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Subsequent Products After Antioxidant Actions of β-Carotene and α-Tocopherol Have No *Salmonella* Mutagenicity

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β-Carotene and α-tocopherol are important antioxidants biologically, but whether their oxidized products are toxic or not remains to be discovered. Here, we chromatographically separated 5 pure products or isomeric mixtures from reaction mixtures of β-carotene and reactive oxygens, and 17 lipid-radical scavenging products of α-tocopherol. The products were tested for mutagenicity using *Salmonella typhimurium* TA98, TA100, TA102, and TA104, in the presence and absence of S9. None showed mutagenicity against any of the four strains, or cytotoxicity that influenced the survival of the bacteria. Lipid-peroxides have been known to increase the formation of mutagens from dietary procarcinogens such as heterocyclic amines. So, we also measured the activity to increase 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) mutagenicity. The products from β-carotene and α-tocopherol did not increase, but rather several of them suppressed, the mutagenicity of Trp-P-2. Thus, the products of β-carotene and α-tocopherol formed after the antioxidant actions were not genotoxic.

Key words: oxidized β-carotene; oxidized α-tocopherol; mutagenicity; antioxidant; oxidative stress

Carotenoids and tocopherols are among the important biological antioxidants.[1,2] They strongly suppress oxidative stress to quench singlet oxygen or to scavenge radicals, and thereby contribute to suppress the occurrence of several diseases such as protoporphyria,[3] photocarcinogenesis,[4] cardiovascular disease,[5] and atherosclerosis.[6,7] In a human intervention study, however, daily supplementation with 20 mg β-carotene or 50 mg α-tocopherol did not reduce the incidence of lung cancer in smokers, and β-carotene instead increased the cancer incidence and total mortality.[8] Another study has found that women with higher concentrations of α-tocopherol and carotenoids in plasma had higher levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine in lymphocytes, correlating positively and significantly.[9] Under high oxygen tension, β-carotene had been shown to cause concentration-dependent DNA breakdown.[10] The finding may indicate that the subsequent products of carotenoids and α-tocopherol formed after the antioxidant actions have genotoxicity.

Here, we examined various oxidation products of β-carotene and α-tocopherol for mutagenicity using *Salmonella typhimurium* TA98, TA100, TA102, and TA104 strains. β-Carotene has been recognized to play an antioxidative role as a quenching and scavenging reactive oxygen species and α-tocopherol as a trapper for lipid-peroxyl radicals.[1,2] Then, we tested major 5 reaction products of β-carotene with reactive oxygen species[11,12] and 17 products from α-tocopherol after the peroxidized lipids were trapped.[13–18] Since physiological levels have been estimated in healthy human blood to be around 0.12–0.89 nmol/ml for β-carotene[19] and 16–31 nmol/ml for α-tocopherol,[20] the concentration of each peroxidation product is considered to rise to above 10 nmol/ml. We then did experiments with a concentration of 10 nmol/plate of each peroxidation product. On the other hand, it has been also reported that lipid peroxides were cofactors in cytochrome P450 (CYP)-mediated oxidation[21] and facilitated metabolic activation of dietary procarcinogens such as heterocyclic amines to their ultimate carcinogenic form.[22,23] Also, peroxyl radicals generated from lipid-peroxides have been known to epoxidize polycyclic aromatic hydrocarbons and to produce ultimate

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Abbreviations: AAPH, 2,2′-azo*bis*(2-amidinopropane)dihydrochloride; AMVN, 2,2′-azo*bis*(2,4-dimethylvaleronitrile); CYP, cytochrome P450 monooxygenases; DMSO, dimethylsulfoxide; HPLC, high-pressure liquid chromatography; ML, methyl linoleate; 13-MLOOH, methyl (9*Z*, 11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate; PLPC, 1-palmitoyl-2-linoleoyl-3-*sn*-phosphatidylcholine; 13-PLPCOOH, 1-palmitoyl-2-[(9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoyl]-3-*sn*-phosphatidylcholine; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole

carcinogenic forms.[24] We examined whether the products of β-carotene and α-tocopherol increased the mutagenicity of 3-amino-1-methyl-5*H*-pyrido [4,3-*b*]indole (Trp-P-2), a typical dietary procarcinogen.[25] None of the oxidation products showed mutagenicity, and interestingly, several of them inhibited the mutagenicity of Trp-P-2.

Materials and Methods

Chemicals. All-*trans*-β-carotene was obtained from Wako Pure Chemical Industries (Osaka, Japan) and recrystallized from benzene. 2*R*,4′*R*,8′*R*-α-Tocopherol was from Sigma Chemical Co. (St. Louis, MO) and was purified by reversed-phase HPLC with methanol as the solvent. Methyl linoleate (ML) was obtained from Tokyo Kasei Co., Tokyo, Japan, and was purified to be peroxide-free by silica gel column chromatography.[15] Linoleic acid (Wako Pure Chemical) was oxidized with soybean lipoxygenase-1 (Sigma) and the resulting hydroperoxide was converted to its methyl ester with diazomethane.[16] The purity of methyl (9*Z*, 11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoate (13-MLOOH) was about 90%, the remainder being the 9-isomer. 1-Palmitoyl-2-linoleoyl-3-*sn*-phosphatidylcholine (PLPC) was synthesized by 2-acylation of 1-palmitoyl-3-*sn*-glycerophosphocholine with linoleic acid anhydride according to the method of Gupta *et al.*[26] 1-Palmitoyl-2-[(9*Z*,11*E*)-(*S*)-13-hydroperoxy-9,11-octadecadienoyl]-3-*sn*-phosphatidylcholine (13-PLPCOOH) was prepared with soybean lipoxygenase-1 by the method of Brash *et al.*[27] Alkylperoxyl radical generators, a lipid-soluble 2,2′-azo*bis*(2,4-dimethylvaleronitrile)(AMVN) and a water-soluble 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), were obtained from Wako Pure Chemical. For *Salmonella* tests, agar and extracts from beef and yeast were purchased from Difco Laboratory (Detroit, MI). Trp-P-2 was from Wako Pure Chemical. All other chemicals were of the highest grade commercially available.

Oxidation products of β-carotene. β-Carotene was incubated with AMVN in benzene at 37°C for 2 h in the dark under air.[11] The subsequent products were separated and collected through HPLC with an Inertsil Prep ODS column (20×250 mm, GL Sciences, Tokyo, Japan) developed with methanol/ethyl acetate (8:2, v/v) at a flow rate of 15 ml/min. Then they were identified as mentioned previously:[11,12] **C1**, a mixture of 12-formyl-11-nor-β,β-carotene and 15′-formyl-15-nor-β,β-carotene; **C2**, 19-oxomethyl-10-nor-β,β-carotene; **C3**, 5,6-epoxy-5,6-dihydro-β,β-carotene; **C4**, a mixture of 13,15′-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene and 15′,13-epoxyvinyleno-13,15′-dihydro-14,15-dinor-β,β-carotene; and **C5**, 11,15′-dihydrooxepin-β,β-carotene. Each of the products was free of other products on HPLC and did not further decompose in chloroform/ethanol (2:1, v/v) at −20°C before it was used at around day 10.

Oxidation products of α-tocopherol. Seventeen oxidation products of α-tocopherol were prepared in six different systems. First, α-tocopherol was photooxidized in the presence of methylene blue as a photosensitizer and the resulting 8a-hydroperoxy-α-tocopherone (**T1**) was purified by HPLC.[13] Second, α-tocopherol was oxidized in phosphatidylcholine liposomes, and three products, α-tocopherylquinone (**T2**), 2,3-epoxy-α-tocopherylquinone (**T3**), and 5,6-epoxy-α-tocopherylquinone (**T4**), were obtained.[17,28,29] Third, the spirodiene dimer of α-tocopherol (**T5**) was synthesized following the procedure of Nelan and Robeson.[30] Fourth, α-tocopherol was left to stand in an autoxidation system of methyl linoleate under air-insufficient conditions. This yielded two isomeric products of α-tocopherol with methyl linoleate radicals, methyl (10*E*,12*Z*)-9-(α-tocopheroxy)-10,12-octadecadienoate (**T6**) and methyl (9*Z*,11*E*)-13-(α-tocopheroxy)-9,11-octadecadienoate (**T7**).[15,31] Alternatively, under air-sufficient conditions, eight isomeric products of α-tocopherol with ML-peroxyl radicals were obtained:[14,15,31] methyl (9*Z*,11*E*)-(*S*)-13-[(*S*)-8a-dioxy-α-tocopherone]-9,11-octadecadienoate (**T8a**), methyl (9*Z*,11*E*)-(*R*)-13-[(*R*)-8a-dioxy-α-tocopherone]-9,11-octadecadienoate (**T8b**), methyl (9*Z*,11*E*)-(*R*)-13-[(*S*)-8a-dioxy-α-tocopherone]-9,11-octadecadienoate (**T8c**), methyl (9*Z*,11*E*)-(*S*)-13-[(*R*)-8a-dioxy-α-tocopherone]-9,11-octadecadienoate (**T8d**), methyl (10*E*,12*Z*)-(*S*)-9-[(*S*)-8a-dioxy-α-tocopherone]-10,12-octadecadienoate (**T9a**), methyl (10*E*,12*Z*)-(*R*)-9-[(*R*)-8a-dioxy-α-tocopherone]-10,12-octadecadienoate (**T9b**), methyl (10*E*,12*Z*)-(*R*)-9-[(*S*)-8a-dioxy-α-tocopherone]-10,12-octadecadienoate (**T9c**), and methyl (10*E*,12*Z*)-(*S*)-9-[(*R*)-8a-dioxy-α-tocopherone]-10,12-octadecadienoate (**T9d**). Fifth, α-tocopherol was reacted with 13-MLOOH in an iron-catalyzed reaction. This yielded a mixture of methyl (*E*)-(12*S*,13*S*)-9-(8a-dioxy-α-tocopherone)-12,13-epoxy-9-octadecenoate and methyl (*E*)-(12*S*,13*S*)-11-(8a-dioxy-α-tocopherone)-12,13-epoxy-9-octadecenoates (**T10**).[16] Sixth, similar products of α-tocopherol were obtained from the reaction with 13-PLPCOOH in the presence of an iron-chelate, Fe(III)-acetylacetonate, at 37°C in ethanol. The reaction products were separated through HPLC with an Inertsil Prep ODS column (20×250 mm) developed with methanol/ethanol (2:3, v/v) at a flow rate of 15 ml/min. The HPLC gave a single peak, which was characterized chemically as a mixture of 1-palmitoyl-2-[12,13-epoxy-11-(8a-dioxy-α-tocopherone)-9-octadecanoyl]-3-*sn*-phosphatidylcholine and 1-palmitoyl-2-[12,13-epoxy-

9-(8a-dioxy-α-tocopherone)-10-ocatadecenoyl]-3-*sn*-phasphatidylcholine (**T11**): UV (ethanol) λ_{max} (ε) 240 nm (11800); and FABMS (glycerol as the matrix) m/z 1235 (MH^+), 806 ([MH − 429]$^+$), 702 ([790 − choline]$^+$, 430 ([α-tocopherone]$^+$), 224 ([CH = CH-CH_2-O-phosphocholine]$^+$), and 184 ([phosphocholine]$^+$).

The α-tocopherol products thus obtained were free of other products on the respective HPLC and were almost completely stable in ethanol at −20°C until used.

Mutagenicity test. Salmonella strains TA102 and TA104 are superior for detecting mutations caused by active oxygen species such as aldehydes and peroxide,[32,33] as is TA100.[34] We used these three strains as well as strain TA98. Tests were done essentially as described previously[35] except for the mixing of products with bacteria. The S9 mix was prepared from the S9 fraction obtained from the liver of Sprague-Dawley rats given intraperitoneally 30 mg/kg/day phenobarbital for 3 days, accompanied by 80 mg/kg/day methylcholanthrene plus 30 mg/kg/day phenobarbital for another 2 days.[36] Bacterial strains were grown overnight in a liquid broth medium at 37°C. A part of the bacterial suspension (0.1 ml) was mixed with peroxidation products of α-tocopherol or β-carotene in 0.1 ml of dimethylsulfoxide (DMSO), and sonicated for 10 sec in order to enter the cells. Sonication for less than 120 sec did not influence the ratios of cells. For example, the surviving cell numbers ($\times 10^7$) of TA98, TA100, TA102, and TA104 after 10-sec sonication were 303 ± 52, 241 ± 17, 623 ± 11, and 817 ± 12, respectively, compared to 165 ± 5, 202 ± 13, 616 ± 14, and 796 ± 11 for untreated ones. The bacterial suspension was incubated at 37°C for 20 min in the presence or absence of the S9 mix (0.5 ml, containing 0.052 nmol of CYP). The incubation mixture was added to 2 ml of molten top agar, poured onto minimal-glucose agar medium, and cultured at 37°C for 2 days. To detect cytotoxicity of the products, surviving cells were enumerated simultaneously. A part of the mixture was washed with 0.4 ml of 0.1 mM sodium phosphate buffer (pH 7.4) by centrifugation at 3000 rpm for 10 min, twice. The bacteria were resuspended in the buffer and diluted 10^6 or 10^7-fold with saline solution. A 0.1-ml portion was cultured on minimal medium together with top agar containing a 5 mM excess of histidine. After the cultivation, the His^+ colonies and surviving colonies (His^-) were enumerated. The tests were done two times with two plates for each product.

Effects on mutagenicity of Trp-P-2. For the antimutagenicity test, *Salmonella* TA98 was used.[37] The bacteria (0.1 ml) was mixed with the products of β-carotene or α-tocopherol, and then incubated with 0.1 nmol of Trp-P-2 in 0.1 ml of water at 37°C for 20 min in the presence of the S9 mix. After the cultivation, the number of His^+ revertant colonies was counted and compared to that of revertant colonies without products. The tests were done two times with three plates for each product.

Results

Evaluation of the products of β-carotene for mutagenicity

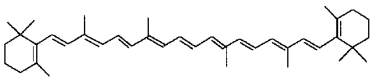
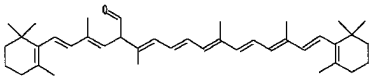
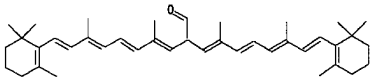
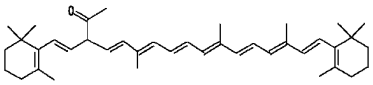
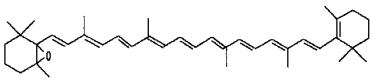
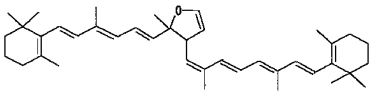
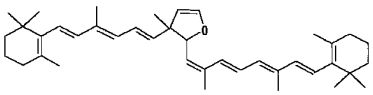
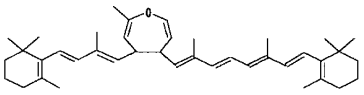
We separated three pure products (**C2**, **C3**, and **C5**) and two isomer-mixtures (**C1** and **C4**) from the reaction mixture of β-carotene and peroxyl radicals generated by AMVN. They were added to the *Salmonella* strains at concentrations of 1, 2, 5, and 10 nmol/plate. Table 1 shows the results at the highest concentrations of 10 nmol/plate (12.5 μM in the bacterial solution), because the products gave similar results at every concentration. Generally, the tested chemicals have been judged to be mutagenic when could give two-fold of revertant numbers or more compared to that of the control (vehicle alone). None of the products or the intact β-carotene increased the revertant numbers of any *Salmonella* strains or influenced cell numbers. In the same strains, positive controls greatly increased the revertant numbers. For example, 0.5 nmol of 1-nitropyrene (Wako Pure Chem. Ind.) gave 4684 ± 266 revertants for TA98 and −S9; 0.1 nmol of Trp-P-2, 1423 ± 87 for TA98 and +S9; 10 nmol of *N*-methyl-*N'*-nitro-nitrosoguanisine (Tokyo Kasei Co.), 4454 ± 536 for TA100 and −S9; 0.5 nmol of aflatoxin B_1 (Makor Chem. Ltd., Israel), 1661 ± 272. Cumene hydroperoxide has been recognized to be mutagenic and gives 246 ± 10 revertants for TA102 and −S9, 321 ± 4 for TA102 and +S9, 583 ± 7 for TA104 and −S9 at 11 nmol of the concentration.[34]

Then, the reaction mixture of β-carotene and AMVN was directly tested with four strains (Table 2). The mixture was not mutagenic, regardless of the presence or absence of S9. These results clearly showed that no products of β-carotene after the reaction with peroxyl radicals were mutagenic or cytotoxic.

Evaluation of the products of α-tocopherol for mutagenicity

α-Tocopherol was incubated with ML under air in the presence of AMVN or AAPH as an initiator, and the incubation mixture was assayed for mutagenicity (Table 2). The mixtures of ML with AMVN and ML plus α-tocopherol with AMVN slightly increased the revertant numbers compared to AMVN alone. We separated 5 oxidation products of α-tocopherol (**T1-T5**) and 12 products of α-tocopherol with lipid-peroxyl or lipid-alkyl radicals (**T6-T11**). Table 3 shows the effects of these products on the four

Table 1. The Effects of Subsequent Products of β-Carotene after the Antioxidant Action on Four *Salmonella* Strains

Product (10 nmol/plate)[a]	TA98		TA100		TA102		TA104		
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9	Surviving cells ($\times 10^7$)[b]
	Revertant number (Mean ± SD, n = 4)								
Vehicle (DMSO)	17 ± 3	24 ± 6	121 ± 27	132 ± 44	196 ± 9	161 ± 14	150 ± 26	156 ± 25	796 ± 11
β-Carotene	18 ± 6	19 ± 3	98 ± 16	93 ± 2	183 ± 16	160 ± 10	180 ± 30	136 ± 29	817 ± 12
									
C1	19 ± 4	22 ± 4	109 ± 6	89 ± 6	188 ± 3	168 ± 23	148 ± 17	66 ± 21	794 ± 14
									
									
C2	19 ± 6	20 ± 3	103 ± 27	87 ± 6	179 ± 15	167 ± 17	161 ± 40	52 ± 3	778 ± 16
									
C3	15 ± 4	22 ± 5	118 ± 12	76 ± 6	172 ± 4	169 ± 9	66 ± 12	59 ± 9	815 ± 11
									
C4	16 ± 3	21 ± 3	113 ± 16	92 ± 11	186 ± 5	176 ± 22	50 ± 2	38 ± 9	793 ± 18
									
									
C5	18 ± 4	21 ± 6	98 ± 34	84 ± 10	189 ± 10	166 ± 22	56 ± 8	84 ± 20	786 ± 11
									

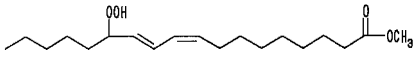
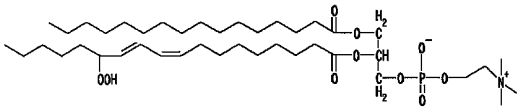
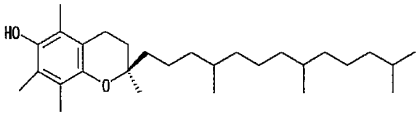
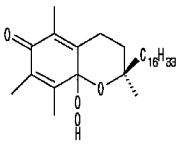
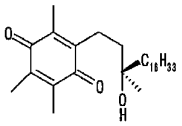
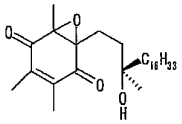
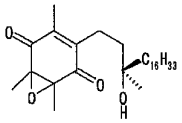
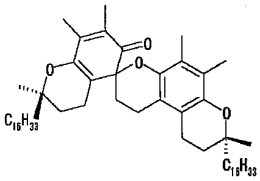
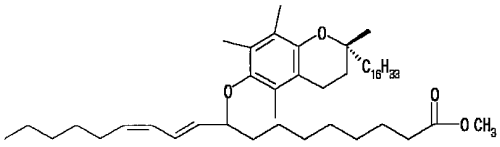
[a] The tests were done at a concentration of 1, 2, 5, or 10 nmol/plate, each of which gave similar results.
[b] Surviving cell numbers remained unchanged under conditions of − and + S9, and in TA98, TA100, and TA102, compared to vehicle alone.

Table 2. Effects of Peroxidizing Mixtures of β-Carotene and α-Tocopherol on Four *Salmonella* Strains

Reaction component[a]	TA98		TA100		TA102		TA104	
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9
	Revertant number (mean ± SD)							
Vehicle (DMSO)	20 ± 4	34 ± 16	99 ± 12	107 ± 22	214 ± 41	217 ± 48	406 ± 129	382 ± 161
AMVN	13 ± 4	21 ± 8	104 ± 5	114 ± 7	206 ± 17	229 ± 13	366 ± 179	295 ± 15
AMVN + β-Carotene	24 ± 4	25 ± 2	98 ± 16	117 ± 15	185 ± 14	211 ± 9	378 ± 54	276 ± 11
AAPH	20 ± 6	23 ± 5	110 ± 6	115 ± 7	221 ± 17	241 ± 9	392 ± 33	258 ± 93
AAPH + ML	15 ± 4	22 ± 4	76 ± 15	100 ± 28	213 ± 18	254 ± 19	206 ± 30	356 ± 49
AMVN + ML	28 ± 5	21 ± 4	93 ± 20	120 ± 33	182 ± 15	267 ± 24	247 ± 37	713 ± 96
AAPH + ML + α-Tocopherol	21 ± 4	14 ± 3	81 ± 19	114 ± 21	250 ± 11	232 ± 10	301 ± 25	448 ± 40
AMVN + ML + α-Tocopherol	7 ± 3	20 ± 5	95 ± 5	97 ± 6	227 ± 27	261 ± 23	723 ± 51	746 ± 66

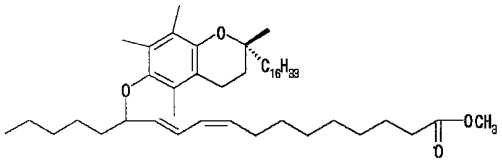
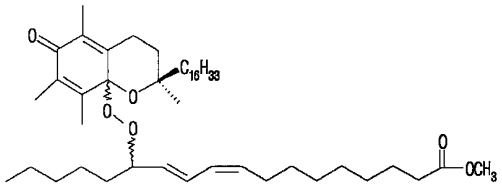
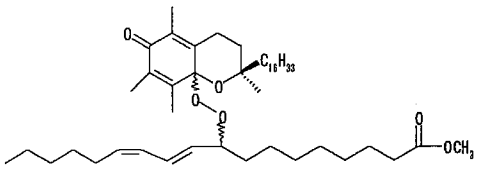
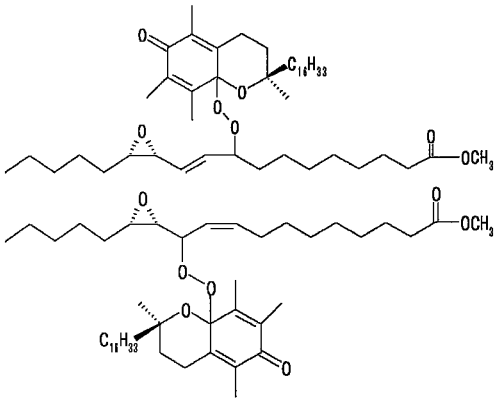
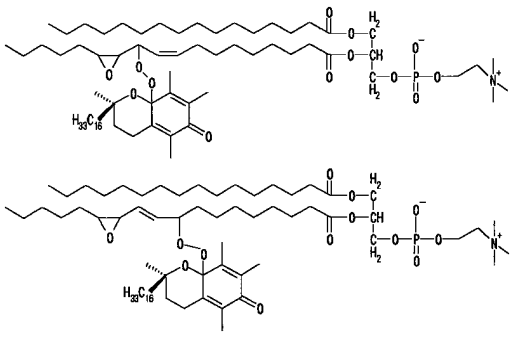
[a] The mixtures in a benzene solution were incubated under air at 37°C for 1 h, dried, resuspended in DMSO, and then added to *Salmonella* cells. The final dose-concentrations/plate: AAPH and AMVN, 1 nmol; ML, β-carotene and α-tocopherol, 10 nmol.

Table 3. The Effects of Subsequent Products of α-Tocopherol, after the Antioxidant Action, on Four *Salmonella* Strains

Product (10 nmol/plate)	TA98		TA100		TA102		TA104		
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9	Surviving cells ($\times 10^7$)[a]
	Revertant number (Mean ± SD, n = 4)								
Vehicle (DMSO)	17 ± 3	24 ± 5	123 ± 22	117 ± 16	260 ± 12	271 ± 19	167 ± 21	173 ± 23	729 ± 25
ML	17 ± 3	22 ± 2	109 ± 15	106 ± 8	258 ± 9	235 ± 11	145 ± 20	83 ± 11	672 ± 30
13-MLOOH	17 ± 3	25 ± 7	95 ± 29	90 ± 10	248 ± 22	312 ± 15	162 ± 24	105 ± 14	703 ± 18
									
PLPC	17 ± 4	22 ± 4	105 ± 4	83 ± 9	239 ± 5	224 ± 21	142 ± 32	54 ± 7	750 ± 8
PLPCOOH	18 ± 3	21 ± 3	77 ± 24	82 ± 6	245 ± 30	221 ± 28	84 ± 11	58 ± 8	724 ± 11
									
α-Tocopherol	20 ± 5	24 ± 6	108 ± 36	83 ± 4	235 ± 14	297 ± 19	152 ± 12	92 ± 25	734 ± 24
									
T1	28 ± 7	28 ± 7	88 ± 10	90 ± 5	237 ± 29	333 ± 18	147 ± 10	89 ± 19	733 ± 20
									
T2	11 ± 2	21 ± 5	98 ± 15	114 ± 7	212 ± 20	296 ± 25	175 ± 14	130 ± 31	652 ± 19
									
T3	12 ± 2	21 ± 4	105 ± 19	115 ± 6	292 ± 32	216 ± 26	186 ± 12	167 ± 13	747 ± 5
									
T4	20 ± 4	20 ± 4	66 ± 14	104 ± 10	244 ± 23	312 ± 37	171 ± 25	85 ± 11	636 ± 29
									
T5	16 ± 3	21 ± 5	62 ± 18	96 ± 4	244 ± 33	312 ± 20	155 ± 15	67 ± 20	628 ± 31
									
T6	15 ± 2	19 ± 3	98 ± 21	88 ± 7	247 ± 23	324 ± 28	190 ± 11	88 ± 14	691 ± 25
									

Contd.

Table 3. Contd

Product (10 nmol/plate)	TA98		TA100		TA102		TA104		
	− S9	+ S9	− S9	+ S9	− S9	+ S9	− S9	+ S9	Surviving cells ($\times 10^7$)[a]
T7	28 ± 5	25 ± 6	68 ± 17	71 ± 3	270 ± 12	273 ± 14	189 ± 35	70 ± 23	673 ± 29
									
T8a-d									
									
T8a	16 ± 4	32 ± 10	82 ± 11	81 ± 9	258 ± 18	331 ± 19	166 ± 18	96 ± 26	648 ± 28
T8b	17 ± 2	21 ± 3	106 ± 5	90 ± 10	293 ± 27	279 ± 22	157 ± 19	133 ± 28	626 ± 23
T8c	27 ± 5	24 ± 5	76 ± 13	87 ± 11	239 ± 31	366 ± 36	169 ± 13	80 ± 20	880 ± 26
T8d	27 ± 6	23 ± 4	86 ± 28	93 ± 6	211 ± 13	281 ± 20	172 ± 10	101 ± 26	689 ± 32
T9a-d									
									
T9a	23 ± 4	25 ± 5	104 ± 19	90 ± 11	244 ± 15	309 ± 10	157 ± 9	141 ± 16	657 ± 23
T9b	13 ± 3	23 ± 4	108 ± 34	100 ± 6	267 ± 31	267 ± 26	178 ± 20	53 ± 9	727 ± 13
T9c	20 ± 5	30 ± 8	74 ± 19	114 ± 7	235 ± 22	258 ± 23	190 ± 15	89 ± 20	632 ± 18
T9d	13 ± 2	18 ± 3	90 ± 11	90 ± 6	239 ± 28	300 ± 45	170 ± 24	76 ± 19	687 ± 22
T10	22 ± 4	16 ± 2	110 ± 30	120 ± 11	227 ± 19	261 ± 25	191 ± 23	249 ± 45	646 ± 44
									
T11	18 ± 4	21 ± 5	102 ± 13	109 ± 4	225 ± 16	252 ± 22	107 ± 16	150 ± 3	734 ± 23
									

[a] Surviving cell numbers remained unchanged under conditions of − and + S9, and in TA98, TA100, and TA102, compared to vehicle alone.

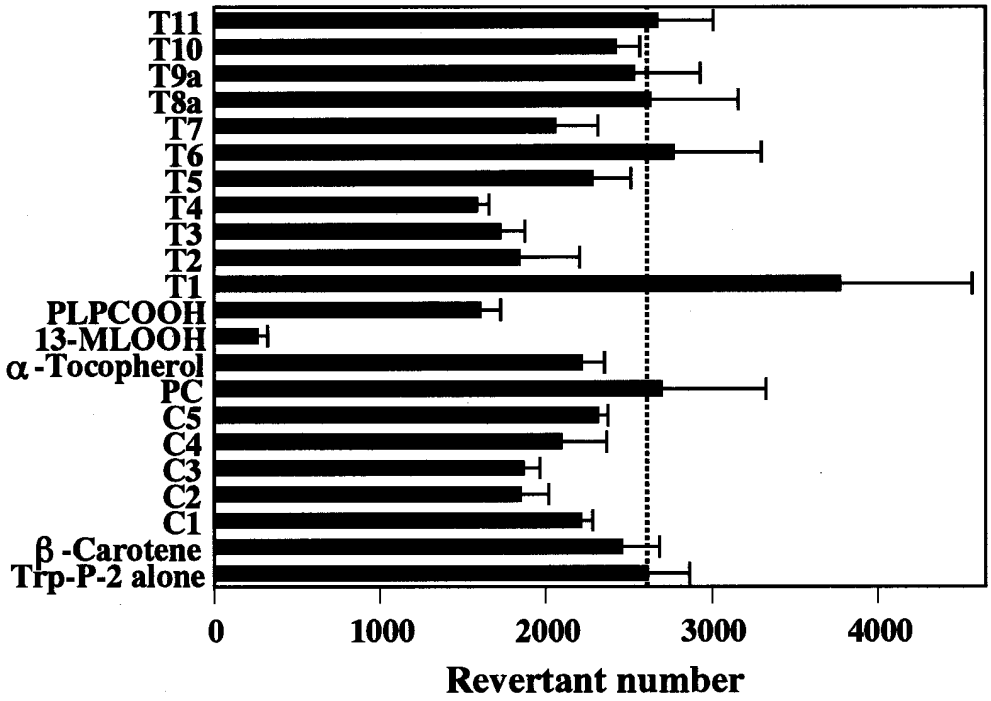


Fig. 1. Effects of Products of β-Carotene and α-Tocopherol after the Antioxidant Actions on Trp-P-2 Mutagenicity.
Against a mutagenicity of 0.1 nmol Trp-P-2, the suppressive effects of the oxidation products (10 nmol) were measured. Data are the mean ± SD for 6 plates tested. No products influenced cell survival compared to the vehicle, as shown in Tables 1 and 3.

Salmonella strains. None of the products increased the revertant numbers or influenced cell survival compared to vehicle, ML, PLPC, or α-tocopherol alone. Thus, the products of α-tocopherol that formed after the antioxidant action had no mutagenicity or cytotoxicity. The slight increases of revertant numbers in Table 2 were considered to be attributed to reaction products of AMVN and ML but not to oxidation products of α-tocopherol.

Effects of products of β-carotene and α-tocopherol on Trp-P-2 mutagenesis

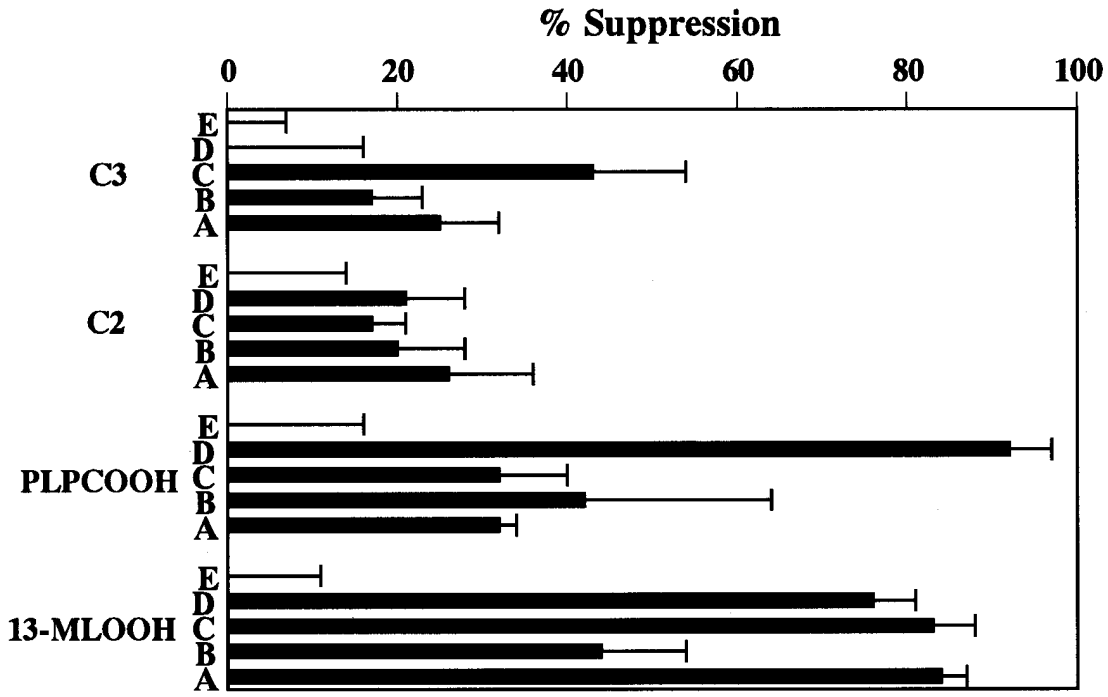
Trp-P-2 becomes mutagenic after being metabolically activated by CYP 1A enzymes, which can be detected with *Salmonella* TA98.[36] The 5 products of β-carotene and 17 products of α-tocopherol were added to the mutagenesis system and the effects on Trp-P-2 mutagenicity assayed (Fig. 1). All of the β-carotene products suppressed mutagenicity to some extent; **C2** and **C3** inhibited it by 29% at 10 nmol compared to Trp-P-2 at 0.1 nmol. Among the 17 α-tocopherol products, 7 suppressed the mutagenicity partly. Lipid hydroperoxides, 13-MLOOH and PLPCOOH, showed marked suppression.

Then, we examined the mechanisms of suppression by 13-MLOOH, PLPCOOH, **C2**, and **C3**, on mixing with Trp-P-2, S9 and TA98, which were set as five experimental designs (Fig. 2). In design A, the peroxidation products were incubated with all three factors, which is the same method used for the test in Fig. 1. In design B, the products were first incubated with Trp-P-2 for 10 min, and with S9 for another 10 min, and then incubated with bacterial cells for 20 min. In design C, products were first incubated with S9, and with Trp-P-2, and then with bacteria. In design D, Trp-P-2 was first activated with S9 following a 10-sec boiling to inactivate the S9 enzymes, and incubated with the products, and then mixed with bacteria. In design E, TA98 cells were first mutated by incubation with Trp-P-2 and S9, and then incubated with the products, as an assay to detect bio-antimutagenicity.[38] Compared to design A, 13-MLOOH showed similar suppression in designs C and D, suggesting that it affected S9 and the activated Trp-P-2. PLPCOOH and **C3** were more suppressive in design D, indicating that they acted on the activated Trp-P-2. None of the four products used here showed any bio-antimutagenicity.

Discussion

This study showed that none of the products of β-carotene and α-tocopherol after acting as antioxidants had mutagenicity against *Salmonella* TA98, TA100, TA102, or TA104. These two biological antioxidants are well known to suppress oxidative stress that is closely associated with degenerative diseases including cancer. The absence of genotoxicity indicates that β-carotene and α-tocopherol should be favored as biological antioxidants.

One question remaining about the method for testing mutagenicity was whether the substances had



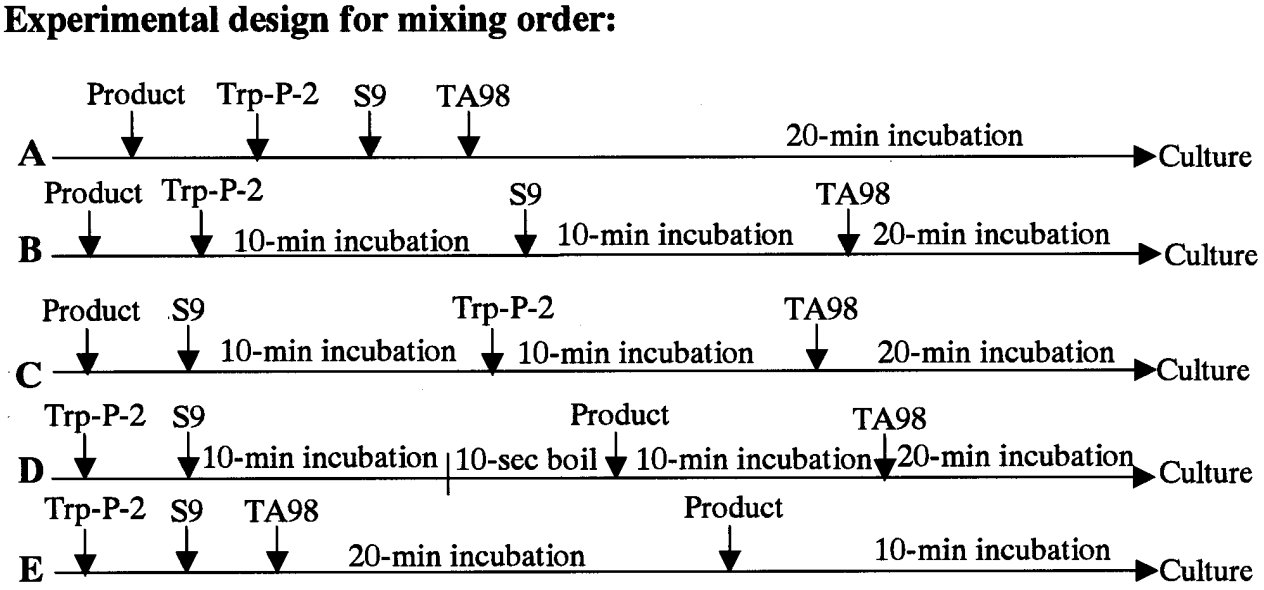


Fig. 2. Elucidation of the Suppressive Mechanism of *Salmonella* Mutation Induced by Trp-P-2.

Products (10 nmol) were added to the 0.1-nmol Trp-P-2-mutagenicity system, in five different mixing orders (experimental design A-E) and incubated at 37°C. The percentage of suppression was calculated as: $[(X\text{-}Y)\text{-}(Z\text{-}W)]/(X\text{-}Y) \times 100$, where X is the number of revertants obtained with Trp-P-2 (2610 ± 254), Y is the number of spontaneous revertants (20 ± 3), Z is the number of revertants obtained with both the products and Trp-P-2, and W is the number of revertants obtained with the products.

been incorporated into the bacterial cells sufficiently. All the products of β-carotene and α-tocopherol tested here are lipophilic. Generally, lipophilic substances can easily pass through the bacterial cell membrane, which is constructed of phospholipids but hydrophilic substances require a transporter protein.[39,40] Further, in this study, we used sonication to mix the products with bacterial cells in order to facilitate their incorporation. The results in Tables 1–3 were obtained following a 10-sec sonication, and were similar to the results without sonication (data not shown). Among the tester strains, Dillon *et al.*[34] who tested several lipophilic aldehydes and peroxides such as benzoyl peroxide and veratraldehyde, described how *Salmonella* TA100, TA102 and TA104 were better for detecting mutagenicity. Therefore, we

believe that the products of β-carotene and α-tocopherol entered the bacterial cells and that they had no mutagenicity.

These products of β-carotene and α-tocopherol were also examined for any increase of the activation of Trp-P-2 to its ultimate mutagenic form. Procarcinogen Trp-P-2 is *N*-hydroxylated by CYP 1As and then acts as a mutagen and carcinogen. In the metabolic activation, peroxidation products of lipid have been known to play a role of cofactor as oxygen donor, and consequently to facilitate the formation of *N*-hydroxyl.[21–23] Thus, the products of β-carotene and α-tocopherol had been predicted to increase the mutagenicity of Trp-P-2. The results in Fig. 1, however, showed that most products of β-carotene and α-tocopherol did not increase but rather suppressed the mutagenicity. Only **T1** slightly increased the revertant number compared to Trp-p-2 alone. **T1** is a hydroperoxide of tocopherone, and may facilitate *N*-hydroxylation of Trp-P-2. Other hydroperoxides, 13-MLOOH and PLPCOOH, suppressed the mutagenicity, and showed obvious activity when previously incubated with S9 or *N*-hydroxy-Trp-P-2 (Fig. 2). Both CYP and *N*-hydroxy-Trp-P-2 have been recognized to be sensitive to reactive oxygen species such as hydroperoxides.[41,42] These hydroperoxides probably inactivated S9 enzymes and/or decomposed the activated Trp-P-2.

We have recently found that α-tocopherol inhibits the formation of 8-hydroxy-deoxyguanosine in the Fenton reaction system with an IC_{50} of 1.5 μM while other antioxidative polyphenols require a concentration of 10 μM or more.[43] Thus, α-tocopherol is considered one of the most important lipophilic antioxidants that scavenges reactive oxygen species without producing any genotoxic products.

Acknowledgments

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