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Interaction of Anthocyanins and Anthocyanidins with α-Hydroxyethyl Radicals

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Abstract—The products of interaction of anthocyanins and anthocyanidins with α-hydroxyethyl radicals have been studied using spectrophotometry and liquid chromatography–mass spectrometry. It has been shown that anthocyanins and anthocyanidins oxidize the hydroxyethyl radical. The anthocyanin transformation products are oxidized with oxygen to the parent anthocyanins. Anthocyanidins irreversibly react to form the corresponding hydroxybenzoic acid and presumably 4-(2-hydroxyethyl)resorcinol.

Keywords: anthocyanins, free radicals, radiolysis
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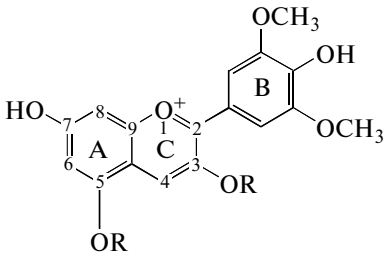
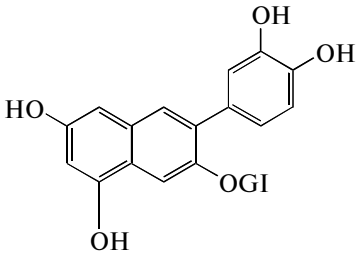
Anthocyanins are a widespread group of natural phenolic pigments. It is now well known that anthocyanins can react with oxygen-centered radicals, such as the superoxide radical anion [1–4], the hydroxyl radical [5, 6], and peroxide radicals [6, 7]. However, very little is known the about interaction of anthocyanins with carbon-centered radicals (CCRs). The reactions of antioxidants with CCRs attracts interest since the in vivo formation of radicals of this type has been detected. For example, Reinke et al. [8] detected α-hydroxyethyl radicals (HERs) in murine liver as intermediate products of ethanol metabolism. The trichloromethyl radical and lipid carbon-centered radicals were detected in the case of carbon tetrachloride metabolism [9], and methyl radicals were found during the metabolism of acetaldehyde [10] or *tert*-butyl peroxide [11]. The spin trapping technique was used in all of these cases, and the fact that the spin traps successfully competed with oxygen for CCRs without creating special conditions (hypoxia) suggests possible involvement of the reactions of antioxidants with CCRs in the mechanism of antioxidant protection.



R=H—malvidin
R=glucose—malvidin-3,5-diglycoside



cyanidin-3-glycoside

Scheme 1.

EXPERIMENTAL

The chemicals used were the anthocyanidins malvidin (Malv) and cyanidin (Cy), the anthocyanins malvidin-3,5-diglycoside (Malv-Gl) and cyanidin-3-glucoside (Cy-Gl) (PHYTOPLAN, Germany), and syringic acid (Sigma, the United Kingdom). The structural formulas are shown in Scheme 1.

To study the interaction of anthocyanins with hydroxyethyl radicals, the radiolysis of aqueous ethanol solutions (alcohol concentration, 5%; hydrochloric acid, 0.1%; anthocyanins, ~10^{-4} mol/L) was performed. Oxygen was removed by threefold freeze–pump–thaw cycles followed by sealing the ampoules under vacuum. The solutions were irradiated at room temperature on an RKhM-γ-20 facility (radiation source ^{60}Co, absorbed dose 4.5 Gy/min according to Fricke dosimeter). Radiation-induced changes in the

electronic absorption spectra of the solutions were recorded on an SF-2000 spectrophotometer in special quartz cells (10 mm in optical path length) connected with an ampoule for evacuation.

The products of radiation-induced transformations of anthocyanins were identified by liquid chromatography–mass spectrometry using an Acquity UPLC chromatograph with a tandem quadrupole detector. The separation was performed on an ACQUITY UPLC® R BEH C_{18} 50 × 2 mm 1.7 μm column in the gradient elution mode as specified in the table. The mobile phase was composed of (A) 0.1% formic acid in water and (B) acetonitrile.

Spectrophotometric scans were in the range of 200–800 nm. Mass spectrometric detection was carried out under the following conditions (found by optimizing the determination of Malv-Gl using the program InteliStart): the ESI+ operating mode, a voltage across the capillary of 3.0 kV, a cone voltage of 30 V, a source temperature of 150°C, a drying gas (nitrogen) flow rate of 1000 L/h, and a collision-cell voltage of 30 V (high-purity argon gas).

Acetaldehyde was determined as its hydrazone with 2,4-dinitrophenylhydrazine using HPLC.

2,4-Dinitrophenylhydrazine, purified by recrystallization from its ethanol solution saturated at a temperature of 50–60°C, was dissolved in acetonitrile to have a concentration of 1 mg/mL. The 2,4-dinitrophenylhydrazine solution and phosphoric acid (1 : 7) in an amount of 0.2 mL each were added to 1 mL of an alcohol solution, and 1 μL of the mixture was injected into the chromatograph after 20 minutes.

The mobile phase was the 60/40 acetonitrile–water blend used at a flow rate of 0.3 mL/min. The ACQUITY UPLC® BEH C_{18} (50 × 2 mm, 1.7 μm) column temperature was 40°C, detection was spectrophotometric at a wavelength of 360 nm, and the sample chamber temperature was 20°C.

RESULTS

Because of the low concentration of alcohol and other solutes, most (~95%) of the ionizing radiation energy incident on the system was absorbed by water, generating a significant amount of free radicals, whose yield is well documented [12]:

$$\begin{gathered} H_2O \rightsquigarrow \bar{e}\,(2.8–2.9), \\ H(0.6),\ \dot{O}H(2.8–2.9), \\ H_2(4.5),\ H_2O_2(0.75). \end{gathered} \quad (1)$$

Since the alcohol and hydrochloric acid concentrations are large compared with the test substances, the hydroxyl radical and the electron react with them, giving ultimately HER:

$$\bar{e} + H^+ \rightarrow H, \quad (2)$$

$$H + C_2H_5OH \rightarrow CH_3\dot{C}HOH + H_2, \quad (3)$$

Gradient elution mode of separation of radiolysis products

Time, min	Flow rate, mL/min	A, %	B, %
0	0.3	95	5
0.25	0.3	95	5
6.50	0.3	30	70
7.60	0.3	0	100

$$\dot{O}H + C_2H_5OH \rightarrow CH_3\dot{C}HOH + H_2O. \quad (4)$$

Thus, it may be assumed that HER is the only radical that can react with the test substance in this system. In addition, the presence of the acid ensures the stability of anthocyanins and anthocyanidins, which degrade in a neutral or alkaline solution. In the absence of additives, HERs are consumed in combination (5) and disproportionation reactions (6) to give 2,3-butanediol and acetaldehyde, respectively:

$$CH_3\dot{C}HOH + CH_3\dot{C}HOH \rightarrow CH_3CH(OH)CH(OH)CH_3, \quad (5)$$

$$CH_3\dot{C}HOH + CH_3\dot{C}HOH \rightarrow CH_3CHO + CH_3CH_2OH. \quad (6)$$

The irradiation of Malv-Gl and Cy-Gl glycoside solutions results in the most significant changes in the visible region of their electronic absorption spectra, as shown in Fig. 1a, and these changes are linearly related to the absorbed dose (Fig. 1b). The disappearance of the absorption band in the visible part of the spectrum implies that the reaction of Malv-Gl with HER destroys conjugation between ring B and the rest of the molecule, since it is this conjugation that is responsible for the electronic transition in anthocyanins at 520 nm [13].

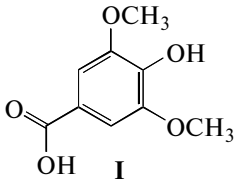
After unsealing the ampoule and saturating the irradiated solution with atmospheric oxygen, the color is restored and the absorption spectra of the initial unirradiated solution and the aerated irradiated solution differ for the most part in the intensity alone, as shown in Fig. 2.

The products of the Malv-Gl reaction with HER were separated chromatographically (Fig. 3a). Two major products with retention times of 2.55 and 2.69 min had the mass spectrometric characteristics similar to those of Malv-Gl ($m/z = 655$). These species were supposed to be protonated ions $(M + H)^+$, not the molecular ions as in the case of Malv-Gl. To verify this hypothesis, the flow from the chromatograph was mixed in the mass spectrometric detector with an ammonia solution, an operation that would lead to the formation of the $(M + NH_3)^+$ ion and an increase in m/z by 16. However, m/z remained unchanged: thus, it

is most likely that this is the molecular ion and the molecular weight of the products is the same as that of Malv-Gl. Similar to the spectrophotometric data, chromatographic results showed the recovery of the original Malv-Gl molecule as a result of contacting the irradiated solution with air (Fig. 4).

The radiolysis of aglycone (Malv) led to a similar change in the spectral characteristics of the solutions, but the color was not restored after contacting the irradiated solution with air, unlike the case of Malv-Gl.

Two products of the reaction of Malv with HER were detected chromatographically, syringic acid (**I**) and the product characterized by ion at m/z 155.



I

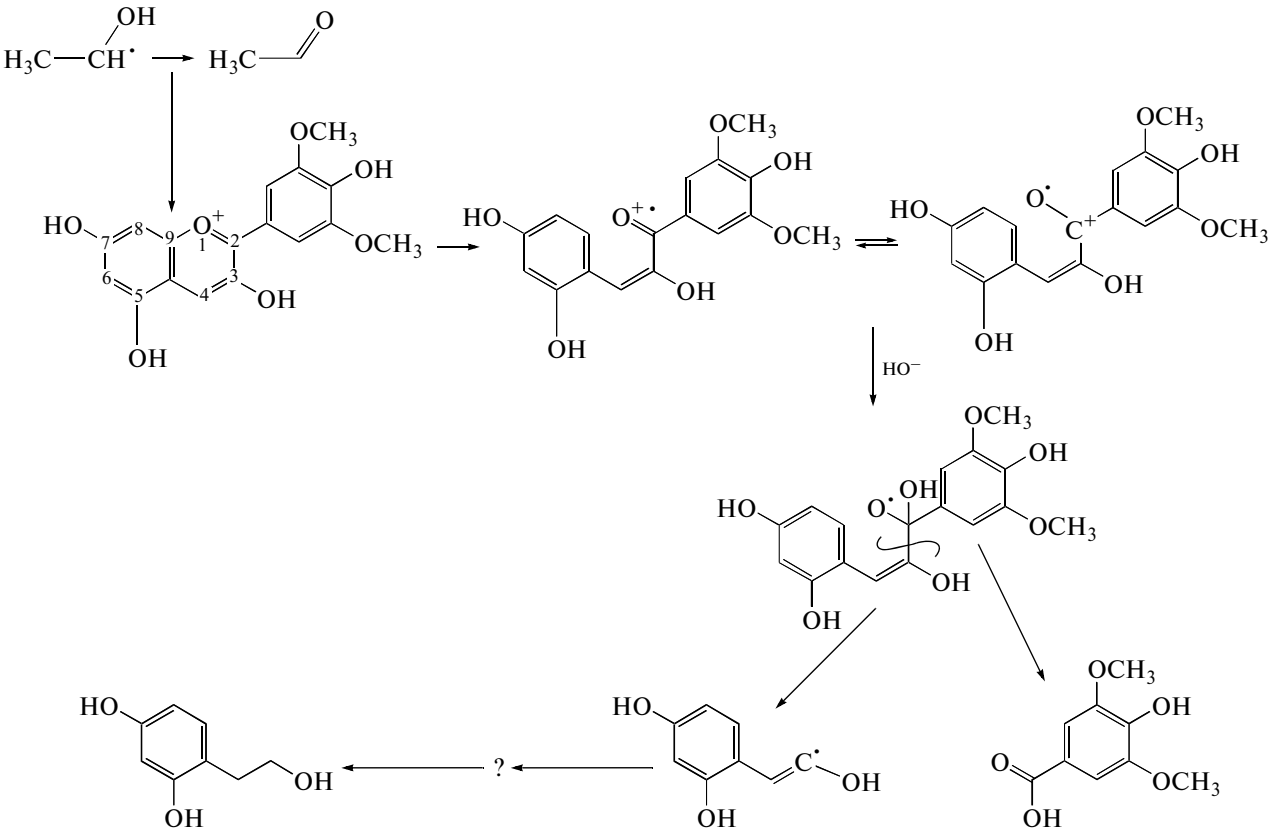
The interaction of anthocyanins with HER can occur via the following routes: (1) reduction of HER, (2) oxidation of HER, and (3) addition of HER.

Our studies showed that the yield of acetaldehyde in the presence of malvidin or malvidin-3,5-diglycoside increases from 1.29 to 2.68 or 3.27 molecule/100 eV, respectively, indicating that anthocyanins oxidize the hydroxyethyl radical.

DISCUSSION

Syringic acid can be formed during the radiolysis of a Malv solution via the fragmentation of an intermediate product of the Malv reaction with HER. This kind of fragmentation was suggested by Lee et al. [14], who observed the formation of methyl phenolcarboxylate during the radiolysis of cyanidin rutinoside in absolute methanol. However, the mechanism proposed by them involves the methoxyl radical, which is known to react with methanol with a large rate constant, thereby casting doubt on the mechanism.

We suggest that anthocyanins oxidize HER via hydrogen transfer from the HER to C9 (Scheme 2) followed by breaking the C9–O bond and nucleophilic addition of the hydroxyl ion to C2. When a medium lacks hydroxyl ions as in absolute methanol, the methoxide ion can add to the resulting cation, a fact that explains the difference in the radiolysis products, phenolcarboxylic acid in our case, and methyl phenolcarboxylate in the cited study.



Scheme 2. Suggested scheme of interaction of anthocyanidins with hydroxyethyl radicals

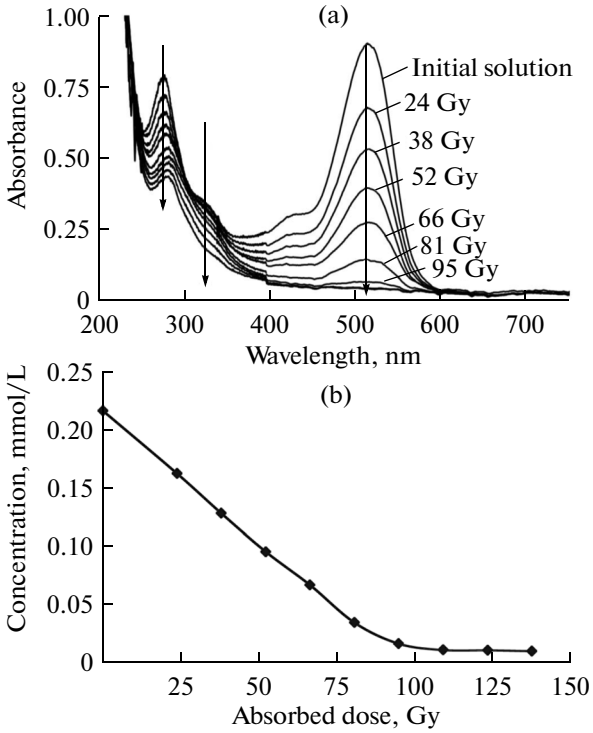
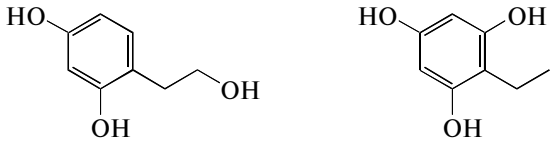


Fig. 1. (a) Change in electronic absorption spectra of Cy-Gl solutions by irradiation with different doses. (b) Dose dependence of the Cy-Gl concentration.

The second product of Malv fragmentation during the reaction with HER has structural formula II or III in the case of cleavage of the C9–O (our assumption) or the C2–O (as assumed in [14]) bond, respectively:



II **III**

In accordance with the mass of the ion, the substance must possess three hydroxyl groups and the saturated ethyl substituent on the benzene ring, suggesting two possibilities, 4-(2-hydroxyethyl)benzyl-1,3-diol (C9–O cleavage) and 4-ethylbenzyl-1,3,5-triol (C2–O cleavage).

ZINDO CI quantum-chemical calculations of the electronic spectra of the suggested entities (with geometry optimized by the B88-PW91 method of density functional theory) did not reveal a significant difference that would give grounds to prefer a particular structure. However, if the C2–O bonds breaks, the formation of phenolcarboxylic acids requires the attachment of two oxygen atoms to C2 and oxygen detachment from C3, whereas the attachment of one oxygen atom to C2 is required and the C3–O bond breaking is unnecessary in the case of C9–O cleavage.

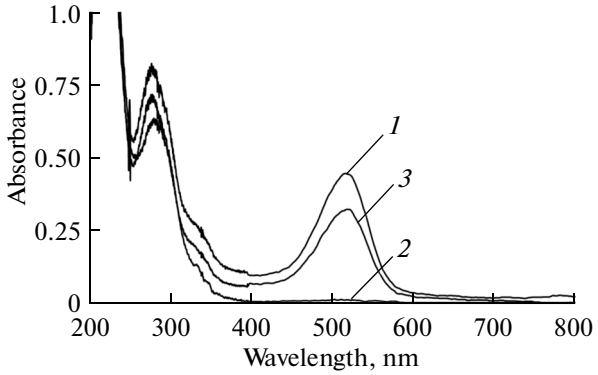


Fig. 2. Electronic absorption spectra of Malv-Gl solutions: (*1*) the solution before irradiation and (*2, 3*) the solution irradiated to a dose of 160 Gy before and after contact with air, respectively.

The reversible degradation of Malv-Gl shows that hydrogen of the hydroxyl group in the 3-position is involved in the fragmentation process, but this mechanism is difficult to explain. The presence or absence of free hydrogen in the hydroxyl group at C-5 has no effect on the reversibility of reduction of anthocyanins, since both cyanidin-3-glycoside and malvidin-3,5-diglycoside are reversibly reduced by HER, as shown in our experiments.

It is also interesting that hydroxyl groups, which exhibit antiradical activity in most phenolic compounds, do not participate in the interaction with HER. The fact that phenolic hydroxyls are not oxidized in the reaction with hydroxyethyl radicals

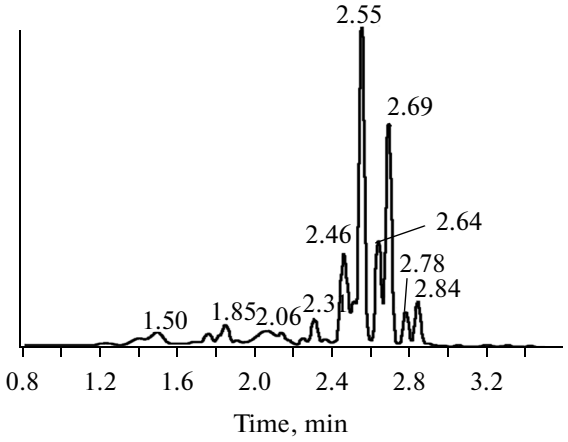


Fig. 3. Chromatogram of an irradiated malvidin-3,5-diglycoside solution in aqueous ethanol. Absorbed dose, 100 Gy; [Mal-Gl] = 0.1 mmol/L.

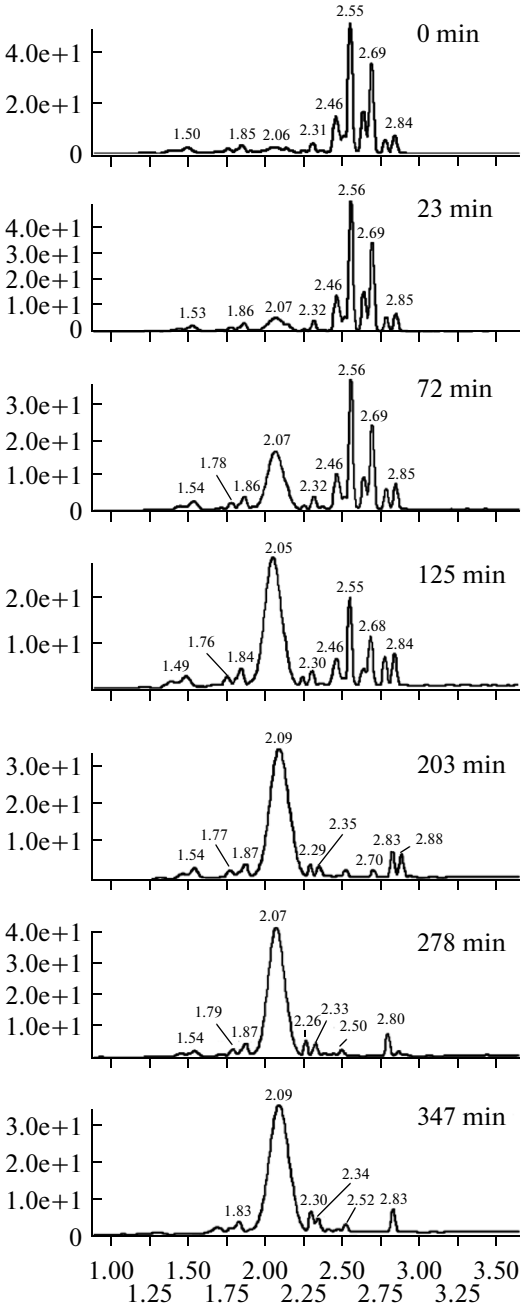


Fig. 4. Time changes in the chromatogram of the irradiated malvidin-3,5-diglycoside solution in aqueous ethanol solution after unsealing to be exposed to air over 0, 23, 72, 125, 203, 278, and 347 min.

explains the lack of inhibition of scavenging by the DPPH radical during irradiation of extracts of anthocyanin-containing plants [15].

CONCLUSIONS

It has been shown that anthocyanins oxidize the hydroxyethyl radical to acetaldehyde so that anthocyanidin-3-glycosides are reversibly reduced but aglycones decompose into the corresponding phenolcarboxylic acid (depending on the substituents on ring B) and, presumably, 4-(2-hydroxyethyl)resorcinol.

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