Synergistic Antioxidant Effect of Nucleic Acids and Tocopherols

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ABSTRACT AND SUMMARY

Nucleic acids acted as synergists with tocopherols in inhibiting the oxidation of methyl linoleate. DNA and RNA enhanced the activity of tocopherols to different extents in the order α->γ->δ-tocopherol. Nucleic acids decreased the rates of consumption of tocopherol in the presence of oxidizing methyl linoleate. Nucleic acids also decreased the rate of oxidation of tocopherols by PbO_2. The synergistic effect of nucleic acids seemed to be caused by hydrogen bond formation with tocopherols which protected tocopherols from direct air oxidation.

INTRODUCTION

Synergistic antioxidant effects have been studied extensively, and synergists may be classified in three groups: 1 – mixtures of antioxidants such as phenols and amines (1); 2 – hydroperoxide eliminators such as sulfur compounds, phenols, and amines (2,3); and 3 – metal chelators such as citric, ascorbic, and phosphoric acids (4-7). Type 1 synergists show low antioxidant activities alone, but are able to reproduce other antioxidants consumed in the reaction AH + ROO·→ A· + ROOH. Type 2 synergists eliminate hydroperoxides necessary to initiate the autoxidation chain. Type 3 synergists tie up metals that catalyze the decomposition of hydroperoxides to initiate free radical chains.

Ehrenberg, Ehrenberg, and Löfroth (8) showed that DNA radicals were formed by irradiating DNA with cobalt-60 γ-rays. At that time, they observed thimine and thymidine radicals and found a striking similarity between the electron spin resonance (ESR) spectra of these radicals.

In this report nucleic acids were evaluated for their synergistic antioxidant activity with tocopherol in a heterogeneous mixture with methyl linoleate, and the mechanism of their activity was investigated.

EXPERIMENTAL PROCEDURE

Materials

Methyl linoleate [99.9% purity as determined by gas liquid chromatography (GLC)] was prepared from safflower oil fatty acids followed by the urea adduct formation. The linoleic acid was esterified and purified by column chromatography on silicic acid to remove a trace of peroxides and pigments. Removal of natural antioxidants and metals was demonstrated by the ferric chloride: 2,2'-bipyridine method (9) and by atomic absorption spectra, respectively. The dl-α-tocopherol (α-T) was a commercial product, and d-γ-(γ-T) and d-δ-tocopherols (δ-T) were obtained from Eisai Co., Ltd., (Tokyo, Japan). All tocopherols were refined by chromatography on alumina before use (10,11), and their purities were demonstrated by elemental analyses, ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), and refractive indices. Deuterized tocopherols were prepared from refined tocopherols using D_2O and HCl in benzene. The extent of deuterization was calculated from IR and NMR spectra by comparing with the calibration curves. Deoxyribonucleic acid (DNA) was a commercial product originating from herring (Nakarai Chemicals, Ltd., Tokyo, Japan; Guaranteed Reagent). The DNA had a molecular weight of about one million by the viscosity method (12). Ribonucleic acid (RNA) was a commercial product prepared from Torula Yeast (Sigma Chemical Co., St. Louis, MO; Grade VI).

Autoxidation Procedure

Methyl linoleate (1.5000-1.5005 g), tocopherols (3.76 x 10^{-6} mole) and nucleic acids (2.00% by wt of linoleate) were added to beakers (4.1 cm diameter) and held at 30 ± 1 C for 1 hr under a vacuum of 10^{-3} mmHg to remove volatiles (13). The samples were incubated at 36.5 ± 0.5 C in air. Oil thickness in the beakers was 1.03 mm. Weight increases during autoxidation of the samples were determined (14).

Analytical Procedure

Residual tocopherols in autoxidized samples were determined by the ferric chloride:2,2'-bipyridine method, after hydroperoxides were removed by potassium iodide treatment. This method will be reported in a separate paper. The detection and measurement of antioxidant and synergist radicals produced during autoxidation were carried out at 20 C using an ESR spectrometer (Japan Electron Optics Lab. Co., Tokyo, Japan; model JES-1X) in 5 mm diameter quartz tubes. Termination by tocopherol of nucleic acid radicals formed during autoxidation of methyl linoleate was determined under the following conditions: tocopherol – 5.00 x 10^{-6} mole/1.5 g methyl linoleate, starting DNA radical concentration – 10.75 x 10^{-6} mole/1.5 g linoleate, starting RNA radical concentration – 4.08 x 10^{-6} mole/1.5 g methyl linoleate, 36.5 ± 0.5 C and 10^{-3} mmHg. The change of DNA and RNA radicals with time was determined by ESR. Infrared, UV, and ESR spectra, peroxide values (PV) (15-17), molecular weights, and refractive indices were determined at various stages in the oxidation of methyl linoleate. These observations allowed determinations of (a). alpha methylene groups, (b) conjugated dienes (18), (c) conjugated cis,trans- and trans,trans- dienes (19), (d). radicals due to the oxidation of methyl linoleate, (e) isolated trans double bond (20), and (f) α,β,γ,δ-unsaturated carbonyls. Tocopherols (4.00 x 10^{-5} mole/ml) were oxidized by PbO_2 (4.00 x 10^{-5} mole) at 36.5 ± 0.5 C under the 10^{-3} mmHg pressure in benzene. Tocopheroxy radicals and residual tocopherols in these oxidized samples were determined by ESR and UV, respectively. Autoxidized methyl linoleate (56.7 mg of weight increase), 2.68 x 10^{-2} g in 1 ml of benzene, was decomposed in the presence of tocopherols (1.56-6.24 x 10^{-5} mole) and nucleic acids (30 mg) at 36.5 ± 0.5 C under the 10^{-3} mmHg pressure. t-Butyl hydroperoxide, 7.61 x 10^{-4} mole in 1 ml of benzene, was also decomposed in the presence of tocopherols (1.20-4.83 x 10^{-4} mole). The ability of tocopherols to form hydrogen bonds with methyl linoleate hydroperoxides or t-butyl hydroperoxide was determined under the IR spectrophotomeric condition of: tocopherols – 2.03 x 10^{-3} mole/1, autoxidized methyl linoleate (56.7 mg of weight gain) – 4.30 g/1, and t-butyl hydroperoxide – 4.12 x 10^{-3} mole/1

in CCl_4 with 9.97 mm NaCl cell. Under this condition, the association between tocopherols or hydroperoxides was not observed.

RESULTS AND DISCUSSION

Synergistic Antioxidant Activity

Weight increases during autoxidation of methyl linoleate containing tocopherols and nucleic acids are shown in Figure 1. The times required to gain 16 mg or attain a peroxide value of 0.70×10^3 meq/kg are compared in Table I. The relative effectiveness of tocopherol-nucleic acid combinations as antioxidants was calculated from these induction periods. DNA and RNA are insoluble in methyl linoleate. They showed slight antioxidant activity in the heterogeneous reaction system. RNA was slightly superior to DNA. The nucleic acids showed marked synergist activity with tocopherols. As a synergist, DNA was superior to RNA. The magnitude of the synergistic effect varied with the kind of tocopherol, decreasing in α->γ->δ-tocopherol.

The maximum value in the weight increase shown in Figure 1 and the slope of the weight increase from the end of each induction period to the maximum were almost the same in samples with an without nucleic acids and tocopherols. The similarity of the maximum value in the weight increase shows that the amount of oxygen absorbed in the autoxidation of methyl linoleate was almost the same regardless of the sorts of antioxidants and synergists. Curves similar to Figure 1 were obtained regardless of the method used to monitor the oxidation, and the same kind of synergistic action between tocopherols and nucleic acids were revealed. If the measures of oxidation such as peroxide value, decrease in α-methylene groups, conjugation, formation of peroxy radicals, formation of isolated trans double bonds, formation of unsaturated carbonyls, molecular weight increase, or refractive index increase were plotted versus the corresponding weight increase instead of time, the curves for all the antioxidant treatments approached that of the control. This indicates that DNA and RNA or tocopherols do not alter the autoxidation mechanism of methyl linoleate.

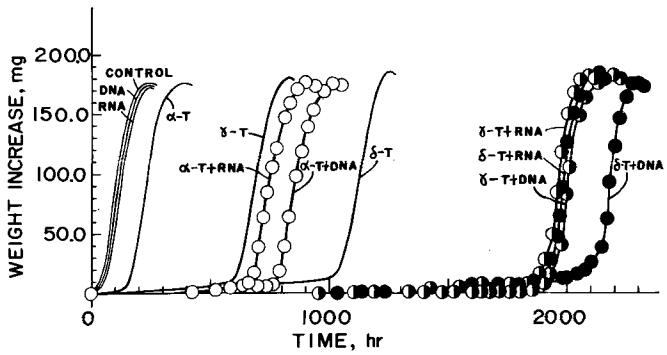

FIG. 1. Weight increase with time in oxidizing methyl linoleate containing nucleic acids and tocopherols. Conditions: 36.5 ±0.5 C, oil thickness – 1.03 mm, nucleic acids – 2.00% by weight, tocopherols – 3.76×10^{-6} mole/1.5 g-methyl linoleate.

Tocopherol Consumption

The change in tocopherol concentration with time during the autoxidation of methyl linoleate plus nucleic acids is shown in Figure 2. Figure 3 shows the tocopherol consumption plotted versus weight gain. Figures 2 and 3 show that the tocopherols were slowly consumed and could not be detected at the end of the induction period. Figure 2 also shows that RNA and DNA delayed the consumption of the tocopherols during the autoxidation of methyl linoleate. Figure 3 shows that at a given degree of oxidation (weight gain) RNA and DNA reduced the percentage of tocopherol consumed during the oxidation of methyl linoleate. Consumption rates of tocopherols decreased in the order α->γ->δ-T with the addition of DNA or RNA, and DNA was more effective than the latter.

The autoxidation of methyl linoleate samples containing tocopherols and nucleic acids induced tocopheroxy and nucleic acid radicals. The ESR signals and g-values of these

TABLE I

Approximate Induction Times of Methyl Linoleate Containing Combinations of Tocopherols, DNA, and RNA

	Time to gain 16 mg[a]			Time to attain a iodometric peroxide value 700[a]		
		Time sample	Time sample		Time sample	Time sample
	Hours	Time control	Time tocopherol	Hours	Time control	Time tocopherol
Control	55	1.0	---	51	1.0	---
DNA	58	1.1	---	60	1.2	---
RNA	64	1.2	---	64	1.3	---
α-Tocopherol	170	3.1	1.0	162	3.2	1.0
Deut. α-T	55	---	---	---	---	---
α-T+DNA	789	14.3	4.6	787	15.4	4.9
Deut. α-T+DNA	162	---	---	---	---	---
α-T+RNA	688	12.5	4.0	687	13.5	4.2
Deut. α-T+RNA	231	---	---	---	---	---
γ-Tocopherol	620	11.3	1.0	610	12.0	1.0
Deut. γ-T[b]	116	---	---	---	---	---
γ-T+DNA	1940	35.3	3.1	1938	38.0	3.2
Deut. γ-T+DNA	287	---	---	---	---	---
γ-T+RNA	1882	34.2	3.0	1881	36.9	3.1
Deut. γ-T+RNA	710	---	---	---	---	---
δ-Tocopherol	1050	19.1	1.0	1030	20.2	1.0
Deut. δ-T	55	---	---	---	---	---
δ-T+DNA	2040	37.1	1.9	2033	39.9	2.0
Deut. δ-T+DNA	240	---	---	---	---	---
δ-T+RNA	1908	34.7	1.8	1904	37.3	1.8
Deut. δ-T+RNA	512	---	---	---	---	---

[a]This is approximately the induction period.

[b]γ-Tocopherol was 91.6% deuterized, the others 100%.

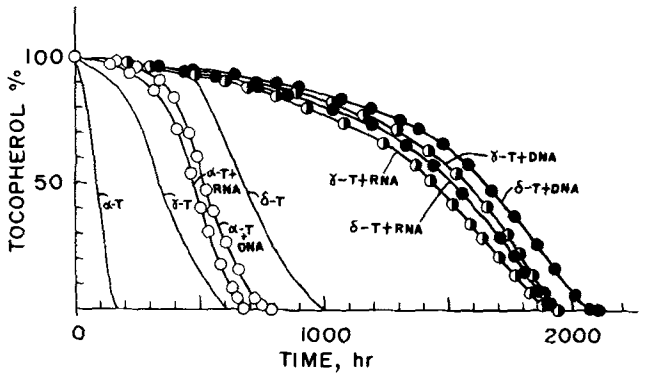

FIG. 2. Change in tocopherol concentration with time in oxidizing methyl linoleate. Conditions the same as in Figure 1.

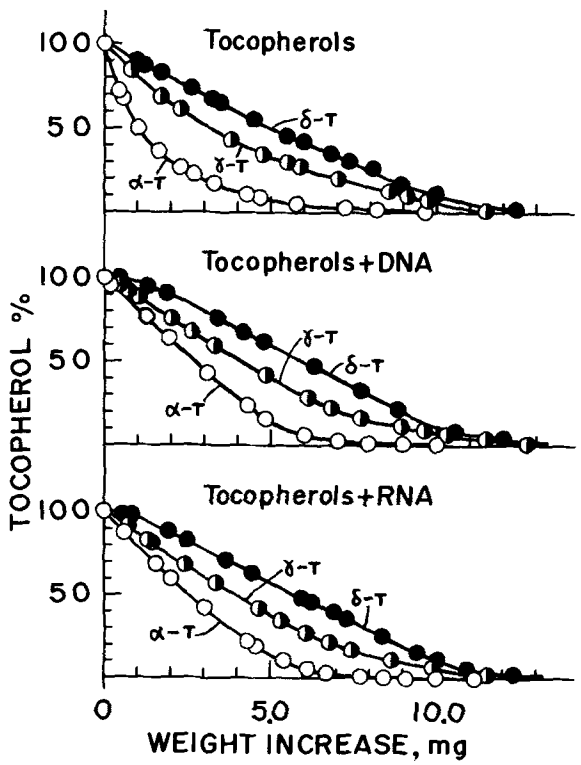

FIG. 3. Tocopherol consumption versus weight increase during the autoxidation of methyl linoleate samples with added nucleic acids and tocopherols.

radicals in methyl linoleate containing both tocopherols and nucleic acids were similar to those observed in samples containing tocopherols or nucleic acids alone. Further information on the ESR signals of tocopheroxy radicals will be reported separately. Changes in the amounts of tocopheroxy radicals with time and weight increase during the autoxidation of methyl linoleate samples are shown in Figures 4 and 5, respectively. The tocopheroxy radicals rise to a maximum and then disappear during the induction period. This would probably be caused by the reactions: ROO· + AH → ROOH + A· and ROO· + A· → ROOA. Figures 4 and 5 show that nucleic acids retarded and decreased the formation of tocopheroxy radical. This effect was in the order α->γ->δ-T and was larger for DNA than RNA. Nucleic acids retarded and decreased the formation of tocopheroxy radicals because they retarded and decreased tocopherol consumptions.

If tocopherols and their radicals are regarded as the active antioxidant components because of their abilities in terminating peroxy radicals, the active components consumed in the autoxidation can be calculated from the difference between the amount of tocopherols consumed (Fig. 3) and tocopheroxy radicals formed (Fig. 5). This is shown in Figure 6. It includes both the tocopherol consumed by the reaction of tocopheroxy radicals with peroxy radicals and by the side reaction of tocopheroxy radicals with each others as 2A· → A-A. Figure 6 shows that the addition of RNA and DNA did not change the shape of the consumption curves. Therefore, the improved antioxidant activities of tocopherols in the presence of nucleic acids were not caused by the effect of nucleic acids on the side reactions of tocopheroxy radicals.

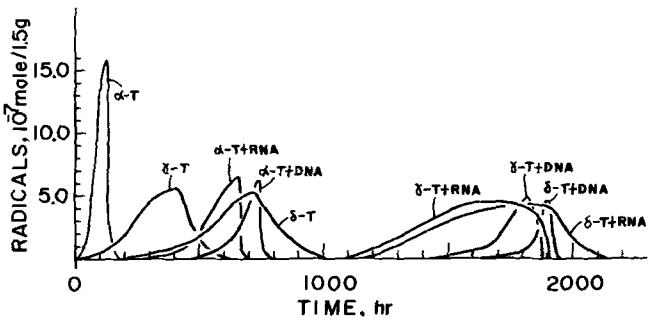

FIG. 4. Change in tocopheroxy radicals (g-values: αT-2.0052, γT-2.0043, and δT-2.0061) with time. Conditions as in Figure 1.

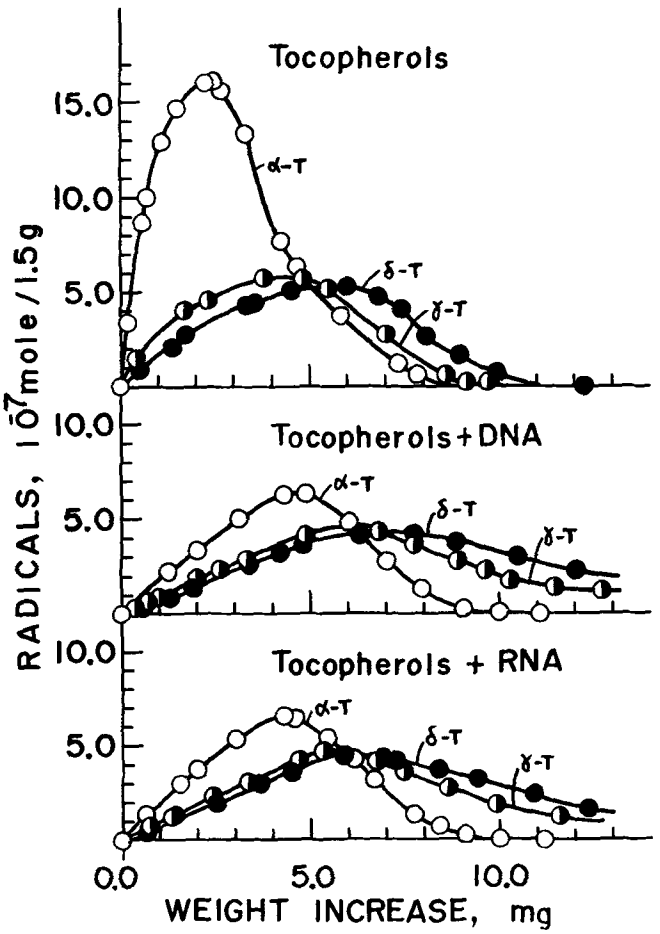

FIG. 5. Change in tocopheroxy radicals with weight increase in oxidizing methyl linoleate.

Change of Nucleic Acids

ESR spectra of DNA and RNA radicals produced in the autoxidation of methyl linoleate are shown in Figure 7. The change in nucleic acid radicals with time and with weight increase during the autoxidation of methyl linoleate are

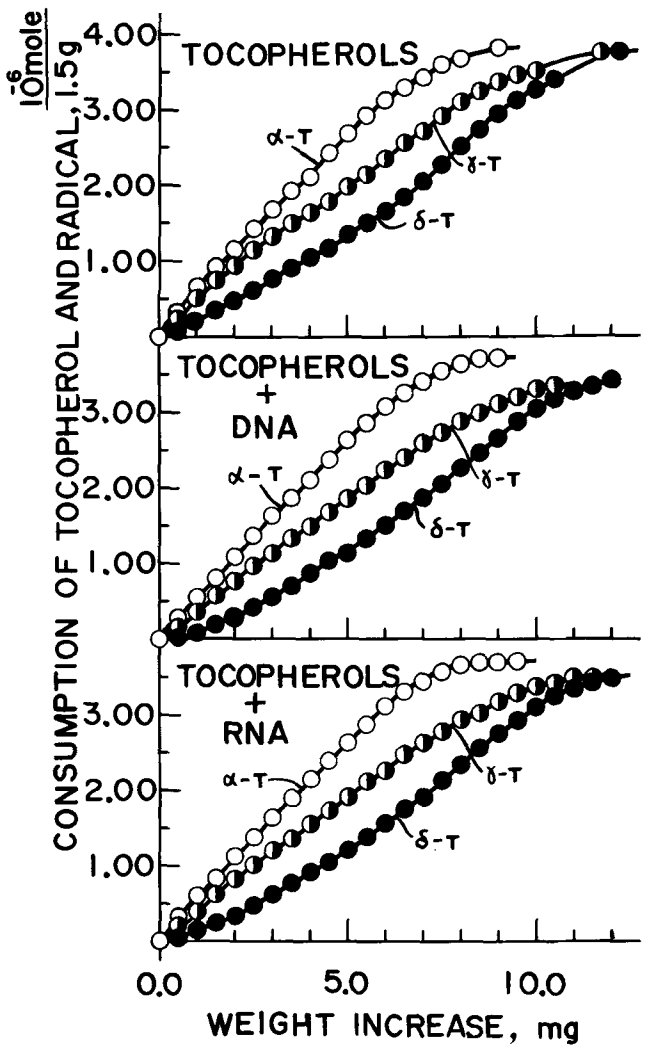

FIG. 6. Consumption of tocopherol and tocopheroxy radical with weight increase.

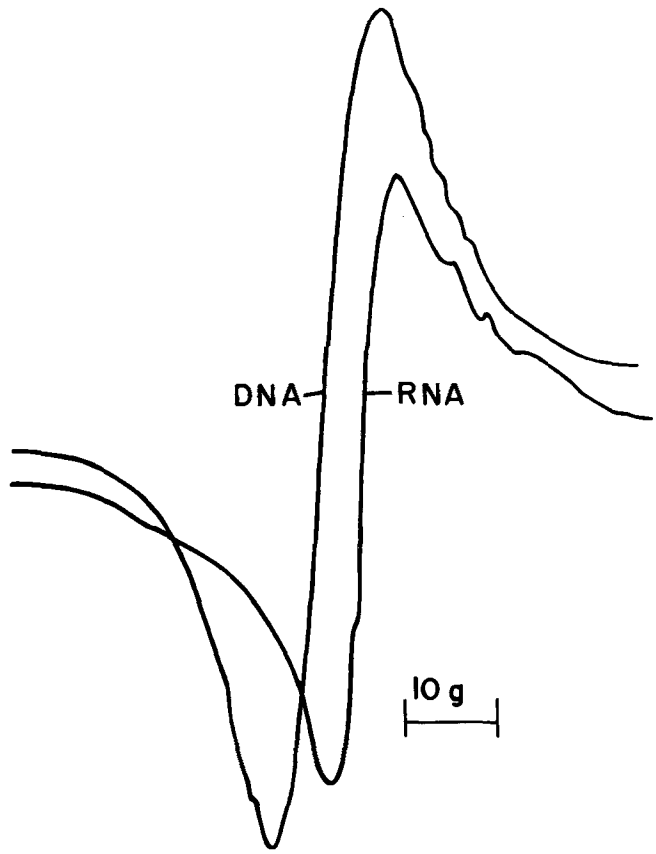

FIG. 7. Electron spin resonance (ESR) spectra of DNA radicals produced during autoxidation of methyl linoleate at weight increase 25.7 mg; g-value: 2.0035 and RNA radicals at weight increase 15.5 mg; g-value: 2.0049.

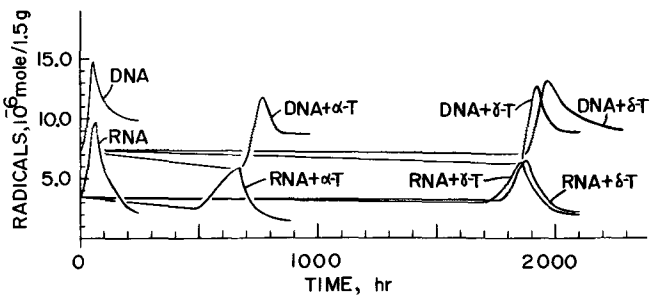

FIG. 8. Nucleic acid radical concentration versus time. Conditions as in Figure 1.

shown in Figures 8 and 9, respectively. The DNA and RNA contained some free radicals before autoxidation began. DNA and RNA radicals increased during the induction period, reached a maximum near the end of the induction periods, and subsequently gradually decreased. The timing of the appearance and disappearance of these nucleic acid radicals suggest hydrogen transfer from DNA and RNA to peroxy radicals formed in the autoxidation of methyl linoleate. But Figure 8 shows that tocopherols retarded and decreased nucleic acid radical formation. Radical formation was lowered in the order α->γ->δ-T, and the order of the maximum amount of radicals, shown in Figure 4, was the same. But, the disappearance of DNA and RNA radicals in the presence of tocopherols was almost identical after the induction period. The same order of effectiveness of the tocopherols, α->γ->δ-T is shown for the termination of DNA and RNA radicals versus time under vacuum in Figure 10. RNA radicals were terminated faster by tocopherols than DNA radicals. Figure 10 shows that DNA and RNA radicals in the absence of tocopherols were very stable under the condition used. The stability of DNA and RNA radicals is also illustrated by their slow decline after the induction period in Figures 8 and 9. Seemingly, DNA and RNA radicals formed in the autoxidation of methyl linoleate are stable and cannot effectively terminate peroxy radicals and lengthen the induction period. Probably the stability of DNA and RNA radicals is caused by the tightly coiled configuration of these large polymers. The radical species of DNA and RNA probably stem from the basic groups of the nucleic acids. We have observed subsequently a synergistic activity of purine and pyrimidines with tocopherol during the autoxidation of methyl linoleate. Comparison of Figure 8 with Figures 2 and 4 shows that nucleic acid radicals were formed after most of the tocopherols and tocopheroxy radicals were consumed, so nucleic acids in the synergist system with tocopherols seem to be protected from direct oxidation by linoleate peroxy radicals.

Synergist Mechanism

One might suggest that tocopherols and nucleic acids act as antioxidants according to the following equations (21-27).

$$ROO^{\bullet} + AH \rightarrow [ROO\text{----}H\text{---}A] \rightarrow ROOH + A^{\bullet} \quad (I)$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \quad (II)$$

$$ROO^{\bullet} + \text{nucleic acids} \rightarrow ROOH + \text{nucleic acid radicals} \quad (III)$$

$$ROO^{\bullet} + \text{nucleic acid radicals} \rightarrow \text{coupling products} \quad (IV)$$

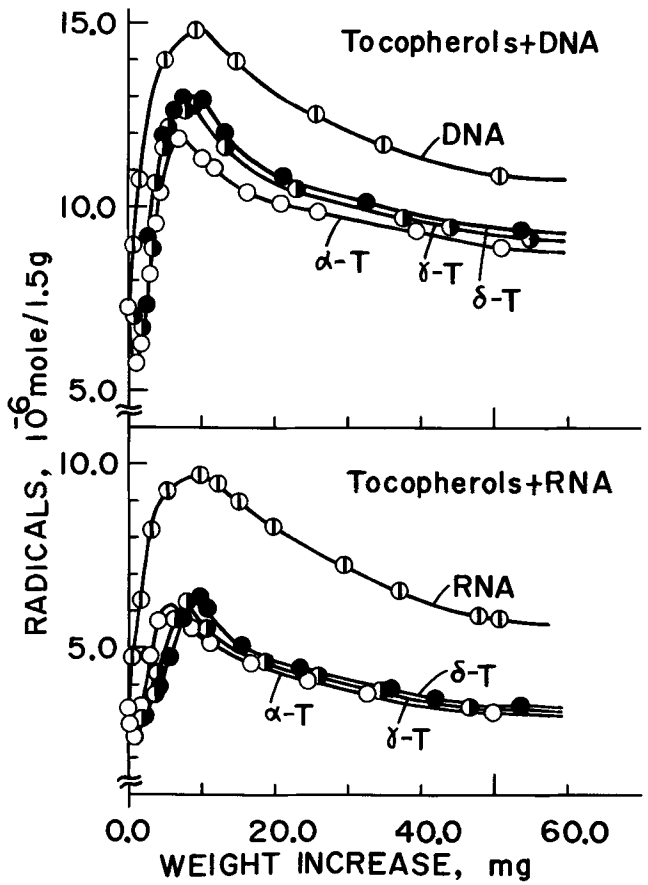

FIG. 9. Change in nucleic acid radicals with weight increase during autoxidation of methyl linoleate.

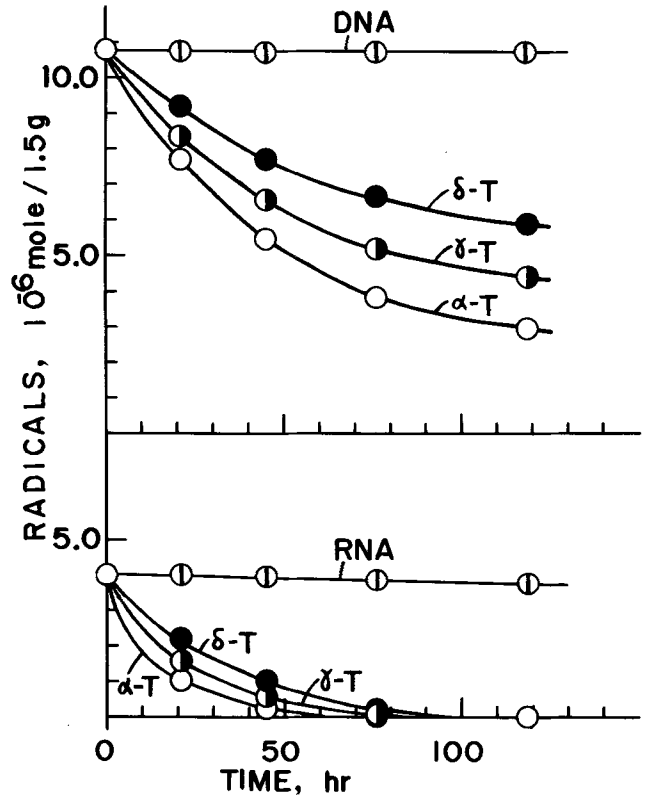

FIG. 10. Termination by tocopherols of DNA and RNA radicals formed in the autoxidation of methyl linoleate. Tocopherols – 5.00 x 10^{-6} mole/1.5 g-linoleate, starting DNA radical concentration – 10.75 x 10^{-6} mole/1.5 g-linoleate; starting RNA radical concentration – 4.08 x 10^{-6} mole/1.5 g-linoleate, reaction temperature – 36.5 ± 0.5 C, pressure – 10^{-3} mm Hg.

But this would give an antioxidant effect equal only to the sum of the activity of the tocopherol and nucleic acids and would not account for synergism.

Nucleic acids might act by chelating metals, but this is unlikely because very little metal is present in the system.

Tocopherols and nucleic acids might eliminate hydroperoxides in the autoxidized methyl linoleate in some way (28). This was investigated, shown in Table II, but no effect was observed. No effect was also observed for the decomposition of t-butyl hydroperoxide. Still, the ability of tocopherols to form hydrogen bonds with methyl linoleate hydroperoxides or t-butyl hydroperoxide was in the order γ->α->δ-T. This observation was supported also by peroxide values which showed that the oxygen absorbed during autoxidation (weight increase), regardless of the addition and sort of tocopherols and nucleic acids, paralleled hydroperoxide formations up to weight increases of 85 mg or PV of 3.2 x 10^3 meq/kg regardless of the antioxidants added.

The synergistic effect of nucleic acids with tocopherols might result from the regeneration of nucleic acids or tocopherols as antioxidants through hydrogen transfer reactions. The data in Figure 10 suggest that hydrogen transfer from tocopherols to nucleic acid radicals occurs and the reverse transfer may be possible. If the synergistic activity envolved this mechanism, consumption of tocopherols or the formation of tocopherol or nucleic acid radicals would be greater when both tocopherol and nucleic acids were present than when only one was present, but this was not observed.

The nucleic acids may protect tocopherols during the autoxidation of methyl linoleate. Tocopherols themselves were autoxidized easily to tocopheroxy radicals, in benzene, cyclohexane, liquid paraffin, and methyl laurate, at 36.5 ± 0.5 C in the order α->γ->δ-T. The reaction is:

$$AH + O_2 \rightarrow [A\cdots H\cdots O_2] \rightarrow A^{\bullet} + {}^{\bullet}OOH \qquad (V)$$

It is quite possible that this occurs during the autoxidation of methyl linoleate. If loss of tocopherols by air oxidation is important, this would explain why the antioxidant activity (α-<γ-<δ-T) of tocopherols observed in this study is in reverse order to that expected from their substituents and from their abilities in terminating nucleic acid radicals (see Fig. 10). As shown in the transition state of Equation I, substituent inducing a high electron density in the hydroxyl group of tocopherols would give the high antioxidant activity. Air oxidation of tocopherols would also explain why the autoxidation of linoleate with only tocopherol entailed greater consumption of tocopherol and more extensive formation of tocopheroxy radicals while inhibiting oxidation less than samples also containing nucleic acids. Possibly antioxidants superior as hydrogen donors are not always superior as antioxidant, because the substituent effects that enhance their antioxidant properties also enhance their air oxidations. The effect of exchanging the hydrogen in the hydroxy group of tocopherols with deuterium is shown in Table I. Obviously, the antioxidant activities of tocopherol-nucleic acid systems, as well as those of tocopherols alone, depend primarily upon the hydrogen transfer as shown in Equation I and deuterated tocopherols are much less effective. The deuterium effect in the synergist system with nucleic acids was sometimes not as significant as with tocopherol alone. This may result from deuterium exchange between deuterized tocopherols and nucleic acids. Moreover, all the analytical data in this study show that the improved antioxidant activities of tocopherols plus nucleic acids resulted from a decreased rate of tocopherol consumption. The low consumption rate of tocopherols and the low tocopheroxy radical formations in the tocopherol-nucleic acid systems

TABLE II

Effects of Tocopherols on the Decomposition of Methyl Linoleate Hydroperoxides in Benzene

	Peroxide value, x 10^3 meq/kg			
	After 0 hr	After 20 hr	After 45 hr	After 70 hr
Control	2.18	1.96	1.68	1.29
DNA	2.18	---	1.70	---
RNA	2.18	---	1.70	---
α-Tocopherol 1.56 x 10^{-5} mole/ml	2.18	---	1.72	---
3.12	2.18	---	1.71	---
6.24	2.18	1.98	1.72	1.35
α-T 6.24 x 10^{-5} mole/ml + DNA	2.18	---	1.71	---
α-T 6.24 + RNA	2.18	---	1.69	---
γ-Tocopherol 1.56 x 10^{-5} mole/ml	2.18	---	1.69	---
3.12	2.18	---	1.69	---
6.24	2.18	1.99	1.75	1.40
γ-T 6.24 x 10^{-5} mole/ml + DNA	2.18	---	1.72	---
γ-T 6.24 + RNA	2.18	---	1.71	---
δ-Tocopherol 1.56 x 10^{-5} mole/ml	2.18	---	1.66	---
3.12	2.18	---	1.74	---
6.24	2.18	1.97	1.70	1.31
δ-T 6.24 x 10^{-5} mole/ml + DNA	2.18	---	1.71	---
δ-T 6.24 + RNA	2.18	---	1.70	---

are accounted for if nucleic acids protect tocopherols from the air oxidation. This protective effect of nucleic acids would result from hydrogen bonding between tocopherols and nucleic acids which inhibits the reaction shown in Equation V. Naturally, for the same reason, the hydrogen transfer reactions from tocopherols to peroxy radicals (Equ. I) would be inhibited also, but this may not matter because the reactivities of peroxy radicals and oxygen are significantly different. Also the strengths of hydrogen bonds of tocopherol with nucleic acid and oxygen may also differ.

The antioxidant activities of tocopherols were improved by additions of nucleic acids in the order α->γ->δ-T. It is likely that this results in the different electron densities on the hydroxy groups of the tocopherols which affects their ability to form hydrogen bonds with nucleic acids.

The conclusion that the synergistic activity of nucleic acids is due to protection of tocopherols from the air oxidation also was supported by the PbO_2 oxidation of tocopherols. These data are shown in Figures 11 and 12. Addition of nucleic acids decreased the rate of oxidation of tocopherols by PbO_2.

This data suggest that antioxidants may be important in suppressing alteration of nucleic acids which could result in mutation and carcinogenesis.

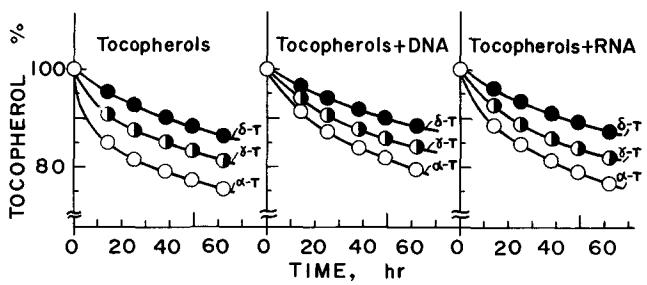

FIG. 11. Oxidation of tocopherols by PbO_2. Residual tocopherol versus time. Tocopherol concentrations – 4.00 x 10^{-5} mole/ml-benzene, nucleic acid concentrations – 0.030 g, PbO_2–4.00 x 10^{-5} mole, temperature – 36.5 ± 0.5 C, pressure – 10^{-3} mmHg.

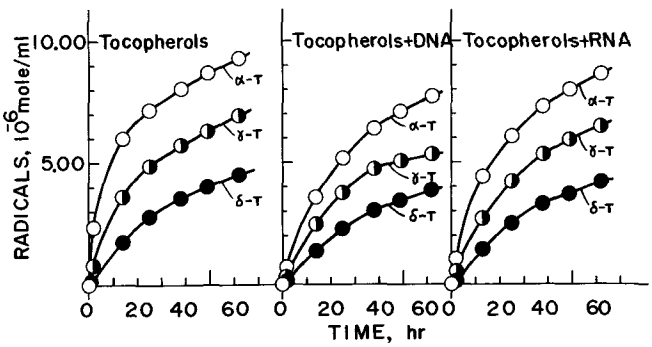

FIG. 12. Oxidation of tocopherols by PbO_2. Tocopheroxy radicals versus time. Conditions the same as Figure 11.

ACKNOWLEDGMENTS

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