# ANTHOCYANINS OF HYBRID HIBISCUSES

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The flowers of many hybrid forms of hibiscus obtained by the interspecies hybridization of the North American species of hibiscus Hibiscus moscheutos, H. militaris, and H. coccineus contain considerable amounts of anthocyanins [2].

From the flowers of H. mutabilis L. we have previously isolated cyanidin 3,5-diglucoside [3], and from the flowers of H. esculentus L. we have isolated cyanidin 4'-glucoside and cyanidin 3-glucosido-4'-glucoside, which are very rarely found in plants [4].

Continuing our study of the anthocyanins of plants of the genus Hibiscus, we have found two anthocyanins in the flowers of hybrid hibiscuses (M. Gor'kii, Gladiolus vidnyi, Yu. Gagarin, Alenushka, Kolkhoznitsa, Krasnyi partizan, and Kyzyl Uzbekistan). However, the amounts of some anthocyanins in different species of hybrid hibiscus are different.

The individual anthocyanins of the hybrid hibiscus M. Gor'kii were separated and isolated on a column of cellulose powder by the method described previously [5]. This gave two crystalline anthocyanin glycosides with mp 239-241°C and 215-217°C (decomp.).

The anthocyanin with mp 239-241°C had the composition $C_{26}H_{29}O_{15}Cl \cdot 4H_2O$; $R_f$ 036 (systems 1 and 2), $\lambda_{max}$ 523 nm ($E^{1\%}_{1cm}$ 2.2636) (0.01% HCl, methanol), and on the addition of a 5% solution of aluminum chloride in ethanol, it had $\lambda_{max}$ 558 nm, which is characteristic for anthocyanins containing free hydroxy groups in the C-3' and C-4' positions [6]. This anthocyanin appeared on a chromatogram in ordinary light in the form of a dark pink spot and in UV light it gave a red fluorescence, while in the presence of ammonia vapor and when the chromatogram was sprayed with a 1% aqueous solution of sodium carbonate it became bright blue.

To determine the nature of this anthocyanin we performed acid, enzymatic, and stepwise acid hydrolyses and also oxidative degradation with hydrogen peroxide. The acid and enzymatic hydrolyses of the anthocyanin formed the aglycone – an anthocyanidin – and sugars – glucose and xylose.

The melting point of the anthocyanidin was above 300°C (decomp.) and it had the composition $C_{15}H_{11}O_6Cl$, $\lambda_{max}$ 535 nm, shifting to 572 nm on the addition of aluminum chloride, which shows the presence of hydroxy groups in its side chain. When the anthocyanidin was chromatographed on paper in systems 3 and 4, its $R_f$ values (0.69 and 0.34) were the same as for the cyanidin isolated from the flowers of the cotton plant.

The alkaline cleavage of the aglycone with 15% barium hydroxide at 100°C for 30 min led to the formation of phloroglucinol and protocatechuic acid, which were identified by paper chromatography in the presence of markers.

The stepwise acid hydrolysis of the substance with 3.5% hydrochloric acid in methanol for 15 minutes gave chrysanthemin and xylose, and further hydrolysis gave cyanidin and glucose.

To determine the ratio of aglycone and sugar in the glycoside, it was subjected to complete acid hydrolysis. The amount of cyanidin (aglycone) formed was determined spectrophotometrically at a wavelength of 535 nm. The calculation of the proportion of aglycone was made by means of a calibration curve plotted for pure cyanidin. It was found that the precentage of cyanidin in the molecule of the glycoside was 52, which corresponds to an aglycone: sugar ratio of 1 : 2.

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The ratio of the sugars to one another was found by the aniline phthalate method [7], for which purpose the hydrolyzate obtained after the complete acid hydrolysis of the glycoside was chromatographed in system 1. The sugars were eluted from the chromatogram with glacial acetic acid. The intensity of the coloration of the solution was measured on an FÉK-M photoelectric colorimeter. The amounts of sugars were determined by means of calibration curves plotted for pure samples of glucose and xylose. It was found that the ratio of glucose to xylose was 1:1.

The results of the determination of the site of attachment of the sugar in the anthocyanin by oxidative degradation [8] showed that the sugar of this anthocyanin is a bioside and is attached to the cyanidin at $C_3$. On further acid hydrolysis, the bioside gave glucose and xylose.

To determine the position of the bond between the sugars in the bioside we used the color reactions of disaccharides [9]. The biose isolated from the anthocyanin under investigation formed with diphenylamine–urea a pink spot which became organge-brown after 30 min. The diphenylamine–p-anisidine spot of the bioside was blue-green, becoming deep blue after 24 h. This shows that the glucose and the xylose are connected in the bioside by a 4→1 bond.

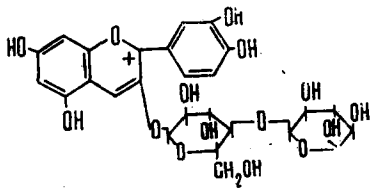
The IR spectrum of the anthocyanin showed absorption bands in the following regions ($cm^{-1}$): 3300-3400 (OH), 1650 (pyran ring), 1610-1430 (aromatic ring), 1200, 1080, and 1040 (C–O), 890 ($\beta$-glycosidic bond).

The differential IR spectrum of the glycoside showed three maxima in the 1010, 1040, and 1080 $cm^{-1}$ regions, which gives grounds for assuming that the sugars are present in the pyranose form.

The enzymatic glycolysis of the anthocyanin with mp 239-241°C by means of the enzyme of Aspergillus oryzae gave a hydrolyzate containing glucose and xylose, which shows $\beta$ linkages between the aglycone and the glucose and between the glucose and the xylose. In addition, the presence of a $\beta$ linkage both between the aglycone and the sugar and also between the monosaccharides was confirmed by the presence of an absorption band in the 890 $cm^{-1}$ region of the IR spectrum of this anthocyanin.

What has been said above enabled the anthocyanin with mp 239-241°C to be characterized as cyanidin 3-$\beta$-D-xylopyranosyl-(1→4)-$\beta$-D-glucopyranoside. This glucoside is a new one which has not been described in the literature, and we have called it gossypicyanin.

The second anthocyanin, with mp 215-217°C (decomp.) has the composition $C_{21}H_{21}O_{11}Cl \cdot 2H_2O$, $R_f$ 0.23 (system 1) and 0.36 (system 2). $\lambda_{max}$ 525 nm ($E_1^{1\%}$ cm 2.2613) shifting on the addition of a solution of aluminum chloride to $\lambda_{max}$ 575 nm. On a chromatogram the anthocyanin gave a pink coloration and in UV light it appeared violet. The spot of the anthocyanin was colored blue by a solution of sodium acetate and by ammonia vapor.



On the basis of acid and enzymatic hydrolyses, oxidative degradation, and the alkaline fusion of its aglycone, and also its UV and IR spectra, the second anthocyanin was identified as chrysanthemin – cyanidin 3-$\beta$-D-glucopyranoside [10]. Chrysanthemin and cyanidin 3-$\beta$-D-xylopyranosyl-$\beta$-D-glucopyranosides have also been isolated from other species of hybrid hibiscuses (Gladiolus vidnyi, Alenushka, Kolkhoznitsa, Krasnyi partizan, Yu. Gagarin, and Kyzyl Uzbekistan).

## EXPERIMENTAL

The following solvent systems were used in the study of the composition of the anthocyanins and in their separation: 1) water–acetic acid–hydrochloric acid (82:15:3); 2) butan-1-ol–acetic acid–water (4:1:5); 3) butan-1-ol–2 N hydrochloric acid (1:1, upper phase); 4) acetic acid–hydrochloric acid–water (5:1:5); 5) ethyl acetate–pyridine–water (2:1:2); and 6) butan-1-ol–benzene–acetic acid–water (2:10:2:1).

Preparation of the Combined Anthocyanins. The air-dry comminuted flowers of hybrid forms of hibiscuses of the variety M. Gor'kii collected at the beginning of August (1 kg) were extracted with chloro-

form (2 × 3 liters) to eliminate fatty and waxy substances and then with methanol containing 1% of hydrochloric acid (3 × 3 liters) at room temperature.

The extract was concentrated under vacuum in a current of nitrogen at 35-40°C to small volume and was left in the refrigerator for 12 h. The white amorphous precipitate was filtered off and a threefold amount of dry peroxide-free ethyl ether was added the filtrate. A red flocculent precipitate was deposited, which was immediately filtered off with suction and was washed with dry ether and with acetone and was then rapidly dried under vacuum over phosphorus pentoxide. This gave 74 g of anthocyanins (7.4% of the weight of the air-dry flowers).

The residue was dissolved in solvent mixture 2 and the resulting solution was passed through a column (60 × 4.5 cm) filled with cellulose powder. The eluate was concentrated under vacuum to small volume and was then treated with five volumes of petroleum ether (bp 40-70°C). The precipitate was separated off, washed with petroleum ether, and dried under vacuum.

The paper chromatography of the combined anthocyanins showed two spots with $R_f$ 0.23 and 0.36 (system 1).

Separation of the Anthocyanins. A solution of 15 g of the combined anthocyanins in solvent system 1 was passed through a column of cellulose powder. Elution was performed with the same mixture until the anthocyanins had been clearly separated. Each band was cut out and was eluted with methanol containing 0.1% of hydrochloric acid. The eluates containing the anthocyanins were filtered from the adsorbent and concentrated under vacuum at 30-35°C in an atmosphere of nitrogen to small volume. The concentrated solutions were treated with ten volumes of dry ethyl ether. The anthocyanins that precipitated were filtered off, washed with ether, and dried under vacuum. The yield of anthocyanin with $R_f$ 0.23 was 3.1 g and that of the anthocyanin with $R_f$ 0.36 was 9.6 g.

Gossypicyanin. The anthocyanin with $R_f$ 0.36 was crystallized from 0.2 N hydrochloric acid in ethanol. The gossypicyanin formed a red-brown microcrystalline powder with a golden tinge which melted at 239-241°C (decomp.), $\lambda_{max}$ 523 nm (0.01% HCl in methanol).

For the acid hydrolysis of gossypicyanin, 0.2 g of the anthocyanin was heated with 10 ml of 7% hydrochloric acid in methanol at 70-75°C in an atmosphere of nitrogen for 60 min. Then 20 ml of water was added to the hydrolyzate and the anthocyanidin aglycone was extracted with isoamyl alcohol. The extract was concentrated under vacuum at 35-40°C in an atmosphere of nitrogen and the anthocyanidin was precipitated with petroleum ether (bp 40-70°C). The precipitate was rapidly filtered off and was washed several times with petroleum ether and was recrystallized from a 0.2 N solution of hydrochloric acid in ethanol. This gave 0.08 g of a cyanidin with mp above 300°C (decomp.).

The aqueous part of the hydrolyzate was treated with activated carbon, and the filtrate was neutralized with $Ba(OH)_2$ solution, evaporated to small volume, and chromatographed on paper in solvent systems 2 and 5. The chromatograms were treated with an ethanolic solution of salicylic acid and o-toluidine. The sugars revealed had the same $R_f$ values as glucose and xylose (0.57 and 0.83 in system 5, and 0.43 and 0.64 in system 2).

Alkaline Cleavage of the Cyanidin. A solution of 0.08 g of the anthocyanidin aglycone in 6 ml of a 15% solution of $Ba(OH)_2$ was heated in the boiling water bath in a current of nitrogen for 45 min. Then the mixture was cooled, acidified with hydrochloric acid, and extracted with ether (2 × 3 ml).

The extract was chromatographed in systems 2 and 6. To reveal the spots, the chromatograms were treated with a 1% solution of vanillin in concentrated hydrochloric acid and with a mixture of equal volumes of 1% solutions of ferric chloride and potassium ferricyanide. The products of alkaline cleavage contained phloroglucinol with $R_f$ 0.16 (system 6) and protocatechuic acid with $R_f$ 0.54 (system 6).

Stepwise Acid Hydrolysis of Gossypicyanin. A solution of 0.05 g of the substance in 2 ml of 3.5% hydrochloric acid in methanol was heated on the water bath in an atmosphere of nitrogen at 70-75°C for 45 min. Samples for analysis were taken every 5 min. After 15-min hydrolysis, the spots of cyanidin 3-$\beta$-D-glucoside, with $R_f$ 0.23 (system 1) and of xylose appeared on the chromatogram. The complete hydrolysis of the glycoside gave cyanidin with $R_f$ 0.34 (system 4), glucose, and xylose with $R_f$ 0.43 and 0.64 (system 2).

Enzymatic Hydrolysis of Gossypicyanin. A solution of 0.050 g of the anthocyanin in 3 ml of water was treated with 0.02 g of the enzyme from the fungus Aspergillus oryzae and was left in the thermostat at 36°C for 36 h. The precipitate that had deposited was filtered off, and the filtrate was mixed with 0.01 g of

activated carbon, filtered, and chromatographed in the systems described above. After the spots had been shown up on the chromatogram glucose and xylose were detected.

Oxidation of Gossypicyanin with Hydrogen Peroxide. A solution of 0.010 g of the glycoside in 2 ml of methanol was treated with 2 ml of 0.1 N ammonia and 1 ml of 30% hydrogen peroxide and was left at room temperature for 4 h. Then a freshly prepared suspension of lead sulfide was added to decompose the excess of hydrogen peroxide. The precipitate was filtered off and washed with water, and the filtrate was treated with 1 ml of ammonia (sp. gr. 0.88) and was heated in the water bath for 5 min. On paper chromatography, a spot appeared with $R_f$ 0.17, which is characteristic for biosides (system 2; chromogenic agent o-toluidine salicylate). On subsequent acid hydrolysis with 2 N HCl for 10 min, the bioside decomposed into glucose and xylose, the formation of which was confirmed by paper chromatography in solvent systems 2 and 5.

Chrysanthemin. The anthocyanin with $R_f$ 0.23 (system 1) was crystallized from the minimum amount of 0.5 N hydrochloric acid in ethanol. The crystals that deposited were filtered off and were dried under vacuum. The anthocyanin isolated consisted of a dark violet microcrystalline powder with a golden tinge, mp 215-217°C (decomp.), $\lambda_{max}$ 525 nm (0.01% HCl, methanol).

Acid Hydrolysis of Chrysanthemin. Chrysanthemin (0.5 g) was hydrolyzed by the method described above for gossypicyanin (see 1st substance). This gave cyanidin with mp 300°C, and glucose was found on a chromatogram.

Enzymatic Hydrolysis of Chrysanthemin. Chrysanthemin (0.05 g) was subjected to enzymatic hydrolysis by the method described above for gossypicyanin, and glucose was identified chromatographically.

Oxidation of Chrysanthemin with Hydrogen Peroxide. The glycoside (0.05 g) was oxidized with hydrogen peroxide in a similar manner to the oxidation of gossypicyanin. The product of oxidative degradation was characterized by paper chromatography in solvent systems 2 and 5. The chromatogram showed a spot corresponding to glucose.

Alkaline Cleavage of the Cyanidin. The process was performed by the method described above for the aglycone of gossypicyanin, and the cleavage products were found to contain phloroglucinol and protocatechuic acid.

## SUMMARY

Two anthocyanin glycosides have been isolated from the flowers of hybrid hibiscuses (M. Gor'kii, Gladiolus vidnyi, Yu. Gagarin, Alenushka, Kolkhoznitsa, Krasnyi partizan, and Kyzyl Uzbekistan). One of them proved to be a new glycoside, which we have called gossypicyanin, and the second the known glycoside chrysanthemin, this being the first time it has been isolated from the flowers of hybrid hibiscuses.

## LITERATURE CITED

1. F. N. Rusanov, Hybrid Hibiscuses [in Russian], Tashkent (1953).
2. Z. B. Rakhimkhanov, A. S. Sadykov, A. I. Ismailov, and A. K. Karimdzhanov, Khim. Prirodn. Soedin., 130 (1970).
3. S. Sankava Subramanian and N. Swamy Naranaya, Current Sci. (India), 33, 112 (1964).
4. P. A. Hedin, Amer. J. Bot., 55, No. 4, 431 (1968).
5. Z. B. Rakhimkhanov, A. S. Sadykov, A. I. Ismailov, and A. K. Karimdzhanov, Khim. Prirodn. Soedin., 723 (1971).
6. E. C. Geissman, Arch. Biochem. and Biophys., 54, 72 (1955).
7. G. I. Zaitseva and T. I. Afanas'eva, Biokhim., 22, No. 6, 1035 (1957).
8. B. V. Chandler and K. P. Harper, Austral. J. Chem., 14, 586 (1961).
9. R. W. Bailey, J. Chrom., 8, 7 (1962).
10. L. Hsia and B. S. Luh, J. Food Sci., 30, 1, 5 (1965).