Inhibitory Effects of Cocoa Flavanols and Procyanidin Oligomers on Free Radical-Induced Erythrocyte Hemolysis

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Excessive peroxidation of biomembranes is thought to contribute to the initiation and progression of numerous degenerative diseases. The present study examined the inhibitory effects of a cocoa extract, individual cocoa flavanols (-)-epicatechin and (+)-catechin, and procyanidin oligomers (dimer to decamer) isolated from cocoa on rat erythrocyte hemolysis. *In vitro*, the flavanols and the procyanidin oligomers exhibited dose-dependent protection against 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis between concentrations of 2.5 and 40 μ*M*. Dimer, trimer, and tetramer showed the strongest inhibitory effects at 10 μ*M*, 59.4%, 66.2%, 70.9%; 20 μ*M*, 84.1%, 87.6%, 81.0%; and 40 μ*M*, 90.2%, 88.9%, 78.6%, respectively. In a subsequent experiment, male Sprague-Dawley rats (~200 g; *n* = 5–6) were given a 100-mg intragastric dose of a cocoa extract. Blood was collected over a 4-hr time period. Epicatechin and catechin, and the dimers (-)-epicatechin-(4β>8)-epicatechin (Dimer B2) and (-)-epicatechin-(4β>6)-epicatechin (Dimer B5) were detected in the plasma with concentrations of 6.4 μ*M*, and 217.6, 248.2, and 55.4 n*M*, respectively. Plasma antioxidant capacity (as measured by the total antioxidant potential [TRAP] assay) was elevated ($P < 0.05$) between 30 and 240 min following the cocoa extract feeding. Erythrocytes obtained from the cocoa extract-fed animals showed an enhanced resistance to hemolysis ($P < 0.05$). This enhanced resistance was also observed when erythrocytes from animals fed the cocoa extract were mixed with plasma obtained from animals given water only. Conversely, plasma obtained from rats given the cocoa extract improved the resistance of erythrocytes obtained from rats given water only. These results show cocoa flavanols and procyanidins can provide membrane protective effects.

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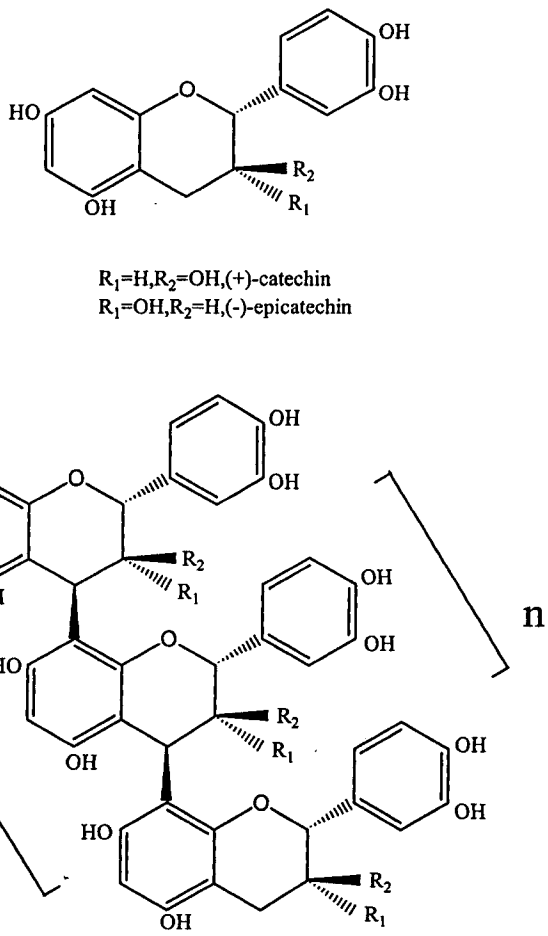
Excessive oxidative damage to cellular membranes is thought to contribute to the initiation and progression of numerous degenerative diseases, including certain cancers and cardiovascular disease (1–6). Erythrocytes are vulnerable to lipid peroxidation due to their high content of polyunsaturated lipids, their rich oxygen supply, and the presence of transition metals (7, 8). Reactive oxygen species (ROS) generated in the aqueous or lipid phase can attack erythrocyte membranes and can induce the oxidation of lipids and proteins, triggering disruptions in the membrane and hemolysis (5–9). Numerous investigations have used erythrocytes as model systems for studying biomembrane oxidative damage (5, 7, 9, 10). In many of these studies, free radical initiators such as 2,2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH) have been used to generate free radicals in the aqueous phase that can attack the erythrocyte membrane and propagate lipid peroxidation, leading to hemolysis (5, 9, 10). The erythrocyte has several membrane systems to protect itself against oxidation damage and hemolysis; these systems include superoxide dismutase, glutathione peroxidase, and catalase. In addition, water-soluble chain-breaking antioxidants such as ascorbic and uric acids can scavenge oxygen radicals residing in the aqueous phase, whereas lipid-soluble scavengers such as α-tocopherol can scavenge radicals within the lipid region of erythrocyte membranes (9). Although the erythrocyte oxidative defense system is robust, deficiencies in the above systems, a large oxidant insult, or certain disorders such as β-thalassaemia, sickle cell anemia, and glucose-6-phosphate dehydrogenase deficiency can increase the susceptibility of the erythrocyte to peroxidation (7).

Plant-derived polyphenols are significant constituents of the human diet. Diets rich in flavonoids (a class of polyphenols) have been associated with a reduced risk for cardiovascular disease (2, 11–16). Using *in vitro* systems, nu-

merous investigators have reported that isolated flavonoids can have antioxidant and anticarcinogenic activities (11, 17–19), and other investigators have reported that the consumption of flavonoid-rich foods and beverages can be associated with increases in plasma antioxidant capacity, reduced platelet aggregation, and improved endothelial function (20–24). Recently, there has been increasing interest in cocoa and chocolate as rich sources of flavonoids. The major flavonoids present in cocoa are the flavan-3-ol monomers, epicatechin and catechin, and procyanidin oligomers (dimer through decamer) built upon these monomeric units (Fig. 1) (25, 26). It has been reported that flavanols and procyanidin oligomers isolated from cocoa and chocolate have a number of antioxidative properties and can protect against the oxidation of human low-density lipoprotein (18, 27–29). Other researchers have reported that cocoa flavanols and the procyanidins can provide protection against peroxynitrite-dependent oxidation of dihydrorhodamine 123 and nitration of tyrosine, *in vitro* (30).

Given previous reports that there can be marked increases in plasma antioxidant capacity following the acute consumption of flavanol-rich foods (23, 31), we hypothesized that the ingestion of cocoa flavanols and procyanidin oligomers would reduce the risk of free radical-induced oxidative damage to the erythrocyte. The present study was designed to examine the concentration of cocoa flavanols and procyanidin oligomers in plasma obtained from healthy rats shortly after they were given a cocoa extract meal, and to characterize the effects of these flavonoids on erythrocyte hemolysis.



R_1=H,R_2=OH,(+)-catechin
R_1=OH,R_2=H,(-)-epicatechin

n: 0(dimer) to 8 (decamer)

Figure 1. Examples of the monomers and procyanidins isolated from cocoa.

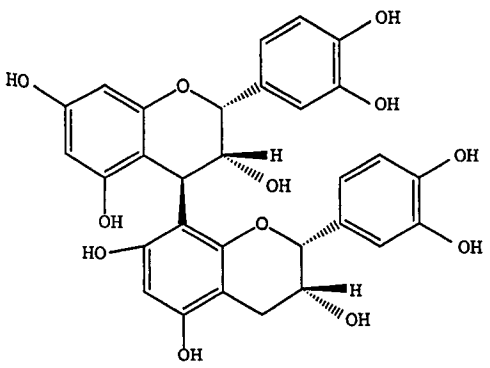
Materials and Methods

Materials. The cocoa extract, as well as monomers and procyanidin oligomers purified from cocoa, were provided by Mars Incorporated (Cocoapro; Hackettstown, NJ) (26). The composition of the cocoa extract was: 11.6% monomers, 8% dimers, 6.8% trimers, 6.5% tetramers, 5.6% pentamers, 5.3% hexamers, 2.7% heptamers, 1.9% octamers, 1.8% nonamers, 0.9% decamers, ~6.7% theobromine, ~1.4% caffeine, ~20%-30% sugar, and ~8%-10% anthocyanidins. The azo compound AAPH (a free radical initiator) was obtained from Polysciences Inc. (Warrington, PA). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

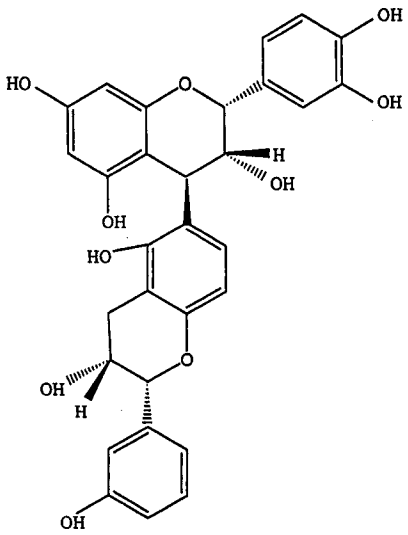
Animals. Male Sprague-Dawley rats (150–200 g) were used for this study. The rats were housed under normal laboratory conditions (21° ± 2°C, 12:12-hr light:dark cycle) with free access to a commercial rat diet for 1 week. The animal protocol used was in accordance with the Guide for the Care and Use of Laboratory Animals, and was approved by the University of California's (Davis) Animal Use and Care Committee.

***In Vitro* Study of the Anti-Hemolysis Activity of Cocoa Flavanols and Procyanidin Oligomers.** Rats were anaesthetised with CO_2 and were exsanguinated. Blood (5–7 ml/rat) obtained from the abdominal aorta was collected into heparinized tubes. Erythrocytes were separated from plasma and the buffy coat, and were washed three times with 5 vol of phosphate-buffered saline (PBS), pH 7.4 (Invitrogen, Carlsbad, CA). During the last wash, the erythrocytes were centrifuged at 3000*g* for 10 min to obtain a packed cell preparation (8). The packed erythrocytes were then suspended in 4 vol of PBS solution.

The peroxidation of erythrocyte membranes can be studied using a variety of agents, including hydrogen peroxide, dialuric acid, xanthine oxidase, and organic hydroperoxides (9, 32). Azo compounds (AAPH) generate free radicals by their unimolecular thermal decomposition in the aqueous region. The radicals produced quickly react with oxygen to produce peroxyl radicals that can attack the erythrocyte membrane. The rate of generation of free radicals can be easily controlled and measured by adjusting the concentration of the initiator. The hemolysis induced by an azo compound can provide a model for studying oxidative membrane damage when the free oxygen radical is present on the outside of the membrane (9, 32). In the present study,



(1) epicatechin (4β>8)epicatechin



(2) epicatechin (4β>6)epicatechin

Figure 2. Chemical structures of (1) epicatechin (4β>8) epicatechin (Dimer B2) and (2) epicatechin (4β>6) epicatechin (Dimer B5).

the method described by Miki *et al.* (8) was used to determine erythrocyte hemolysis mediated by AAPH. The addition of AAPH to the suspension of washed erythrocytes induces the oxidation of membrane lipids and proteins, resulting in hemolysis. Two milliliters of the erythrocyte suspension was mixed with 2 ml of PBS solution containing varying amounts of cocoa monomers and procyanidin oligomers (2.5–40 μ*M*). Two milliliters of 200 m*M* AAPH in PBS was then added to the mixture. The reaction mixture was shaken gently while being incubated at 37°C for 3 hr. After incubation, the reaction mixture was diluted with 8 vol of PBS and was centrifuged at 3000*g* for 5 min. The absorbance (*A*) of the supernatant fraction at 540 nm was recorded in a DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). The percentage of inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = [A_{AAPH} - A_{Oligomer}]/A_{AAPH} \quad (1)$$

Where $A_{Oligomer}$ is the absorbance of the sample containing either the cocoa monomer or procyanidin oligomers, and A_{AAPH} is the absorbance of the sample containing neither the cocoa monomer nor cocoa oligomers. L-ascorbic acid was used as a positive control. Five to six replicates were performed for each monomer and procyanidin, and the whole experiment was repeated at least three times on different days.

***In Vivo* Study of Anti-Hemolysis Activity of Cocoa Flavanols and Oligomers.** Rats (n = 5–6) were given, by gastric intubation, 5 ml of distilled water with or without 100 mg of the cocoa extract. The rats were anaesthetised with CO_2 and were exsanguinated 0, 30, 60, 120, 180, or 240 min after dosing. Blood from the aorta was collected into heparinized tubes. Erythrocytes from each rat were separated from the plasma by centrifugation at 3000*g* for 5 min at 4°C. The plasma was removed from the erythrocytes. After removing the buffy coat, the remaining erythrocytes were resuspended in the plasma. The reconstituted blood (erythrocytes plus plasma) was subjected to the hemolysis assay by adding 2 ml of AAPH solution and 2 ml of PBS followed by incubation at 37°C for 3 hr. As described above, 8 vol of PBS solution was added to the incubation mixture and this mixture was centrifuged at 3000*g* for 5 min. The absorbance of the supernatant fraction at 540 nm was measured and the percentage of inhibition induced by feeding the cocoa extract was calculated by Equation 2:

$$\% \text{ Inhibition} = [A_{CTL} - A_{COCOA\text{-}G}]/A_{CTL} \quad (2)$$

Where $A_{COCOA\text{-}G}$ is the absorbance of the reconstituted blood obtained from rats receiving intragastrically a solution containing 100 mg of the cocoa extract in 5 ml of distilled water, and A_{CTL} is the absorbance of the reconstituted blood obtained from rats receiving 5 ml of distilled water intragastrically.

High-Performance Liquid Chromatography (HPLC) Analysis of Monomers and Oligomers in Plasma. Plasma samples were extracted as described by Richelle *et al.* (33) and Rein *et al.* (31). The resulting solution was filtered with a 0.22-μm Ultrafree-MC low-binding Durapore centrifugal filter (Millipore, Bedford, MA). Fifty microliters of the filtered solution was analyzed for catechin, epicatechin, and procyanidin dimer by reversed-phase HPLC with coulometric multiple-array detection.

Chromatography was carried out as described by Holt *et al.* (33) using a 1100 HPLC system equipped with Chemstation software and a quaternary pump (Hewlett-Packard, Wilmington, DE), and a CoulArray 5600 coulometric electrochemical array detector (ESA, Chelmsford, MA). Separation was achieved using a reverse-phase Alltima C18 column (5 μm, 150 × 4.6 mm; Alltech, Deerfield, IL) with a C18 5-μm guard column. The mobile phase was mixed with the following two solvents: solvent A, 40% methanol and 60% 50 m*M* sodium acetate (EM Sciences, Darmstadt, Ger-

many) in water, pH 5.0; and solvent B, 7% methanol and 93% 100 m*M* sodium acetate in water, pH 5.2. A gradient elution was employed at a flow rate of 1 ml/min with the initial concentration of A set at 20%, which was held for 2 min. This was followed by a linear increase to 40% A for 10 min, immediately followed with another linear increase to 85% A for 13 min. The system was held at 85% of A for 17 min when at that time a linear increase to 100% A was achieved for 20 min. The system was then linearly decreased to 40% A for 23 min followed by another linear decrease to 20% A for 25 min.

Coulometric electrochemical array detection was carried out using the following cell settings: −50, +65, +150, +200, +250, +300, +700, and +800 mV. The resulting chromatographs were analyzed using CoulArray for Windows software (ESA). Epicatechin and catechin peak identification at +150 mV was based on co-elution with authentic standards and was quantified using external standards. Procyanidin dimer peak identification at +700 mV was based on co-elution with authentic standards and was quantified using external standards extracted from cocoa (Cocoapro; Mars Incorporated) (25).

Confirmation of Monomers and Dimers Using LC-Mass Spectrometry (MS). Monomers and dimers (Figs. 1 and 2) were confirmed in the plasma using HPLC-MS. HPLC-MS analyses were performed on an Agilent 1100 Series HPLC (Agilent, Palo Alto, CA) equipped with an auto-injector, quaternary HPLC pump, column heater, and diode array detector, and interfaced to an Agilent Series 1100 MS trap. Reverse-phase separations of the plasma extracts were performed on a Phenomenex (Torrance, CA) 5-μm Kingsorb C18 column (250 × 4.6 mm). UV detection was recorded at λ = 280 nm. The binary mobile phase consisted of A, 0.5% acetic acid in water; and B, 0.5% acetic acid in acetonitrile. Separations were effected by a series of linear gradients of B into A at a flow rate of 0.8 ml/min as follows: elution starting with 5% B in A, 0 to 5 min; 5% to 50% B in A, 5 to 40 min; 50% to 95% B in A, 40 to 45 min; 45 to 50 min isocratic.

Methanol was added via a tee in the eluant stream of the HPLC just prior to mass spectrometer and was delivered with an Agilent 1100 series HPLC pump at 0.2 ml/min. Conditions for analysis in the negative ion mode include a capillary voltage of 3 kV, the skim 1 at −34 V, the capillary exit offset at −60 V, the drying gas temperature at 350°C, and the drying gas flow at 9.0 liters/min. Spectra were scanned over a mass range of *m/z* 100 to 1000.

Determination of Plasma Antioxidant Capacity. Plasma antioxidant capacity was determined as previously described (23, 35) with some modification. Plasma samples were diluted 1:10 with 1 m*M* PBS, pH 7.4 and they were added to a solution containing 20 m*M* AAPH and 16.6 μ*M* 5-amino-2, 3-dihydro-1, 4-pthalazinedione (luminol). The chemiluminescence was recorded for 20 min using a HTS 7000 Series Bio Assay Reader (Perkin Elmer, Buckinghamshire, UK). The plasma antioxidant capacity value was calculated as the lag time before an increase in the chemiluminescence was observed. This lag time is proportional to the cumulative amount of antioxidants present in the samples (35). A reference lag time was obtained by using a known amount of 6-hydroxy-2, 5, 7, 8-tetramethoxychroman-2-carboxylic acid (Trolox; Aldrich Chemical Co., Milwaukee, WI). Plasma antioxidant capacity is expressed as Trolox equivalents.

Statistics. Results are expressed as means ± SEM. Data for hemolysis inhibition were subjected to analysis of variance (ANOVA), and the means were compared between treatments by paired *t* test. Statistical significance was assessed at the 5% level. Analyses were performed using routines available in Staview for Windows (version 5.0.1.; SPSS Institute Inc., Cary, NC).

Results

The Effect of Cocoa Flavanols and Procyanidin Oligomers on *In Vitro* Erythrocyte Hemolysis. The influence of the cocoa extract on *in vitro* erythrocyte hemolysis was examined by incubating rat erythrocytes in the presence of 200 m*M* AAPH as an initiator of oxidation. The cocoa extract provided a strong inhibitory effect against erythrocyte hemolysis. On a weight basis, the inhibitory effects of the cocoa extract were almost twice that of ascorbic acid (Fig. 3).

All 10 fractions tested (monomers through decamers) inhibited *in vitro* AAPH-induced red blood cell hemolysis in a dose-dependent manner (concentrations ranged from 2.5 to 40 μ*M*), but the antioxidative activity of the individual fractions differed significantly (Table I). The dimer, trimer, and tetramer fractions demonstrated the strongest

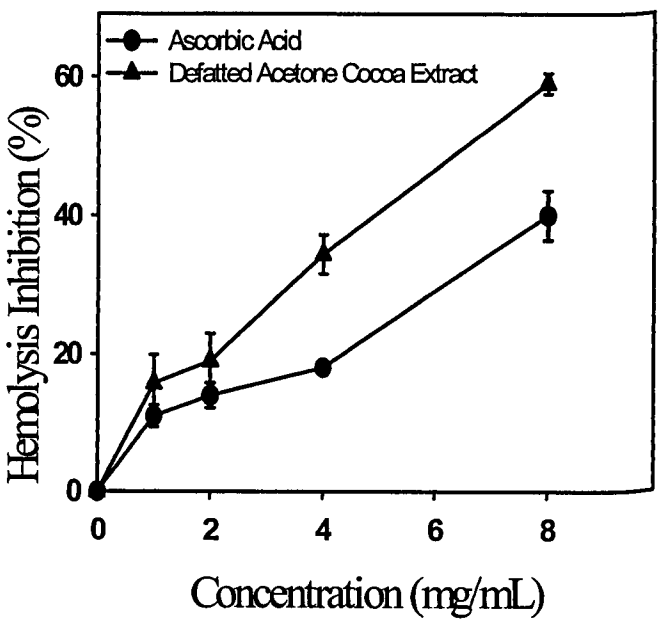


Figure 3. Inhibitory effects of cocoa extract on AAPH-induced hemolysis of rat erythrocytes *in vitro*. Data are expressed as mean ± SEM of *n* = 6 samples.

Table I. Inhibitory Effect (%) of Individual Cocoa Procyanidins on AAPH-Induced Hemolysis of Rat Erythrocytes *in Vitro*[a]

	2.5 μM	5 μM	10 μM	20 μM	40 μM
Monomer	4.87 ± 0.64[b]	12.61 ± 1.02[b]	25.20 ± 1.25[b]	55.56 ± 2.55[b]	80.80 ± 3.51[b]
Dimer	11.88 ± 1.61[c]	31.86 ± 3.66[c]	59.43 ± 2.25[c]	84.11 ± 1.35[c]	90.24 ± 0.99[c]
Trimer	22.59 ± 2.56[d]	40.90 ± 3.01[d]	66.22 ± 2.54[c]	87.58 ± 1.65[c]	88.88 ± 1.74[c]
Tetramer	25.75 ± 1.21[d]	46.35 ± 3.23[d]	70.94 ± 0.29[d]	80.98 ± 0.11[d]	78.56 ± 1.56[b]
Pentamer	30.50 ± 2.84[e]	49.78 ± 3.60[d]	55.81 ± 3.87[c]	62.21 ± 1.87[b]	70.10 ± 2.65[b]
Hexamer	35.99 ± 2.66[e]	44.20 ± 2.80[d]	46.33 ± 4.87[d]	60.94 ± 2.54[b]	66.45 ± 2.95[b]
Heptamer	35.78 ± 1.96[e]	36.59 ± 4.99[d]	40.49 ± 3.32[d]	54.64 ± 1.53[b]	70.07 ± 2.10[b]
Octamer	30.15 ± 2.74[e]	33.64 ± 3.34[c]	44.74 ± 2.32[d]	55.38 ± 1.56[b]	75.21 ± 3.21[b]
Nonamer	29.14 ± 2.60[e]	28.80 ± 3.56[c]	42.32 ± 2.32[d]	50.27 ± 2.63[b]	72.07 ± 0.60[b]
Decamer	27.07 ± 1.70[d]	31.26 ± 2.12[c]	42.65 ± 1.89[d]	48.50 ± 3.21[b]	72.92 ± 3.75[b]
Ascorbic acid	8.93 ± 2.30[b]	8.49 ± 2.50[b]	15.32 ± 0.80[e]	13.05 ± 2.76[e]	31.71 ± 2.65[d]

[a] Data are expressed as mean ± SEM of n = 5–6.
[b,c,d,e] In the same column with different superscripts differ significantly at $P < 0.05$.

protective activity toward erythrocyte hemolysis. The monomers and pentamer to decamer fractions had similar protective effects. All of the fractions provided a protective effect that exceeded that of ascorbic acid on a weight basis (Table I).

Identification and Confirmation of Monomers and Dimers in Plasma. Plasma obtained from rats fed the cocoa extract (~0.5g/kg body weight) contained measurable concentrations of both monomers and dimers. Using LC-CoulArray, the peaks eluting at 16 and 22 min (Fig. 4) were identified as catechin (CT) and epicatechin (EC), respectively, based on retention time compared with authentic compounds. These identifications were confirmed using LC-MS (Fig. 5, peaks 1 and 3) based on molecular weight ($[M-H]^-$ 289) and retention time with authentic standards. Besides the two monomer peaks, two additional peaks were detected in the LC-CoulArray chromatograph (Fig. 4, peaks at 19 and 24 min). Using LC-MS, these peaks were identified as dimers based on molecular weight ($[M-H]^-$ 577) and retention time compared with purified standards (Fig. 5, peaks 2 and 4). We later characterized the dimers as epicatechin-(4β>8)-epicatechin (Dimer B2), and (-)-epicatechin-(4β>6)-epicatechin (Dimer B5) (36). The concentrations of epicatechin and catechin and both dimers increased in the plasma 30 min after dosing with the cocoa extract, with maximal concentrations reached between 60 and 120 min (Fig. 6). The average peak plasma concentrations of epicatechin and catechin were 6.4 μM and 217.6 nM, respectively, whereas the two dimers, B2 and B5, reached average peak concentrations of 248.2 and 55.4 nM, respectively.

The Effect of Cocoa Extract on *In Vivo* Erythrocyte Hemolysis. Plasma antioxidant capacity as measured by the total antioxidant potential (TRAP) assay significantly increased following administration of the cocoa extract (Fig. 7a). Within 3 hr after the consumption of cocoa extract, plasma antioxidant capacity increased more than 2-fold and remained unchanged for 4 hr. Consistent with the above, oral administration of the cocoa extract reduced the extent of AAPH-induced hemolysis (Fig. 7b).

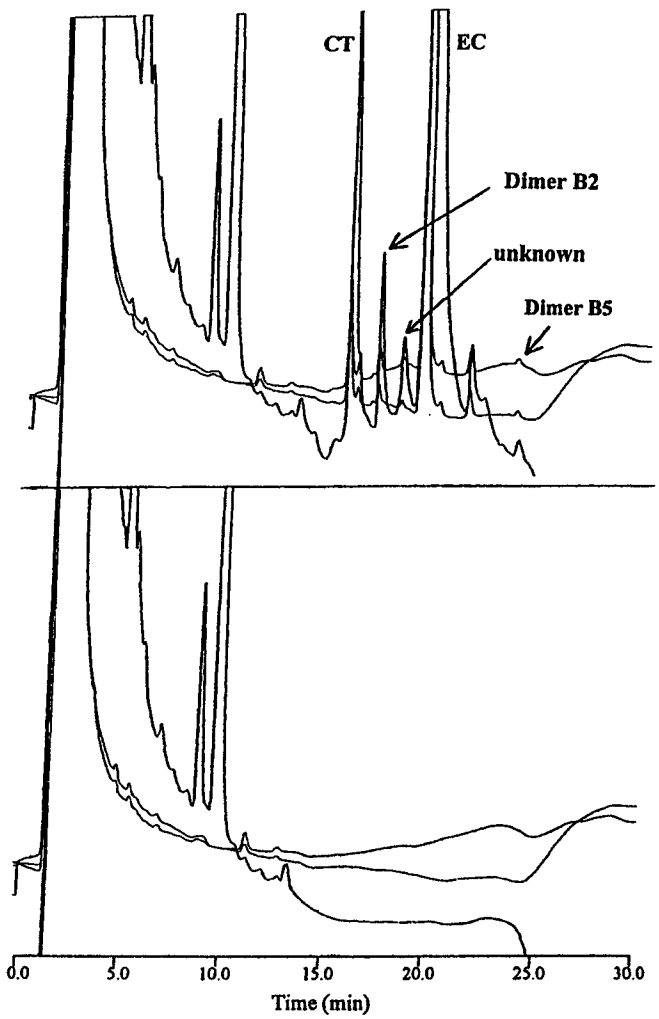


Figure 4. Typical HPLC chromatograms of plasma extracts from rats given either 100 mg of cocoa extract in 5 mL of distilled water (top), or 5 mL of distilled water alone as a control (bottom). The data shown represents results at 1 hr post-feeding.

Given the results in Figure7b, a second set of animals was studied. For this work, erythrocytes from rats fed the cocoa extract were incubated either in plasma from rats given water only, or in plasma from rats given the cocoa

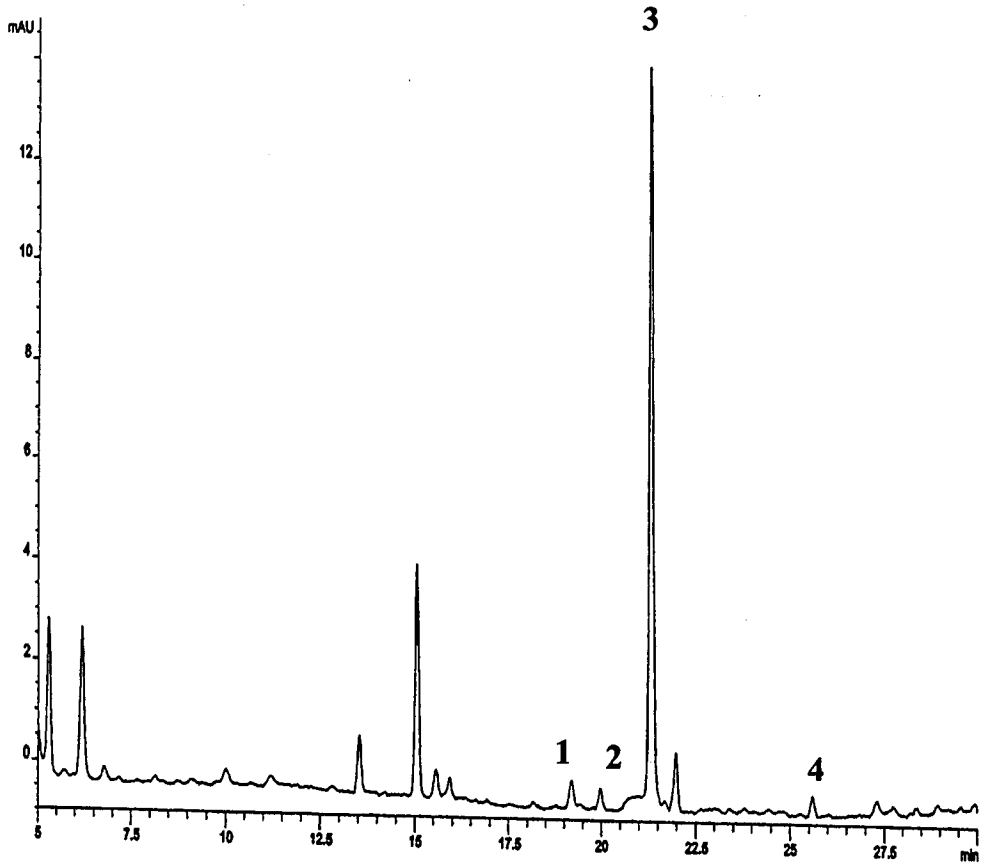


Figure 5. Typical LC-MS chromatograms for rat plasma obtained 1 hr after the oral administration of a cocoa extract (0.5 g/kg body weight, with enzyme treatment using β-glucuronidase). The molecular ion [M-H] peaks obtained at *m/z* 289 (1 and 3) and at *m/z* 577 (2 and 4) were identified as 1, (+)-catechin; 2, Dimer B2; 3, (-)-epicatechin; and 4, Dimer B5, respectively.

extract. Finally, erythrocytes from the rats given water were incubated in plasma from rats given the cocoa extract. As is depicted in Figure 7, cells obtained from rats that were fed the cocoa extract showed the greatest resistance to hemolysis when they were incubated in plasma obtained from rats also given the cocoa extract (Fig. 7b). However, protection was observed when erythrocytes from rats fed the cocoa extract were incubated in plasma from rats given water (Fig. 7c), as well as when erythrocytes from rats given water only were incubated in plasma from rats fed the cocoa extract (Fig. 7d).

Discussion

Cocoa and chocolate are rich sources of natural polyphenol antioxidants (37, 38). Prominent among these are the monomeric flavanols, epicatechin and catechin, and the procyanidin oligomers made from these monomeric units. In a recent Dutch survey, tea was reported to be the most important source of catechins in the diet (55% of total intake), whereas chocolate contributed approximately 20% of the total intake (38). However, this study took into account only the monomers (flavanols). The inclusion of the oligomers (dimers through decamers) into their calculations would have markedly increased the calculated contribution of chocolate to dietary flavonoid intake (39).

The excessive peroxidation of biomembranes is accepted as one of the processes by which tissues can be damaged during ischemia/reperfusion, inflammation and aging (1–9). The peroxidation of erythrocyte membranes and hemolysis induced by various agents such as hydrogen peroxide, dialuric acid, xanthine oxidase, organic hydroperoxides, and AAPH (9, 32) has been extensively studied as a model for membrane-peroxidative damage. In the present study, we investigated the antioxidative activity of a cocoa extract. A strong dose-dependent protection was demonstrated toward the hemolysis of red blood cells *in vitro* (Fig. 3). The inhibitory effect of the cocoa extract was stronger than ascorbic acid, which has been shown to act as an antioxidant against human low-density lipoprotein oxidation (40).

When the individual cocoa monomer and oligomer fractions were tested, all of the fractions were highly effective in inhibiting AAPH-induced hemolysis. Interestingly, the antioxidative activities of the oligomers, dimer to decamer, were higher than that of those of the monomers (Table I). This is in agreement with recent studies that have shown differential effects of the procyanidin oligomers. For example, procyanidin oligomers (dimers and trimers) isolated from apple juice were found to exhibit higher growth-promoting activities for hair epithelial cells than monomeric

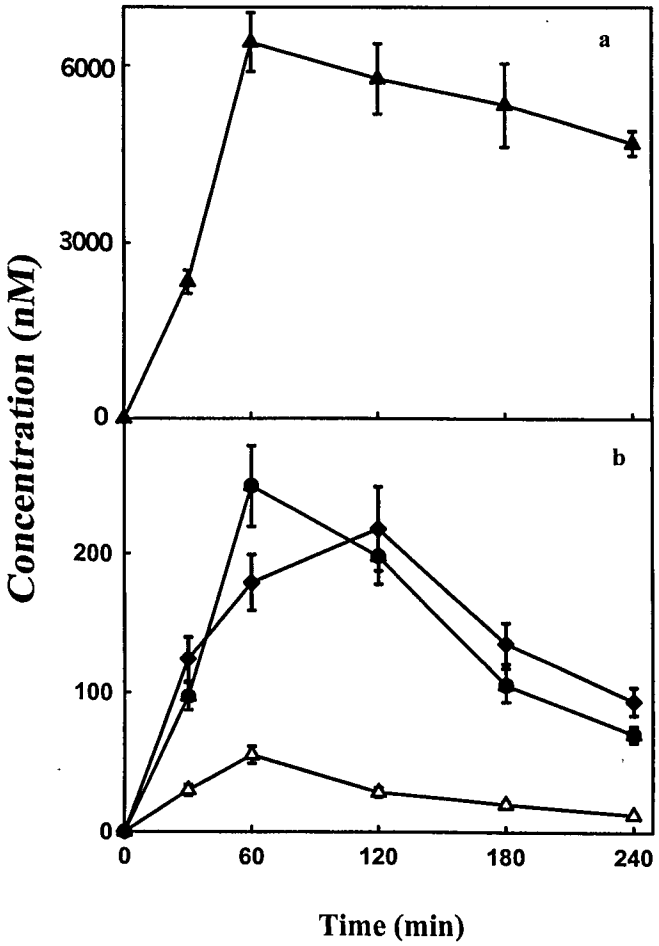


Figure 6. Changes in plasma concentrations of (a) (-)-epicatechin (▲-▲) and (b) (+)-catechin (◆-◆) and the procyanidin dimers, dimer B2 (●-●) and dimer B5 (△-△) in rats given an oral dose of 100 mg of cocoa extract. Data are expressed as means ± SEM of n = 5–6 rats.

epicatechin (41). Similarly, Arteel and Sies (30) reported that cocoa dimers, trimers, and tetramers were more effective against peroxynitrite-dependent oxidation than the larger oligomers. Lotito *et al.* (18) also reported that the dimers and trimers were stronger antioxidants than the larger oligomers in a liposome model. The reasons for these differential effects deserve further study; they most likely include chemical properties such as differences in partition coefficients, differences in the three-dimensional structure of the oligomers, and in their ability to chelate ions and stabilize free radicals. It is important to note that the first two factors would influence the ability of the compounds to interface with biological membranes. In this regard, Tsuchiya (42) has reported that tea catechin and epicatechin have markedly different effects on liposome membranes.

Potential mechanism(s) by which cocoa flavanols and procyanidins may protect erythrocytes from hemolysis include the following: (i) The flavanols and procyanidins may function as primary antioxidants by directly reducing the formation of free radicals mediated by AAPH; (ii) they may spare, maintain, or regenerate α-tocopherol and other antioxidants by donating a hydrogen to the α-tocopherol radicals (43); and (iii) they may function as chelators and bind redox active metals, including $Fe^{2+}$and Cu^{2+}, involved in the initiation of free radicals.

A better understanding of the protective role of dietary antioxidants *in vivo* requires quantitative data on their absorption (44). Until recently, only limited information has been available concerning the absorption of cocoa flavanols and its oligomers. To our knowledge, the current paper represents the first report of the presence of the two procyanidin dimers, Dimer B2 and Dimer B5, in plasma following the feeding of cocoa in a rodent model. When the rats were given 100 mg of the cocoa extract (~0.5g/kg body weight) intragastrically, Dimer B2 and Dimer B5 reached maximum concentrations 1 to 2 hr post-administration. In the present study, a single procyanidin intake of 51 mg from 100 mg of cocoa extract resulted in plasma concentrations of epicatechin, catechin, Dimer B2, and Dimer B5 of 6.4 μ*M*, and 217.6, 248.2, and 55.4 n*M*, respectively. It is interesting to note that the ratio of epicatechin to catechin (EC:CT) in plasma is significantly different from the profile of the two monomers in the cocoa extract, which is approximately 2:1 (36). Baba *et al.* (44) has also reported an EC:CT in cocoa of about 2. However, in marked contrast to the EC:CT in cocoa, the EC:CT in plasma was close to 30 (Fig. 6). It is also interesting to note that although the ratio of Dimer B2 to Dimer B5 is approximately 2 in the cocoa extract (36), the measured ratio of the two dimers in rat plasma exceeded 4 (Fig. 6b). The reasons for these marked differences in the ratios deserve further study, but may be due to preferential absorption, *in vivo* isomerization, or the partial decomposition of the higher molecular weight procyanidins into monomers and dimers (34, 46). Given that the different isomers can have different biological effects, this is an area that merits further investigation.

The present study shows that the ingestion of a cocoa extract elicited a rise in plasma antioxidant capacity (Fig. 7a). This is consistent with reports that acute consumption of flavonoid-rich cocoa and chocolate can result in increased plasma antioxidant activity in healthy human adults (23). These observations are also consistent with Lotito's (47) report that addition of catechin to human plasma can prevent the accumulation of 2-thiobarbituric acid-reactive substances, and can delay the depletion of α-tocopherol and β-carotene oxidized by AAPH or 2,2'-azobis (2,4-valeronitrile).

A recent editorial by Nestel (48) brought into question the robustness of using total antioxidant capacity in biological fluids as a biomarker. That the increase in plasma antioxidant capacity observed following cocoa feeding is functionally significant is suggested by our erythrocyte hemolysis data. When we mixed erythrocytes from rats given water with plasma collected at different time points from rats fed the cocoa extract, the degree of hemolysis inhibition was related to the concentration of monomers and dimers in the plasma (Fig. 7d). In contrast, when plasma obtained

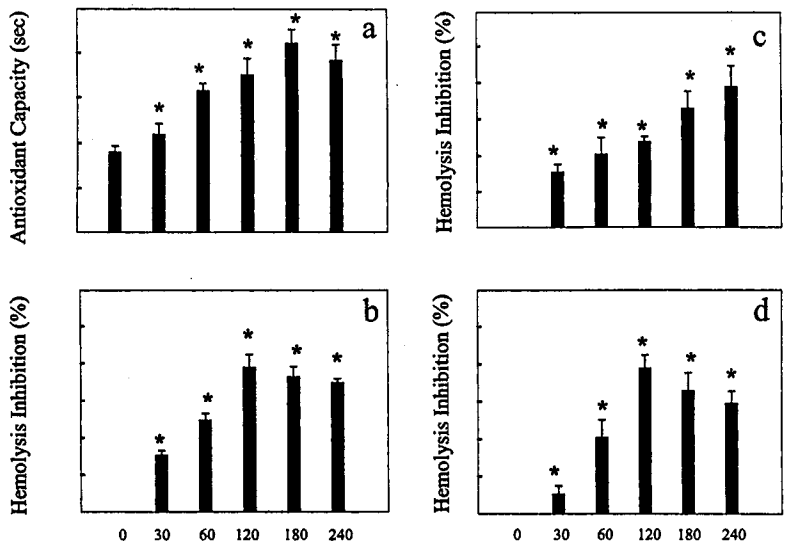


Figure 7. (a) The antioxidant capacity (as measured by the TRAP assay) of plasma obtained from rats 0, 30, 60, 120, 180, and 240 minutes after they were given an intragastric dose of 100 mg of cocoa extract in 5 mL of distilled water. The inhibition of AAPH-induced hemolysis of (b) red blood cells from rats given the cocoa extract and plasma from rats given the cocoa extract (c) red blood cells from rats given the cocoa extract mixed with the plasma from control (water only) rats (d) red blood cells from control rats mixed with the plasma from rats given the cocoa extract. Data are expressed as mean ± SEM of $n = 5–6$ rats. Mean values with an asterisk are significantly different from baseline values: $^*P < 0.05$.

from rats given water alone was mixed with erythrocytes obtained from rats given the cocoa extract, the protective effects of the cocoa feeding on the erythrocytes continued to increase at 180 and 240 min, whereas the plasma monomers and dimers started to decrease in concentration after 120 min (Figs. 7c and 6). The above data suggest that some polyphenols are binding to the erythrocytes during this time period. This hypothesis may also explain the observation that the protection in plasma mixed with erythrocytes, both from the blood of different time points, peaked at 120 min and remained almost unchanged over the 4-hr test period (Fig. 7b). Further work involving the characterization of the membrane binding and membrane action of catechin, epicatechin, and the procyanidin oligomers is clearly warranted.

In conclusion, the cocoa extract and the individual cocoa flavanols and procyanidin oligomers demonstrated a dose-dependent antioxidant activity against *in vitro* erythrocyte hemolysis. Epicatechin, catechin, Dimer B2, and Dimer B5 were detected in plasma within 30 min following the feeding of a cocoa extract. Concordant with this rise, we observed an increase in plasma antioxidant capacity, and a concurrent decrease in erythrocyte hemolysis. Thus, the flavanols catechin and epicatechin, and the procyanidin oligomers (at least the dimers) from flavanol-rich foods (e.g., cocoa and chocolate) can be absorbed. The absorption of these compounds is associated with an increase in plasma antioxidant capacity and a reduction in the susceptibility of erythrocyte membranes to oxidation. The results support the concept that the consumption of flavonoid-rich foods can be associated with improvements in oxidant defense mechanisms. We suggest that the above contributes to the reported positive vascular health benefits of flavonoid-rich foods.

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1. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signalling, and cell injury. Free Rad Biol Med **28:**1456–1462, 2000.
2. Morton LW, Caccetta RA-A, Puddey IB, Croft KD. Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. Clin Exp Pharmacol Physiol **27:**152–159, 2000.
3. Morena M, Cristol J, Canaud B. Why hemodialysis patients are in a prooxidant state: What could be done to correct the pro/antioxidant imbalance. Blood Purification **18:**191–199, 2000.
4. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med **119:**598–620, 1992.
5. Niki E, Komuro E, Takahashi M, Urano S, Ito E, Terao K. Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J Biol Chem **263:**19809–19814, 1988.
6. Kris-Etherton PM, Keen CL. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol 13(1):41–49, 2002.
7. Chiu D, Lubin B, Shohet S. Peroxidative reactions in red cell biology. In: Pryor W, Ed. Free Radicals in Biology. New York: Academic Press, Vol V:pp115–160, 1982.
8. Puppo A, Halliwell B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Biochem J **249:**185–190, 1988.
9. Miki M, Tamai H, Mino M, Yamamoto Y, Niki E. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch Biochem Biophys **258:**373–380, 1987.
10. Mabile L, Piolot A, Boulet L, Fortin L-J, Doyle N, Rodriquez C, Davignon J, Blache D, Lussier-Cacan S. Moderate intake of n-3 fatty acids is associated with stable erythrocyte resistance to oxidative stress in hypertriglyceridemic subjects. Am J Clin Nutr **74:**449–456, 2001.
11. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev **52:**673–751, 2000.
12. Rimm EB, Ascherio A, Giovannucci E, Spiegelman D, Stampfer MJ, Willett WC. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men. J Am Med Assoc **275:**447–451, 1996.
13. Liu S, Manson JE, Lee I-M, Cole SR, Hennekens CH, Willett WC, Buring JE. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. Am J Clin Nutr **72:**922–928, 2000.
14. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet **342:**1007–1011, 1993.

15. Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M, Simic BS, Toshima H, Feskens EJ, Hollman PC, Katan MB. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med **155:**381–386, 1995.
16. Knekt P, Jarvinene R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Finland: a cohort study. Br Med J **312:**478–481, 1996.
17. Rice-Evans C, Nicholas J, Molwell P, Bramley P, Pridham J. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radic Res **22:**375–383, 1995.
18. Lotito SB, Actis-Goretta L, Renart L, Caligiuri M, Rein D, Schmitz HH, Steinberg FM, Keen CL, Fraga CG. Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun **276:**945–951, 2000.
19. Stoner G, Mukhtar H. Polyphenols as cancer chemopreventive agents. J Cell Biochem **22:**169–180, 1995.
20. Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, Keen CL. Cocoa inhibits platelet activation and function. Am J Clin Nutr **72:**30–35, 2000.
21. Freedman JE, Parker CI, Li L, Perlman JA, Prei B, Ivanov V, Deak LR, Iafrati MD, Folts JD. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation **103:**2792–2798, 2001.
22. Schramm DD, Wang JF, Holt RR, Ensunsa JL, Gonsalves JL, Lazarus SL, Schmitz HH, German JB, Keen CL. Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. Am J Clin Nutr **73:**36–40, 2001.
23. Wang JF, Schramm DD, Holt RR, Ensunsa JL, Fraga CG, Schmitz HH, Keen CL. A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. J Nutr **130:**2115S–2119S, 2000.
24. Duffy S, Keaney JJ, Holbrook M, Gokce N, Swerdloff PL, Frei B, Vita JA. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. Circulation **104:**151–156, 2001.
25. Hammerstone JF, Lazarus SA, Mitchell AE, Rucker R, Schmitz HH. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. J Agric Food Chem **47:**490–496, 1999.
26. Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, Kremers BG, Hammerstone JF, Rucker RB, Ritter KA, Schmitz HH. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J Agric Food Chem **47:**4184–4188, 1999.
27. Waterhouse AL, Shirley JR, Donovan JL. Antioxidants in chocolate. Lancet **348:**834, 1996.
28. Kondo K, Hirano R, Matsumoto A, Igarashi O, Itakura H. Inhibition of LDL oxidation by cocoa. Lancet **348:**1514, 1996.
29. Pearson DA, Schmitz HH, Lazarus SA, Keen CL. Inhibition of in vitro low-density lipoprotein oxidation by oligomeric procyanidins present in chocolate and cocoas. In: Packer L, Ed. Methods in Enzymology. New York: Academic Press, pp350–360, 2001.
30. Arteel GE, Sies H. Protection against peroxynitrite by cocoa polyphenol oligomers. FEBS Lett **462:**167–170, 1999.
31. Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. J Nutr **130:**2109S–2114S, 2000.
32. Hatherill R, Till G, Ward P. Mechanisms of oxidant-induced changes in erythrocytes. Agents Actions **32:**351–358, 1991.
33. Richelle M, Tavazzi I, Enslen M, Offord E. Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr **53:**22–26, 1999.
34. Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, Fraga CG, Schmitz H, Keen CL. Procyanidin dimer B2 (epicatechin-(4β-8)-epicatechin) in human plasma after the consumption of a flavanol-rich cocoa. Am J Clin Nutr (in press) 2002.
35. Lisse E, Salim-Hanna M, Pascual C, del Castillo MD. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med **18:**153–158, 1995.
36. Zhu QY, Holt RR, Lazarus SA, Ensunsa JL, Hammerstone JF, Schmitz HH, Keen CL. Stability of flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. J Agric Food Chem **50:**1700–1705, 2002.
37. Rice-Evans C. Flavonoid antioxidants. Curr Med Chem **8:**797–807, 2001.
38. Arts I, Hollman P, Kromhout D. Chocolate as a source of tea flavonoids. Lancet **354:**488, 1999.
39. Lazarus SA, Hammerstone JF, Schmitz HH. Chocolate contains additional flavonoids not found in tea. Lancet **354:**1825, 1999.
40. Ma Y, Stone W, Leclair I. The effects of vitamin C and urate on the oxidation of human low-density lipoprotein. Proc Soc Exp Biol Med **206:**53–59, 1994.
41. Takahashi T, Kamiya T, Hasegawa A, Yokoo Y. Procyanidin oligomers selectively and intensively promote proliferation of mouse hair epithelial cells *in vitro* and activate hair follicle growth *in vivo*. J Invest Dermatol **112:**310–316, 1999.
42. Tsuchiya H. Stereospecificity in membrane effects of catechins. Chem Biol Interact **134:**41–54, 2001.
43. Zhu QY, Huang Y, Tsang D, Chen ZY. Regeneration of α-tocopherol in human LDL by green tea catechins. J Agric Food Chem **47:**2020–2025, 1999.
44. Hollman P, Katan MB. Absorption, metabolism and health effects of dietary flavonoids in man. Biomed Pharmacother **51:**305–310, 1997.
45. Baba S, Osakabe N, Yasuda M, Takizawa T, Nakamura T, Terao J. Cocoa powder enhances the level of antioxidative activity in rat plasma. Br Med J **84:**673–680, 2000.
46. Spencer JP, Chaudry F, Pannala AS, Srai SK, Debnam E, Rice-Evans C. Decomposition of cocoa procyanidins in the gastric milieu. Biochem Biophys Res Commun **272:**236–241, 2000.
47. Lotitio SB, Fraga CG. (+)-Catechin prevents human plasma oxidation. Free Radic Biol Med **24:**435–441, 1998.
48. Nestel PJ. How good is chocolate? Am J Clin Nutr **74:**563–564, 2001.