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High Molecular Weight Plant Polyphenolics (Tannins) as Biological Antioxidants

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Representative condensed and hydrolyzable tannins and related simple phenolics were evaluated as biological antioxidants using cyclic voltammetry, the metmyoglobin assay, and the deoxyribose assay. The redox potentials of the tannins were similar to those of structurally related simple phenolics. However, the tannins were 15−30 times more effective at quenching peroxyl radicals than simple phenolics or Trolox. One of the tannins, polygalloyl glucose, reacted an order of magnitude more quickly with hydroxyl radical than mannitol. These results suggest that tannins, which are found in many plant-based foods and beverages, are potentially very important biological antioxidants.

Keywords: *Tannins; plant phenolics; antioxidant; total antioxidant activity; oxidative damage; dietary antioxidant*

INTRODUCTION

Phenolic compounds are among the most widely distributed plant secondary products and are found in many plants used as foods and feeds. Recently, the ability of phenolic compounds to serve as antioxidants has been recognized (Decker, 1995), leading to speculation about the potential benefits of ingesting plant phenolics. The ability of small phenolics including flavonoids and phenolic acids to act as antioxidants has been extensively investigated (Rice-Evans et al., 1996), but the high molecular weight phenolics known as tannins have been largely neglected. Tannins are found in grains and legumes (Salunkhe et al., 1990), fruits (Foo and Porter, 1981), herbs (Haslam et al., 1989), and in beverages derived from plants. The average human consumption of tannins in U.S. is estimated to be at least 1 g/day (Pierpoint, 1990), so they could be a significant source of dietary antioxidants.

Tannins are naturally occurring phenolic compounds which precipitate protein. In general, tannins are high molecular weight (M_r > 500) and have many phenolic groups (Hagerman et al., 1997). There is significant chemical heterogeneity among the tannins, as illustrated by the representative compounds shown in Figure 1. Compounds typical of the three principle groups of tannins, the condensed tannins (proanthocyanidins), the hydrolyzable tannins, and the phlorotannins are depicted. Phlorotannins are found only in marine brown algae (Ragan and Glombitza, 1986) and are not widely consumed by humans. Our study was therefore limited to condensed tannins and hydrolyzable tannins.

For monomeric phenolics, the ability to act as antioxidants is dependent on extended conjugation, number and arrangement of phenolic substitutents, and molecular weight. For example, the flavonoids with the most hydroxyl groups were most easily oxidized (Hodnick et al., 1988), and for simple flavonoid oligomers, the degree of polymerization was correlated with the ability to scavenge free radicals (Ariga and Hamano, 1990). We hypothesized that tannins, which are highly polymerized and have many phenolic hydroxyl groups, are likely to be very effective antioxidants. To test this hypothesis, we evaluated representative tannins using methods which provide insights into both efficacy and mechanism of potential antioxidants.

Nonenzymatic antioxidants can either prevent free radical formation (e.g., by chelating redox active metal ions) or inhibit free radical chain-propagation reactions (e.g., by reacting with peroxyl radical to form stable free species) (Simic and Jovanovic, 1994). We evaluated tannins electrochemically to determine their ability to participate in biological redox reactions (Hodnick et al., 1988), with the deoxyribose assay system to determine their reactivity in Fenton (metal ion-catalyzed) systems (Aruoma, 1994), and with the metmyoglobin method to rank their abilities to react with active oxygen species represented by the stable radical cation $ABTS^{\bullet+}$ (Rice-Evans and Miller, 1994).

MATERIALS AND METHODS

Chemicals and Reagents. Methyl gallate, catechin, myoglobin, deoxyribose, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid, trichloroacetic acid, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Chemicals (St. Louis, MO). All other chemicals were reagent grade. Procyanidin (PC) was purified from Sorgum grain (*Sorghum biocolor* Moench variety IS8260, obtained from John Axtell, Purdue University, W. Lafayette, IN) according to Hagerman and Butler (1980). HPLC of phloroglucinol degradation products (Koupai-Abyazani et al., 1992) and C-13 NMR (Porter, 1989) were used to establish that the material was a B-1 type procyanidin comprised of 16 epicatechin extender units and a catechin terminal unit with average M_r of 4930 (Schofield et al., 1998). β-1,2,3,4,6-Penta-*O*-galloyl-D-glucose (PGG) (M_r 940) was purified from commercial tannic acid by solvent extraction (Hagerman et al., 1997) and was characterized by proton NMR and negative ion FAB-MS (Porter, 1989; Self et al., 1986). Al-

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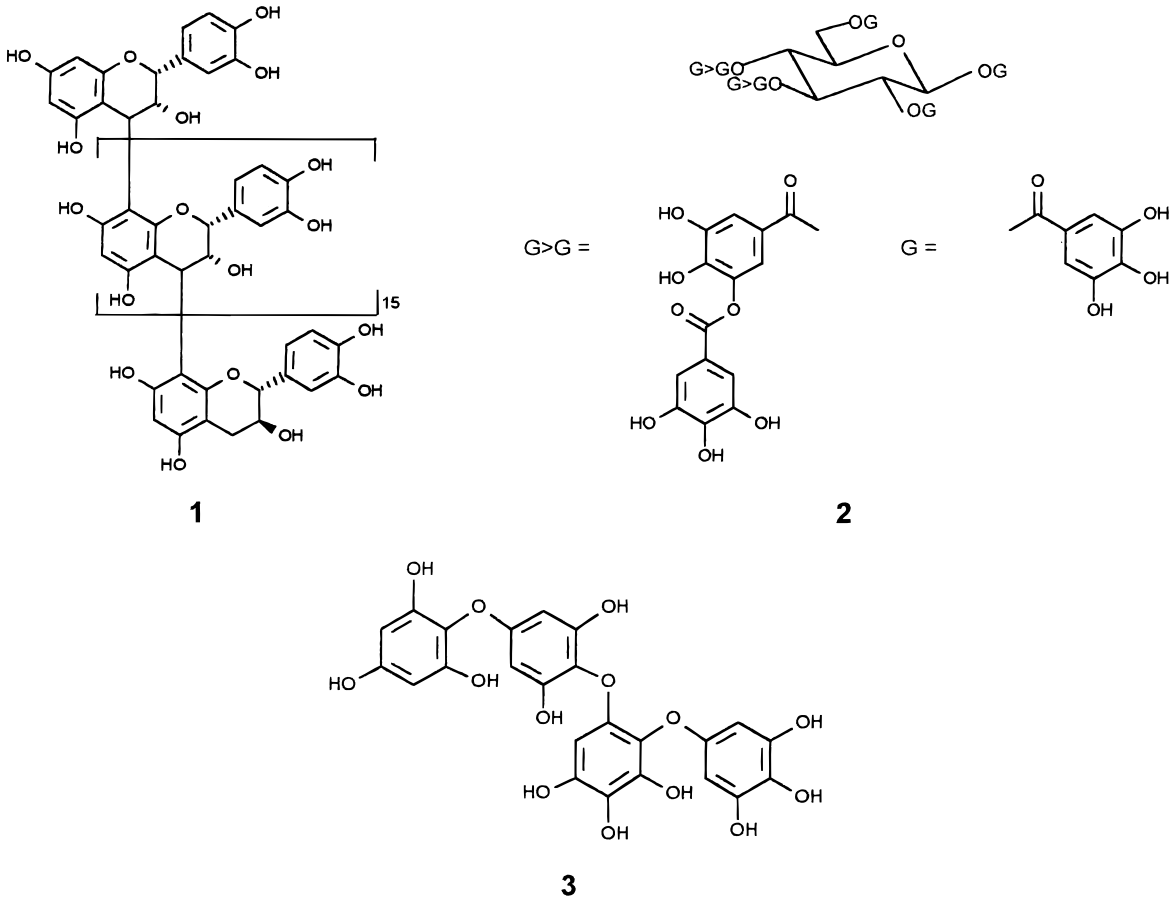

Figure 1. Typical tannins (polyphenolics). **1** is a typical procyanidin or condensed tannin (PC), made up of catechin and epicatechin. **2** is a hydrolyzable tannin (polygalloyl glucose), made up of a glucose core esterified with gallic acid residues. **3** is a phlorotannin (tetrafuhalol A), made up of phloroglucinol subunits.

though the redox potential of PGG was assessed, PGG is virtually water insoluble so it could not be tested in the biological antioxidant assays. Instead, polygalloyl glucose (polyGG) was prepared by Sephadex LH-20 chromatography of commercial tannic acid (Hagerman and Klucher, 1986) and was characterized by normal-phase HPLC (Hagerman et al., 1992). PolyGG used in these experiments was a mixture of penta- through decagalloyl glucoses with average M_r of 1351, corresponding to 7.64 galloyl/glucose.

Electrochemistry. Cyclic voltammetry was performed using an EG&G Princeton Applied Research model 173 Potentiostat/Galvanostat and a model 175 Universal Programmer following the method of Hayes et al. (1987). The cell consisted of a glassy carbon working electrode, a platinum wire counter electrode, and a Ag/AgCl reference electrode. The working electrode was polished with alumina between runs. The scan rate was 100 mV/s using 1×10^{-4} M samples in 1:1 (v/v) 0.05 M phosphate buffer:MeOH as the supporting electrolyte. The pH of the buffer was adjusted to 3.03, 4.41, 6.24, or 7.95. Solutions were purged with oxygen-free nitrogen before adding the phenolic, which was prepared as a concentrated solution in methanol. Samples were continuously blanketed with nitrogen during the runs. Redox potentials from the voltammograms were converted to values vs SHE by adding 221 mV to the values obtained vs Ag/AgCl (Cadenas and Ernster, 1990).

Deoxyribose Assay. The deoxyribose method for determining the rate of reaction of hydroxyl radical with a putative antioxidant was performed as described by Aruoma (1994). All solutions were prepared in metal-free water (NANOpure System, Barnstead/Thermolyne, Dubuque, IA). Reactions were performed in 10 mM phosphate buffer, pH 7.4, containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 μM $FeCl_3$, 100 μM EDTA, and the test material. Reactions were started by adding ascorbic acid to a final concentration of 100 μM. After incubating for 1 h at 37 °C, the color was developed by adding thiobarbituric acid followed by trichloroacetic acid and heating in a boiling water bath for 20 min. Samples were allowed to cool, and the chromophore was extracted into *n*-butanol before reading the absorbance at 532 nm. Absorbances were compared to control samples not containing test material. Control experiments were done with tetraethoxypropane to ensure that the test substances did not interfere with the color development stage of the assay. For pH experiments, the pH 7.4 phosphate was replaced with pH 6.4 phosphate or pH 8.4 phosphate. For some experiments, ascorbic acid was omitted and the reactions were started by addition of the test compound. For other experiments, EDTA was omitted.

Metmyoglobin Assay. The metmyoglobin method was performed as described in Miller et al. (1993). Metmyoglobin was prepared fresh daily by mixing equal volumes of 2 mg/mL myoglobin and 0.73 mM potassium ferricyanide and immediately applying the reaction mixture to a 20 cm Sephadex G-10 column equilibrated in 0.05 M phosphate buffer, pH 7.4, containing 0.145 M NaCl. The concentration and purity of the metmyoglobin in the first brown peak to elute from the column were assessed spectrophotometrically (Whitburn et al., 1982). The 520 μL reaction mixture contained 6.1 μM metmyoglobin, 610 μM ABTS, and an appropriate amount of the putative antioxidant. The absorbance at 600 nm was recorded, and the reaction was then started by adding hydrogen peroxide to make a final concentration of 250 μM. Exactly 3 min later, the absorbance was recorded again, and the change in absorbance was calculated.

RESULTS AND DISCUSSION

The cyclic voltammograms were used to estimate the electrochemical redox potentials of various phenolics

Table 1. Redox Potentials for Tannins and Simple Phenolics at Four pH Values[a]

	E (mV vs SHE)			
compd	pH 3.03	pH 4.41	pH 6.24	pH 7.95
catechol	881	796	696	596
catechin B ring	881	796	671	546
PC B ring	946	871	746	none
phloroglucinol	1121	1096	971	846
catechin A ring	1071	1046	946	796
PC A ring	1121	1096	996	896
pyrogallol	771, 971	721, 896	621, 796	496, 771
methyl gallate	771, 971	721, 946	571, 821	471, 596
PGG	796, 1046	721, 996	571, 971	721, 1021

[a] Redox potentials were determined by cyclic voltammetry in 1:1 methanol:phosphate buffer at the indicated pH values. The potentials were obtained using a silver/silver chloride reference electrode and were converted to SHE values by adding 221 mV (Cadenas and Ernster, 1990).

(Table 1). For all of the phenolics tested, only the oxidation half-reaction was observed, presumably because the scan rate was relatively slow and the oxidized species were quite reactive (Hodnick et al., 1988). Thus, the redox process is irreversible.

Each of the polymeric phenolics had several redox potentials. The functional group responsible for each redox potential was identified by cyclic voltammetry of simple phenolic compounds (Lunte et al., 1988). For example, the potential of catechol was similar to one of the potentials found for the flavonoid catechin and the flavonoid polymer PC (Table 1). We thus attributed that potential to the ortho-disubstituted B ring of the flavonoids (Figure 1). Similarly, the potentials that were similar to those of phloroglucinol were assigned to the meta-substituted A ring of the flavonoids (Figure 1). We believe that the redox potentials obtained for PGG are representative of those for other galloyl glucoses including the mixture of galloyl glucoses (polyGG) that we used in the biological antioxidant assays.

On the basis of their redox potentials, tannins are equivalent to or slightly worse than monomeric phenolics as reducing agents over the pH range 3−8. As expected (Simic and Jovanovic, 1994), the redox potentials of tannins were more negative at higher pH, with the single exception of the redox potential of PGG at pH 7.95 (Table 1). To inhibit free radical chain-propagation reactions, an antioxidant needs to have a redox potential which is more negative than about 1000 mV, the potential of the peroxyl radical at pH 7 (Simic and Jovanovic, 1994). Simple phenolics and tannins have potentials at pH 6−8 which are substantially below 1000 mV, indicating that they are thermodynamically able to reduce the peroxyl radical. None of the phenolics examined had potentials low enough to reduce α-tocopherol (E_7 480 mV) or ascorbic acid (E_7 280 mV) (Simic and Jovanovic, 1994).

Most redox active compounds react rapidly ($k = 10^9$−10^{10} M^{-1} s^{-1}) with the hydroxyl radical ($OH^•$), which is an extremely reactive free radical (E_7 2310 mV) (Simic and Jovanovic, 1994). The deoxyribose method provides a convenient way to determine the rate constant of water soluble compounds with $OH^•$. Using that method, we obtained a rate constant for mannitol (Figure 2, inset) of 1.1×10^9 M^{-1} s^{-1}, consistent with the literature value (Halliwell et al., 1987). The hydrolyzable tannin polyGG was a potent inhibitor of color formation in the deoxyribose assay (Figure 2). The calculated rate constant for reaction of polyGG with $OH^•$, 3.1×10^{11} M^{-1} s^{-1} at pH 7.4, was similar to the value reported for proteins (2.43-2.97 $\times 10^{11}$ M^{-1} s^{-1}) (Smith et al., 1992). In contrast, methyl gallate, the small phenolic that is structurally analogous to polyGG, is a pro-oxidant at pH 7.4. Low levels of methyl gallate *increase* oxidative damage to deoxyribose in the presence of the Fenton reagents (Figure 2) and thus methyl gallate is classified as a pro-oxidant (Aruoma et al., 1993). Therefore, the redox activity of hydrolyzable tannins cannot be predicted from the activity of related simple phenolics.

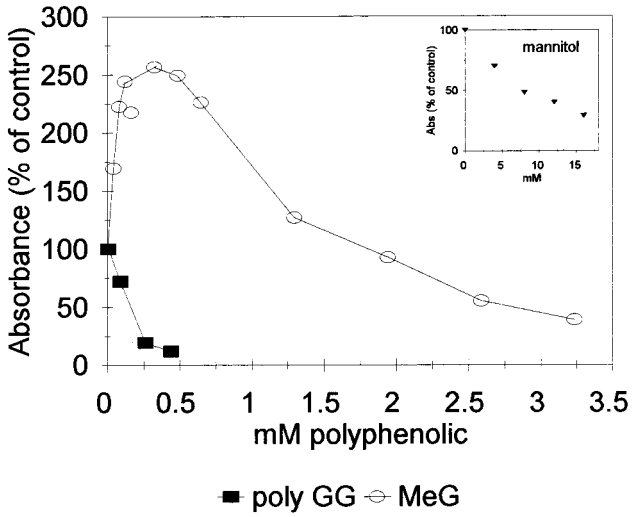

Figure 2. PolyGG and methyl gallate in the deoxyribose assay. The indicated concentrations of polyGG or methyl gallate were used as inhibitors in the standard deoxribose assay (Aruoma, 1994). The inset shows typical data obtained with mannitol, a standard inhibitor in this method. Points shown are the mean of 3 determinations. The standard deviation averaged 4.5% of the mean for these experiments.

Table 2. Rate Constants for Reaction of Phenolics with Hydroxyl Radical[a]

	rate constant (M^{-1} s^{-1})		
compd	pH 6.4	pH 7.4	pH 8.4
polyGG	3.0×10^{10}	3.1×10^{11}	9.3×10^{10}
methyl gallate	redox inactive	pro-oxidant	pro-oxidant
PC	redox inactive	weak pro-oxidant	4.5×10^{10}
catechin	9.2×10^9	inactive	weak pro-oxidant

[a] The deoxyribose method was used to determine rate constants (Aruoma, 1994). The values reported are based on linear regressions of triplicate values.

Similarly, activity of the PC polymer was different from that of catechin, a typical monomeric flavan-3-ol (Table 2). Catechin was inactive in the deoxyribose assay at pH 7.4; samples containing up to 0.5 mM catechin yielded absorbances identical to those of the controls. PC was a weak pro-oxidant, since as much as 0.5 mM PC in the deoxyribose assay at pH 7.4 increased the absorbance only to about 125% of the control; similar amounts of methyl gallate increased the absorbance as much as 250% (Figure 2). Using a slightly modified deoxyribose assay in which hydrogen peroxide was generated in situ, daSilva et al. (1991) found that catechin, catechin dimers, and catechin trimers reacted with hydroxyl radicals with a rate constants of about (2−3) $\times 10^9$ M^{-1} s^{-1} at pH 7.4.

We predicted that the ability of the phenolics to react with hydroxyl radical would be pH dependent, since

Table 3. Absorbances Obtained in the Deoxyribose Assay in the Absence of Ascorbic Acid (-AA) or EDTA (-EDTA) at Three pH Values[a]

condition	no test substance (control)	catechin 0.37 mM	PC 0.10 mm	methyl gallate 0.37 mM	polyGG 0.37 mM
-AA pH 6.4	0.101	0.211	0.555	0.151	0.119
-AA pH 7.4	0.185	0.266	0.41	1.132	0.198
-AA pH 8.4	0.215	0.301	0.083	1.218	0.243
-EDTA pH 6.4	0.699	0.444	0.311	0.309	0.248
-EDTA pH 7.4	0.469	0.361	0.209	0.185	0.314
-EDTA pH 8.4	0.480	0.217	0.132	0.204	0.383

[a] The deoxyribose assay (Aruoma, 1994) was performed with the indicated concentration of test substances at pH 6.4, 7.4, or 8.4. Values shown are average absorbances for three replicate determinations. The average standard deviation for these experiments was 8.6% of the mean.

phenolics are generally more easily oxidized at higher pH (Table 1) (Simic and Jovanovic 1994). Consistent with this idea, we found that PC reacted rapidly with $OH^{\bullet}$ at pH 8.4 (Table 2). Similarly, we found that polyGG was a better antioxidant at pH 7.4 or 8.4 than at pH 6.4 (Table 2). Reaction of simple monomeric phenolics in the deoxyribose assay did not follow the pH trends noted for electrochemical redox potential. Catechin, which was redox inactive at pH 7.4, was a very weak pro-oxidant at pH 8.4 and an antioxidant at pH 6.4 (Table 2). Methyl gallate was redox inactive at pH 6.4, and pro-oxidant at pH 7.4 and 8.4 in the deoxyribose assay (Table 2), as reported by Moran et al. (1997).

Compounds which act as pro-oxidants are thought to be detrimental since they may enhance oxidative damage. Some compounds are pro-oxidants because they are able to redox cycle the metal ion required for $OH^{\bullet}$ generation, and thus increase the radical concentration. We modified the deoxyribose method by omitting ascorbic acid (Aruoma, 1991) to evaluate the potential of tannins to behave as pro-oxidants. In this modification of the method, pro-oxidants substitute for ascorbic acid in the Fenton reaction and increase color formation over the ascorbic acid-free controls. We found that methyl gallate was a very effective substitute for ascorbic acid at pH 7.4 and 8.4, but was unable to substitute at pH 6.4 (Table 3). These results are consistent with its pro-oxidant activities in the standard deoxyribose assay (Figure 2 and Table 2) and consistent with other reports (Moran et al., 1997). Both catechin and PC were poor substitutes for ascorbic acid at the lower pH values (Table 3), consistent with their tendency to be redox inactive or weak pro-oxidants in the standard deoxyribose assay (Table 2). PolyGG was unable to substitute for ascorbic acid at any pH and is thus not a pro-oxidant (Table 2).

Some compounds inhibit color formation in the deoxyribose assay, not by reacting with $OH^{\bullet}$ but by chelating iron(III) and preventing $OH^{\bullet}$ formation (Aruoma, 1994). To identify compounds which chelate metals, the deoxyribose assay is performed without EDTA (Aruoma et al., 1987). In the absence of metal chelating test compounds, iron(III) is complexed to deoxyribose and causes "site specific" hydroxyl radical damage. When an iron-chelating test substance is present, hydroxyl radical damage and the accompanying color production is diminished. Moran et al. (1997) established that a wide range of low molecular weight phenolics chelate Fe(III) at pH 5.8 and 7.4, and we obtained similar results with

Table 4. Ability of Polyphenolics To Quench Radicals in the Metmyoglobin Method[a]

test substance	TEAC[b]	concn (mM) to give 50% inhibition	Trolox ratio[c]
catechin	2.61	0.86	2.64
methyl gallate	2.60	0.87	2.61
PC	≫3	0.080	28.4
polyGG	≫3	0.15	15.1
Trolox	1.0	2.27	1.0

[a] Triplicate determinations were fit with linear regression to calculate TEAC or concentration for 50% inhibition. The average SD for these determinations was 1.9% of the mean. [b] TEAC is the concentration of Trolox required to give the same inhibition as 1 mM test substance (Rice-Evans and Miller, 1994). [c] Trolox ratio is the concentration of Trolox to give 50% inhibition divided by the concentration of test substance to give 50% inhibition when the metmyoglobin method is run for a fixed time of 3 min.

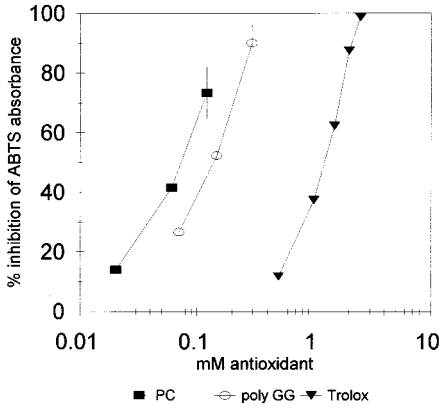

Figure 3. PolyGG and PC in the metmyoglobin assay. The indicated concentrations of polyGG, PC, or Trolox were used in the standard metmyoglobin assay. Points represent the mean of triplicate determinations, error bars indicate standard deviation. If no error bar is shown, the standard deviation was smaller than the marker used in the graph.

both the monomeric and polymeric phenolics tested (Table 3). It was notable that the interaction between methyl gallate and iron in the absence of EDTA was strong enough to prevent methyl gallate from acting as a pro-oxidant in the absence of EDTA.

To identify compounds which may have biologically relevant antioxidant activities, it is essential to measure reactivity with radicals less reactive than the hydroxyl radical. In the metmyoglobin assay, the reactivity of antioxidants with the stable radical cation $ABTS^{\bullet+}$ is compared to that of the standard antioxidant Trolox by calculation of the TEAC value (Trolox equivalent antioxidant activity) (Rice-Evans and Miller, 1994). We demonstrated that, like other antioxidants examined with this assay (Miller and Rice-Evans, 1997), monomeric and polymeric phenolic antioxidants react directly with $ABTS^{+\bullet}$ by showing that the phenolics could bleach preformed ABTS radical cations.

Using the metmyoglobin method, we obtained TEAC values for catechin and methyl gallate (Table 4), which were very similar to those reported by Rice-Evans et al. (1996). However, PC and polyGG were much more effective quenchers of the $ABTS^{\bullet+}$ than Trolox (Figure 3). TEAC values could not be calculated for PC and polyGG because 1 mM of either of these tannins completely quenched the $ABTS^{\bullet+}$. We developed a new parameter, the Trolox ratio, to allow us to express the antioxidant activity of tannins in the metmyoglobin assay. We have defined the Trolox ratio as the concentration of Trolox required for 50% inhibition of $ABTS^{\bullet+}$

color divided by the concentration of test substance required for 50% inhibition of $ABTS^{\bullet+}$ color when the metmyoglobin method is run for a fixed time of 3 min. For the antioxidants we tested, color formation is inversely related to the amount of test substance (average $r^2 = 0.99$ for linear regressions). The amount of test substance required for 50% inhibition of $ABTS^{\bullet+}$ color was calculated from the linear fit. Comparison to the value obtained for Trolox compensates for day-to-day variation in the assay. The ratio is very similar to the TEAC value for any compound which has potency similar to Trolox (Table 4), but has the advantage of allowing quantitative comparisons of compounds which are more active than Trolox. We found that PC and polyGG were far better inhibitors in the metmyoglobin than any other compounds tested (Table 4).

The ability of high molecular weight PC to effectively quench free radicals is consistent with trends noted by Ariga and Hamano (1990). They tested several oligomeric flavonoids and found that the ability to scavenge azobis-generated peroxyl radicals is proportional to degree of polymerization, so very effective scavenging by PC is not surprising. PC is a polymer based on flavan-3-ol subunits and is structurally quite different from the polyester polyGG (a hydrolyzable tannin). The very effective scavenging of $ABTS^{\bullet+}$ radicals by these two different types of tannin suggests that high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for free radical scavenging by tannins than specific functional groups.

When considering the likely role of dietary components, it is important to assess not only in vitro activity but also to consider the likelihood that the compound will be active after ingestion. Tannins are high molecular weight, form strong complexes with protein, and are resistant to degradation, so it is likely that they remain in the digestive tract and are not absorbed and transported to other tissues (Jimenez-Ramsye et al., 1994). We propose that because they are localized in the digestive tract, tannins may have unique roles as antioxidants. For example, they may spare other antioxidants and thus indirectly increase antioxidant levels in other tissues, or they may protect proteins, carbohydrates, and lipids in the digestive tract from oxidative damage during digestion (Marshall and Roberts, 1990). Our studies to date have focused on purified tannins with well-defined structures, but it will be important to conduct similar tests of the ability of tannin−protein complexes to inhibit oxidative damage. In preliminary experiments, we used soluble PC−gelatin or polyGG−gelatin complexes as antioxidants in the metmyoglobin method and found that the tannin−protein complex retained at least half the activity of the free tannin. It is possible that tannin-complexed proteins may be less susceptible to oxidative damage than free proteins and that tannins may be particularly important as an antioxidant for this important class of biomolecules.

Our data suggests that tannins, or polymeric polyphenolics, may be much more potent antioxidants than are simple monomeric phenolics. Furthermore, the tannins we tested had little or no pro-oxidant activity, although many small phenolics are pro-oxidants. Tannins are widespread in many plant-derived foods and thus may be important dietary antioxidants. The potential of tannins to diminish nutrient digestibility (Salunke et al., 1990) must be balanced against their potential to serve as biological antioxidants.

ABBREVIATIONS USED

PC, procyanidin (epicatechin$_{16}$−catechin); PGG, β-1,2,3,4,6-penta-*O*-galloyl-D-glucose; polyGG, mixture of penta- through decagalloyl glucoses with average M_r of 1351, corresponding to 7.64 galloyl/glucose; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); SHE, standard hydrogen electrode; TEAC, Trolox equivalent activity.

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