Studies of the Biological Function of Vitamin E

II. THE NATURE OF THE SPECIFIC ACTivating EFFECT OF TOCOPHEROL IN AGED PREPARATIONS OF CYTOCHROME REDUCTASES

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Recent work has indicated that evidence supporting a role for tocopherol in electron transport should be re-evaluated. The direct demonstration of the inhibitory nature of solvents on enzymes of the respiratory chain (1) and nonspecific restoration of this activity by various lipids (2, 3) are the major objections to studies which employed isooctane extraction. However, other evidence based on the specific activating effect of tocopherol on aged preparations of cytochrome reductases does suggest a role for tocopherol in the respiratory chain. Thus Nason and Lehman (4), Donaldson et al. (2), and Vasington et al. (5) have shown that under certain conditions of aging, with or without isooctane extraction, some enzyme preparations are activated by the addition of tocopherol. Tocopheryl acetate, tocopherylquinone, and other lipids which reversed the solvent-inhibited enzyme preparations are activated by tocopherol in whole tissues or enzyme fractions. In the present report, data are presented which are consistent with the view that (a) inhibitory products are present in aged enzyme preparations, and (b) that tocopherol and other hydroxylated compounds reactivate these preparations by removing the inhibitory products. We have examined the specific activating effect of tocopherol on some aged preparations of chick heart muscle.

EXPERIMENTAL

Because rigid experimental conditions must be adhered to for greatest reproducibility, some of the conditions previously described (8) are given here in greater detail. For the DPNH oxidase assay, the system employed consisted of 0.3 ml of DPNH (1.15 μmoles per ml), 0.1 ml of a 1% solution of cytochrome c, 0.030 to 0.050 ml of enzyme solution, and enough 0.1 M potassium phosphate buffer, pH 7.5, to make a total volume of 3.0 ml. The reaction was started by adding DPNH. The first reading, at 340 mμ, was taken 30 seconds after the addition of DPNH and at 1-minute intervals for 5 minutes. In the DPNH-cytochrome c reductase assay, the system contained 0.1 ml of a 2% solution of cytochrome c, 0.3 ml of 0.01 M KCN, varying amounts of enzyme solution (0.020 to 0.120 ml), 0.1 ml of DPNH of the same concentration that was used in the DPNH oxidase assay, and enough 0.1 M potassium phosphate buffer, pH 7.5, to make a total of 3.0 ml. Readings were made at 550 mμ at zero time and at 30-second intervals after the addition of DPNH. In the succinate-cytochrome c reductase assay, 0.2 ml of 0.1 M disodium succinate was substituted for DPNH. In experiments in which dibenzoyl peroxide was used, the peroxide in 0.01 ml of absolute ethanol was allowed to incubate for 3 minutes with the reaction mixture in the absence of DPNH or in the absence of succinate.

In attempts to reverse the peroxide inhibition, tocopherol, tocopherylquinone, tocopheryl acetate, vitamin K₁, or naphthotocopherol in bovine plasma albumin-ethanol-buffer suspension were allowed to incubate with the inhibited enzyme 5 additional minutes before the assay.

Since some aged preparations showed increased activity after simply standing in the reaction mixture for 3 to 5 minutes, all experiments were done after a 5-minute incubation of the aged enzyme in the reaction mixture, with or without compounds being tested. Control and "no addition" experiments reported in this paper represent the activity of the enzymes in the presence of the carriers used to suspend the compounds being tested. In some instances it was necessary to increase the cytochrome c concentration of control and experimental samples to assure linearity of cytochrome c reduction with time.

The aged preparations used in this study were prepared as follows. Heart muscle from chicks was homogenized with 10 times its weight of 0.1 M phosphate buffer, pH 7.5, first in a VirTis homogenizer and then in a TenBroeck hand-driven homogenizer. The homogenate was centrifuged at approximately 3,000 × g for 10 minutes and the supernatant fluid dialyzed against 10 volumes of 0.01 M phosphate buffer, pH 7.5, for 1 hour. The suspension was centrifuged for 10 minutes at 3,000 × g to remove any precipitate formed during the dialysis. The supernatant fluid was centrifuged at 25,000 × g for 30 minutes. The resulting pellet was resuspended in 2% digitonin solution.
solution in 0.1 M phosphate buffer, pH 7.5, by vigorously homogenizing with a hand-driven TenBroeck homogenizer. The volume of digitonin solution was equal to or 1.5 times the original weight of the tissue. The digitonin suspension was centrifuged at 25,000 × g for 30 minutes and the clear supernatant solution was used for aging. Usually over 90% of the original DPNH-cytochrome c reductase activity was recovered in the supernatant solution. These procedures are based on the methods of Donaldson et al. (2).

Preparation of the digitonin-solubilized enzyme solution and aging were carried out at approximately 4°C. An effect of tocopherol on succinate-cytochrome c reductase was observed in most preparations upon aging for 1 day. Longer periods of aging, up to 10 days, were required before an effect of tocopherol on DPNH-cytochrome c reductase could be observed.

The dibenzoyl peroxide was obtained from the Fisher Scientific Company. The 5-pentadecyl resorcinol was kindly supplied by Dr. E. G. High. The DPD was a specially purified product of the B. F. Goodrich Chemical Company. SANTOQUIN (2,2,4-trimethyl, 6-ethoxy, 1,2-dihydroquinoline) was obtained from the Monsanto Chemical Company. Coenzyme Q was kindly provided by Dr. Karl Folkers of the Merck Sharp and Dohme Research Laboratories. d,α-Tocopherol was obtained from Distillation Products Industries; dl-α-tocopherol and dl-α-tocopherol acetate were obtained from Merck and Company. Naphthotocopherol was prepared by the method of Tishler et al. (9). Reduced vitamin K and reduced coenzyme Q were prepared by reduction of the quinones with KBH₄ in ethanol (10).

The hydroquinones were extracted into hexane, and the hexane extracts were washed with water and dried over anhydrous sodium sulfate. The hexane was evaporated to dryness under vacuum and the hydroquinones were weighed. Only freshly prepared solutions of the hydroquinones were used. Tocopherylquinone was prepared by the oxidation of tocopherol with dibenzyl peroxide in ethanol. It was purified by chromatography on alumina with 3% acetone in hexane as developing agent. The tocopherol, vitamin K, and coenzyme Q derivatives were added to the reaction mixtures in a bovine plasma albumin-ethanol-buffer suspension. The suspensions contained 5 mg of the vitamin products or coenzyme Q derivatives, 2 mg of albumin, and 0.1 ml of ethanol per ml of suspension. The suspensions were not centrifuged. From 0.040 to 0.080 ml of the suspensions was used in attempting to activate enzyme preparations. The nontocopherol antioxidants were added to the reaction mixtures dissolved in 0.01 to 0.014 ml of absolute ethanol.

Vitamin E-deficient chicks were produced by feeding day-old chicks a synthetic diet (C47A) containing 4% of vitamin E-free lard (8). The same diet containing 1% of vitamin E-free lard was used to produce chicks which were marginally deficient in essential fatty acids. Chicks were maintained on these diets for at least 4 weeks.

RESULTS

Evidence for Existence of Inhibitor(s) in Aged Preparations; Kinetics of Cytochrome c Reduction by Aged Preparations which Respond to Tocopherol—A striking phenomenon observed with some aged preparations in cytochrome c reduction was the absence of proportionality between enzyme concentration and activity. This was in contrast to linear relationships obtained with freshly prepared solutions. The reduction of cytochrome c by both aged and freshly prepared enzyme solutions was linear with time. Fig. 1 shows the effect of increasing enzyme concentration on the initial rate of reduction of cytochrome c by typical freshly prepared or aged enzyme preparations. Table I shows the effect of increasing enzyme concentration on the response of succinate-cytochrome c reductase activity after the addition of tocopherol. Results similar to those in Table I and Fig. 1 were obtained with DPNH-cytochrome c reductase of aged preparations. It is evident from the combined data in Table I and Fig. 1 that (a) the rate of reaction did not increase linearly upon the addition of increasing amounts of enzyme solution, and (b) the tocopherol effect was more noticeable at high concentrations of the enzyme than at low concentrations. Furthermore, it was observed that some loss of activity often occurred when higher concentrations of the enzyme were used (for example, Preparation 4, Table I), suggesting the presence of an inhibitor (11).

Increasing the concentration of the substrates had no effect on the activity of these preparations.

* Activity = ΔA₄₅₀ mV X 10⁻² minutes.
† At the lowest concentration, 1 time, the volume of enzyme varied from 0.020 to 0.080 ml with different preparations.
Inhibition of enzymes by dibenzoyl peroxide and its reversal by tocopherol and other antioxidants

<table>
<thead>
<tr>
<th>Experiment</th>
<th>System</th>
<th>Control</th>
<th>Activity* With peroxide†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No add</td>
<td>Vitamin E and ethanol</td>
</tr>
<tr>
<td>A</td>
<td>Succinate-cytochrome c reductase</td>
<td>229</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>Succinate-cytochrome c reductase</td>
<td>168</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>DPNH-cytochrome c reductase</td>
<td>110</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>DPNH oxidase</td>
<td>217</td>
<td>7</td>
</tr>
</tbody>
</table>

* Activity: Cytochrome c reductase = ΔA340 × 10^9/2 minutes.
† 0.8 to 1.6 µg of dibenzoyl peroxide per ml.
‡ 3.6 µg of Santoquin or DPD per ml. All of these experiments were done on particulate preparations of beef heart (Fraction IV) (2).

If the action at the lowest level of enzyme concentration in the absence of added tocopherol was mediated by endogenous tocopherol, it would appear that increasing the enzyme concentration would also increase the activity, with an increase in endogenous tocopherol concentration, rather than create a tocopherol "requirement" as shown above.

Possible Model System of Aged Preparations—The deleterious effect of products derived from fatty acid peroxidation is well known. The prevention of peroxidation by tocopherol has also been widely studied. Recently, Tappel and Zalkin (12) demonstrated that tocopherol stabilizes DPNH-cytochrome c reductase of mitochondria undergoing peroxidation. These workers did not attempt to demonstrate an effect of tocopherol in activating DPNH-cytochrome c reductase of peroxidized mitochondria. Table II gives representative data on the effect of dibenzoyl peroxide on enzymes of the respiratory chain of beef heart muscle. Similar results were obtained with preparations of heart and skeletal muscle from other species. The data in the table demonstrate that the addition of very small quantities of dibenzoyl peroxide inhibited DPNH oxidase, DPNH-cytochrome c reductase, and succinate-cytochrome c reductase of various preparations. This inhibition was reversed by the addition of tocopherol, but tocopheryl acetate and vitamin K, had no effect. Vitamin K, hydroquinone effectively reversed the peroxide inhibition with low concentrations of peroxide. In a limited number of experiments, the addition of tocopheryl-quinone, coenzyme Q₀, or coenzyme Q₁ reduced by potassium borohydride had no effect on the inhibition.

That dibenzoyl peroxide is not a general enzyme inhibitor was shown by its inability to inhibit rat liver microsomal or chick liver mitochondrial DPNH-cytochrome c reductase and rat heart muscle lactic dehydrogenase. There was no evidence of inhibition even with four times the concentration of dibenzoyl peroxide usually used to inhibit the chick heart preparations. The respiratory enzyme systems from muscle were consistently inhibited by the addition of dibenzoyl peroxide. The enzyme systems of liver mitochondria were only slightly affected even when solutions of digitonin-solubilized mitochondria were used. Lineweaver-Burk plots of the dibenzoyl peroxide inhibition of chick muscle DPNH-cytochrome c reductase revealed that the inhibition was noncompetitive with DPNH. When 2,6-dichlorophenol-indophenol was used as electron acceptor from DPNH, an inhibition was also obtained with dibenzoyl peroxide. This system appeared more resistant to the action of dibenzoyl peroxide, but this may be a reflection of the ability of the 2,6-dichlorophenol-indophenol to destroy dibenzoyl peroxide.

Although from the data in Table II the inhibition might appear to be an all-or-none phenomenon, partial inhibition was obtained when smaller quantities of dibenzoyl peroxide were used. In other experiments ditertiary butyl peroxide and tertiary butylbenzene peroxide were not inhibitory to the systems shown in Table II.

Table II also shows the effectiveness of the antioxidants Santoquin and DPD in reversing the dibenzoyl peroxide-inhibited inhibition of succinate-cytochrome c reductase. The antioxidants were slightly inhibitory to the succinate-cytochrome c system and were very inhibitory in the DPNH-cytochrome c reductase system of muscle. The latter effect was observed by Nason and Lehman (13). Therefore we have not been able to reverse the peroxide-inhibited DPNH-cytochrome c reductase system with antioxidants. In general, under the conditions of these experiments, Santoquin was the least inhibitory of the antioxidants on the succinate-cytochrome c reductase system. It was also the most effective antioxidant in reversing the dibenzoyl peroxide inhibition of this system. Other antioxidants such as \( N,N'\text{-}N,N'\text{-}bis(1\text{-}methylheptyl)-p\text{-}phenylenediamine, N,N'\text{-}bis(1\text{-}ethyl-3\text{-}methylpentyl)-p\text{-}phenylenediamine, dibutyl cresol, propylparasert, and hydroquinone showed no reversal of the peroxide effect or were inhibitory when used in concentrations comparable to the concentration of Santoquin and DPD.

As mentioned above, several of the antioxidants are inhibitory to muscle DPNH-cytochrome c reductase. This might appear important in view of the present interest in antioxidants. We have observed, however, that the DPNH-cytochrome c reductase of freshly prepared liver mitochondria is resistant to inhibition by DPD; digitonin-solubilized solutions of mitochondria are more easily inhibited. Preliminary evidence indicates that the digitonin-solubilized liver mitochondrial enzyme is still more resistant to inhibition by DPD than muscle preparations. Therefore, in addition to permeability, other factors may be involved.

Some of the antioxidants reduce cytochrome c nonenzymatically. This could not account for the reversal of the inhibition reported in this paper, since the concentration of antioxidants was very low and all of the reduction of cytochrome c by these substances was completed during the 5-minute incubation period before measurement of enzyme activity. Also, the antioxidants in the concentrations used had no effect on uninhibited, freshly-prepared enzyme solutions.

Activation of Succinate-Cytochrome c Reductase of Aged Preparations by Antioxidants—Since Santoquin was found to be as effective as tocopherol in reversing the dibenzoyl peroxide inhibition of succinate-cytochrome c reductase, it was reasoned that Santoquin might be effective in aged preparations which gave a response to tocopherol. Table III shows the response of some aged preparations to Santoquin, tocopherol, and to 5-pentadecyl resorcinol. As can be seen, Santoquin was quite effective in activating succinate-cytochrome c reductase. More recently we...
have observed that tocopherol is effective in activating some aged preparations where Santoquin is not. There is no obvious explanation for this difference.

Unlike the inconsistent activation obtained with Santoquin in the succinate system of aged preparations, 5-pentadecyl resorcinol showed consistent activating effects when tested in the succinate-cytochrome c reductase system of aged preparations. The effects parallel those of tocopherol in this system. At the concentration used in these experiments, the compound had no effect on succinate-cytochrome c reductase of preparations not showing a tocopherol effect. DPNH-cytochrome c reductase was inhibited by the substance; therefore it was not effective in this system.

Response of Cytochrome c Reductases of Aged Preparations to Other Hydroxylated Lipid Compounds—Naphthotocopherol, formed by the reductive cyclization of vitamin K1, and reduced vitamin K1, were just as effective as tocopherol in activating cytochrome reductases of aged preparations. This is shown in Table IV. The ineffectiveness of tocopherylquinone is also demonstrated. Vitamin K1 had no effect in any aged system tested.

**DISCUSSION**

Several recent studies on the effect of aging of enzyme preparations on electron transport and oxidative phosphorylation indicate that a variety of complex reactions may occur during the process. Consequently interpretations must be difficult when aged enzymes are studied. The success reported here in obtaining preparations which responded to tocopherol probably is due to the use of heart muscle from chicks on selected dietary regimens. It was found that preparations of heart muscle from chicks fed either a tocopherol-deficient diet or a diet low in polyunsaturated fatty acids (1 2% of lard) consistently showed a response to the addition of tocopherol after aging. It has been shown by Tappel and Zalkin (12) that tissues from animals deficient in vitamin E form more peroxides when incubated at 37° for short periods than do tissues from animals whose diet is supplemented with the vitamin. This has also been observed in our laboratory with vitamin E-deficient chick tissues. It was reasoned that if the aging process produced peroxides, or other oxidation products which acted as inhibitors, then tissues deficient in the vitamin should be more suitable for use in studying the aging effect. The interrelationship of tocopherol deficiency and low unsaturated fatty acid intake in creating the tocopherol effect in aged preparations is not clear. However, it is interesting to note that increased oxygen consumption of tissues is found in the deficient state of both nutrients. Furthermore, reduced P:O ratios have been observed in vitamin E deficiency and in essential fatty acid deficiency (14, 15).

The data in Table I relating to the kinetics of cytochrome reduction by aged preparations appear to support the hypothesis that an inhibitor or inhibitors are present. It is important to note with respect to the preparations in Table I that (a) no tocopherol could be found in these preparations (8), (b) no significant effect could be demonstrated with freshly prepared enzyme solutions, and (c) tocopherol had little or no effect at low concentrations of the enzyme. It is highly probable that more than one inhibitor may be formed when such preparations are aged. Our observations indicate that at least three distinct activations may be recognized; (a) an activation by plasma albumin, (b) an activation by Santoquin, and also by certain hydroxylated lipid substances, and (c) an activation which is specific for the hydroxylated lipid substances. The activating effect of bovine serum albumin in oxidative phosphorylation of insect mitochondria has been ascribed to the removal of inhibitors (10).

The nonspecificity of the stimulation of aged preparations of cytochrome reductases is illustrated by the effectiveness of reduced vitamin K1, 5-pentadecyl resorcinol, and naphthotocopherol in addition to tocopherol. It would appear highly improbable that these substances participate in electron transport. The presence of hydroxyl group and a long side chain are characteristics of these substances in common with tocopherol. The reason why reduced coenzyme Q10, which also has these characteristics, was inactive in aged preparations, is unclear.

Any discussion of the nature of the inhibition in aged preparations or of the mechanism of the removal of the inhibitor by the active substances must be seriously restricted until the inhibitor or inhibitors are isolated and identified.

Because some differences exist between the dibenzoyl peroxide inhibition and the inhibition observed in aged preparations, the dibenzoyl peroxide effect can only be considered as evidence that
an inhibition due to peroxides can occur which may be reversed by tocopherol and other substances.

Since peroxides in general did not cause inhibition of enzymes of the respiratory chain, it follows that these compounds may not necessarily be the inhibitors in aged preparations. However, there may be a special structural requirement in the peroxide for it to be an inhibitor. The occurrence of peroxides, as measured by the thiobarbituric acid test (17), in many of the aged preparations reported here has been demonstrated. The possibility remains, however, that the presence of peroxides may be fortuitous with respect to the activity.

The following relationships appear to exist between the studies reported here and those from other laboratories, especially in instances where isooctane extraction was used. Isooctane and other solvents inhibit enzymes of the respiratory chain; this inhibition responds to the addition of tocopherol and a variety of lipids (13). Aging produces an inhibition which responds to tocopherol and, as has been shown in this paper, to other hydroxylated lipid substances. In those instances where other neutral lipids were reported to be as effective as tocopherol in activating cytochrome reductases after isooctane extraction, the inhibition was due solely to isooctane; in instances where tocopherol was found to be somewhat more effective than other lipids, a mixed inhibition occurred. Finally, upon aging after isooctane extraction, in which case tocopherol was the specific activator, sufficient isooctane had been removed from the enzymes and the inhibition was due to aging.

The present experiments demonstrate that the activation of cytochrome c reductases of aged preparations is not specific for tocopherol. It has been suggested that the stimulation involves the removal of inhibitors; although this may be a biological function of the vitamin in the intact animal, it is probably closely related to its action as an antioxidant. These and other data suggest that tocopherol does not actively participate in electron transport.

**SUMMARY**

The activating effect of tocopherol on aged preparations of chick heart cytochrome c reductases has been examined. Experiments with increasing concentrations of the aged enzyme preparations demonstrated that linearity of activity was not achieved with increasing amounts of enzyme. Linearity was obtained with freshly prepared enzyme solutions. The existence of an inhibitor, or inhibitors, in aged preparations is postulated to account for the behavior of aged preparations.

The stimulating effect of tocopherol on aged preparations is shown to be nonspecific. The activating effect of the antioxidant, SANTOQUIN, on succinate-cytochrome c reductase of some aged preparations is described. The consistent activating effect of naphthotocopherol and 5-pentadecyl resorcinol, also an antioxidant, on succinate-cytochrome c reductase of aged preparations which responded to tocopherol is also described. The efficacy of naphthotocopherol and reduced vitamin K in activating reduced diphenylpyridine nucleotide-cytochrome c reductase of aged preparations is demonstrated to be of the order of that of tocopherol.

The inhibitory action of dibenzoyl peroxide on enzymes of the respiratory chain from muscle and the reversal of the inhibition by tocopherol and other antioxidants are demonstrated.

**REFERENCES**

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