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Studies on the Antioxidants. XIII.[1] Hydrogen Donating Capability of Antioxidants to 2,2-Diphenyl-1-picrylhydrazyl

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The hydrogen donating capabilities of antioxidants, such as butylated hydroryanisole (BHA), butylated hydroxytoluene (BHT), *dl*-α-tocopherol (Toc), sesamol and ethyl protocatechuic acid (EP), to a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), were investigated. Toc, sesamol and EP donated two hydrogen atoms, BHA donated 0.75—1.2 hydrogen atoms, and BHT donated much less hydrogen. A synergistic effect on hydrogen donation to DPPH was observed with the combinations of Toc+BHT and BHA+BHT. The synergistic effect observed in the reaction between BHA+BHT and DPPH might arise from the regeneration of BHA from its oxidized intermediate, with the loss of BHT.

Keywords——antioxidants; butylated hydroxyanisole; butylated hydroxytoluene; 2,2-diphenyl-1-picrylhydrazyl; synergistic effect

It is known that oils and fats are autoxidized *via* free radicals and that phenolic antioxidants act as chainblockers by donating hydrogen atoms to the intermediate peroxy free radicals.[3,4] Our previous paper[5] demonstrated that photoxidation of butylated hydroxyanisole (BHA) produced dimers *via* the free radicals.

A stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), has been used as a hydrogen acceptor, and its ability to accept hydrogen from phenols has been demonstrated.[6–14] DPPH was therefore expected to act as a hydrogen acceptor for antioxidants by analogy with the reactions between peroxy free radicals and antioxidants. This paper deals with the hydrogen donating capability of antioxidants towards DPPH. The hydrogen donating capabilities of BHA, butylated hydroxytoluene (BHT), *dl*-α-tocopherol (Toc), sesamol and ethyl protocatechuic acid (EP) were compared, and the effects of combinations of these hydrogen donors were investigated.

Experimental

Materials——DPPH was used after repeated recrystallization of the commercial product (Tokyo Kasei Kogyo Company, Ltd.) from benzene–ether mixture.[10] BHA and BHT were supplied by Nikki-Universal Company, Ltd. BHA was recrystallized from petroleum ether to remove the 3-isomer[5] and

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BHT was recrystallized from ethanol. Sesamol and Toc were obtained from Aldrich Chemical Company, Inc. and Tokyo Kasei Kogyo Company, Ltd., respectively. EP was synthesized from protocatechuic acid according to the method of Hesse.[15]

Estimation of Decrease in DPPH Concentration——A solution of 0.87—0.92 mM DPPH and solutions of 0.1 and 1.0 mM antioxidant (and solutions of 50 mM BHA and BHT) in benzene were prepared separately. Solutions of DPPH and antioxidant were mixed and the mixtures were permitted to stand at 20° in a thermostat. The concentration of DPPH in the reaction mixture was determined at selected times. The concentration of DPPH was calculated from the absorbance at 520 nm according to the equation reported by Boguth and Repges.[16] The absorbance was measured with a Hitachi 101 spectrophotometer, or a Shimadzu UV-200S double-beam spectrophotometer.

Time Course of the Changes of BHA and BHT Concentration——Solutions of DPPH, BHA and BHT were mixed and the mixtures were treated at 20°. The concentrations of BHA and BHT were determined by high performance liquid chromatography according to the method described previously,[1] and by gas chromatography using a Yanaco G 80 gas chromatograph equipped with a hydrogen flame ionization detector and a glass column (3 mm I.D. × 2 m) of Silicone OV-17 on Chromosorb W AW. The chromatograph was operated isothermally at 140° (column temperature) and 170° (injection temperature) with a nitrogen gas flow of 20 ml/min. BHA and BHT were determined by comparing the peak areas of samples with those of authentic standard solutions (0.1 mM).

Confirmation of BHA by Combined Gas Chromatography and Mass Spectrometry——The identity of BHA in the reaction mixture was confirmed by mass spectrometry using a Hitachi RMU-7L double focusing mass spectrometer after chromatographic separation using a Hitachi K-53 gas chromatograph with a Silicone OV-17 column.

Results and Discussion

Phenolic antioxidants in common use such as BHA, BHT, Toc and EP, and a naturally occurring monophenolic antioxidant, sesamol, were reacted with a 3.7-fold molar excess of DPPH at 20° in benzene. The time course of the decrease in DPPH concentration is illustrated in Fig. 1. The DPPH concentration was decreased most rapidly by Toc, reaching a minimum at 5 min after Toc addition, when 2 mol of DPPH were lost. BHA rapidly consumed about one mol of DPPH during 10 min, and further reagent was gradually lost thereafter. Sesamol and EP gradually consumed DPPH during 90 min, reaching a

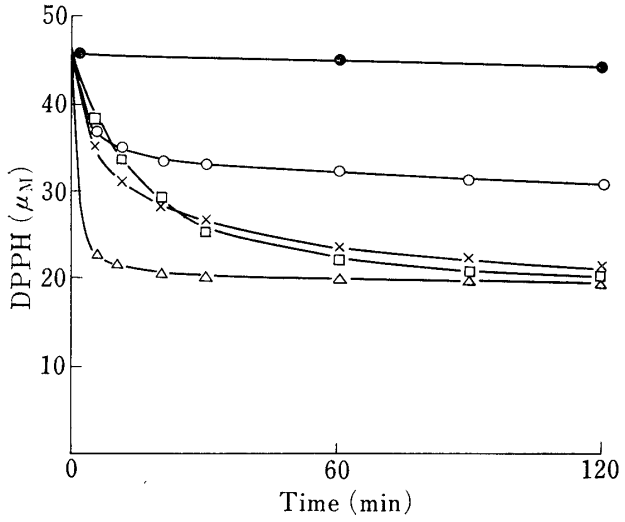

Fig. 1. Time Course of the Decrease in DPPH Concentration caused by the Antioxidants

Mixtures of DPPH (46 μM) and each of the antioxidants (12.5 μM) were treated at 20°.
○, BHA; ●, BHT; △, Toc; ×, sesamol and □, EP.

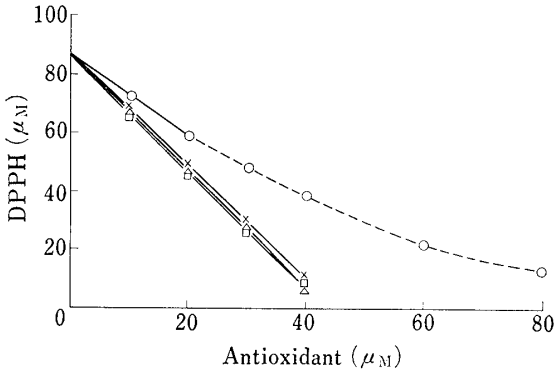

Fig. 2. Effect of Antioxidant Concentration on the Decrease in DPPH Concentration

Mixtures of DPPH (87 μM) and each antioxidant were treated at 20° for 60 min (BHA and Toc) or 120 min (sesamol and EP).
○, BHA; △, Toc; ×, sesamol and □, EP.

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maximal loss of 2 mol of the reagent. The loss of DPPH with BHT was the slowest; less than 0.2 mol of the reagent was lost after 120 min.

DPPH was treated with each antioxidant for 60 min (BHA and Toc) or 120 min (EP and sesamol) and the antioxidant concentration dependencies of the decrease of DPPH were investigated (Fig. 2). When DPPH was treated with Toc, sesamol and EP, the decrease in DPPH concentration was proportional to the concentration of each antioxidant, and 1 mol of each antioxidant consumed 2 mol of DPPH. In the reaction between DPPH and BHA, the DPPH concentration decreased in proportion to the concentration of BHA, and 1 mol of BHA consumed 1.2 mol of the reagent when the ratio of the concentration of BHA to that of DPPH was less than one-third. However, the DPPH concentration decrease was no longer proportional when the ratio was higher than one-third. The hydrogen donating reaction of BHA might thus differ from those of Toc, sesamol and EP.

Table I. Effects of Antioxidant Combinations on DPPH Loss

	BHT	Toc	Sesamol	EP
BHA	2.78	0.98	1.05	1.16
BHT		1.97	1.15	1.03
Toc			0.99	1.07
Sesamol				1.05

Two antioxidants, separately or combined in an equivalent ratio (total concentration: 12.5 μM), were treated with 44—46 μM DPPH for 60 min at 20°. The ratios of the loss of DPPH caused by the combined antioxidants to the mean value of the loss obtained with each antioxidant alone are tabulated.

A solution of each antioxidant or a solution of two antioxidants combined in an equivalent ratio was treated with a 3.5-fold molar excess of DPPH, and the decrease in DPPH concentration caused by two antioxidants in combination was compared with that caused by each of the two antioxidants alone. The ratio of the decrease in DPPH concentration caused by the combined antioxidants to the mean value of the decreases caused by each of the two antioxidants separately was calculated and the results are listed in Table I. Among 10 combinations, BHA+BHT and Toc+BHT exhibited ratios much higher than 1. These two combinations were tested in varying ratios, and the results are shown in Fig. 3. The synergistic effect of Toc+BHT was marked in the range of (Toc/Toc+BHT) ratio from 0.2 to 1.0, and in this range 2 mol of DPPH was removed by 1 mol of the combined antioxidants. The results coincide with the observations of Boguth *et al.*[17] who demonstrated that the synergistic effect of Toc and BHT arose as a result of catalytic activation of the hydrogen donating capability of inactive BHT by Toc. The synergistic effect of BHA+BHT was most marked at a (BHA/BHA+BHT) ratio of 0.2. At this ratio, 1.8 mol of DPPH was removed by 1 mol of the combined antioxidants, whereas BHA and BHT consumed only 1.2 and 0.1 mol of DPPH, respectively, when used separately.

The time courses of the decreases in DPPH concentration by combined antioxidants, Toc+BHT and BHA+BHT, were followed (Fig. 4). The addition of BHT to the reaction mixture of Toc and DPPH at the start of the reaction decreased the DPPH concentration rapidly, whereas the addition of BHT 30 min after the reaction decreased the DPPH concentration very slowly (Fig. 4A). The addition of BHT to the reaction mixture of BHA and DPPH at the start of the reaction decreased the DPPH concentration rapidly, and the addition of BHT to the reaction mixture after 30 min led to loss of the reagent as rapidly as BHT addition at the start of the reaction (Fig. 4B).

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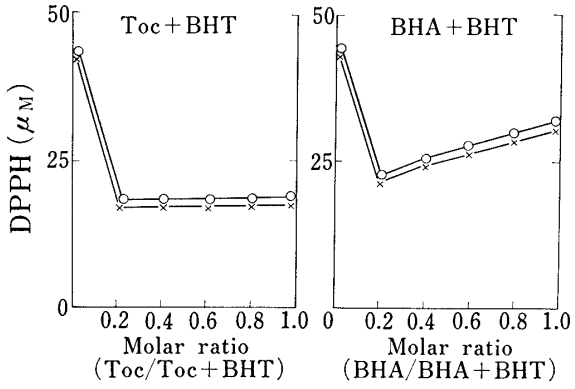

Fig. 3. Effect of the Combination Ratio of Antioxidants on the Decrease in DPPH Concentration

Mixtures of DPPH (44 μM) and combined antioxidants (total concentration: 12.5 μM) were treated for 60 min (○) and 120 min (×) at 20°.

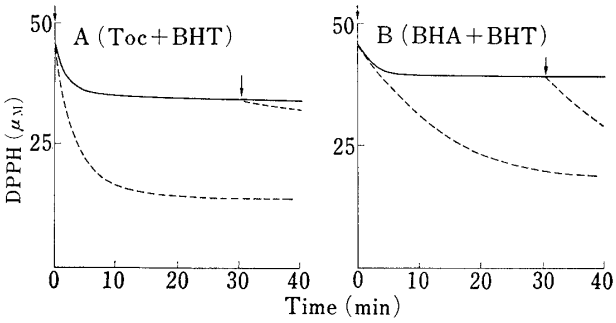

Fig. 4. Time Course of the Decrease in DPPH Concentration Caused by Combined Antioxidants

A 20 ml solution of Toc (or BHA) and DPPH was treated at 20° (——). A 20 ml solution containing Toc (or BHA), BHT and DPPH was treated at 20° (----); a 1 ml solution of BHT was added to a 19 ml solution of Toc (or BHA) and DPPH at the times indicated by arrows. Toc, 5 μM; BHA, 5 μM; BHT, 10 μM and DPPH, 45 μM.

The time course of the loss of antioxidants in the reaction between BHA+BHT and DPPH was followed by both high performance liquid chromatography and gas chromatography (Fig. 5, 6). When BHA (1 mol) was treated with DPPH (2 mol), BHA was rapidly lost and the amount of BHA remaining was 0.48 mol at 10 min and 0.38 mol at 40 min. When BHT (5 mol) was added 10 min after this reaction, 0.90 mol of BHA was recovered. High recovery of BHA was also obtained when BHT was added 30 min after the reaction. These results suggest that BHT might have reacted with a possible intermediate between DPPH and BHA to regenerate BHA. When BHT was added at the start of the reaction, 0.96 mol of BHA was recovered at 7 min, although only 0.75 mol was recovered after 3 min. The curve may be explained as a result of the simultaneous progress of loss and regeneration of BHA. The regeneration of BHA was confirmed by combined gas chromatography and mass spectrometry.

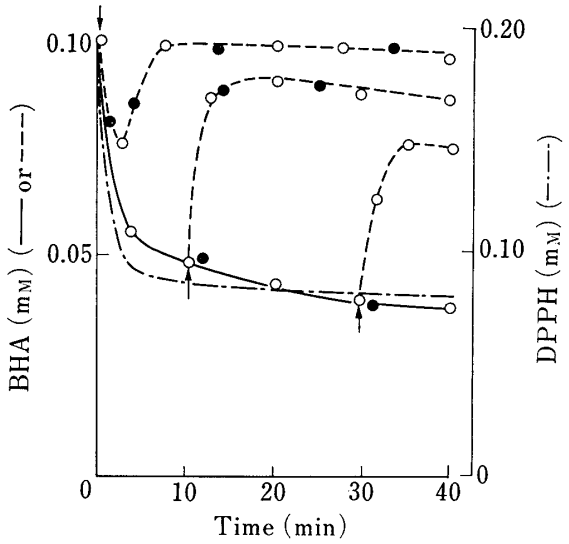

Fig. 5. Effect of BHT on the Recovery of BHA in the Reaction with DPPH

A 3 ml solution of BHA and DPPH (—— and ----) was supplemented with a 0.03 ml solution of BHT (----) at the times indicated by arrows. Final concentrations of BHA, BHT and DPPH were 0.10, 0.50 and 0.19 mM, respectively. The concentration of BHA was determined by high performance liquid chromatography (○) and gas chromatography (●).

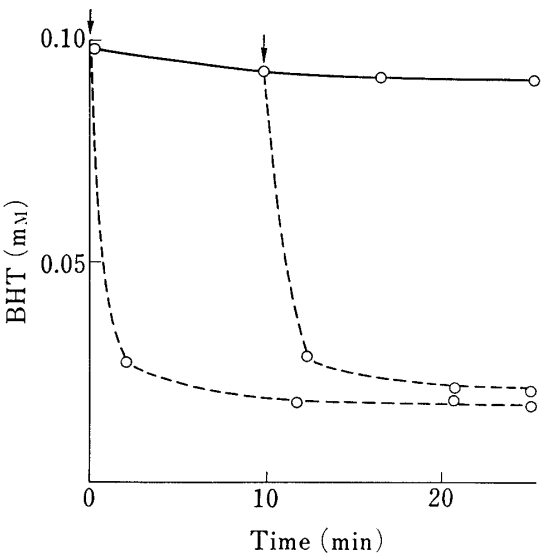

Fig. 6. Effect of BHA on the Decrease in BHT Concentration in the Reaction with DPPH

A 3 ml solution of BHT and DPPH (——) was supplemented with a 0.03 ml solution of BHA (----) at the times indicated by arrows. Final concentrations of BHA, BHT and DPPH were 0.50, 0.10 and 0.19 mM, respectively.

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The molecular ion peak (M^+ 180) and fragment ion peaks of the compound corresponding to BHA in the gas chromatogram of the reaction mixture before and after addition of BHT were compared with those of authentic BHA.

When BHT (1 mol) was treated with DPPH (2 mol) (Fig. 6), the loss of BHT was very small. When BHA (5 mol) was added to the reaction mixture at 0 and 10 min, BHT was rapidly lost.

The hydrogen donating capabilities of DPPH differed among the antioxidants; Toc, sesamol and EP donated 2 hydrogen atoms, BHA donated 0.75—1.2 hydrogen atoms, and BHT donated much less hydrogen. Synergistic effects in the reaction with DPPH were observed in the combinations of Toc+BHT and BHA+BHT. The results obtained in this study indicate that the synergistic effect of BHA+BHT might arise from the regeneration of BHA from a possible intermediate formed from DPPH and BHA, with enhanced loss of BHT. Boguth *et al.*[17] have suggested on the basis of kinetic experiments that the synergistic effect of Toc+BHT arose as a result of the rapid donation of hydrogen atoms from BHT to the tocopheryl radical produced by the interaction between Toc and DPPH. The results described in this paper may help to cast light on the mechanisms of the complex actions of antioxidants on oils and fats.

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