Bioflavonoid Rescue of Ascorbate at a Membrane Interface

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In aqueous solution, ascorbate potently prevents bleaching of cytochrome *c* on exposure to excess H_2O_2 or *t*-butyl hydroperoxide. Ascorbate failed to protect cytochrome *c* in the presence of liposomes of mitochondrial membranelike composition. Like the redox mediator *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), however, the bioflavonoids epicatechin and quercetin restored the protection afforded by ascorbate in the presence of liposomes and gave further protection. The quercetin glycoside, rutin, was much less effective, as was the vitamin E analog Trolox. In the presence of liposomes, quercetin alone was relatively ineffective, but cooperated with ascorbate to extend protection synergistically. The results bear specific implications in antioxidant protection of cytochrome *c* and in moderation of its hydroperoxidase activities in biological membranes. The data also reveal a situation where ascorbate is without effect except in the presence of a bioflavonoid, and substantiate a possibly vital role for certain bioflavonoids in mediating electron transfer from ascorbate into a hydrophobic environment.

KEY WORDS: Ascorbate; bioflavonoid; cytochrome *c* bleaching; peroxide; membrane interface; electron transfer.

INTRODUCTION

The bioflavonoids were initially suggested as a vitamin principle in fruits and vegetables that spared ascorbate and extended the lifespan of scorbutic guinea pigs (Rusnyák and Szent-Györgyi, 1936; Bentsáth *et al*., 1936). However, the bioflavonoids were found to represent a large class of plant phenolics with diverse pharmacological effects (Kühnau, 1976; Havsteen, 1983) and no bioflavonoid-dependent role in animal metabolism was established. Nevertheless, several studies support ascorbate protecting or sparing and other semiessential effects of bioflavonoids, especially potent antioxidant activities (Rice-Evans *et al*., 1996). In mitochondria and other biological membranes, several bioflavonoids give more potent protection against lipid peroxidation than vitamin E (Bindoli *et al*., 1977; Okuda *et al*., 1983). The association of ascorbate with biomembranes is re-stricted by its solubility, but it interacts with some surface components (Beyer, 1994; Rubinstein, 1994; Gomez-Diaz *et al*., 1997).

The mitochondrial hemoprotein, cytochrome *c* reacts readily with hydroperoxides, producing peroxyl, oxyl, and alkyl radicals (Tappel, 1953; Davies, 1988; Barr and Mason, 1995), and can promote peroxidation of biological membranes and other organic substrates (Tappel, 1953; Cadenas *et al*., 1980a; Radi *et al*., 1991a,b, 1993; Evans *et al*., 1994). Conversely, cytochrome *c* is sensitive to damage by peroxides (Desai and Tappel, 1963, O'Brien, 1966; Cadenas *et al*., 1980b; Florence, 1985; Harel *et al*., 1988), producing a radical species (Barr *et al*., 1996). Factors that influence the reaction of hydroperoxides with cytochrome *c*, therefore, bear importance both to cytochrome *c* destruction and to propagation of damage.

In studies of the reaction of cytochrome *c* with H_2O_2 and *t*-butyl hydroperoxide (*t*-buOOH), we find that while ascorbate efficiently protects cytochrome *c* in aqueous solution, as noted previously (Florence, 1985; Harel *et al*., 1988), it fails to protect in the presence of cytochrome *c*-affinic liposomes (results herein). This result is consistent with the inability of ascorbate to reduce mitochondrial or liposome-bound cytochrome *c*,

except in the presence of the redox mediator *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) (Slater, 1949; Jacobs, 1960; Nicholls and Malviya, 1973; Nicholls *et al.*, 1980). We report that certain bioflavonoids can effectively mediate electron transfer in the presence of liposomes to rescue protective activity of ascorbate.

MATERIALS AND METHODS

Materials

Cytochrome *c* (horse heart, type VI), ascorbic acid, (-)-epicatechin, quercetin, rutin, *N*-(2-hydroxyethyl) piperazine-*N'*-(ethanesulfonic acid) (HEPES), phosphatidylcholine (type XVI-E, egg yolk), phosphatidylethanolamine (type III, egg yolk), cardiolipin (bovine heart), and *t*-buOOH were from Sigma (St. Louis). Hydrogen peroxide, TMPD, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and potassium phosphate buffer salts were from Merck. All aqueous solutions were made using distilled, MilliQ-deionized water. Solutions of ascorbate and TMPD were prepared in N_2-saturated water and kept on ice in septum-stoppered vials with a slight positive pressure of N_2. Bioflavonoids and Trolox were dissolved in dimethyl sulfoxide (DMSO), and 5–20 μl aliquots added to reaction mixtures. Additions of DMSO alone had no effect.

Liposome Preparation

In preparation of phosphatidylcholine/phosphatidylethanolamine/cardiolipin (PC/PE/C) liposomes, the lipids were first dissolved in chloroform in a 15/12/9 proportion, the chloroform evaporated with N_2, and the resulting test tube film stored at $-20°C$ until use. Prior to experiments, cold phosphate buffer was added to the tube, mixed with a vortex, and sonicated at 60 W on ice, with a microtip-equipped Cole-Parmer 4710 series ultrasonic homogenizer, in eight 15-s bursts with 1-min cooling intervals. Liposome preparations were centrifuged at 10,000 × g, 4°C for 40 min to sediment Ti introduced by the sonicator tip.

Bleaching of Cytochrome *c* Soret Absorbance

Cytochrome *c* bleaching experiments were conducted in 10 mM HEPES, 50 mM phosphate buffer or 50 mM phosphate buffer at pH 7.4, 37°C, in the presence and absence of 1 mM liposomes. Concentrations of H_2O_2 and *t*-buOOH were determined from their absorbances

at 240 nm using extinction coefficients of 42 M^{-1} cm^{-1} for H_2O_2 (Beers and Sizer, 1952) and 18.8 M^{-1} cm^{-1} for *t*-buOOH (determined from a fresh stock solution). Cytochrome *c* concentrations were determined from the Soret absorbance at 408 nm, using $\varepsilon = 104.6 \, mM^{-1} \, cm^{-1}$ (Margoliash and Frohwirt, 1959). In kinetic measurements of cytochrome *c* bleaching, cytochrome *c* Soret absorbance was monitored at 408 nm in the absence of a reductant, or, due to a shift in the absorbance peak, at 415 nm in the presence of a reductant.

Data Analyses

Representative kinetic traces and scans are shown. Reactions were performed in triplicate or more. Half-times ($t_{1/2}$ s) for loss of cytochrome *c* Soret absorbance were calculated for the different conditions. For reactions where bioflavonoids absorbed significantly at the cytochrome *c* Soret wavelength, after determining that the bioflavonoids oxidized before cytochrome *c*, the $t_{1/2}$ was calculated by subtracting the value of cytochrome *c* Soret absorbance from the end absorbance after bleaching.

RESULTS AND DISCUSSION

Protection by Ascorbate on Peroxide Bleaching of Cytochrome *c*

The protection by ascorbate against *t*-buOOH-induced bleaching of cytochrome *c* in aqueous solution is shown in Fig. 1. While protecting cytochrome *c* ascorbate rapidly oxidized, evident from loss of its absorbance at 265 nm. Upon exhaustion of ascorbate, cytochrome *c* rapidly oxidized and the Soret band (408–415 nm) bleached, indicating damage to the Soret band chromophore. The cytochrome *c* half-life was negatively dependent on hydroperoxide concentration (0.5–30 mM) and increased with increasing ascorbate (50–400 μM) (not shown). Similar results were obtained with H_2O_2 instead of *t*-buOOH, except that the protection given by ascorbate was of shorter duration with H_2O_2 due to more rapid ascorbate oxidation.

These experiments showed ascorbate as a remarkably efficient protector of aqueous cytochrome *c* from peroxide damage (Fig. 1). Even at millimolar concentrations of hydroperoxide, 200 μM ascorbate was able to almost completely protect cytochrome *c* from damage until ascorbate had been consumed to very low levels. The current results are consistent with those of a previous study on H_2O_2-induced release of iron from hemoproteins, including cytochrome *c*, under conditions of low-level
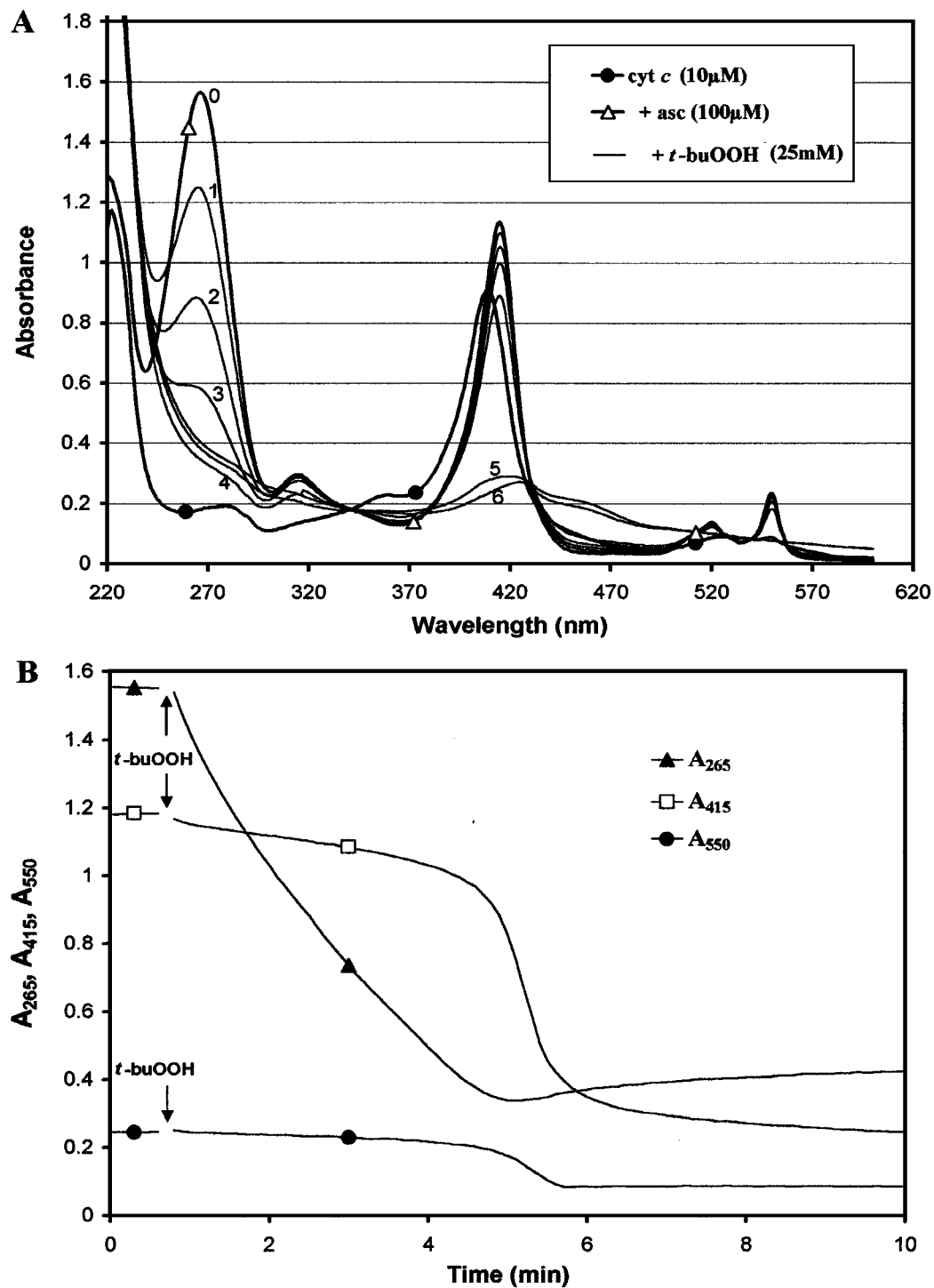
Fig. 1. Oxidation of ascorbate and bleaching of cytochrome c on addition of t-buOOH. The reaction was conducted in 10 mM HEPES, 50 mM phosphate buffer, at pH 7.4, 37°C, with 10 μM cytochrome c (cyt c) and 100 μM ascorbate (asc), and initiated by addition of 25 mM t-butyl hydroperoxide (t-buOOH). (A) Scans of absorbance changes taken before and at 1-min intervals after addition of t-buOOH. The sequence (min) after addition of t-buOOH is indicated by the numerals. (B) Kinetics of absorbance changes on addition of t-buOOH. Bleaching of the Soret absorbance of ferrocytochrome c in the presence of ascorbate was followed at 415 nm. The concurrent oxidation of ascorbate was followed at 265 nm, and the oxidation of ferrocytochrome c at 550 nm in separate reactions.

continuous generation of H_2O_2 (1 nmol/min/ml) (Harel *et al.*, 1988), as encountered physiologically. Considering physiological concentrations of ascorbate intracellularly of 0.5–2.0 mM (Hornig, 1975), the current and previous study show that ascorbate offers considerable capacity to protect cytochrome *c* from peroxide damage in an aqueous environment. In addition to protecting the cytochrome, addition of ascorbate has been observed to protect against cytochrome c/H_2O_2-mediated peroxidation of arachidonic acid, proteins, and other substrates (Evans *et al.*, 1994), as it does with myoglobin (Galaris *et al.*, 1989; Galaris and Korantzopoulos, 1997).

However, the ability of ascorbate to protect cytochrome *c* from peroxide damage was substantially lost in the presence of liposomes of mitochondrial membrane-like composition (Fig. 2). Similar results were found with H_2O_2 or *t*-buOOH. The amphiphilic electron transfer reagent TMPD, however, while only slightly slowing Soret bleaching itself, substantially rescued the protective effect of ascorbate in the presence of liposomes (Fig. 2), consistent with its effect as a redox mediator from ascorbate to cytochrome *c* in mitochondrial preparations.

Rescue of Ascorbate Protection by Bioflavonoids

Addition of the bioflavonoid epicatechin, a bioflavonoid of green tea (Scheme 1), also markedly restored protection by ascorbate, to almost that in the absence of liposomes (Fig. 3). Upon exhaustion of ascorbate, absorbance at 415 nm initially declined and then increased again, thereafter slowly declining (Fig. 3, filled triangles). Scans showed initial loss of ascorbate and then formation of a complex with a broad absorbance at 405–450 nm (Fig. 4), which does not form with ascorbate alone (Fig. 1). With epicatechin in the absence of ascorbate, this absorption complex formed rapidly on addition of hydroperoxide (H_2O_2 or *t*-buOOH), in the presence (Fig. 3, unfilled triangles) or absence of liposomes.
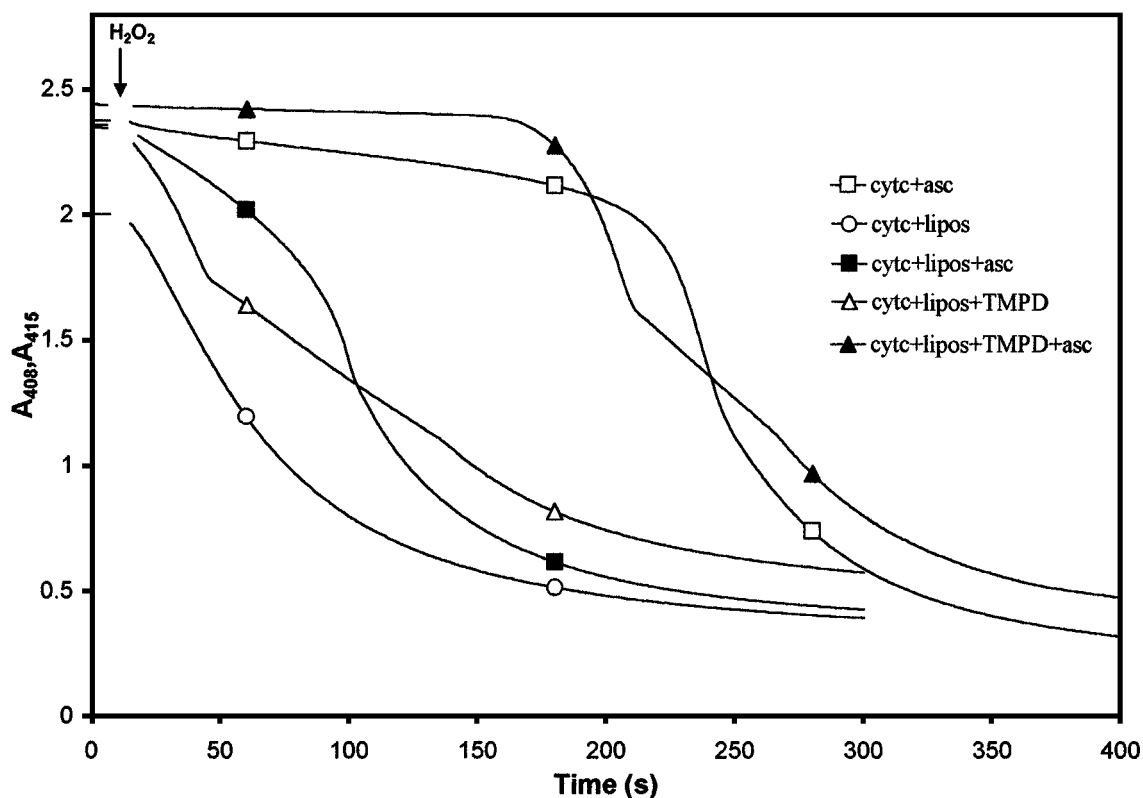
Fig. 2. Effect of liposomes on protection of cytochrome *c* by ascorbate and rescue by TMPD. Reactions were conducted in 50 mM potassium phosphate buffer, at pH 7.4, 37°C, with 20 μM cytochrome *c* (cytc) in the presence and absence of 1 mM, 15/12/9, phosphatidylcholine/phosphatidylethanolamine/cardiolipin liposomes (lipos), and initiated by addition of 10 mM H_2O_2. Ascorbate (asc, 200 μM) and TMPD (150 μM) were included where indicated in the legend. Reaction mixtures were equilibrated for 3 min prior to addition of H_2O_2. Reactions were initiated by addition of 10 mM H_2O_2 and the changes in Soret absorbance of cytochrome *c* followed at 415 nm (presence of ascorbate or TMPD) or 408 nm (absence of ascorbate or TMPD).

Quercetin   (R = H)

Rutin   (R = rutinose)
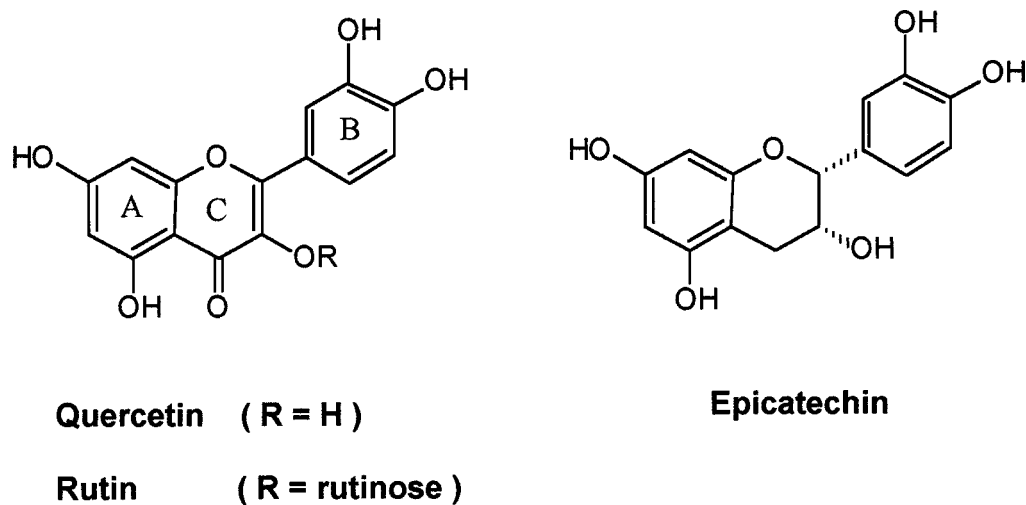
Scheme 1. Structures of quercetin, rutin and epicatechin.

In characterizations of the absorption complex formed with epicatechin, further addition of ascorbate did not return the original cytochrome c spectrum. In the absence of cytochrome c, incubation of epicatechin, or epicatechin plus ascorbate with H_2O_2 for 15 min produced no noticeable oxidation of epicatechin or absorbance in this region. The yet unidentified chromophore precipitated on addition of trichloroacetic acid and failed to solubilize
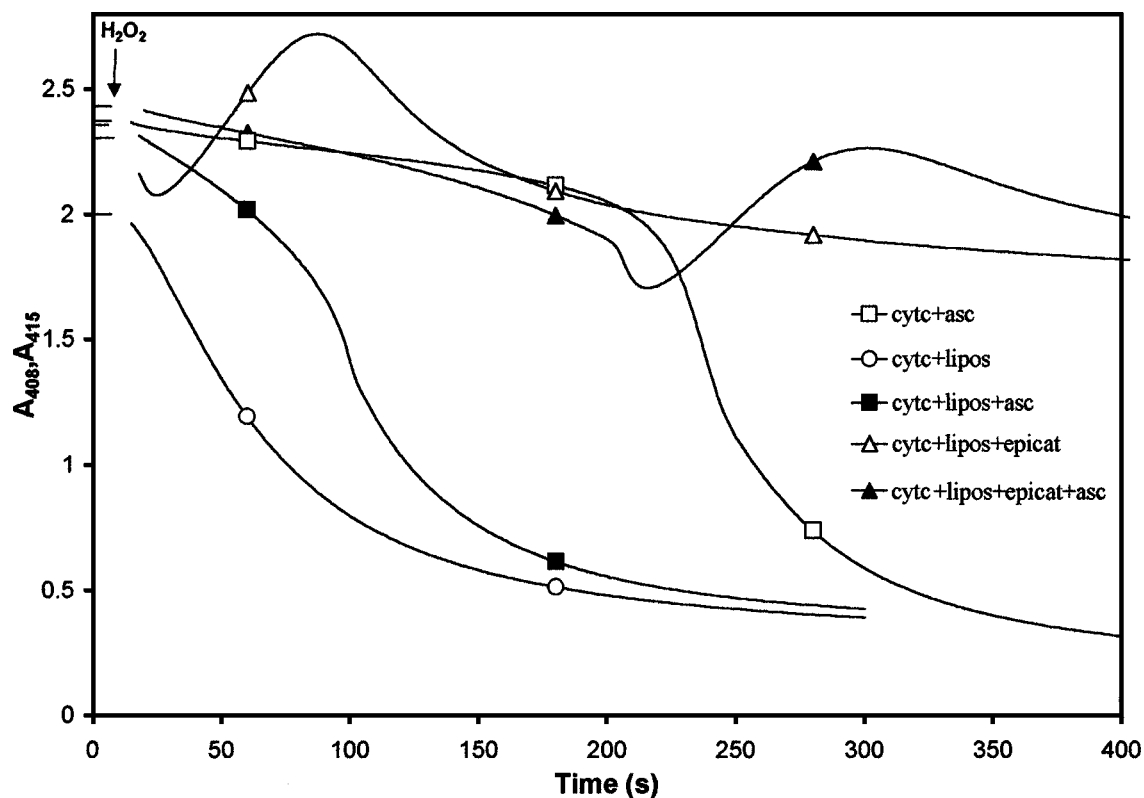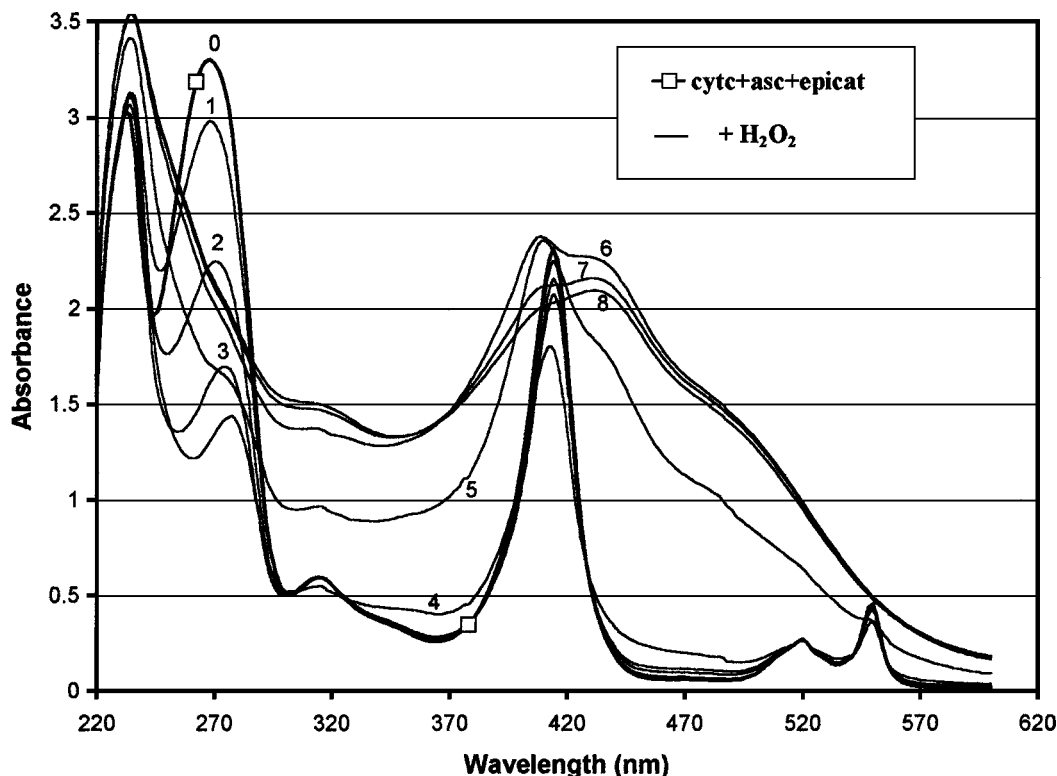

Fig. 3. Rescue of ascorbate protection in the presence of liposomes by epicatechin. Reactions were conducted as in Fig. 2 in the presence and absence of ascorbate (asc, 200 μM) and epicatechin (epicat, 200 μM), and initiated by addition of 10 mM H_2O_2.

Fig. 4. Absorbance scans of cytochrome c plus epicatechin and ascorbate before, and on addition of H_2O_2. Reaction conditions were as in Fig. 3, here shown in the absence of liposomes. Scans were taken before (scan 0), and at 1-min intervals (scans 1–8) after addition of 10 mM H_2O_2.

into ethanol, suggesting formation of a covalent derivative between epicatechin and cytochrome c. Notably, the absorption complex did not form while ascorbate was present (Fig. 3, filled triangles), even in the presence of liposomes, and together ascorbate and epicatechin protected the cytochrome.

Quercetin, although adding mildly to the absorbance at 415 nm, could be observed to extend protection by ascorbate in the presence of liposomes to an even greater extent than epicatechin (Fig. 5). At 100 μM, in the presence of liposomes, quercetin cooperated synergistically to extend the $t_{1/2}$ of cytochrome c bleaching with 200 μM ascorbate from 1.28 min, to 10.40 min, while quercetin alone provided a $t_{1/2}$ of 1.65 min (Fig. 5; Table I). Similar results were observed with cytochrome c bleaching by H_2O_2. Scans over time revealed that ascorbate oxidized first, followed by quercetin, and then bleaching of the cytochrome (not shown). After exhaustion of ascorbate, quercetin slowed the bleaching without forming the derivative seen with epicatechin. In the absence of liposomes, quercetin protected cytochrome c with similar efficiency to ascorbate (not shown) and, together with ascorbate, gave greater than additive protection. Quercetin,

therefore, lost protective ability alone in the presence of liposomes, but, together with ascorbate, offered synergistic protection.

Rutin, in contrast, was much less effective than quercetin (Fig. 5). With otherwise identical structures, the rutinose glycoside group at position 3 of ring C of rutin obviously accounts for this difference. The glycosidic group makes rutin more hydrophilic than quercetin and also adds a bulky group that lacks the redox activity of the 3-OH group in quercetin. Unexpectedly, rutin alone did not significantly protect cytochrome c in the absence of liposomes, although it is a stronger reductant than quercetin (Bors *et al.*, 1995) and reacts more efficiently with aqueous $O_2^{\bullet-}$ (Jovanovic *et al.*, 1994). Thus, the differences in effectiveness between rutin and quercetin may be due to one or more of a difference in lipid partitioning, in interaction with cytochrome c, or in hydrophobic free radical reactivity.

The vitamin E analog Trolox, more amphiphilic than tocopherols, slightly cooperated with ascorbate in protecting cytochrome c in the presence of liposomes, with 100 μM extending the $t_{1/2}$ from 1.28 to 2.00 min (Table I). In the absence of ascorbate, Trolox gave no
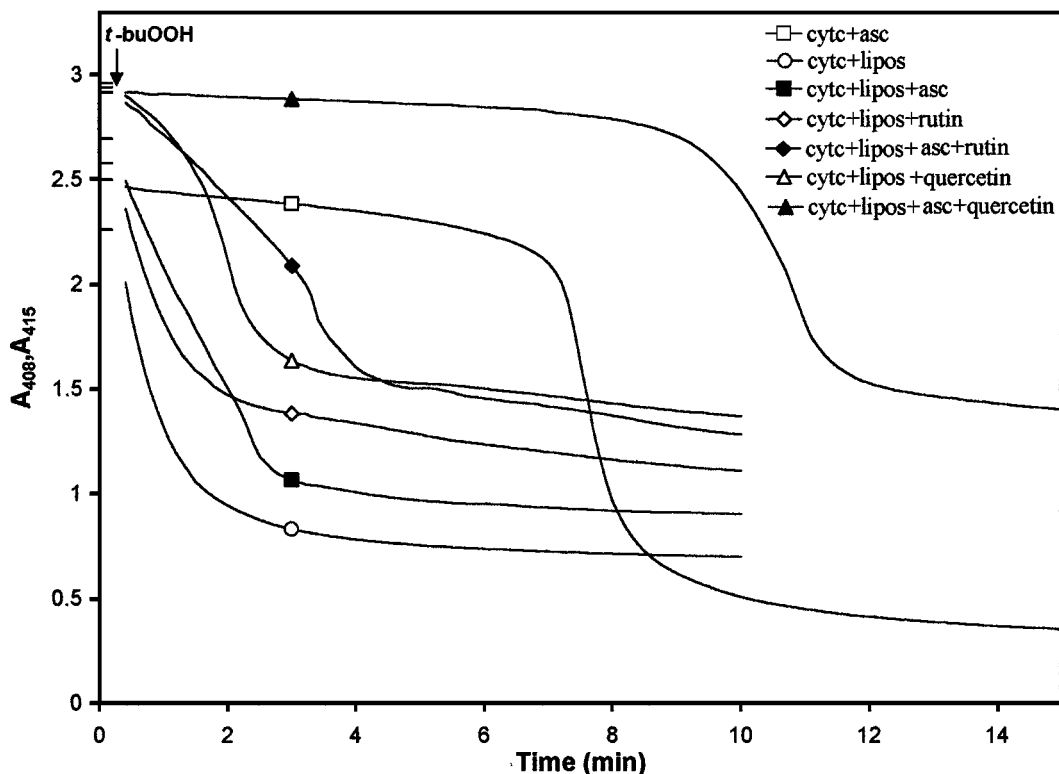
Fig. 5. Effects of quercetin and rutin on ascorbate protection of cytochrome *c* in the presence of liposomes. Reactions were conducted as in Figs. 2 and 3, and initiated by addition of 20 mM *t*-buOOH. Ascorbate (asc) was at 200 μM; rutin and quercetin were at 100 μM.

protection, either in the presence (Table I) or absence of liposomes.

The bioflavonoids, found along with vitamin C in fruits and vegetables, represent a class of phenolic com-

Table I. Comparison of Ascorbate-Mediating Abilities of Trolox, Rutin, and Quercetin in Protecting Liposome-Bound Cytochrome *c* from t-BuOOH[a]

Cyt *c*/*t*-BuOOH (20 mM) additions	$t_{1/2}$ (min)	+asc (200 μM)
None	0.50	7.04
Liposomes	0.48	1.28
Liposomes + Trolox	0.55	2.00
Liposomes + rutin	0.50	2.67
Liposomes + quercetin	1.65	10.40

[a]Reactions were conducted as in Fig. 5, in the presence or absence of 200 μM ascorbate (asc). Phosphatidylcholine/phosphatidylethanolamine/cardiolipin liposomes (liposomes) were at 1 mM. Trolox, rutin, and quercetin were at 100 μM. The half-times ($t_{1/2}$) for bleaching of cytochrome *c* are tabulated. The mean standard deviation of replicates ($N \geq 3$) was ±5%.

pounds of wide redox properties and solubilities. The observation that epicatechin and quercetin, like TMPD, markedly rescued the protection by ascorbate on cytochrome *c* in the presence of liposomes (Figs. 3 and 5) shows that certain bioflavonoids can conduct electrons from ascorbate into a hydrophobic environment. This property appears to be noteworthy to bioflavonoids, since the amphiphilic vitamin E analog Trolox was relatively ineffective (Table I).

A recent study of the reduction of cytochrome *c* by bioflavonoids (Moini *et al.*, 2000) showed that some could reduce cytochrome *c* in mitochondrial cytochrome *c* oxidase, but that the rate was insufficient to support significant respiration, unlike with TMPD. In the current studies, the lack of protection of cytochrome *c* by TMPD alone (Fig. 2), despite efficiently reducing the heme iron, suggests that the effectiveness of the bioflavonoids is due to their radical reactivity, rather than simple iron-reducing ability.

The current results are similar to the regeneration of membranous vitamin E from the chromanoxyl radical by ascorbate (Tappel, 1968; Packer *et al.*, 1979; Niki *et al.*, 1984; Chan *et al.*, 1991), invoking the special redox properties and membrane partitioning of flavonoids.
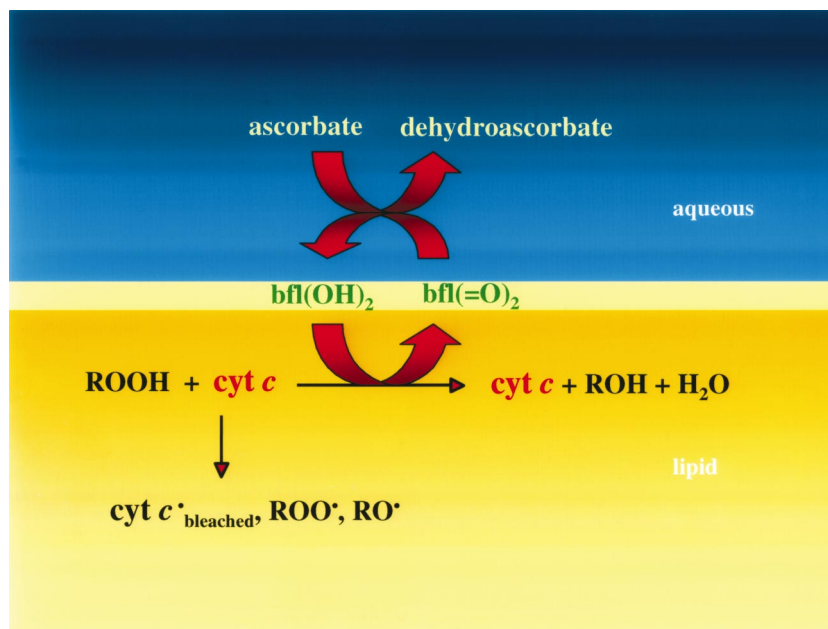
Fig. 6. Cooperation of bioflavonoids and ascorbate in protecting liposome-bound cytochrome *c* from peroxide damage. In the absence of bioflavonoid, cytochrome *c* (cyt *c*) reacts with hydroperoxides (ROOH) to produce bleached cytochrome *c* (cyt $c_{bleached}^{\bullet}$) and radical products (ROO$^{\bullet}$, RO$^{\bullet}$). In cooperation with ascorbate, a dihydroxy amphiphilic bioflavonoid, such as epicatechin or quercetin (bfl(OH)$_2$), can mediate electron transfer to liposome-bound cytochrome *c*, reducing hydroperoxide to ROH and protecting cytochrome *c*.

Experiments with phospholipid vesicles and lipid bilayers indicate that quercetin inserts between the acyl chains, as well as interacts with the polar phospholipid head groups (Movileanu *et al.*, 2000; van Dijk *et al.*, 2000). A number of observations of cooperation between bioflavonoids and ascorbate have been reported, but in most cases the results can be interpreted as protection or sparing of ascorbate or binding of metals by the bioflavonoid. The current observations in a biphasic membrane system show that in some situations bioflavonoids may be well suited to mediate electron transfer from ascorbate to an acceptor in a hydrophobic environment. In such a cooperation, the flavonoid, at lower concentration, can rescue the reducing power of ascorbate. The suggested interaction in the current system is shown in Fig. 6.

In a similar system of hematoporphyrin-photosensitized photoperoxidation and lysis of erythrocytes, ascorbate was shown to potentiate inhibition by quercetin and suppress quercetin oxidation, although alone giving prooxidant effects (Sorata *et al.*, 1988). The authors suggested that ascorbate regenerated the quercetin flavonol, likely at the membrane interface (Sorata *et al.*, 1988).

Biological Perspectives

In vivo, ascorbate and bioflavonoids act in an antioxidant network. Ascorbate can be regenerated by glutathione (Meister, 1994), and aqueous bioflavonoids can help stabilize ascorbate (Kühnau, 1976; Cossins *et al.*, 1998). Membrane studies with ascorbate (e.g., Sorata *et al.*, 1988, current results), and those showing that bioflavonoids can spare vitamin E (Jan *et al.*, 1991; Terao *et al.*, 1994; van Acker *et al.*, 2000), suggest that bioflavonoids can occupy intermediate positions in the antioxidant network, both from their redox reactivity and their amphiphilicity.

In several instances, cooperation between ascorbate and bioflavonoids could be noted in protecting mitochondria (Chamrai, 1969; Bindoli *et al.*, 1977; Das and Ratty, 1986; Ratty and Das, 1988) or other biomembranes (Sorata *et al.*, 1988; Jan *et al.*, 1991) from peroxidation. Also, *in vivo* or in cultured cells, ascorbate alone sometimes does not protect against oxidative stress-induced cell death or mitochondrial damage, but a combination of ascorbate and bioflavonoid inhibits synergistically (Chamrai, 1969; Skaper *et al.*, 1997). Notably, aglycones are more protective against mitochondrial lipid peroxidation than the corresponding glycones (Haraguchi

et al., 1986; Das and Ratty, 1986; Ratty and Das, 1988; Miyahara *et al.*, 1993). Considering the activity of cytochrome *c* in catalyzing peroxidations, inhibition of mitochondrial membrane peroxidation by bioflavonoids may partially reflect the current observations. To the extent that an ascorbate-mediating activity of bioflavonoids at membrane interfaces occurs *in vivo*, it adds to original suggestions of a vital cooperation between bioflavonoids and ascorbate (Rusnyák and Szent-Györgyi, 1936; Bentsáth *et al.*, 1936).

ACKNOWLEDGMENTS

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