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# Oxidative Cyclization, 1,4-Benzothiazine Formation and Dimerization of 2-Bromo-3-(glutathion-S-yl)hydroquinone

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## SUMMARY

Several lines of evidence suggest that the renal-specific toxicity of quinol-linked GSH conjugates is probably a result of their metabolism by γ-glutamyl transpeptidase and selective accumulation by proximal tubular cells. Transport of the resultant quinol-cysteine and/or cystein-*S*-ylglycine conjugate followed by oxidation to the quinone may be important steps in the mechanism of toxicity of these compounds. Factors modulating the intracellular and/or intralumenal concentration of the cystein-*S*-yl and cystein-*S*-ylglycine conjugate will, therefore, be important determinants of toxicity. We have now studied the γ-glutamyl transpeptidase-mediated metabolism of 2-bromo-3-(glutathion-*S*-yl)hydroquinone. The product of this reaction, 2-bromo-3-(cystein-*S*-ylglycyl)hydroquinone, undergoes an intramolecular cyclization to yield a 1,4-benzothiazine derivative that retains the glycine residue. A similar cyclization reaction occurs with 2-bromo-3-(cystein-*S*-yl)hydroquinone, which is unstable in aqueous solutions and undergoes a pH-dependent rearrangement that requires initial oxidation to the quinone. UV spectroscopy revealed that, at neutral pH, further reaction results in the formation of a chromophore, consistent with 1,4-benzothiazine formation. This product arises via cyclization of the cysteine residue via an intramolecular 1,4 Michael addition. Further reaction results in the precipitation of a pigment that exhibits properties of a pH indicator. The pigment undergoes a marked pH-dependent bathochromic shift (~100 nm); it is red in alkali ($\lambda_{max}$, 480 nm) and violet in acid ($\lambda_{max}$, 578 nm). These properties are similar to those of the trichochrome polymers that are formed during melanin biosynthesis from *S*-(3,4-dihydroxyphenylalanine)-L-cysteine. Because the intramolecular cyclization reactions remove the reactive quinone moiety from the molecules, they may be regarded as detoxication reactions. 1,4-Benzothiazine formation represents a novel pathway that diverges from the usual route of mercapturic acid synthesis and may represent previously unrecognized and important products of quinone metabolism *in vivo*.

We have recently provided evidence that supports the hypothesis that the renal necrosis observed after bromobenzene administration to rats is caused by the formation of GSH conjugates of 2-BrHQ (1-3). In particular, 2-Br-[diGSyl]HQ is a potent renal proximal tubular toxicant (2, 3). This disubstituted GSH conjugate is about 30 times more toxic than the parent hydroquinone and between 5- and 10-fold more toxic (on a molar equivalent basis) than three monosubstituted isomers. Physiological, biochemical, and electrochemical factors appear to contribute to the enhanced nephrotoxicity of the disubstituted conjugate (3).

The mechanism of quinol-linked GSH conjugate-mediated nephrotoxicity remains unclear. However, metabolism of these conjugates by γ-GT appears to be essential for their expression of toxicity, because inhibition of this enzyme with AT-125 completely protected animals from the proximal tubular necrosis caused by quinol-GSH conjugates (3, 4). The activity of γ-GT is probably required either for the uptake of the resulting quinol-cystein-*S*-yl and/or cystein-*S*-ylglycine conjugates into renal proximal tubular cells (3) and/or to facilitate oxidation of the hydroquinone moiety (5, 6); 2-bromo-3-(cystein-*S*-yl)hydroquinone is more readily oxidized than 2-bromo-3-(glutathion-*S*-yl)hydroquinone. Thus, the accumulation of 2-Br-(diG[$^{35}$S]yl)HQ by freshly isolated renal cortical slices was inhibited by AT-125 (7) and the *in vivo* covalent binding of [$^{14}$C]2-BrHQ to renal macromolecules was also significantly inhibited by pretreatment of rats with AT-125 (8).

A number of factors modulate the intracellular concentration of cysteine conjugates. The conjugate may be excreted unchanged into urine or it can be converted to its mercapturic

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**ABBREVIATIONS:** 2-BrHQ, 2-bromohydroquinone; AT 125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; 2-Br-(diGSyl)HQ, 2-bromo-(diglutathion-*S*-yl)hydroquinone; β-lyase, cysteine conjugate β-lyase; γ-GT, γ-glutamyl transpeptidase; DOPA, 3,4-dihydroxyphenylalanine; HPLC, high performance liquid chromatography.

acid derivative by *N*-acetyltransferase (9). In turn, the mercapturate may then be excreted in urine or the cysteine conjugate regenerated by the action of a deacetylase enzyme (10). Thus, the amount of cysteine conjugate will depend upon the relative activities of these two enzymes. Alternatively, the cysteine conjugate may be converted to a potentially reactive thiol by $\beta$-lyase (11). Thus, a number of enzymatic factors contribute to the intracellular concentration of cysteine conjugates. We now report additional factors that may contribute significantly to the intracellular and/or intralumenal concentration of quinol-linked cysteine-*S*-yl and cystein-*S*-ylglycine conjugates, namely, oxidative cyclization, 1,4-benzothiazine formation, and dimerization. The possible toxicological implications of these reactions are also discussed. The data also reveal some potentially important new insights into the biochemistry of melanin synthesis.

## Materials and Methods

**Chemicals.** $\gamma$-GT (EC 2.3.2.2; bovine, 5.6 units/mg) was purchased from the Sigma Chemical Co. (St. Louis, MO). 2-Bromo-3-(glutathion-*S*-yl)hydroquinone was synthesized according to previously published methods (2, 3) and structural identification was confirmed by $^1$H and $^{13}$C NMR spectroscopy. 2-Bromo-3-(cystein-*S*-yl)hydroquinone was synthesized according to previously published methods (2, 3) and purified by HPLC. Structural identification was confirmed by $^1$H and $^{13}$ NMR (to be published elsewhere). All other reagents were of the highest grade commercially available.

**$\gamma$-GT-mediated metabolism of 2-bromo-3-(glutathion-*S*-yl)hydroquinone.** 2-Bromo-3-(glutathion-*S*-yl)hydroquinone (15 mg) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.4. To this solution was added 0.5 units of $\gamma$-GT (3.9 $\mu$g/$\mu$l; 0.5 unit/11 $\mu$l) in 0.1 M phosphate buffer (pH of final solution, 6.7), and the reaction was allowed to stand at 25° for 3 hr. At this time, 1.0 ml of Tris buffer (0.1 M, pH 9.0) and 1.0 unit of $\gamma$-GT were added (pH of final solution, 7.5), and the reaction was allowed to continue for an additional 2 hr. The solution turned progressively from colorless to yellow to brown. An additional 9 units of $\gamma$-GT were added and the solution then turned from brown to green during the following 3.5 hr. The reaction was terminated by freezing the mixture in dry ice. The reaction products were analyzed and purified by HPLC (Shimadzu LC-6A). A 1.0-ml aliquot of the reaction mixture was injected onto a Magnum 9 ODS-3 (Whatman) reverse phase semipreparative column and was eluted with a linear gradient of methanol/water/formic acid (10:89.6:0.4 to 100:0:0) at a flow rate of 3 ml/min, over 60 min; the eluate was monitored at 280 nm. Several UV-absorbing peaks were eluted from the column, with the major peak eluting at 33.4 min (Fig. 1). Individual peaks were collected from several injections of the mixture and combined. The methanol content of each fraction was removed by evaporation under vacuum. The remaining aqueous fractions were then frozen in dry ice/acetone and lyophilized. The major product eluting at 33.4 min was analyzed by $^1$H and $^{13}$C NMR spectroscopy and $^{252}$Cf plasma desorption mass spectroscopy.

**Structural identification.** NMR spectra were determined on solutions of 2 mg of compound dissolved in 0.5 ml of deuteromethanol. Spectra were recorded on a Varian XL200 spectrometer at 200 MHz ($^1$H, 64 free induction decays) and 50 MHz ($^{13}$C, 61,000 free induction decays). Mass spectra were obtained on a $^{252}$Cf plasma desorption mass spectrometer (12).

**UV spectroscopy studies.** All spectra were recorded on a Hewlett-Packard 8450 UV-visible recording spectrophotometer at 25°. 2-Bromo-3-(cystein-*S*-yl)hydroquinone was dissolved at a concentration of 50 $\mu$g/ml (0.16 mM) in 0.1 M Tris buffer at various pH values, and spectra were recorded at various time intervals.

## Results

**$\gamma$-GT-mediated metabolism of 2-bromo-3-(glutathion-*S*-yl)hydroquinone.** Fig. 1A shows the HPLC profile of the reaction products obtained after incubation of 2-bromo-3-(glutathion-*S*-yl)hydroquinone in the presence of $\gamma$-GT for 5 hr. Two UV-absorbing peaks, with retention times of 14.8 and 16.2 min, were observed. The latter peak represents the substrate, 2-bromo-3-(glutathion-*S*-yl)hydroquinone. The earlier peak probably represents 2-bromo-3-(cystein-*S*-ylglycyl)hydroquinone, because the intensity of this peak decreases concomitantly with the increase in the major product (peak 3) eluting with a retention time of 33 min (Fig. 1B). Fig. 2 shows the UV spectrum of this product, with maxima at 250, 270, and 345 nm. Purification of this product by preparative HPLC and analysis by $^1$H (Fig. 3) and $^{13}$C NMR spectroscopy support the following structural assignment. The $^1$H spectrum showed H-5 and H-6 as doublets, $J$ = 8.4 Hz, at 6.83 and 7.35 ppm; two singlets of two-proton strength appeared, corresponding to H-2 at 4.06 and $\alpha$-glycine at 3.67 ppm. The $^{13}$C spectrum showed: 173.1 ppm (COOH), 165.0 (amide CO), 156.5 (C-3), 148.4 (C-7), 137.1 (C-4*a*), 130.6 (C-5), 114.3 (C-6), 109.6 (C-8), 42.3 ($\alpha$-glycine), and 21.8 (C-2). The absence of a peak corresponding to C-8*a* is presumably due to its long relaxation time, a consequence of its being separated by several bonds from the closest proton. The plasma desorption mass spectrum showed quasi-molecular ions at $m/z$ 389 and 391, corresponding to $C_{11}H_8BrN_2O_4S \cdot Na_2$. Based upon $^1$H and $^{13}$C NMR and $^{252}$Cf plasma desorption mass spectroscopy, the compound was, therefore, identified as 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine. When the compound was dissolved in 1%

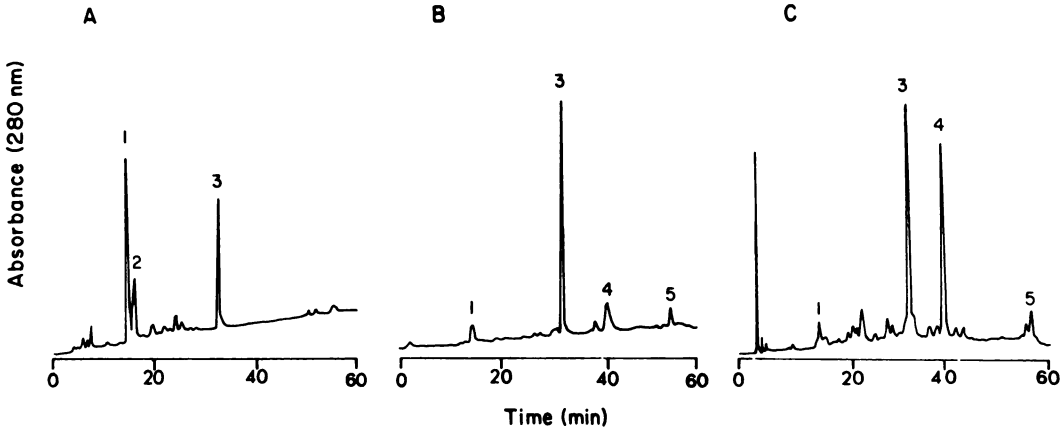


**Fig. 1.** HPLC elution profile of the products formed from the $\gamma$-GT-catalyzed hydrolysis of 2-bromo-3-(glutathion-*S*-yl) hydroquinone after 5 hr (A), 23 hr (B), and 27 hr (C). Chromatographic conditions are as described in Materials and Methods.

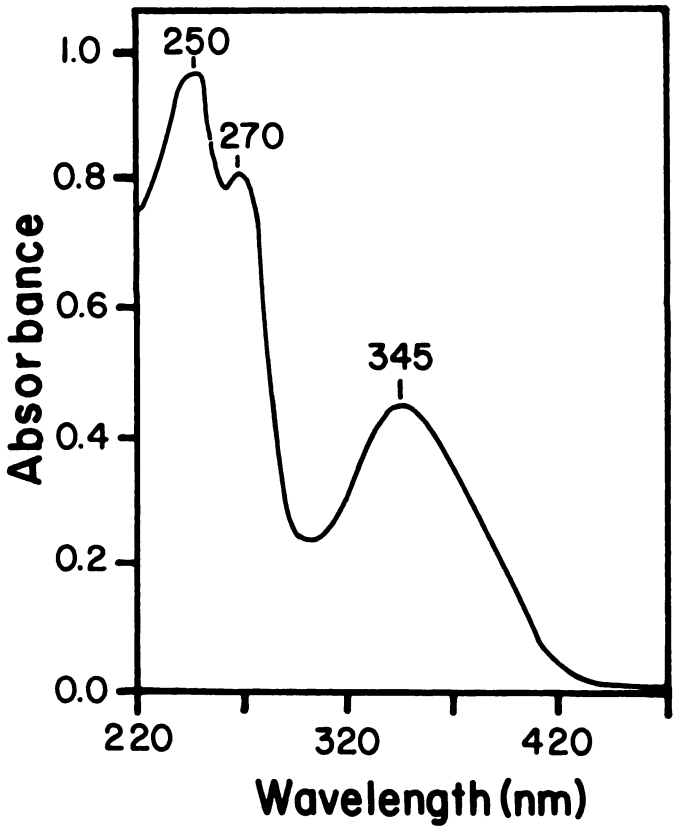


**Fig. 2.** UV spectrum of HPLC peak 3 described in Fig. 1. UV spectra were recorded on a Hewlett-Packard 8450 UV/visible spectrophotometer at 25°.

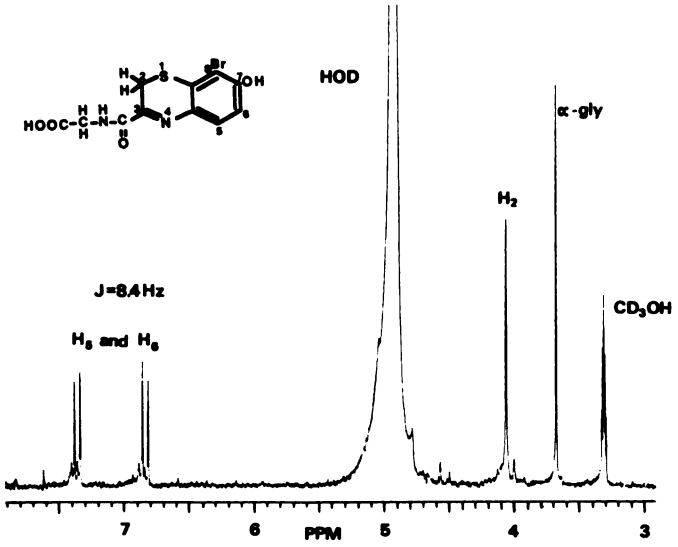


**Fig. 3.** 200 MHz $^1$H NMR spectrum of 2*H*-(3-glycinyl)-7-hydroxy-8-bromo-1,4-benzothiazine (HPLC peak 3 described in Fig. 1).

deuteroacetic acid in deuteromethanol, acid-catalyzed hydrolysis was shown by the appearance of signals in the $^1$H NMR spectrum that could be attributed to free glycine.

When the γ-GT-containing reaction mixture was allowed to stand at room temperature for 24 hr, a black precipitate formed. The HPLC profile of an aliquot of the supernatant showed numerous UV-absorbing peaks (Fig. 1C). Although the major product remained the 1,4-benzothiazine derived from 2-bromo-3-(cystein-*S*-ylglycyl)hydroquinone, additional minor products (as determined by the yield of material following lyophilization) were observed with retention times of 39.9 and 57 min. Both these products exhibited an intense yellow color. Several additional minor UV-absorbing peaks were also observed. Addition of 1 N NaOH to peak 5 shifted the $\lambda_{max}$ from 326 and 468 nm to 343 and 543 nm, with the solution turning from yellow to violet. The pH-indicator properties of this product and its longer retention time upon reverse phase HPLC suggest that it exhibits structural similarities with the trichochrome pigments and, therefore, probably represents dimeric and/or polymeric forms of the 1,4-benzothiazine.

**UV spectroscopic studies.** Fig. 4 shows the UV spectra of 2-bromo-3-(cystein-*S*-yl)hydroquinone dissolved in either 1 N HCl or 1 N NaOH. In acid, the compound was very stable and exhibited UV maxima at 210, 260, and 310 nm. A similar spectrum was observed using 40% ethanol as the solvent. Base-catalyzed oxidation of the conjugate with 1 N NaOH gave rise to the immediate appearance of a yellow chromophore with a $\lambda_{max}$ at 356 nm. Oxidation of 2-bromo-3-(cystein-*S* -yl)hydroquinone occurred rapidly at pH 10 in 0.1 M Tris buffer (Fig. 5). In addition, the spectrum underwent a rather slow red-shift, which was presumably due to cyclization of the quinone. This shift could be accelerated by dissolving the compound in distilled water and oxidizing it with sodium periodate (data not shown) but, at pH 7.4, oxidation to the quinone was slower (Fig. 6). The UV spectroscopy studies are consistent with the structural assignment for these pigments. The close similarity of the behavior of this chromophore to that of some model compounds (13–15) confirmed the presence in the molecule of a chromophoric system in which the nitrogen and sulfur atoms are linked by a chain of conjugated carbon atoms, —S—[C═C—C]═N—. Thus, like the natural melanin polymers trichochromes E and F, the pigment resulting from the oxidative cyclization of 2-bromo-3-(cystein-*S*-yl)hydroquinone showed a marked bathochromic shift (~100 nm) in acid solution (Fig. 7).

## Discussion

We have demonstrated that 2-bromo-3-(glutathion-*S*-yl)hydroquinone and 2-bromo-3-(cystein-*S*-yl)hydroquinone

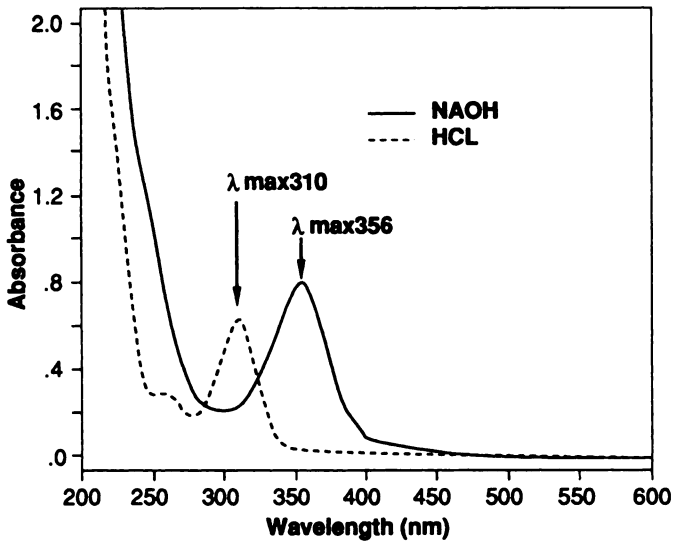


**Fig. 4.** UV spectra of 2-bromo-3-(cystein-S-yl)hydroquinone under acidic and basic conditions. The $\lambda_{max}$ at 310 nm represents the reduced form of the conjugate, whereas the $\lambda_{max}$ at 356 nm represents the oxidized form of the conjugate.

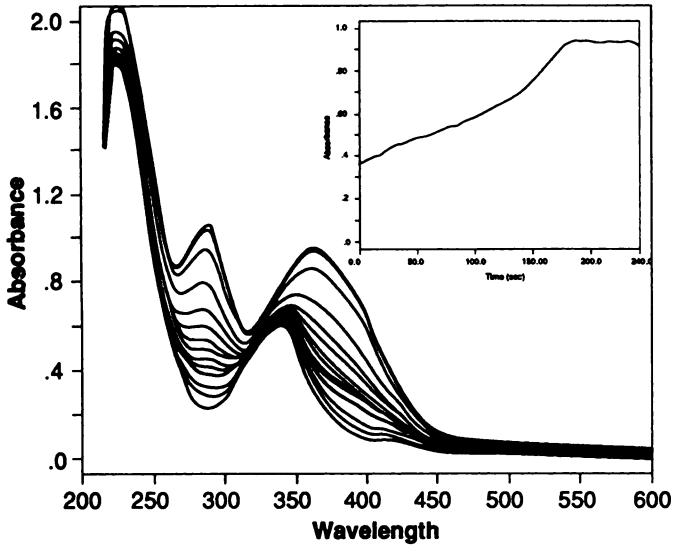


**Fig. 5.** Base-catalyzed oxidation of 2-bromo-3-(cystein-*S*-yl)hydroquinone (50 $\mu$g/ml in 0.1 M Tris, pH 10). Spectra were recorded at 30-sec intervals over a period of 4 min; *bottom trace*, time zero; *top trace*, 4 min. *Inset*, the increase in absorbance at 356 nm with time.

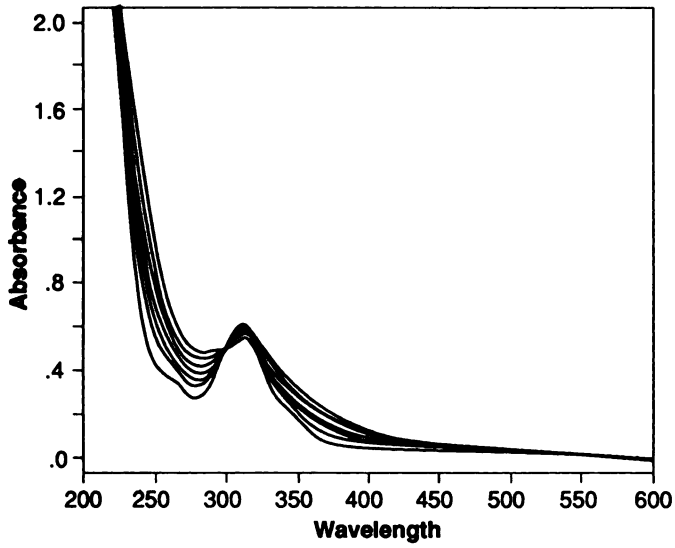


**Fig. 6.** Autooxidation of 2-bromo-3-(cystein-*S*-yl)hydroquinone (50 $\mu$g/ml in 0.1 M Tris, pH 7.4) Spectra were recorded at 30-sec intervals over a period of 4 min.

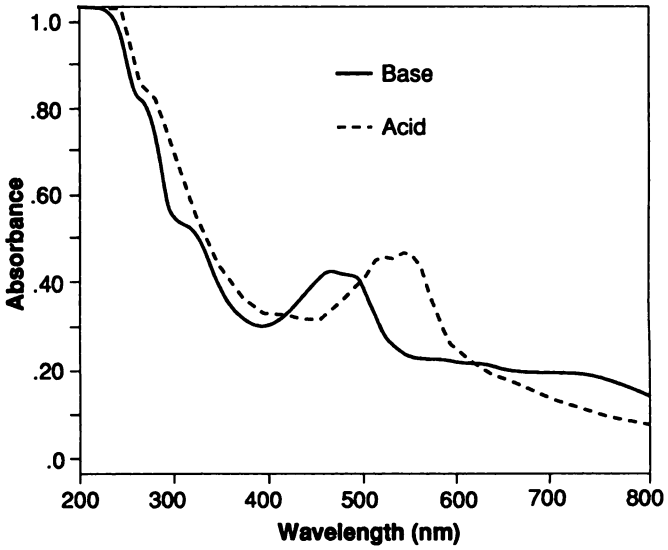


**Fig. 7.** UV spectra of the pigment formed via the oxidative cyclization of 2-bromo-3-(cystein-*S*-yl)hydroquinone in acid and base. The pigment demonstrates pH-indicator properties, being violet in acid and red in base.

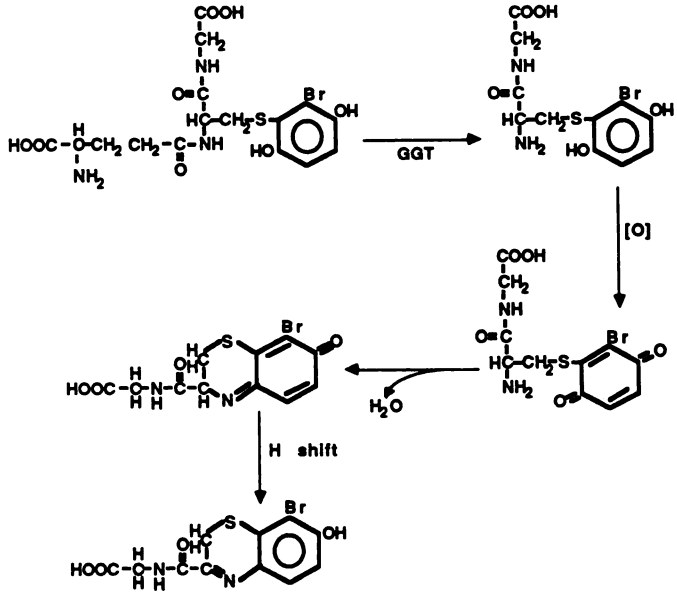


**Fig. 8.** Suggested pathway(s) of the $\gamma$-GT-mediated metabolism of 2-bromo-3-(glutathion-*S*-yl)hydroquinone followed by subsequent rearrangement via oxidative cyclization, resulting in 1,4-benzothiazine formation.

undergo oxidative cyclization reactions that result in 1,4-benzothiazine formation (and subsequent dimerization) (Fig. 8). The addition of cysteine to 1,4-benzoquinone has previously been shown to be followed by subsequent oxidation and cyclization (16), although the biological significance of this reaction has not been investigated. A similar cyclization reaction has been shown to occur with 2-methyl- and 2,5-dimethyl-1,4-benzoquinone and 1,4-naphthoquinone (17). The addition of cysteine to 2-methyl-1,4-naphthoquinone (menadione) also takes place but the cyclization step has yet to be reported (18).

Oxidation of 2-bromo-3-(cystein-*S*-yl)hydroquinone can be base catalyzed and, therefore, occurs more rapidly at pH 10 than at pH 7.2 (Figs. 5 and 6). However, the cyclization of the cystein-*S*-ylquinone requires both the initial protonation of the quinone and elimination of water and, thus would be acid catalyzed (19). The reacting species involves a deprotonated amine on the cysteine side chain. Although at neutral pH neither the conditions for oxidation nor those for subsequent cyclization are optimal, conditions for the overall reaction sequence are ideal. The end products of both 2-bromo-3-(cystein-*S*-ylglycyl)hydroquinone and 2-bromo-3-(cystein-*S*-yl)hydroquinone rearrangement possess pH-indicator properties (Fig. 7) similar to those of the trichochromes, which are formed during melanin biosynthesis from cystein-*S*-yl-DOPA (20). Because oxidative cyclization, 1,4-benzothiazine formation, and dimerization occur readily at neutral pH, such reactions should occur readily within proximal renal tubule cells. Moreover, although this reaction removes the reactive quinone moiety from the molecule and might, therefore, be considered

an intramolecular detoxication reaction, the subsequent formation of insoluble polymers, either within proximal tubule cells or within the tubular lumen itself, may contribute to the toxicity of these compounds. Which of these alternatives occurs as a consequence of 1,4-benzothiazine formation *in vivo* is unclear, but recent evidence suggests that these reactions constitute a detoxication pathway (6, 21).

1,4-Benzothiazine formation from biogenic quinone-thioethers has only been shown to occur with the cystein-*S*-yl precursor. The present data demonstrate for the first time that the building blocks for sulfur-containing phaeomelanin synthesis can arise without the obligatory formation of the free cystein-*S*-yl-linked quinone. The major product formed following the γ-GT-mediated hydrolysis of 2-bromo-3-(glutathion-*S*-yl) hydroquinone is a 1,4-benzothiazine retaining the glycine moeity, which is eliminated only after cyclization (Fig. 9). The significance of these observations is unclear, but they may be pertinent to the current debate on the relative importance of glutathion-*S*-yl-DOPA and cystein-*S*-yl-DOPA in phaeomelanin synthesis (22).

Polymerization of the 1,4-benzothiazines arising from the oxidative cyclization of cystein-*S*-yl-DOPA has been proposed to occur via oxidative coupling of the carbon atoms *ortho* to the sulfur atom [i.e., 2,2-bis(1,4-benzothiazine)] (13–15, 22–26). In contrast, one report suggested that 1,4-benzothiazine may give rise to a mixture of polymeric products that are coupled via the carbon atom *ortho* to the sulfur in one benzothiazine and *ortho* to the nitrogen atom in the second benzothiazine [i.e., 2,3-bis(1,4-benzothiazine)] (27). A similar mechanism of 1,4-benzothiazine coupling has recently been reported (28). Based upon current evidence, we propose that the acid-catalyzed poly(di)merization of the 1,4-benzothiazines arising from the oxidative cyclization of both 2-bromo-3-(cystein- *S*-yl) hydroquinone and 2-bromo-3-(cystein-*S*-ylglycyl) hydroquinone occurs via the mechanism shown in Fig. 9. Evidence for steps 1–3 is provided by the observation that during NMR analysis, under acid conditions, glycine is readily

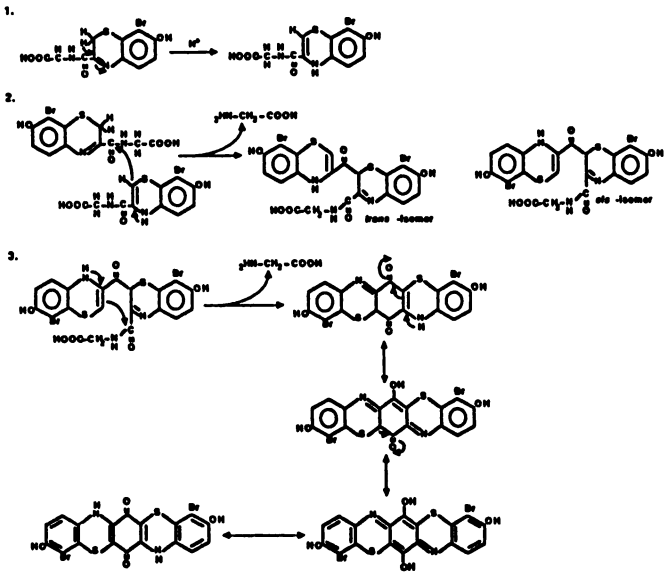

**Fig. 9.** Acid-catalyzed elimination of glycine with subsequent dimerization of the 1,4-benzothiazine derived from 2-bromo-3-(cystein-*S*-ylglycyl)hydroquinone.

eliminated from the molecule and can be detected free in solution. Elimination of glycine also occurs slowly in deuteromethanol. Further reaction of the dimeric product shown in Fig. 9 can give rise to the complex polymers that probably constitute the insoluble precipitate (melanin) that is formed as the end product of these reactions.

Conjugation with GSH and the subsequent formation of the corresponding cysteine conjugates have been implicated in the mutagenicity and nephrotoxicity of a variety of halogenated alkanes and alkenes (29). For example, *S*-(1,2-dichlorovinyl)-L-cysteine (30) and the cysteine and *N*-acetyl cysteine conjugates of hexachloro-1,3-butadiene (31–34) cause renal proximal tubular necrosis when administered to rats. *S*-(1,2-Dichlorovinyl)-L-cysteine is an excellent substrate for the enzyme β-lyase (11) and is metabolized to a sulfur-containing alkylating metabolite that reacts covalently with tissue components (35–38). β-Lyase has also been suggested to play a role in the nephrotoxicity of the cysteine conjugate of hexachloro-1,3-butadiene (32). Although substantial evidence supports a crucial role for β-lyase in the nephrotoxicity of halogenated alkanes and alkenes (39), this enzyme does not appear to play a major role in the nephrotoxicity of quinol-linked GSH conjugates. For example, the selective nephrotoxicity of 6-bromo-2,5-dihydroxythiophenol, a putative β-lyase-catalyzed metabolite of 2-bromo-3-(glutathion-*S*-yl)hydroquinone, was shown to be dependent upon the quinone function (40). 2-, 3-, and 4-Bromothiophenol, which lack the dihydroxy (hydroquinone) moiety, did not produce any signs of renal damage. In addition, aminooxyacetic acid, which has been shown to inhibit β-lyase activity and protect against the nephrotoxicity of *S*-(1,2-dichlorovinyl)-L-cysteine, had little effect on either 2-Br-(diGSyl)HQ- (2)- or 2,3,5-(triglutathion-*S*-yl)hydroquinone-mediated toxicities (4). The present data may help to further explain the inability of aminooxyacetic acid to inhibit the *in vivo* toxicity of quinol-GSH conjugates. Once the γ-glutamyl residue is removed from these conjugates, oxidative cyclization and 1,4-benzothiazine formation can occur without cysteine conjugate formation. Thus, it is possible that quinol-GSH conjugates form few, if any, free cysteine conjugates. This is supported by the observation that neither cystein-*S*-yl nor *N*-acetylcystein-*S*-yl conjugates could be detected as major *in vivo* metabolites of 2-BrHQ (8).

Finally, the mechanisms most frequently suggested to explain quinone-mediated toxicities generally involve either sulfhydryl arylation and/or oxidation or oxidative stress as a consequence of redox cycling. Oxidation of protein sulfhydryl groups may result in the reversible inhibition of sulfhydryl-dependent enzyme activity, whereas quinone-mediated inhibition of sulfhydryl-dependent enzymes is often irreversible, a consequence of covalent interaction. Although sulfhydryl addition to quinones occurs readily, the ability of compounds containing basic nitrogen atoms to undergo Michael addition with a variety of quinones has been documented for many years (41). The present data suggest that some quinones may "cross-link" proteins by reacting with both sulfhydryl and amino groups of amino acids. Thus, certain quinones may also irreversibly inhibit non-sulfhydryl-dependent enzymes, should the cross-linked amino group reside at the active site of the enzyme. Quinones may also be mutagenic and carcinogenic (42). Whether quinones can react with DNA in a similar manner and cause DNA/protein cross-links is not known, and further studies would be

required to either support or refute this possibility. However, the GSH conjugate(s) of *N*-(4-ethoxyphenyl)-*p*-benzoquinone imine has been shown to bind to DNA (43).

In conclusion, we have shown that hydrolysis of 2-bromo-3-(glutathion-*S*-yl)hydroquinone by γ-GT gives rise to a labile cystein-*S*-ylglycine conjugate that readily undergoes oxidative cyclization, resulting in the formation of a 1,4-benzothiazine. Subsequent poly(di)merization of the 1,4-benzothiazine presumably gives rise to insoluble pigments (melanins), exhibiting pH-indicator properties, that are analagous to the trichochromes formed during phaeomelanin synthesis from cystein-*S*-yl-DOPA. 2-Bromo-3-(cystein-*S*-yl)hydroquinone undergoes a similar rearrangement. 1,4-Benzothiazine formation, thus, represents a novel metabolic pathway that diverges from the classical route of mercapturic acid synthesis following the conjugation of xenobiotics with GSH and may represent an important and heretofore unrecognized pathway of quinone metabolism *in vivo*.

**Acknowledgments**

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