the extensive work with tocopherol inhibition of heme-catalyzed oxidation (see reviews by Tappel, 1961, 1962), we know of no mention of a reduction of the heme protein. In fact, Tappel (1955) concluded that there was no valence change of the heme iron.

[2] Abbreviation used: NADH, reduced nicotinamide-adenine dinucleotide.

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Formation of Ammonia by Insertion of Molecular Nitrogen into Metal–Hydride Bonds. III. Considerations on the Properties of Enzymatic Nitrogen-Fixing Systems and Proposal of a General Mechanism*

Hans Brintzinger

ABSTRACT: Based upon results previously obtained with a nonenzymatic nitrogen-fixing reaction, a general mechanism is proposed for reactions of this type. Double insertion of a N_2 molecule into two metal–hydride bonds of a dimeric μ-hydrido complex, containing the metal in a reduced state, allows for the simultaneous uptake of six reduction equivalents by the N_2 molecule, thus avoiding energetically unfavorable intermediates. If applied to enzymatic nitrogen-fixing reactions, this mechanism would account for hitherto unexplained properties of these systems. In addition, experiments are suggested which would allow proper evaluation of the aptness of this hypothesis.

It is now well established that the reduction of molecular nitrogen to ammonia by aerobic and anaerobic microorganisms occurs through the combined mediation of two nonheme iron particles, one of which contains, besides iron, molybdenum (Bulen, 1966; Mortenson, 1966). Investigations into the dependence of these enzyme systems on various kinds of reducing and phosphorylating agents (Mortenson, 1964; Hamilton *et al.*, 1964; Bulen *et al.*, 1965; Hardy and D'Eustachio, 1964), and into their interaction with inhibitors and pseudosubstrates (Lockshin and Burris, 1965; Schöllhorn and Burris, 1966; Hardy and Knight, 1966) have provided considerable information concerning the nature of the enzymatic reaction paths. To date, however, no satisfactory mechanism has been proposed to account for the observed properties of this interesting reaction. Indeed it is not easy to visualize how the necessary total of six electrons can be transferred from the reducing systems onto the N_2 molecule without the occurrence of energetically very unfavorable intermediates (diimine, etc.). It is the purpose of the present series of publications to draw the attention of the biochemists working in this field, to a coordination-chemical mechanism which could account for the peculiarities of the enzymatic nitrogen reduction, and to demonstrate with suitable model systems the possibility and the conditions of its operation.

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Proposed Mechanism

The N_2 molecule, like other unsaturated systems, should in principle be capable to undergo insertion into metal–hydride bonds (for a review on insertion reactions in metal complexes see Heck, 1964). In fact, an example has been given in preceding publications of this series (Brintzinger, 1966), where such an insertion of N_2 into metal–hydride bonds is very likely to have occurred. It was pointed out that a simultaneous insertion of N_2 into two metal–hydride bonds containing the metal in a reduced state makes indeed possible a very smooth transfer of six reduction equivalents. A catalytic cycle based on this mechanism,