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BIOACCUMULATION AND BIOTRANSFORMATION OF FLAVONOLS BY ERYTHROCYTES

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A model for the complete system of bioaccumulation, transport, and biotransformation of polyphenolic compounds (flavonols) that includes hemoglobin-containing red blood cells and serum albumin was proposed. The distribution of flavonols between the erythrocyte fraction and albumin was studied. Hemoglobin was shown to play a role in the biotransformation of flavonols. The formation of several intermediate and final products of pseudoperoxidase oxidation of flavonols catalyzed by methemoglobin was established by UV spectrophotometry and RP-HPLC.

Key words: flavonols, quercetin, fisetin, morin, rutin, hemoglobin, serum albumin, erythrocytes, pseudoperoxidase oxidation.

Pseudoperoxidase oxidation of several natural flavonols involving hemoglobin as a catalyst has been demonstrated in our previous work [1]. It has been reported that hemoglobin can non-enzymatically oxidize quercetin, although with exceedingly low efficiency [2]. However, these processes have until now been poorly studied, even for the most common and studied flavonol, quercetin. Only single and often incomplete reports about the biotransformation pathways of other flavonols have been published.

Therefore, our goal was to investigate the bioaccumulation and biotransformation of flavonols by erythrocytes, including an analysis of the distribution of flavonols between the blood components serum albumin and erythrocytes, and to identify the products of pseudoperoxidase oxidation of flavonols involving hemoglobin using UV spectrophotometry and RP-HPLC. We selected the structurally related flavonols quercetin (3,5,7,3′,4′-pentahydroxyflavone), fisetin (5-deoxyquercetin), morin (3,5,7,2′,4′-pentahydroxyflavone), and rutin (3-rutinoside of quercetin). This assumed that there was a structure—property relationship among the related flavonols.

The study of the accumulation kinetics of flavonols in erythrocytes showed that ~88% of quercetin and ~83% of fisetin transferred from the extracellular medium into the erythrocytes in the first 5 min of incubation at 37°C (100% was taken as the initial extracellular flavonol concentration). Erythrocytes absorbed morin more slowly, up to ~68% in 20 min. The rutin content in the extracellular medium remained constant regardless of the incubation time. Flavonols are rather hydrophobic substances and penetrate rather easily through the lipid bilayer of the erythrocyte cell membrane. The proposed mechanism of accumulation of phenolic compounds through erythrocyte biomembranes is passive diffusion [3]. The bulky carbohydrate unit of rutin makes it much more hydrophilic than the other studied flavonols and prevented it from penetrating through the membrane by passive diffusion.

Morin absorption by erythrocytes depended on the temperature of the incubation medium. At 5°C, ~30% of morin was absorbed in 30 min whereas at 37°C the amount of morin accumulated in erythrocytes reached ~68%. At 5°C, quercetin and fisetin precipitated in the incubation medium. This made it impossible to determine quantitatively the accumulation of these flavonols in erythrocytes.

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TABLE 1. Specific Activity of Various Forms of Hemoglobin in Pseudoperoxidase Oxidation of Flavonols (nmol/min per 1 mg hemoglobin)

Flavonol	Methemoglobin	Oxyhemoglobin	Deoxyhemoglobin
Fisetin	39±3	24±2	36±3
Quercetin	35±2	29±2	17±1
Morin	30±2	26±2	21±2
Rutin	14±1	9±1	8±1

Accumulation of flavonols by erythrocytes decreased sharply to ~27% of quercetin, ~22% of fisetin, and ~17% of morin of the initial flavonol concentration if bovine serum albumin (BSA) was added at 2.5 mg/mL to the incubation medium. This was probably due to competition between albumin and erythrocytes for binding to the flavonols. This hypothesis was confirmed by results from a study of the ability of quercetin, fisetin, and morin to desorb from erythrocytes previously loaded with flavonols into the external medium in the presence of increasing concentrations of BSA. Thus, the flavonol concentration in the medium increased from ~11.8% to ~61% for quercetin; from 31.5 to ~61, for morin; and from 16.7 to ~51, for fisetin upon adding BSA to a final concentration of 2.5 mg/mL to a suspension of erythrocytes. The flavonol content in the medium with BSA concentration 25 mg/mL increased even more, to ~88% for quercetin, ~77.7 for morin, and ~68 for fisetin. Reverse diffusion of flavonols from erythrocytes into the medium was not observed without serum albumin.

The Stern—Volmer method [4] was used to determine the binding parameters of flavonols with BSA and hemoglobin and to confirm that flavonols interacted with erythrocytes and serum albumin. Based on an analysis of the fluorescence quenching of protein tryptophans in the presence of flavonols, it was found that BSA binds flavonols much more effectively than methemoglobin. Quercetin interacts with BSA 27.7 times stronger than with methemoglobin; fisetin, 8.2; morin, 22.5; and rutin, 2.3 [4].

In general the results suggest that there is a system of flavonol transport and bioaccumulation that includes serum albumin, erythrocytes, and hemoglobin. Distribution of flavonols among the following fractions occurs in this system: free flavonols in blood plasma; flavonols bound to serum albumin; intraerythrocyte pool including free flavonols in erythrocyte stroma and those bound to hemoglobin. The aglycons quercetin, fisetin, and morin are bound primarily to serum albumin but can penetrate into erythrocytes and interact with hemoglobin whereas rutin with the carbohydrate unit can contact only extracellular blood components.

Flavonols can have a modifying action by penetrating into erythrocytes and binding to hemoglobin. Thus, we found that quercetin and to a lesser extent fisetin could change the absorption spectrum of methemoglobin to produce maxima characteristic of oxyhemoglobin [1].

Oxidation of flavonols involving various forms of hemoglobin and H_2O_2 that occurs at physiological pH values and reaction-medium ionic strength was used as a model reaction to study biotransformation of flavonols by erythrocytes. Table 1 lists the catalytic activity of the most important functional forms of hemoglobin. The activity of hemoglobin forms decreased in the order methemoglobin > oxyhemoglobin > deoxyhemoglobin for oxidation of quercetin, morin, and rutin. Rutin was oxidized 2.1-4.5 times slower than quercetin, morin, and fisetin. In comparison, the specific activity of horseradish peroxidase (HP) for oxidation of quercetin and morin was 28-33 times faster than for rutin [5-9].

Catalytically active hemoglobin derivatives that can catalyze flavonol oxidation are formed by the reaction of H_2O_2 with hemoglobin [10]. The pseudoperoxidase oxidation must occur on the surface of the protein globule and involve a π-cation-radical of the protein because the size of the heme active site is too small to accommodate the flavonol molecule [11].

It is interesting that flavonols not only are substrates of the pseudoperoxidase reaction but also can have a modifying action on the catalyst. They possess reducing properties and can reduce highly oxidized states of heme iron. This reduces the catalytic activity of hemoglobin. Thus, we found that quercetin and fisetin reduced Fe_3^+ of methemoglobin to Fe_2^+ [1].

Although the extent of pseudoperoxidase oxidation of flavonols was two orders of magnitude less than their oxidation by true peroxidases (HP and thyreoperoxidase) [6], hemoglobin was involved in metabolic transformations of the flavonols. Taking into account the high hemoglobin content in mammals, the fraction of hemoglobin-dependent pseudoperoxidase biotransformation of flavonols may be exceedingly significant. Calculations showed that quercetin (100 mg) can be completely oxidized in 1 h in the presence of an amount of hemoglobin that is about 2% of its total content in the human body. The limiting factor of this reaction is the rate of H_2O_2 production in erythrocytes.

TABLE 2. Chromatographic (HPLC) and Spectrophotometric Properties of Flavonols and Products of Their Pseudoperoxidase Oxidation Catalyzed by Methemoglobin

Flavonol	Retention time, min	Absorption maximum, nm	Flavonol	Retention time, min	Absorption maximum, nm
Ouercetin	1.68	292	Fisetin	8.1	232, 285, 321
	2.72	292		13.41	274, 320, 360
	3.01	258, 293		15.61	283
	4.56	292		15.95	285, 322, 347
	14.83	292		16.59	283
	15.08	255, 370		22.26	224
	16.47	269, 302, 363	Morin	1.74	282
Fisetin	1.74	280		2.71	282
	2.75	280		4.45	252, 293
	3.02	259, 293		10.06	293
	4.45	252, 293		14.25	251, 296, 350
	7.49	258, 293	Rutin	4.88	255, 353

A spectrophotometric analysis of the course of the pseudoperoxidase reaction indicated that several intermediate and final products were formed. Characteristic bands I and II with absorption maxima at 375 and 267 nm, respectively, disappeared upon incubation of quercetin in medium containing methemoglobin and H_2O_2. A new absorption maximum at 333 nm (band III) appeared. Its intensity at first increased and then decreased with further oxidation. During the course of the reaction, two isosbestic points at 283 and 351 nm appeared in the first phase when the primary product with the chromophore at 333 nm accumulated. Two new isosbestic points at 300 and 400 nm appeared with further incubation while the absorption maximum at 333 nm decreased and the maximum at 253 nm increased simultaneously. Two new weak absorption maxima at 444 and 473 nm were observed in the red region of the spectrum.

Bands I and II at 392 and 269 nm, respectively, disappeared and band III at 330 nm grew during oxidation of morin, like for quercetin. Isosbestic points at 283, 358, and 455 nm were observed. Band III gradually strengthened on further reaction whereas the absorption at 250 and 475 nm decreased.

For pseudoperoxidase oxidation of fisetin, band I decreased and shifted simultaneously to shorter wavelength. A double absorption maximum at 294 and 327 nm and maxima at 250 and 442 nm appeared.

Therefore, similar changes in the absorption spectra occurred upon pseudoperoxidase oxidation of quercetin, morin, and fisetin. Characteristic bands I and II (phenylchromane structure [12, 13]) disappeared. Band III (modified phenylchromane structure [12, 13]) appeared and then decreased. An absorption maximum at 250 nm (monophenolic structure [12, 13]) appeared.

Rutin oxidation was accompanied by a characteristic decrease of band I and II absorption maxima at 366 and 269 nm, respectively. However, band III did not appear. Band II shifted to shorter wavelength from 269 to 263 nm. The absorption intensity in the red region (450-550 nm) increased. Isosbestic points appeared at 280, 305, and 437 nm.

The similarity of the spectral changes during peroxidase [7-9, 14] and pseudoperoxidase oxidation of flavonols suggested strongly that similar intermediates and final oxidation products were formed.

We used RP-HPLC for more accurate identification of the reaction products (Table 2). Using spectrophotometric data as a guide, pseudoperoxidase oxidation of flavonols was stopped at the moment of greatest accumulation of the intermediate product with an absorption maximum in the range of band III and after disappearance of band III and formation of the final products. The incubation time of the reaction mixture was 2.5 and 15 min for quercetin, 2.5 and 10 for fisetin, 5.5 and 15 for morin, and 5 and 15 for rutin.

RP-HPLC of Quercetin Oxidation Products. The peak with retention time 15.08 min corresponded to starting quercetin (λ_{max}, nm: 255, 370). The quercetin content decreased by four times. Several products with retention times 1.68, 2.72, 4.56, and 3.01 min (λ_{max}, nm: 258, 293); 14.83 min (λ_{max}, nm: 292); and 16.47 min (λ_{max}, nm: 269, 302, 363) appeared. The quercetin peak and that with retention time 3.01 min practically disappeared after incubation for 15 min. The peaks with retention times 1.68 and 16.47 min increased. Peaks with retention times 2.72, 4.56, and 14.83 min decreased.

RP-HPLC of Fisetin Oxidation Products. The peak with retention time 13.41 min corresponded to fisetin. Compared with the control sample, many new peaks, those with retention times 8.1, 15.61, 15.95, and 16.59 min disappearing upon

further incubation and those with retention times 1.74, 2.75, 4.45, 7.49, and 22.26 min increasing, appeared in the chromatogram after incubation of fisetin with methemoglobin and H_2O_2. Like for quercetin, a peak at 3.02 min (λ_{max}, nm: 259, 293) appeared and one at 15.95 min (λ_{max}, nm: 285, 322, 347) disappeared after 10 min.

RP-HPLC of Morin Oxidation Products. The peak with retention time 14.25 min corresponded to morin. Several new peaks with retention times 1.74, 2.71, 4.45, and 10.06 min arose during the reaction whereas the morin peak decreased. Like for quercetin and fisetin oxidation, oxidation products with retention times 10.06 min (λ_{max}, nm: 293) and 4.45 min (λ_{max}, nm: 252, 293) were formed. The amount of product with retention time 10.06 decreased whereas those with retention times 1.74, 2.71, and 4.45 min increased upon further oxidation.

RP-HPLC of Rutin Oxidation Products. Rutin is a more hydrophilic compound and had a retention time of 4.88 min. However, products analogous to those formed by oxidation of the other flavonols could not be found for it.

Thus, UV spectroscopy and RP-HPLC were used to show that several intermediate and final products from pseudoperoxidase oxidation of flavonols catalyzed by methemoglobin were formed. The results agree with the previously proposed mechanism of peroxidase oxidation of flavonols [7-9, 14, 15] that includes 1) one-electron oxidation at OH groups of the B-ring to form semiquinones and *o*-benzoquinones (quercetin, fisetin, rutin) and C-centered radicals (morin); 2) formation of mesomeric methylenequinones; 3) hydroxylation of the γ-pyrone ring at C2; 4) opening of the γ-pyrone ring at the C3–C4 and/or C2–O bond. This first formed chalcone-like metabolites and then a mixture of phenolcarboxylic and dihydroxybenzoic acids, transformation products of rings A and B, overall more than 20 different products [7-9, 14, 15].

The flavonol structures had certain hydroxyls that were more easily oxidized by the action of various oxidants [12, 13]. Selective oxidation at these groups formed products with similar structures and spectral properties. The primary target was most frequently hydroxyls (especially catecholic) in ring B. Oxidation of them formed short-lived semiquinone anion-radicals and then the corresponding *o*-benzoquinones. Apparently oxidation of the ring B catechol increased absorption in the red region (~500 nm) [7-9, 14, 15] that was also recorded during pseudoperoxidase oxidation of quercetin, fisetin, and morin involving methemoglobin. The nature of pseudoperoxidase oxidation of rutin involving methemoglobin was substantially different. The lack of band III upon rutin oxidation, for which the C3 OH group is blocked, suggests that this OH is involved in the complex process of intramolecular transformations that was induced by oxidation of the ring B OH group [16]. This factor was critical for the opening of the γ-pyrone ring upon oxidation by both peroxidase [14] and by the pseudoperoxidase pathway. It prevented cleavage of ring C and formation of hydroxybenzoic acids upon rutin oxidation.

The presence of a carbohydrate radical on C3 of ring C in the flavonols not only prevented penetration through the erythrocyte membrane but also hindered substantially the biotransformation during pseudoperoxidase oxidation involving hemoglobin.

The results demonstrate that erythrocytes, hemoglobin, and serum albumin play signficiant roles in the bioaccumulation and biotransformation of the studied flavonols.

EXPERIMENTAL

We used flavonols quercetin, fisetin, morin, and rutin (Sigma, USA) and bovine serum albumin (BSA, Reanal, Hungary). Methemoglobin, oxyhemoglobin, and deoxyhemoglobin were obtained and characterized by the usual methods [1, 17].

Fresh human erythrocytes were obtained from regional center Hematology and Transfusiology, Ministry of Health, Republic of Belarus. A suspension of erythrocytes for the experiments was prepared by the standard method [15]. For this, erythrocytes were centrifuged for 10 min at 3,000 rpm. The preservative, leucocyte film, and about 15% of the upper volume of the erythrocyte precipitate were removed. Then erythrocytes were rinsed three times (alternating suspension and centrifugation) with two volumes of a cooled solution containing NaCl (0.15 M) and sodium phosphate buffer (5 mM, pH 7.4), which was used in all experiments with erythrocytes. All spectrophotometric measurements were made on a Cary 50 spectrophotometer (Varian). Flavonol concentrations were determined using molar extinction coefficients [6] ε_{374} = 19,529 for quercetin; ε_{363} = 19,872 for rutin; ε_{393} = 18,501 for morin; and ε_{365} = 22,245 for fisetin.

Bioaccumulation of the flavonols was studied in *in vitro* experiments by incubating erythrocytes at 37°C in buffered physiological solution in the presence of the flavonols (50 μM). Flavonols were added to the incubation medium as solutions (10 mM) in DMSO, the amount of which in the incubation medium was <0.5%. The optimal experimental conditions were selected in preliminary *in vitro* tests with erythrocytes. The incubation medium in a typical experiment on flavonol accumulation

contained a suspension (10%) of erythrocytes and flavonols (50 μM). The degree of hemolysis in the control samples that was determined spectrophotometrically was <0.1%.

Erythrocytes were precipitated by centrifugation at 3,000 rpm for 1 min at certain time intervals of incubation. The flavonol content in the supernatant was determined spectrophotometrically using the molar extinction coefficients. Bioaccumulation of flavonols by erythrocytes was characterized in percent as the ratio of the difference between the starting flavonol content and that in the incubation medium at each time point to the starting content of added flavonol. BSA was added to final concentrations 2.5 and 25.0 mg/mL to the incubation medium in order to determine the effect of BSA on accumulation of flavonols by erythrocytes.

Pseudoperoxidase oxidation of flavonols by various forms of hemoglobin was carried out in sodium phosphate buffer (0.05 M, pH 7.4). The reaction medium contained hemoglobin (5 μM), H_2O_2 (500 μM), and flavonols (50 μM, quercetin, fisetin, morin, rutin). The reaction was started by adding flavonol. Absorption spectra were recorded in the range 230-600 nm. The reaction was stopped by adding HCl to 0.2 M. Substrates and formed reaction products were extracted by an equal volume of cooled ethylacetate. The ethylacetate extracts were evaporated in a rotary evaporator and then dissolved in CH_3OH (60 μL). RP-HPLC analysis of substrates and products from pseudoperoxidase oxidation was performed on a Shimadzu LCMS-QP8000α chromatograph in an Allure C-18 column (150 × 4.6 mm; 5 μm, Restec, USA). Test samples (20 μL) were placed on the column. The separation conditions were flow rate 0.75 mL/min, 40°C, CH_3CN in CH_3CO_2H (1%) gradient: 0-5 min, 20% CH_3CN; 5-15 min, 20-100% linear CH_3CN gradient; 15-18 min, 100% CH_3CN, 18-30 min, 20% CH_3CN. Spectra of the chromatographic peaks were recorded in the range 220-500 nm.

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