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# Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation

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## Abstract

Background

When an antioxidant scavenges a reactive species, i.e. when it exerts it antioxidant activity, the antioxidant is converted into potentially harmful oxidation products. In this way the antioxidant quercetin might yield an *ortho*-quinone, denoted as QQ, which has four tautomeric forms, i.e. the *ortho*-quinone and three quinonmethides. We evaluated the interaction of QQ with ascorbate or glutathione (GSH).

Methods

The reaction of QQ with several compounds was monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

Ascorbate recycled QQ to the parent compound quercetin, while GSH formed two adducts with QQ, i.e. 6-GSQ and 8-GSQ. When both GSH and ascorbate were present, QQ was converted exclusively into GSQ. In the absence of GSH, protein thiols became arylated by QQ. This protein arylation could not be prevented by ascorbate.

Conclusions

Thiol arylation by quinones and quinonmethides can impair several vital enzymes. This implies that the product formed when quercetin displays its antioxidant scavenging effect is toxic in the absence of GSH. Therefore, an adequate GSH level should be maintained when quercetin is supplemented.

# Introduction

Antioxidants have the annotation of being healthy and safe. Many diseases are associated with oxidative stress (1-3) and the use of antioxidant-rich food or antioxidant supplements is often recommended to preserve or regain good health (4,5). Of the dietary antioxidants, flavonoids have gained much interest. One of the most prominent flavonoids is quercetin.

It is not generally recognized that antioxidants may also display adverse effects. Without proper knowledge on side effects, biotransformation or biokinetics relatively high dosages of antioxidants are taken by large populations. Supplements containing up to 500 mg quercetin are being marketed. It should, however, be noted that once an antioxidant such as quercetin exerts its presumed protective effect, i.e. when it neutralizes a reactive species, it is converted into potentially harmful oxidation products (6,7).

Antioxidants do not act in isolation; together they form an intricate antioxidant network. For example, ascorbate can regenerate oxidized vitamin E and glutathione (GSH) can regenerate oxidized ascorbate, as demonstrated by the pioneering work of Hopkins and Morgan (8) and of Slater and coworkers (8,9).

The aim of the present study is to determine how quercetin fits in the antioxidant network. More specifically, we evaluated the interaction of oxidised quercetin with GSH or ascorbate.

# Materials and Methods

## Materials

Quercetin, reduced GSH, ascorbate, tyrosinase, horseradish peroxidase (HRP) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide was obtained from Merck (Darmstadt, Germany).

## Methods

The oxidation of quercetin was performed at 37°C in a 143 mM phosphatebuffer pH 7.4 that contained 50 μM quercetin as well as 25 U/ml tyrosinase, or both 1.6 nM HRP and 33 μM $H_2O_2$, unless otherwise noted. The reactions were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Quercetin consumption was determined at 400 nm, ascorbate consumption at 270 nm. Spectra were recorded with a scanspeed of 480 nm/min. Based on HPLC analysis it was concluded that in the incubations without GSH or ascorbate, the spectral changes were only due to the conversion of quercetin into its quinone QQ. Therefore, it is assumed that in these incubations QQ formation equalled quercetin consumption. In the incubations with ascorbate, spectral changes due to ascorbate oxidation could be found. When GSH was present, quercetin was converted only into two glutathionylquercetin adducts (GSQ), based on HPLC analysis. Therefore, it is assumed that in these incubations GSQ formation equalled quercetin consumption.

HPLC of the incubation mixtures was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). Analytical separations were performed according to Awad *et al*. with minor modifications (10). The column was eluted with a mixture of distilled water containing 0.1% (v/v) trifluoroacetic acid and acetonitrile. From 0 to 10 minutes a linear gradient to 5% acetonitrile was applied, followed by a gradient from 10 to 25 minutes to 15% acetonitrile, from 25 to 28 minutes to 35% acetonitrile and from 28 to 40 minutes to 60% acetonitrile. Based on identical elution profiles and UV spectra compared to other studies, it was concluded that the first and lower GSQ peak is 8-GSQ and the second and higher peak is 6-GSQ. QQ was identified using the diode array spectra.

In order to quantify the GSQ adducts, 50 μM quercetin was oxidized by 25 U/ml tyrosinase in the presence of 100 μM GSH. After approximately half of the quercetin was oxidized (the exact degree of quercetin oxidation was determined spectrophotometrically), the solution was acidified in order to stop the reaction. The solution was directly injected on the HPLC system. The peak area of both GSQ adducts (6-GSQ and 8-GSQ) was determined (290 nm) and the concentration of the adducts was calculated assuming that (i) the consumed quercetin was converted quantatively into GSQ under these conditions and that (ii) the response factor of both GSQ adducts was identical. QQ was identified and quantified using HPLC (290 nm) according to a procedure identical to that of GSQ, albeit that the incubation did not contain GSH. Here only one product was formed. Attempts to synthesize QQ and GSQ failed, probably due to instability of these compounds. Quercetin was quantified using external calibrators (290 nm).

Thiol content of the blood plasma was measured using DTNB. After preincubating 50 μM and 100 μM quercetin with respectively 25 U/ml and 100 U/ml tyrosinase, blood plasma of a healthy 29 year old female volunteer was added in a tenfold dilution with or without 100 μM ascorbate. This reaction mixture was incubated for another 5 min, after which 0.6 mM DTNB

was added. The reduction of DTNB was measured spectrophotometrically at 412 nm.

All experiments were performed, at least, in triplicate. Data are given as mean ± SD or as a typical example.

## Results

Figure 3.1A shows the changes in the UV spectrum during the oxidation of 50 μM quercetin by the enzyme horseradish peroxidase (HRP).

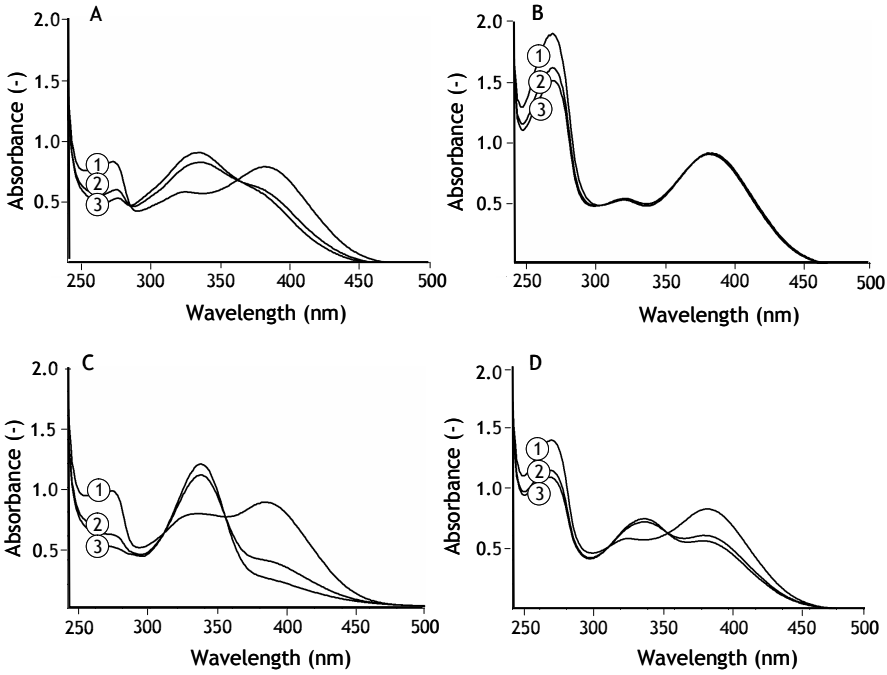


**Figure 3.1** UV scans of the incubation mixture containing 1.6 nM HRP, 50 μM quercetin and 33 μM $H_2O_2$ (A). The reaction was started by the addition of horseradish peroxidase.The same experiment was carried out in the presence of 40 μM ascorbate (B), 100 μM glutathione (C) and both 40 μM ascorbate and 100 μM glutathione (D). The UV scans were started 35 sec (1), 205 sec (2) or 370 sec (3) after the addition of HRP. A typical example is shown.

In the spectrum two isosbestic points are observed at 280 and 360 nm, suggesting the conversion of quercetin into one oxidation product. This is confirmed by HPLC analysis of the incubation mixture, showing the formation

of only one product (data not shown). Based on analogy with other studies (11,12) and on the recorded UV spectrum, it is concluded that the product is the *o*-quinone/quinonmethide of quercetin (QQ). The rate of quercetin oxidation and also of QQ accumulation is 15±2 μM/min.

Addition of 40 μM ascorbate to the incubation mixture containing quercetin and peroxidase prevents the accumulation of QQ. The spectrum of the parent compound, quercetin, is preserved throughout the experiment. At the same time the ascorbate concentration decreases, as determined spectrophotometrically at 270 nm (Figure 3.1B). The rate of ascorbate oxidation is 3.4±1.0 μM/min. In the absence of quercetin the decline in ascorbate concentration is lower (0.35±0.00 μM/min). These data suggest that quercetin is oxidized into QQ and that the QQ formed is immediately regenerated at the expense of ascorbate. The net consumption of ascorbate that is induced by quercetin (3.1±1.0 μM/min) is lower than the formation of QQ in the absence of ascorbate (15±2 μM/min). Assuming that ascorbate and QQ react 1:1 and since no QQ accumulates in the incubation containing ascorbate, the difference in rate without ascorbate (15±2 μM/min) and with ascorbate (3.4±1.0 μM/min) can be attributed to inhibition of HRP by ascorbate. This inhibition depends on the concentration of ascorbate as can be inferred from the data depicted in Figure 3.2. An ascorbate concentration of 25 μM causes 25% inhibition while 40 μM causes 74% inhibition of HRP. Ascorbate concentrations above 40 μM are therefore not tested. It should be noted that ascorbate in a concentration of 25 μM cannot completely prevent a net consumption of quercetin after 60 seconds (Figure 3.2).

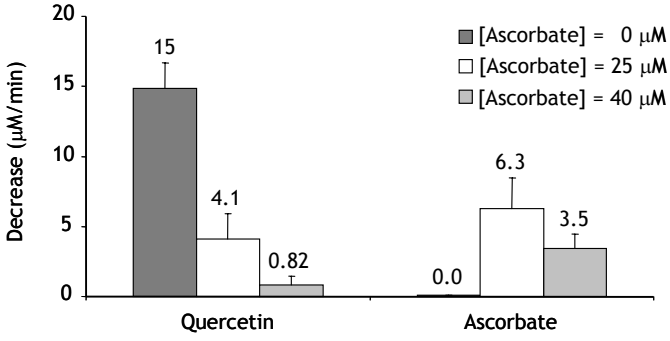


**Figure 3.2** The decrease in 60 seconds of the concentration of quercetin and ascorbate determined spectrophotometrically. The incubation mixture contained 50 μM quercetin, 1.6 nM HRP and 33 μM $H_2O_2$ in the presence of 0, 25 and 40 μM ascorbate. The reaction was started by the addition of HRP. Spontaneous oxidation of ascorbate was neglectable, namely 0.35 μM/min at an ascorbate concentration of 40 μM. All measurements were carried out, at least, in triplicate and expressed as mean ± SD.

Addition of 100 μM GSH to the incubation mixture containing quercetin and peroxidase gives two isosbestic points at 310 nm and 355 nm in the UV spectrum (Figure 3.1C). Since these isosbestic points differ from the isosbestic points observed during QQ formation, it is concluded that another product is formed in the presence of GSH. HPLC analysis demonstrates the formation of two products which can be identified as 6-glutathionylquercetin (6-GSQ) and 8-GSQ in accordance to Awad *et al.* (10) (Figure 3.3). During the incubation the ratio of the concentration of both adducts remains constant, i.e. 6-GSQ:8-GSQ=1.14:1. This indicates that the rate of formation of 6-GSQ is somewhat faster than the rate of the formation of 8-GSQ. The rate of quercetin consumption is not affected by the presence of 100 μM GSH (rate with GSH 15.0±0.2 μM/min, rate without GSH 15±2 μM/min), indicating that GSH does not inhibit HRP.

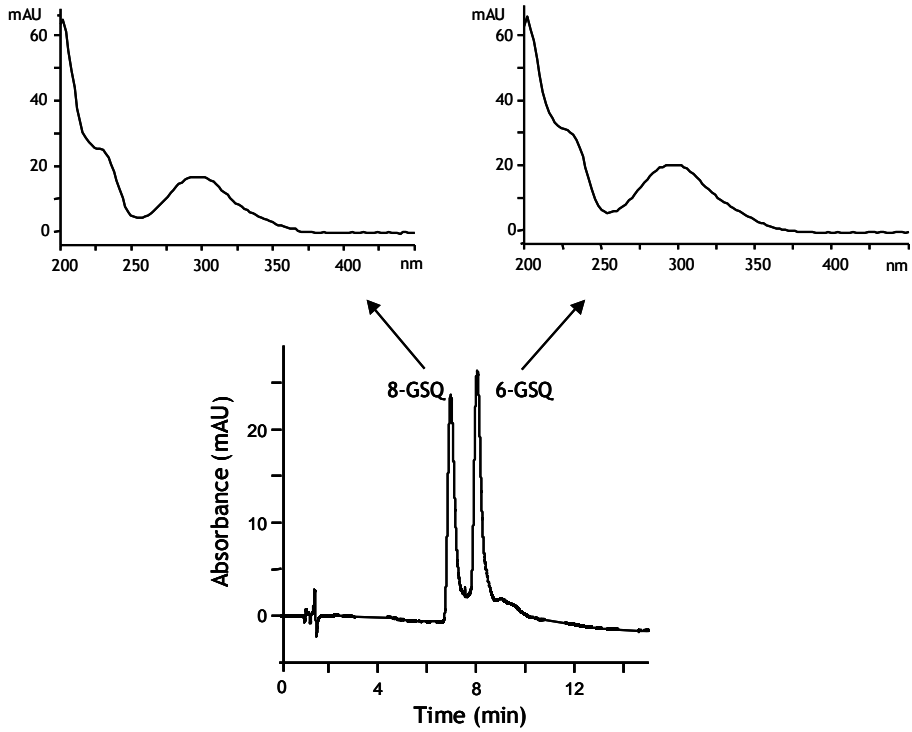


**Figure 3.3** HPLC-chromatogram at t=5 min of the incubation mixture containing 1.6 nM HRP, 33 μM $H_2O_2$, 50 μM quercetin and 100 μM GSH. The spectra of the GSQ adducts with a retention time of 7.0 min and 8.3 min are shown. Based on a comparable elution profile it was concluded that the peak at 7.0 min is 8-GSQ and the peak at 8.3 min is 6-GSQ. The reaction was started by the addition of HRP. A typical example is shown.

*In vivo* both GSH and ascorbate can react with oxidized quercetin. To determine the relative contribution of both reactions, experiments were performed including both GSH and ascorbate.

The UV spectrum of the incubation mixture containing HRP, 50 μM quercetin and both 100 μM GSH and 40 μM ascorbate showed the two isosbestic points at 310 and 355 nm that are indicative for the formation of GSQ (Figure 3.1D). HPLC analysis of the incubation mixture confirms this conclusion since only 6-GSQ and 8-GSQ are detected (data not shown). The rate of this GSQ formation (7.2±1.7 μM/min) is slower than that of GSQ formation when only GSH is present (15.0±0.2 μM/min). This demonstrates that the presence of ascorbate inhibits GSQ formation (Figure 3.4A). This inhibition by ascorbate can be achieved either (i) by competition with GSH for QQ or (ii) by inhibition of HRP. As mentioned above, 40 μM ascorbate reduces the enzyme activity to 3.4±1.0 μM/min. The rate of GSQ formation in presence of both GSH and ascorbate (7.2±1.7 μM/min) is not lower than the enzyme activity with only ascorbate; it is even higher. This suggests that the inhibition of GSQ formation by 40 μM ascorbate proceeds by inhibition of HRP. The lower inhibition of HRP by ascorbate in the presence of GSH suggests that GSH can protect against the inhibition of HRP by ascorbate. Monitoring of ascorbate oxidation in the presence of GSH to quantify ascorbate mediated regeneration is not possible due to the reaction of GSH with oxidized ascorbate to form ascorbate (8). Based on the results it is concluded that 100 μM GSH reacts much faster than 40 μM ascorbate with QQ.

The inhibitory effect of ascorbate on HRP hampers a straightforward interpretation of our results. Moreover, only relatively low ascorbate concentrations compared to the GSH concentration can be used in the experiments. We therefore tested another oxidizing enzyme, i.e. tyrosinase.

As depicted in Figure 3.4B, tyrosinase is also capable of oxidizing quercetin into QQ. Ascorbate in a concentration of 40 μM prevents the apparent quercetin consumption for the greater part, confirming that ascorbate is capable of regenerating quercetin out of QQ. Ascorbate oxidation observed at 270 nm demonstrates that inhibition of tyrosinase by 40 μM ascorbate is much lower than that of HRP (figure not shown).

The presence of GSH during the tyrosinase mediated quercetin oxidation resulted in the formation of 6-GSQ and 8-GSQ in the same ratio as with HRP, based on the two isosbestic points at 310 nm and 355 nm as well as on HPLC analysis. The rate of quercetin consumption in the presence of GSH (6.6±0.6 μM/min) is equal to that in the absence of GSH (5.9±0.4 μM/min), suggesting that all the QQ is converted into GSQ and that GSH also does not inhibit tyrosinase.

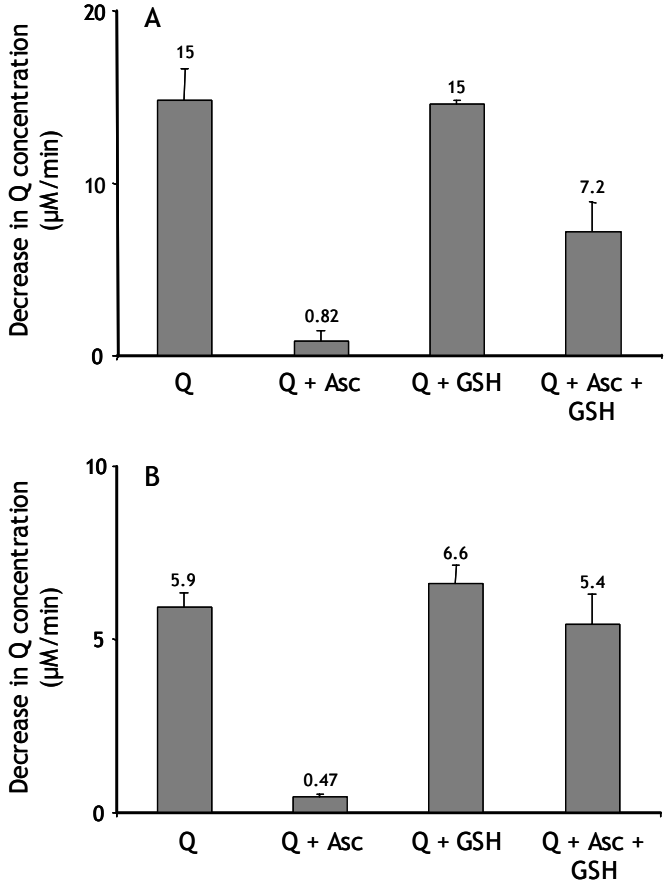


**Figure 3.4** The decrease in 60 seconds of the quercetin (Q) concentration, determined spectrophotometrically. The incubation mixture contained 50 μM quercetin and (A) 1.6 nM HRP and 33 μM $H_2O_2$ or (B) 25 U/ml tyrosinase in the presence of either 40 μM ascorbate (Asc), 100 μM glutathione (GSH) or both 40 μM ascorbate and 100 μM glutathione. All measurements were carried out in triplicate and expressed as mean ± SD.

Addition of both 40 μM ascorbate and 100 μM GSH together to quercetin and tyrosinase again results in the formation of GSQ. Contrary to the experiments with HRP, no significant difference in the rate of GSQ formation can be seen between the incubation without (6.6±0.6 μM/min) or with (5.4±0.9 μM/min) ascorbate. This again indicates that ascorbate does not affect tyrosinase acitivity substantially. Therefore, experiments with higher ascorbate concentrations were performed in order to further examine the competition between GSH and ascorbate for QQ.

Ascorbate at a concentration of 100 μM inhibits GSQ formation by 11%. This implies that GSH (the concentration is 100 μM) reacts at least 10 times faster with QQ than ascorbate. Increasing the ascorbate concentration up to 1 mM results in an inhibition of GSQ formation of 30% (Figure 3.5). This suggests that GSH reacts at least 20 times faster with QQ than ascorbate. Since we expect that the major part of the effect of ascorbate is due to inhibition of tyrosinase, the reaction rate of QQ with GSH surpasses that of QQ with ascorbate by far.
In blood plasma GSH is practically absent and ascorbate concentrations are 40 to 60 μM (normal range). Preincubation of blood plasma with 50 μM quercetin and 25 U/ml tyrosinase causes a reduction of the protein thiol content of 25±6%. This reduction is 50±11 % when the blood plasma is preincubated with 100 μM quercetin and 100 U/ml tyrosinase. Thiol consumption after addition of 100 μM ascorbate to the incubation with 50 μM or 100 μM quercetin is 27±4% or 46±3% respectively. Apparently, protein thiol consumption is not affected by the presence of ascorbate, implying that ascorbate does not protect protein thiol groups against QQ.

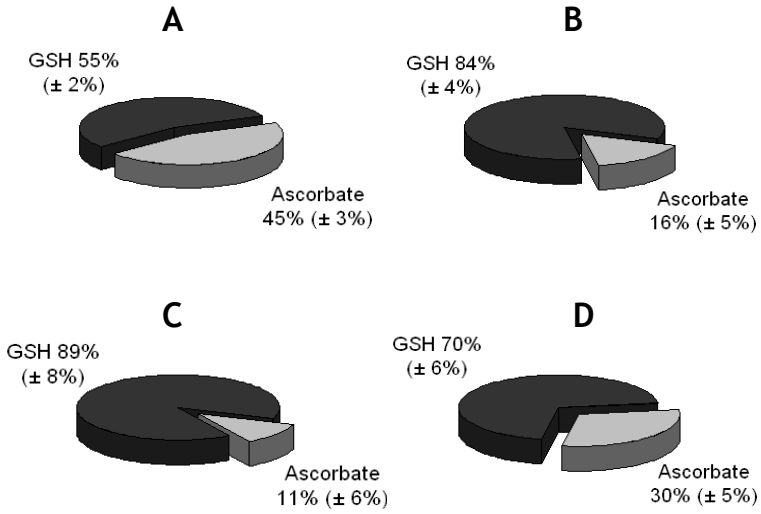


**Figure 3.5** Inhibition by ascorbate of GSQ formation. Quercetin at a concentration of 50 μM was oxidised by either 1.6 nM HRP and 33 μM $H_2O_2$ (A) or by 25 U/ml tyrosinase (B-D). The concentration of ascorbate was 40 μM (A-B), 100 μM (C) or 1 mM (D). The incubation mixture was analysed 5 minutes after the incubation was started by the addition of the enzyme. GSQ formation (the sum of 6-GSQ and 8-GSQ) was quantified using HPLC. The inhibition by ascorbate was calculated by relating GSQ formation in the presence of ascorbate to that in the absence of ascorbate. Experiments were carried out, at least, in triplicate and expressed as mean ± SD.

## Discussion

Free radical scavengers become oxidised when they exert their actual antioxidant effect. This formation of oxidised antioxidants is considered to be an important mechanism for antioxidant toxicity (13). Two electron oxidation of the flavonoid quercetin yields an oxidation product, denoted as QQ, that has four tautomeric forms, i.e. an *ortho*-quinone and three quinonmethides (11,14,15) (Figure 3.6). Oxidised antioxidants can be recycled in an interplay with other antioxidants such as ascorbate and GSH, called antioxidant networking (16,17). In this study we investigated the reaction of QQ with GSH or ascorbate.

The results of this study show that ascorbate recycles QQ to the parent compound quercetin, while GSH forms two adducts with QQ, i.e. 6-GSQ and 8-GSQ (Figure 3.6). In the present study it is shown that GSH reacts much faster than ascorbate with QQ. In other words, when GSH is present QQ will react with GSH.

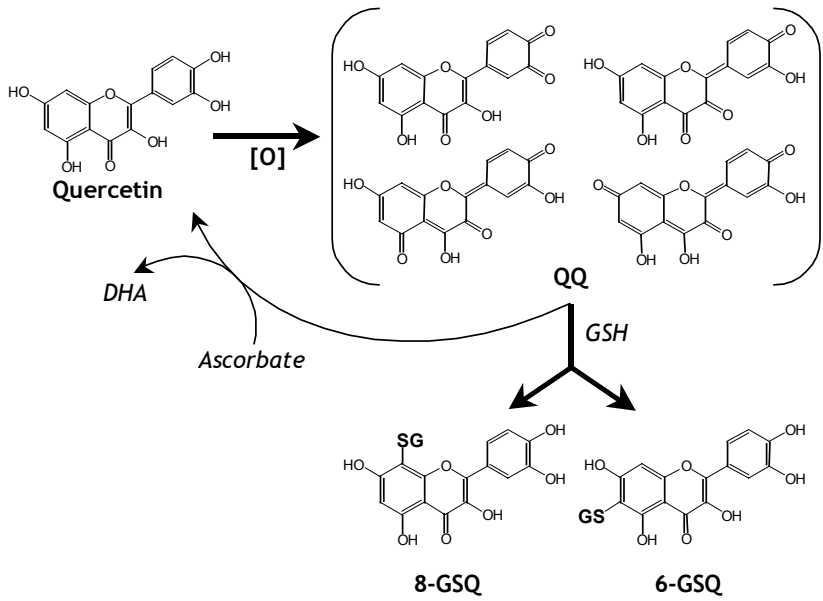


**Figure 3.6** Interplay between quercetin and ascorbate or GSH.
When quercetin becomes oxidized, an *o*-quinone/quinonmethide, referred to as QQ, can be formed. In the figure the 4 tautomeric forms of QQ are given. Ascorbate can recycle QQ to quercetin. During this reaction ascorbate becomes oxidized giving DHA (dehydroascorbate). QQ can also react with GSH, thereby forming 6-GSQ as well as 8-GSQ. QQ reacts much faster with GSH than with ascorbate.

*In vivo* most cells and body fluids contain relatively high levels of GSH. Under these circumstances GSH will capture QQ resulting in the formation of GSQ. This will be the case in epithelial lining fluid (ELF) with GSH and ascorbate concentrations that are identical to those initially used in the present study, i.e. 100 μM GSH and 40 μM ascorbate (18,19). In most cells the GSH concentration is higher, up to 1 mM in erythrocytes and up to 10 mM in hepatocytes (19,20). Furthermore, the formation of GSQ might be further enhanced by the presence of the glutathione-S-transferases, enzymes that catalyse the reaction of GSH with electrophiles such as QQ (21). This means that in most body compartments GSH will react with the formed QQ, resulting in the formation of GSQ adducts.

In blood plasma GSH is practically absent while the concentration of ascorbate is 40 to 60 μM (22). These conditions suggest that in blood ascorbate might react with QQ, thereby regenerating quercetin so that the flavonoid becomes available again for the antioxidant network. However, in blood plasma ascorbate has to compete with protein thiols. Addition of ascorbate (100 μM) does not alter the reduction of thiol content of blood plasma caused by preincubation with QQ, implying that QQ will react rather with the protein thiols than with ascorbate.

Recently, Van Zanden *et al.* (23) found that QQ arylates cysteine 47 of glutathione-S-transferase-P1-1, thereby inhibiting this isoenzyme. Not only GSH but remarkably, also ascorbate was reported to prevent this inhibition. This protective effect of ascorbate might have been due to the high and non-physiological concentration of ascorbate (1 mM) that was used in this study or to a relatively low reactivity of the thiol group of cysteine 47 towards QQ.

A large body of evidence demonstrates that quercetin can protect against oxidative stress by efficiently scavenging free radicals (24). During this "protection" the antioxidant is converted into reactive products. The present study indicates that in this way oxidative damage is shifted toward thiol arylation. Ascorbate cannot prevent this thiol arylation. We have previously shown that quinones similar to the oxidation product of quercetin can reduce the thiol content of microsomes and inactivate vital enzymes that contain critical thiolgroups (6). Interestingly, Yen *et al.* (25) recently reported that treatment of lymphocytes with quercetin reduced the protein thiol content of the cells. This can also be attributed to oxidation products such as QQ formed during the aerobic incubation of the cells.

In summary, when GSH is present oxidised quercetin reacts with GSH. This reaction however eliminates both the flavonoid and GSH from the antioxidant network. When GSH is absent, protein thiols are arylated by the oxidised antioxidant. Thiol arylation by quinones and quinonmethides can impair several vital enzymes. This implies that the product formed when quercetin

displays its antioxidant scavenging effect is toxic in the absence of GSH. Therefore, an adequate GSH level should be maintained when quercetin is supplemented.

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