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AN EVALUATION OF THE ANTIOXIDANT AND POTENTIAL PRO-OXIDANT PROPERTIES OF FOOD ADDITIVES AND OF TROLOX C, VITAMIN E AND PROBUCOL

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The food additives propyl gallate and vanillin inhibited iron ion-dependent lipid peroxidation in rat-liver microsomes, but stimulated formation of a deoxyribose degrading species, probably hydroxyl radical, from ferric-EDTA and hydrogen peroxide. Propyl gallate accelerated DNA damage by the anti-tumour antibiotic bleomycin, although vanillin did not. The water-soluble vitamin E analogue Trolox C also stimulated bleomycin-dependent DNA damage, but not hydroxyl radical generation from ferric-EDTA and H_2O_2. Indeed, Trolox C was found to be a powerful scavenger of hydroxyl radical (rate constant $> 10^{10} M^{-1} s^{-1}$). Probucol did not stimulate oxidative damage in any of the systems tested, and is a powerful inhibitor of lipid peroxidation in rat liver microsomes. The ESR spectrum of the radical produced by one electron-oxidation of probucol in ethanol is described.

KEY WORDS: Food additives, pro-oxidant, anti-oxidant, bleomycin, DNA damage, Trolox C, probucol.

INTRODUCTION

The role played by free radicals and other reactive oxygen species in the pathogenesis of human disease, in the mechanism of action of toxins and in the deterioration of foods is an area of increasing interest (reviewed[1-3]). Species of importance in such systems include hydroxyl radical (·OH), superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals ($RO_2^·$) and hypochlorous acid (HOCl). All these species are probably produced *in vivo*,[1,2,4,5] and this can lead to oxidative damage to many biomolecules, including DNA, proteins and membrane lipids.[1-5] Formation of hydroxyl radicals, and probably additional powerfully-oxidizing species, from O_2^- and H_2O_2 requires catalytic metal chelates, of which iron ion complexes are particularly important *in vivo*.[5,6] Iron ions bound to the synthetic chelating agent EDTA are especially effective in promoting ·OH generation *in vitro*.[5-8] Transition metal ions can stimulate the free radical chain reaction of lipid peroxidation, both by generating species capable of first-chain initiation (such as ·OH) and also by accelerating the breakdown of lipid hydroperoxides to peroxyl and alkoxyl radicals capable of hydrogen atom abstraction (reviewed in[2,5,6]).

Lipid peroxidation can be temporarily slowed by addition of chain-breaking antioxidants, which intercept chain-propagating peroxyl and alkoxyl radicals. α-Tocopherol is an important chain-breaking antioxidant *in vivo* (reviewed in[9]); it is the major,[10,11] although not the only,[11] lipid-soluble chain-breaking antioxidant in humans. Many chain-breaking antioxidants are, or have been, used as food additives.[3] These include the now-banned nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate. Their use is important because end-products of lipid peroxidation are toxic" and can impart a rancidity, or "off-flavour", to foods.[3]

There has also been considerable interest in the use of chain-breaking antioxidants in the treatment of human disease or of poisoning by various toxins. For example, probucol has been shown to inhibit atherogenesis in a rabbit model system[12]: development of the atherosclerotic lesion is thought to involve peroxidation of lipoproteins in the arterial wall (discussed in[11,12]). Vitamin E has been tested for effectiveness against the cardiotoxicity of anthracycline anti-tumour drugs, which is thought to be mediated by increased free radical reactions, although the results were disappointing (reviewed in[13]). A water-soluble vitamin E analogue, Trolox C, has also been developed and its radical-scavenging properties studied.[14] NDGA has been claimed to inhibit lung fibrosis induced by the antibiotic bleomycin:[15] bleomycin damages DNA by forming a reactive bleomycin-iron ion-oxygen complex upon the DNA molecule (reviewed in[16]).

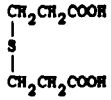
However, two points must be borne in mind before promoting the widespread testing of chain-breaking and other antioxidants upon humans. The first is that oxidant damage *in vivo* is not necessarily mediated via lipid peroxidation: damage to DNA and/or proteins can be equally or more important.[1,2] Secondly, several phenolic chain-breaking antioxidants (including α-tocopherol[9]) are capable of binding iron(III) and reducing it to iron(II), which can result in a pro-oxidant action under certain circumstances,[17,18] especially if non-lipid systems are examined.[18] Thus Gutteridge and Xaio-Chang[18] found that BHA, BHT and propyl gallate accelerate iron-ion dependent DNA damage by bleomycin. Similarly, several complex phenols of plant origin were found to be powerful chain-breaking antioxidant inhibitors of lipid peroxidation, but they *accelerated* both bleomycin-dependent damage to DNA, and $\cdot OH$ generation from H_2O_2 in the presence of iron ion complexes.[19] Radical generation from the synthetic phenolic antioxidants BHA and BHT has been suggested to contribute to some of their reported toxic actions *in vivo*.[20,21,38]

In view of these observations we decided to screen α-tocopherol, Trolox C, probucol, and various food additives for pro-oxidant and anti-oxidant effects. Figure 1 shows the structures of the compounds that we have tested, for reference purposes.

MATERIALS AND METHODS

Reagents

Reagents were of the highest quality available from Sigma Chemical Corp. or from BDH Chemicals Ltd. Poole, Dorset, UK., unless otherwise stated. Trolox C was from Aldrich. Probucol was a gift from the Merrell Dow Research Institute, Reading Berks.



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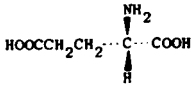

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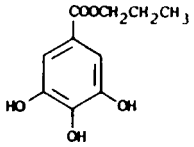

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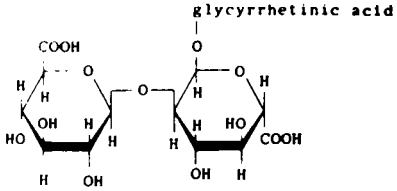
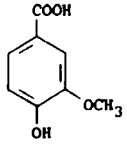
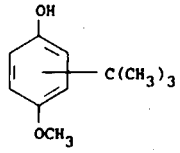


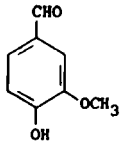
Glycyrrhizic Acid.



Vanillic Acid.



Butylated Hydroxyanisole.



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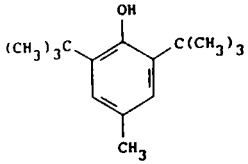

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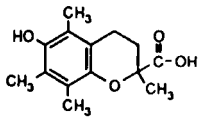
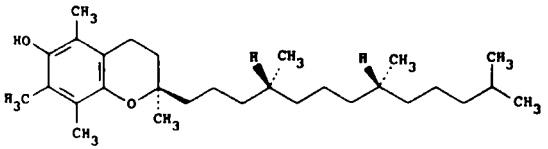
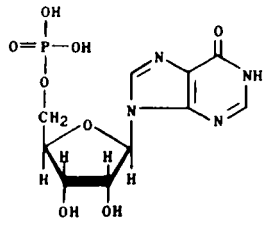


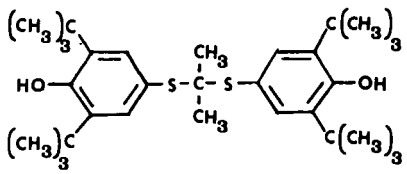
Trolox®



Vitamin E.



Inosinic Acid.



Probucol

FIGURE 1 Structures of some of the compounds referred to in the text.

Methods

Degradation of deoxyribose by ·OH at pH 7.4 in the presence of ascorbate, H_2O_2 and $FeCl_3$ (with or without EDTA) was measured as described in.[22] Rate constants for reaction with ·OH were calculated as in[23] from the equation

$$\frac{1}{A} = \frac{1}{A^\circ}\left(1 \frac{+ k_s[S]}{k_{DR}[DR]}\right)$$

where A is the absorbance in the presence of a scavenger S at concentration $[S]$ and A° the absorbance in the absence of scavenger. [DR] is deoxyribose concentration. Hence a plot of $1/A$ against [S] gives a straight line of slope $(k_s/k_{DR}[DR]A^\circ)$ where k_S is the rate constant for reaction of scavenger with ·OH. Solutions of compounds to be tested were made up immediately before use in water, unless otherwise stated. Glycyrrhizic acid was prepared by dissolving 210 mg of solid in 20 ml of double-distilled water containing 3 drops of concentrated ammonia solution. Control experiments showed that this preparatory step did not affect the degradation of deoxyribose. α-Tocopherol, BHA, BHT and probucol were dissolved in ethanol.

Microsomes from rat liver were prepared by differential pelleting as in[24]; the final microsomal pellet was washed and suspended in 0.25 M NaCl. Peroxidation was measured by the thiobarbituric acid (TBA) test.[24] Ethanol (in which some compounds were dissolved) had no effect on peroxidation under our reaction conditions.

The bleomycin assay was conducted as previously described[25] with a few modifications. Where indicated, ascorbic acid was omitted in order to test the ability of compounds to reduce a bleomycin-iron ion complex and facilitate DNA damage. 0.5 ml DNA (1 mg/ml) was mixed with bleomycin-iron mixture (0.05 ml bleomycin sulphate [1.5 units/ml, approx 0.6 mM]/0.1 ml $FeCl_3$[0.5 mM]) and 0.05 ml Tris-HCl buffer (1.0 M pH 7.4). The reaction was started by adding 0.1 ml of either ascorbic acid (7.5 mM) freshly dissolved in water or 0.1 ml of solutions of compounds under test. Control experiments showed that ethanol (in which some compounds were dissolved) did not affect the bleomycin assay. Tubes were incubated at 37°C for 1 hour, followed by addition of 1 ml TBA reagent (1% [w/v] TBA in 0.05 M NaOH) and 1 ml 25% (v/v) HCl. Colour was developed by heating the tubes at 100°C for 10 minutes. A_{532} was measured after extracting the MDA-$(TBA)_2$ chromogen into butan-l-ol.

DNA damage by hydroxyl radicals was measured by gas chromatography/mass spectrometry with selected ion monitoring essentially as described in.[26,27] Reaction mixtures contained in a final volume of 1.2 ml, the following reagents at the final concentrations stated: DNA (0.5 mg/ml), KH_2PO_4/KOH buffer (10 mM), H_2O_2 (2.8 mM), $FeCl_3$ (100 μM), EDTA (120 μM), vanillin (20 mM) and ascorbate (where indicated, 100 μM). Mixtures were incubated at 37°C for 1 hour and then extensively dialysed against water. The stock solution of calf thymus DNA (1 mg/ml) had been treated with chelex resin (Bio Rad Laboratories) and centrifuged to remove the resin. The pH of the resulting solution was adjusted to 7.4 using chelex-treated HCl (1 mol/dm^3) immediately before use. Vanillin was premixed with iron ions, prior to addition to the appropriate reaction mixture, when ascorbic acid was omitted. After dialysis, the absorbance of each sample was measured at 260 nm in order to calculate the amount of DNA (an absorbance of one corresponds to 50 μg/ml DNA). The samples were subsequently hydrolysed and derivatized and then subjected to gas chromatography and mass spectrometry.[26,27] ESR studies were carried out on a Joel RE IX ESR spectrometer, as described in the legend to Figure 4.

TABLE I

Actions of food additives and antioxidants on rat liver microsomal peroxidation mediated by the ascorbate/$FeCl_3$ system. Reaction mixtures contained, in a final volume of 1 ml, the following reagents: 0.5 ml phosphate saline buffer (4 mM Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl pH 7.4), 0.25 mg microsomal protein, $FeCl_3$ (100 μM), EDTA (where used) (100 μM), food additives (tested at 2.4 mM) and ascorbate (100 μM). Peroxidation was started by adding ascorbate. Tubes were incubated at 37°C for 2 hrs. Peroxidation was measured by the thiobarbituric acid (TBA) method; the reaction mixture was heated at 100°C for 15 min in the presence of 0.5 ml 1% (w/v) TBA and 0.5 ml 2.8% (w/v) trichloroacetic acid. The chromogen was extracted into butan-1-ol and the absorbance of the upper (organic) layer measured at 532 nm. Typical results are shown. BHA and BHT were added dissolved in ethanol, which, in the amounts used, had no effect on the peroxidation process.

	Amount of peroxidation A_{532}	
Food additives	no EDTA	plus EDTA
None	1.02	0.25
Propyl gallate	0.16	0.15
Vanillin	0.38	0.21
Vanillic acid	0.33	0.16
Glycyrrhizic acid	0.64	0.19
3,3'Thiodipropionic acid	1.18	0.24
Inosinic acid	1.12	0.25
BHT	0.19	0.14
BHA	0.18	0.15

RESULTS

Inhibition of lipid peroxidation

Peroxidation of rat-liver microsomes in the presence of iron ions was studied, using ascorbate to stimulate the peroxidation process. As expected, BHT, BHA and propyl gallate inhibited lipid peroxidation, whether or not EDTA was present (Table 1). Under these reaction conditions, EDTA itself decreased the rate of peroxidation, as reported previously.[28] Vanillin, vanillic acid and glycyrrhizic acid also inhibited peroxidation, but thiodipropionic acid and inosinic acid had no effect, presumably because of the lack of easily-donatable hydrogen atoms in their structures (Figure 1). Trolox C and α-tocopherol also inhibited peroxidation, as expected (data not shown).

Figure 2 shows the effect of increasing concentrations of vanillin on microsomal lipid peroxidation. Progressive inhibition was seen over a concentration range up to 1.8 mM, with 50% inhibition at about 1.2 mM. Inclusion of vanillin in the TBA assay did not interfere with measurement of the TBA-malondialdehyde adduct at 532 nm, but did cause formation of chromogens with a λ_{max} of about 435–450 nm (line F in Figure 2). Vanillic acid behaved similarly (data not shown).

Since probucol has been claimed to act as a chain-breaking anti-oxidant *in vivo*[12] and with isolated low-density lipoproteins,[29] it was of interest to test it on the microsomal system. 50 μM probucol inhibited microsomal peroxidation in the presence of Fe(III)/ascorbate by > 95%: a similar inhibition was seen if peroxidation was accelerated by adding Fe(III)-ADP and NADPH to the microsomes (for reaction conditions see[24]). Figure 3 shows the effect of 10 μM probucol on microsomal lipid peroxidation in the presence of Fe(III) and ascorbate.

Probucol presumably acts by donating a hydrogen atom to intermediate peroxyl

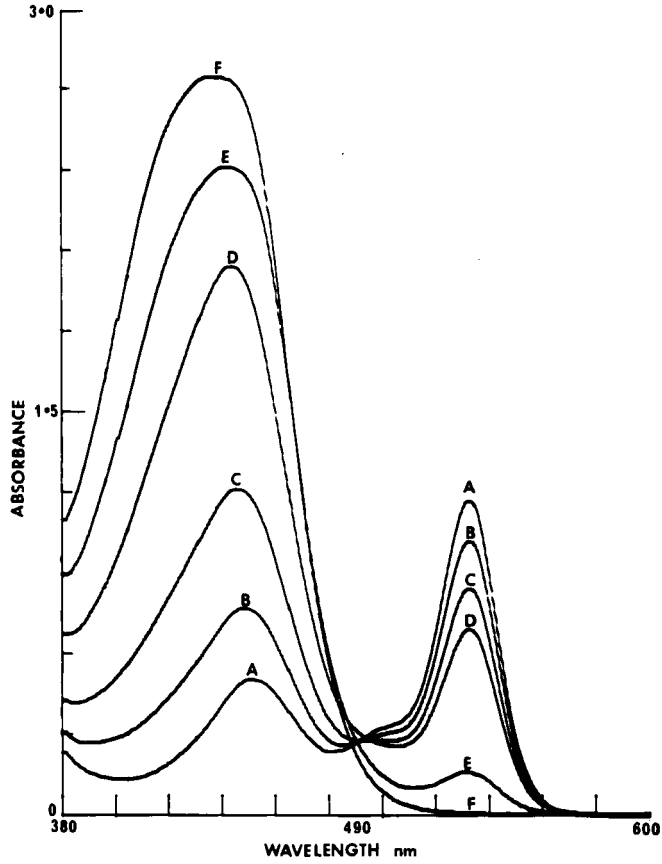


FIGURE 2 Effect of increasing concentrations of vanillin on rat liver microsomal peroxidation mediated by ascorbate and $FeCl_3$. The composition of the assay mixtures was as shown in the legend to Table 1. The TBA reactive material was extracted into butan-l-ol at the end of the experiment and a spectrum of each mixture was taken. Line A, no vanillin; line B, 0.24 mM; line C, 0.6 mM; line D, 1.2 mM; line E, 1.8 mM. In line F, 2.4 mM vanillin was incubated with the TBA reagents in the absence of microsomes, iron and ascorbate.

and/or alkoxyl radicals within the membrane interior. This would result in formation of probucol radicals. Figure 4 shows the ESR spectrum of probucol radicals, as generated by oxidation of an ethanolic solution with silver(I) oxide. The solution developed a pink colour after Ag_2O addition. The half-time of the radical decay is approximately 30 sec at 23°C: as it decays, the pink colour turns yellow. Addition of a large excess of ascorbic acid did not accelerate disappearance of the radical species.

Studies of pro-oxidant action

1. Iron ion-dependent formation of hydroxyl radicals A mixture of ascorbic acid, H_2O_2 and Fe(III)-EDTA at pH 7.4 generates ·OH, which can be measured by its ability to degrade the sugar deoxyribose into fragments that, on heating with thiobarbituric acid at low pH, generate a pink chromogen.[22,23] The ability of a scavenger to

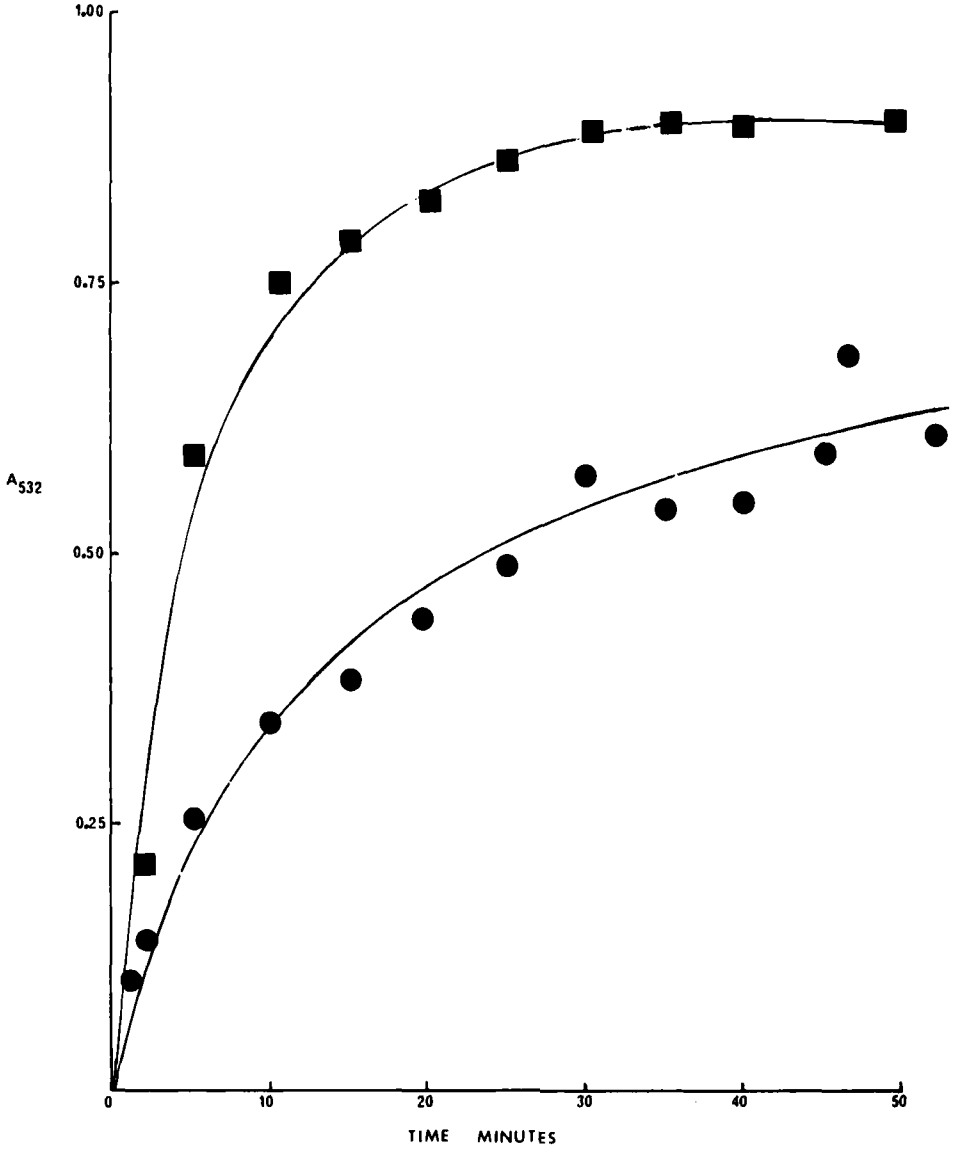


FIGURE 3 Inhibition of lipid peroxidation by probucol. Peroxidation was measured by the TBA test as described in the legend to Table 1, except that aliquots were removed for assay at the times stated. Where indicated, probucol was present in the reaction mixture at a final concentration of 10 μM. Control experiments showed that probucol added with the TBA and acid had no inhibitory effect, i.e. probucol does not interfere with the TBA test. ■ No probucol, ● probucol present.

inhibit deoxyribose degradation in this system depends only on its concentration relative to deoxyribose and on its rate constant for reaction with ·OH. This is because any ·OH escaping reaction with EDTA is equally accessible to deoxyribose and to the scavenger.[23] Most of the compounds tested in this study efficiently scavenged ·OH,

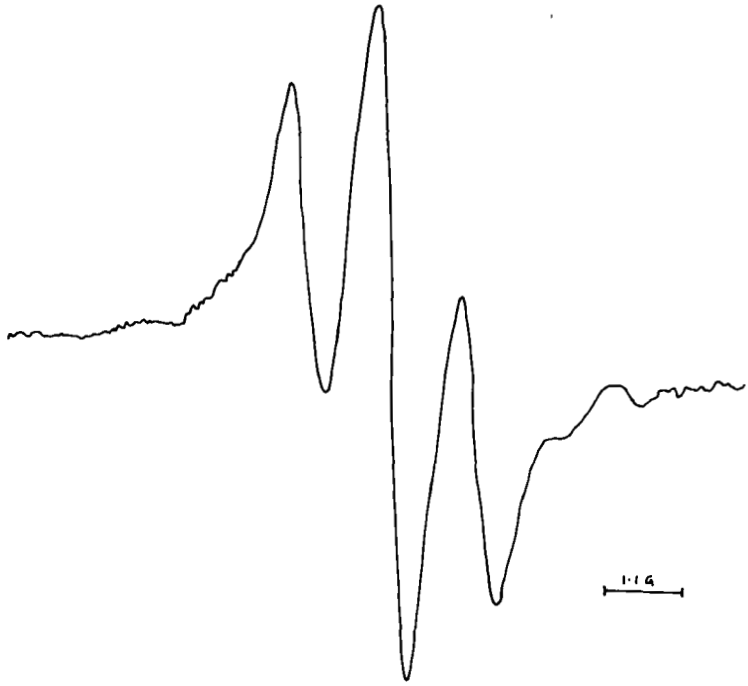


FIGURE 4 Radical formation by one-electron oxidation of probucol. A 5 mM solution of probucol in ethanol at 23°C gave no ESR signal. Addition of solid Ag_2O caused development of the signal shown, which decayed with a half-life of approximately 30 sec. Spectra were determined on a Jeol RE IX ESR spectrometer (microwave power 2 mWatt, modulation amplitude 0.01 mT, Time constant 0.03 sec, Gain $6.3.10^2$, field set 325 mT). The most likely radicals to form arise by conversion of one or both -OH groups (see Figure 1) into phenoxyl radicals (-O˙).

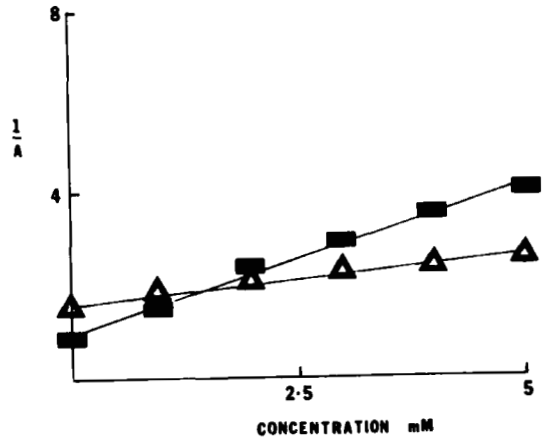


FIGURE 5 Inhibition of hydroxyl radical-dependent deoxyribose degradation by 3,3′ dithiopropionic acid. Experiments were conducted as described in Halliwell *et al.*[25] The rate constants k_s (detailed in Table 2) were determined from the slope of the lines obtained in the presence of EDTA using the equation given in the Materials and Methods section. The deoxyribose concentration [DR], was 2.8 mM. △ Iron added to the reaction mixture as $FeCl_3$ (20 μM), ■ Iron added to the reaction mixture as $FeCl_3$ (20 μM) – EDTA (100 μM) premixed prior to addition.

TABLE 2

Rate constants obtained by the deoxyribose method. Reaction mixtures contained in a final volume of 1.2 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), KH_2PO_4/KOH buffer pH 7.4 (10 mM), iron ions (20 μM $FeCl_3$, or 20 μM $FeCl_3$ plus 100 μM EDTA premixed prior to addition to the reaction mixture), H_2O_2 (2.8 mM), compound to be tested, and ascorbate (100 μM). Reaction mixtures were incubated at 37°C for 1 hour and the deoxyribose degradation measured as formation of TBA-reactive material.[23] Rate constants were calculated from the slopes of competition plots as described in the legend to Figure 5. Values are the means of at least four observations that differed by no more than 10%.

Compounds	Concentration range studied	Second order rate constants for reaction with ·OH $M^{-1}s^{-1}$	
		EDTA present	EDTA absent
Monosodium glutamate	(0–12.0 mM)	3.1×10^8	5.2×10^8
Propyl gallate	(0–0.5 mM)	stimulation	3.2×10^{10}
Vanillin	(0–5.0 mM)	stimulation	3.1×10^8
Vanillic acid	(0–5.0 mM)	1.6×10^{10}	9.6×10^9
Glycyrrhizic acid	(0–1.0 mM)	1.3×10^{10}	6.2×10^9
Thiodipropionic acid	(0–5.0 mM)	6.4×10^9	8.3×10^8
Inosinic acid	(0–5.0 mM)	3.9×10^9	9.3×10^8
Trolox C	(0–0.3 mM)	8.5×10^{10}	8.1×10^{10}

so inhibiting deoxyribose degradation. From the kinetics of the inhibition, second-order rate constants for reaction of the compounds with ·OH were calculated as described in the Materials and Methods section. Figure 5 shows a representative example of the kinetic plots, and Table 2 summarizes the results obtained from several experiments. Monosodium glutamate, at the concentrations studied, was a fairly poor scavenger of ·OH (rate constant about $10^8 M^{-1}s^{-1}$). Glycyrrhizic, thiodipropionic

TABLE 3

An examination of the ability of propyl gallate, vanillin and vanillic acid to replace ascorbate in the deoxyribose degrading system. The experiments were conducted as described in the legend of Table 2. Ascorbic acid was omitted where indicated in order to examine the ability of propyl gallate, vanillin or vanillic acid to reduce iron(III) in the presence of EDTA. Values are the means of at least 3 readings that varied by no more than 10%. Concentrations stated are the final concentrations in the reaction mixture.

Additions to the reaction mixtures	Concentration	Extent of deoxyribose degradation A_{532}	
		Omit ascorbate	Plus ascorbate
None	–	0.29	1.99
Propyl gallate	0.01 mM	0.66	1.89
	0.05 mM	0.92	1.99
	0.20 mM	1.48	1.90
	0.50 mM	1.78	2.17
Vanillin	0.10 mM	0.73	1.98
	0.50 mM	1.01	1.81
	2.00 mM	1.41	1.60
	5.00 mM	1.30	1.23
Vanillic acid	0.10 mM	0.07	1.29
	0.50 mM	0.08	1.03
	2.00 mM	0.08	0.51
	5.00 mM	0.07	0.27

TABLE 4

Action of hydroxyl radical scavengers and other antioxidants on the degradation of deoxyribose in the presence of propyl gallate or vanillin. Experiments were carried out as described in the legend to Table 2. All reaction mixtures contained $FeCl_3$-EDTA and H_2O_2. Where indicated, ascorbic acid (100 μM), propyl gallate (500 μM), vanillin (2 mM) and scavengers were added to the reaction mixture to give the final concentrations stated.

	Extent of deoxyribose degradation A_{532}		
Scavenger added	ascorbate	propyl gallate	vanillin
None	1.52	1.87	1.87
Mannitol (50 mM)	0.26	0.39	0.78
Urea (50 mM)	1.61	1.91	1.79
Dimethylsulphoxide (50 mM)	0.16	0.21	0.28
Sodium formate (50 mM)	0.26	0.30	0.56
Superoxide dismutase (100 units/ml)	1.34	1.89	1.90
Albumin, bovine serum (1 mg/ml)	0.87	0.38†	1.40

†The reason for this inhibition (reproducible in 4 experiments) has not yet been established.

inosinic acids were all good scavengers of ·OH, as was Trolox C. α-Tocopherol, BHA, probucol and BHT could not be adequately tested in this assay as they are insoluble in water, and organic solvents such as ethanol are themselves good scavengers of ·OH. Addition of tocopherol, BHA, probucol or BHT to the reaction mixtures as emulsions had no significant effect.

When propyl gallate and vanillin were tested in the $FeCl_3$-EDTA/H_2O_2/ascorbate system, they were sometimes observed to *stimulate* ·OH generation slightly. This stimulation became especially clear if ascorbate was omitted from the reaction mixture: Table 3 shows a representative experiment. By contrast, vanillic acid had no stimulatory action on deoxyribose degradation.

Deoxyribose degradation in the presence of $FeCl_3$-EDTA, H_2O_2 and vanillin or propyl gallate was inhibited by several well-established scavengers of ·OH, including mannitol, formate and dimethylsulphoxide. Table 4 shows a representative result. Urea, which scavenges ·OH poorly, did not inhibit significantly.

It was noted that stimulation of ·OH generation by vanillin or propyl gallate was seen only when iron ions were added to the reaction mixture as a complex with EDTA. When iron was added as a ferric salt ($FeCl_3$), propyl gallate and vanillin *inhibited* the deoxyribose degradation, as did the other compounds tested. Table 2 (right hand column) summarizes some of the results obtained. It should be noted that the rate constants are given only to illustrate the magnitude of inhibition achieved and cannot be interpreted in terms of ·OH scavenging. The ability of a compound to inhibit deoxyribose degradation in the absence of EDTA relates to its iron-chelating ability and to the ability of its iron complex to participate in Fenton chemistry.[22,23,31]

2. Bleomycin-induced DNA damage The anti-tumour antibiotic bleomycin binds iron ions and a bleomycin-iron complex degrades DNA in the presence of O_2 and a reducing agent such as ascorbic acid (reviewed in[16]). Table 5 shows that, in the absence of ascorbate, Trolox C and propyl gallate could accelerate DNA damage by a Fe(III)-bleomycin complex, but vanillin and vanillic acid had no effect. α-Tocopherol, added dissolved in ethanol, also stimulated the DNA degradation, but probucol

TABLE 5

Bleomycin-iron-induced damage to DNA. Experiments were carried out as described in the Materials and Methods section. $FeCl_3$ was pre-mixed with bleomycin before addition to the reaction mixture. Ascorbic acid or other reagents were added to give the final concentrations stated. Probucol and α-tocopherol were dissolved in ethanol, which itself had no effect on the DNA degradation under our reaction conditions.

Addition to reaction mixture	Concentration	Extent of DNA damage A_{532}
None	-	0.12
Ascorbate	100 μM	2.07
Trolox C	5 μM	0.58
	10 μM	1.27
	20 μM	1.95
	60 μM	2.30
Propyl gallate	10 μM	0.21
	50 μM	0.97
	200 μM	0.60
	500 μM	0.16
Vanillin	100 μM	0.03
	500 μM	0.04
	2 mM	0.02
	5 mM	0.08
Vanillic acid	100 μM	0.05
	500 μM	0.04
	2 mM	0.09
	5 mM	0.17
Probucol	14 μM	0.08
	28 μM	0.09
	55 μM	0.07
	80 μM	0.09
α-Tocopherol	50 μM	0.17
	100 μM	0.26
	200 μM	0.50
	400 μM	0.97

(dissolved in ethanol) did not. If α-tocopherol was added to the reaction mixture in the absence of ethanol it had no stimulatory effect, presumably due to immiscibility with water.

3. Damage to DNA bases by hydroxyl radicals: effect of vanillin Incubation of calf-thymus DNA with a system producing ·OH radicals gives rise to extensive chemical modification of the DNA bases, in a way that appears to be diagnostic for ·OH radical.[26,27] Products formed include thymine glycol, 8-hydroxyguanine, dihydroxycytosine and formamidopyrimidines.[27] Table 6 shows that incubation of DNA with Fe(III)-EDTA, ascorbate and H_2O_2 led to significant rises in the amounts of several oxidatively-modified bases: this is characteristic of attack by ·OH.[26,27] Modification was inhibited by ·OH scavengers such as mannitol, dimethylsulphoxide and hypotaurine[30] (Table 6). Trolox C, a good scavenger of ·OH (Table 2) also inhibited the base modification (Table 6). Omission of ascorbate from the reaction mixture greatly decreased the DNA base modification (Table 6, first line) but vanillin restored some of it (Table 6, last line).

TABLE 6

Damage to DNA bases by hydroxyl radicals generated from Fe(III)-EDTA, H_2O_2 and ascorbate or vanillin at pH 7.4. Experiments were carried out as described in the Materials and Methods section. Criteria for the identification of the base products formed can be found in references.[26,27] Values for the products formed are the means of 3 determinations that agreed to within 10%. Control experiments showed that Trolox C, mannitol, hypotaurine, vanillin or dimethylsulphoxide (DMSO) did not themselves cause any base modification. RM-reaction mixture; RMA-reaction mixture plus ascorbate

Systems tested		Products monitored (nmol/mg DNA) A	B	C	D	E	Total
DNA,Fe^{3+}-EDTA,H_2O_2(RM)		0.08	0.02	0.06	0.10	0.43	0.69
RM + ascorbate	100 μM (RMA)	1.13	0.98	1.76	8.20	2.94	15.01
RMA + mannitol	100 mM	0.23	0.06	0.12	0.33	0.31	1.05
RMA + trolox C	20 mM	0.31	0.11	0.17	0.88	0.21	1.68
RMA + DMSO	100 mM	0.14	0.01	0.08	0.19	0.15	0.57
RMA + hypotaurine	20 mM	0.19	0.04	0.18	0.41	0.42	1.24
RM + vanillin	20 mM	0.40	0.18	0.26	1.20	0.50	2.30

Key: A = Thymine glycol B = 5,6-Dihydroxycytosine; C = 4,6-Diamino-5-formamidopyrimidine; D = 2,6-Diamino-4-hydroxy-5-formamidopyrimidine; E = 8-Hydroxyguanine.

DISCUSSION

Both propyl gallate and vanillin (but not vanillic acid in which the reducing -CHO group has been replaced by -COOH; Figure 1) are able to accelerate formation of a reactive species, capable of degrading the sugar deoxyribose, from Fe(III)-EDTA and H_2O_2. This species has been identified as $\cdot OH$, both on the basis of experiments with scavengers and on the pattern of damage to DNA bases that it produces (Tables 4 and 6), the latter being characteristic of attack by $\cdot OH$ under physiological conditions.[26,27] The stimulatory effect of propyl gallate and vanillin was seen only when EDTA was present: with iron(III) ions alone, these two compounds *inhibited* the deoxyribose degradation. In the deoxyribose assay, the ability of a compound to inhibit in the absence of EDTA is partly due to its action in binding iron ions, withdrawing them from the deoxyribose and so preventing site-specific attack by $\cdot OH$ upon the deoxyribose molecule.[22,23,31] What then is the significance of the pro-oxidant properties of propyl gallate and vanillin demonstrated in the presence of EDTA? Although EDTA is not synthesized by living organisms, it is frequently used to fortify foods, often as an Fe(III)-chelate.[32] Propyl gallate has been used as a food antioxidant, e.g. in vegetable oils, margarine, instant mashed potato and breakfast cereals.[3] Vanillin is a widely-used flavouring agent, e.g. in ice cream, cakes, syrups and chocolate. It has been suggested to protect food lipids from oxidation when added at high concentrations,[33] a proposal consistent with the results in the present paper. However, if foods were simultaneously treated with iron-EDTA and vanillin or propyl gallate, it is possible that pro-oxidant effects could occur, attacking proteins and DNA in the food. This can result in formation of toxic products.[11]

Propyl gallate, but not vanillin, accelerates bleomycin-induced damage to DNA (Table 5), in support of previous studies.[18] Treatment of cancer patients with bleomycin sometimes results in severe lung fibrosis, in which the involvement of oxidative

damage has been suggested.[16,34] Factors influencing development of fibrosis include the availability of iron ions[34,35] and of reducing agents;[34–36] they could also conceivably include the content of certain food additives in the diet (Table 5). α-Tocopherol accelerated bleomycin-induced DNA damage, presumably because of its capacity to reduce Fe(III) ions to Fe^{2+}. It is doubtful that this has physiological significance, in view of the insolubility of α-tocopherol in aqueous solution. However, use of the water-soluble α-tocopherol analogue Trolox C as an "antioxidant" to treat bleomycin-induced lung fibrosis would not seem to be a good strategy (Table 6). Indeed, the water-soluble reducing agent N-acetylcysteine, a powerful antioxidant in several systems,[37] actually *accelerates* bleomycin-induced lung damage.[36] It is interesting to note that, despite its stimulatory action in the bleomycin assay, Trolox C did not accelerate ·OH formation in the Fe(III)-EDTA/H_2O_2 system: indeed, it was found to be a good scavenger of ·OH (Table 2).

Probucol has been proposed for use *in vivo* as an antioxidant.[12] It is interesting to note that it had no pro-oxidant effects on ·OH generation or DNA degradation by bleomycin and it is a powerful inhibitor of lipid peroxidation, presumably by acting as a chain-breaking antioxidant. Our description of the ESR spectrum of the probucol radical (Figure 4) should facilitate studies of its mechanism of action.

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