[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE GEORGIA AGRICULTURAL EXPERIMENT STATION]

The Anthocyanin Pigment of the Hunt Muscadine Grape[1]

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The pigment of the dark blue European grape, *Vitis vinifera*, has been shown by Willstätter and Zollinger[2] to consist largely of the monoglucoside oenin, isolated in crystalline form as oenin chloride. Later it was found[3] that some varieties of grapes probably contain, in addition to the monoglucoside oenin, a small amount of a diglucoside.

In a study of the anthocyanin pigment of the Norton grape, Anderson[4] reported, in addition to oenin, a small amount of an anthocyanin which appeared to be a diglycoside, but which was not investigated further. In later studies on the pigment of several varieties of American grapes and crosses of American and European grapes, values were reported[5] for methoxyl lower than that found by Willstatter and Zollinger[2,3] for oenin, and, while too high for one methoxyl group, the values were too low for two methoxyl groups. The conclusion reached was that the anthocyanins of the American grapes examined were mixtures of oenin and monomethoxydelphinidin monoglucoside. However, the values reported for methoxyl might just as readily be accounted for by a mixture of oenin and delphinidin monoglucoside, or by a mixture of oenin, delphinidin monoglucoside and monomethoxydelphinidin monoglucoside. Levy, Posternack and Robinson[6] found, in addition to oenidin, a small amount of delphinidin and its 3′-methyl ether in the Fogarina grape.

So far as is known, no examination has ever been made of the anthocyanin pigment of the muscadine grapes, *Vitis rotundifolia* Michx. [*Muscadinia rotundifolia* (Michx.) Small], which are native to the southern part of the United States. A preliminary examination of the anthocyanin in the skins of the dark purple Hunt variety, which is the result of a cross between a white male and Flowers variety,[7] indicated that the anthocyanin differed from that found in the *Vitis vinifera*, or European wine grape, and American grapes such as the Concord, *Vitis labrusca*, in several characteristics. This was not unexpected, for the grapes differ widely in many respects. Using methods developed by Robinson and Robinson,[8] an extract of the skins of the Hunt grape appeared to contain a diglycoside of petunidin. There was no evidence of the presence of monoglycosidyl anthocyanidin.

Owing to the increasing interest in the muscadine grapes grown in the South and in order to obtain additional information on the differences between this and other groups of grapes, the anthocyanin was isolated and its composition and probable structure were determined.

The data presented here indicate that the anthocyanin of the Hunt muscadine grape is probably a 3,5-diglucoside of 3′-O-methyldelphinidin. While petunin[9] was identified as a diglucoside of 3′-O-methyldelphinidin,[10] it was not determined whether the two glucose residues are attached to the anthocyanidin as a 3-bioside or as a 3,5-diglucoside. Since certain definite differences are apparent between petunin and the pigment isolated from the Hunt grape, the name *muscadinin* is suggested.

The skins of muscadine grapes, compared to flower petals, have a lower anthocyanin content and contain a relatively large amount of substances, including phlobaphenes, which make purification of the pigment difficult. Attempts at purification through the lead salt failed, probably due to muscadinin being much less stable than most of the other anthocyanins.

Muscadinin was isolated as the chloride in much the same manner as in the method used by Willstätter and Burdick[9] for the isolation of petunin from the violet flowers of the Rathaus petunia. Losses on purification were relatively great. However, a small amount of pure muscadinin was obtained which was sufficient for the characterization of the pigment.

No picrate was obtained, which is evidence that neither oenin nor malvin is present. Both oenin

(1) Published with the approval of the Director as paper No. 72, Journal Series.

(2) Willstätter and Zollinger, *Ann.*, **408**, 83 (1915).

(3) Willstätter and Zollinger, *ibid.*, **412**, 195 (1917).

(4) Anderson, *J. Biol. Chem.*, **57**, 795 (1923).

(5) (a) Anderson and Nabenhauer, *ibid.*, **61**, 97 (1924); (b) Anderson, *ibid.*, **61**, 685 (1924); (c) Anderson and Nabenhauer, THIS JOURNAL, **48**, 2997 (1926); (d) Shriner and Anderson, *J. Biol. Chem.*, **80**, 743 (1928).

(6) Levy, Posternack and Robinson, *J. Chem. Soc.*, 2701 (1931).

(7) Stuckey, *Georgia Agr. Expt. Sta. Bull.*, 133 (1919).

(8) Robinson and Robinson, *Biochem. J.*, **25**, 1687 (1931).

(9) Willstätter and Burdick, *Ann.*, **412**, 217 (1917).

(10) Bradley, Robinson and Schwartzenbach, *J. Chem. Soc.*, 793 (1930).

and malvin give picrates of relatively low solubility.

Experimental

The skins of well-ripened Hunt grapes were separated from the pulp by hand and pressed in a hydraulic press. The pressed skins, 3.6 kg., were ground in a food chopper and the resulting pulp was mixed with 5 liters of 2% methyl alcoholic hydrogen chloride. After twenty hours the alcoholic extract was filtered and the pulp mixed with 2 liters of 1% methyl alcoholic hydrogen chloride. After standing for thirteen hours the second extract was filtered and the pulp washed with a liter of 1% methyl alcoholic hydrogen chloride. The combined extracts, 7.6 liters, were treated with 28 liters of ether, precipitating a dark red sirup. After standing overnight, the ether–alcohol was decanted, and the sirup was filtered with suction. The solid material was washed with a small amount of methyl alcohol, then dissolved in hot 1% methyl alcoholic hydrogen chloride and filtered hot. After standing for some time, some crystals separated out. These were filtered off; air-dry weight 2 g.

To the alcoholic solution were added three volumes of ether. After standing several hours, most of the ether–alcohol was decanted and the precipitated crude pigment was filtered with suction; air-dry weight 2.4 g.

The above-mentioned filtered sirup, which contained much pigment, stood for several days, but no further pigment crystallized out. The sirup was treated with enough 20% hydrochloric acid to bring the hydrochloric acid concentration up to 7%. Fine crystals separated out which after several hours were filtered with suction and washed with 6% hydrochloric acid. The crude pigment was dissolved in hot 0.06% hydrochloric acid and filtered. The hydrochloric acid concentration was increased to 3% and allowed to stand. After some time, crystals separated out which were filtered with suction; air-dry weight 10 g.

The pigment fractions, which contained a large amount of impurities, were ground and the pigment was dissolved in hot 0.5% aqueous hydrochloric acid, centrifuged and then filtered. The residue was treated in the same manner three additional times. Concentrated hydrochloric acid was added to the solutions of the pigment to bring the hydrochloric acid concentration up to 2%. After several days, amorphous material separated out from the last three fractions, but not the first one. This amorphous substance, which contained very little pigment, was filtered and rejected. The hydrochloric acid content of the pigment fractions was further increased to 3.5%. After several days, the muscadinin chloride separated out in brown crystals with a metallic coppery reflex. The pigment fractions were filtered with suction and washed with a small amount of 5% hydrochloric acid.

The first fraction, which was fairly pure, had an air-dry weight of about 1 g. The other fractions yielded smaller amounts of slightly less pure pigment. The purest fraction was recrystallized for analysis by dissolving in a small amount of hot 0.5% hydrochloric acid, filtering and allowing the solution to stand for some time. The fine copperybrown crystals were filtered and dried in the air at room temperature; weight 0.7 g. Muscadinin chloride sinters at 181° (cor.) and melts at 184° (cor.) with decomposition.

The anthocyanin was not extractable from a 0.5% hydrochloric acid solution by isoamyl alcohol. It gave a blue color with sodium carbonate, with sodium hydroxide a blue which turned quickly to green and yellow. Muscadinin chloride gave a blue color with aqueous sodium acetate and a blue precipitate with lead acetate. With ferric chloride in aqueous solution it gave a violet color, quickly fading, and in methyl alcohol a beautiful violet which faded slowly to red-brown. No precipitate separated on treating a solution of the pigment with picric acid. Muscadinin chloride reduced Fehling solution in the cold.

The color reactions in buffered solutions of graded *p*H similar to those used by Robertson and Robinson[11] agreed very closely with those found for the synthetic petunidin 3,5-diglucoside chloride prepared by Bell and Robinson.[12] Their synthetic petunidin diglucoside chloride contained about 43.5% of inorganic salt, but this should not affect the color reactions.

The distribution ratio of muscadinin chloride in mutually saturated *n*-butyl alcohol and 0.5% aqueous hydrochloric acid was determined according to the method of Robinson and Todd.[13] With 3.7 mg. of the pure anthocyanin chloride in 50 ml. of the mixed solvents, a distribution ratio of 13.1 was found on making the second extraction. This compares quite closely with a distribution ratio of 13.6 found by Bell and Robinson[12] for synthetic petunidin 3,5-diglucoside chloride.

Anal. Calcd. for $C_{28}H_{33}O_{17}Cl\cdot 2.5H_2O$: C, 46.57; H, 5.30; Cl, 4.91; OCH_3, 4.30; H_2O, 6.24. Found: C, 46.65; H, 5.46; Cl, 4.61; OCH_3, 4.47; H_2O, 6.66.

Water of crystallization was determined by drying over phosphorus pentoxide at 100° at a pressure of 4 mm. for three and one-half hours. The loss in weight was 7.72%. The dried anthocyanin chloride on analysis gave, for Cl, 3.55%, based on the air-dry weight. Therefore the water of crystallization is considered to be 6.66%. Other investigators have reported the loss of hydrogen chloride on drying certain anthocyanins. After drying petunin chloride in a desiccator at room temperature, Willstätter and Burdick[9] found on analysis: Cl, 1.99%, which is a much greater loss of hydrogen chloride than was found for muscadinin chloride at 100° and 4 mm. pressure.

Calculation of the percentage composition of the water-free glucoside from the experimental values gave: C, 49.96; H, 5.06; Cl, 4.94; and OCH_3, 4.78. $C_{28}H_{33}O_{17}Cl$ requires C, 49.67; H, 4.91; Cl, 5.24; and OCH_3, 4.58.

Quantitative Hydrolysis of Muscadinin.—The glucoside pigment, muscadinin chloride, 0.4008 g. (0.3758 g. anh.), was hydrolyzed by dissolving in 8 ml. of 0.01% hydrochloric acid, adding 12 ml. of concentrated hydrochloric acid and boiling the acid solution for two minutes and twenty seconds. The solution was immediately cooled and the anthocyanidin chloride began to crystallize out shortly afterward. The pigment solution was placed in the refrigerator overnight. The anthocyanidin chloride was collected in a weighed sintered-glass bottom crucible, washed with 10 ml. of cold 10% hydrochloric acid and dried over calcium chloride; recovery of the air-dried anthocyanidin chloride, 0.2157 g. The pigment remaining in the

(11) Robertson and Robinson, *Biochem. J.*, **23**, 35 (1929).
(12) Bell and Robinson, *J. Chem. Soc.*, 1604 (1934).
(13) Robinson and Todd, *ibid.*, 2293 (1932).

mother liquor and washings goes over quantitatively into isoamyl alcohol on extraction and amounted to 0.0031 g., determined colorimetrically. Total anthocyanidin chloride amounted to 0.2188 g. or 53.81%. $C_{28}H_{33}O_{17}Cl\cdot 2.5H_2O$ theoretically yields 55.06% of $C_{16}H_{13}O_7Cl\cdot 2.5H_2O$.

The filtrate from the crystallized anthocyanidin chloride was extracted several times with isoamyl alcohol to remove the residual pigment from the sugar solution. The alcohol was extracted from this solution with ether, the sugar solution was neutralized with solid lead carbonate, the lead chloride precipitate was filtered and the excess lead was removed from the filtrate by precipitation with hydrogen sulfide. The filtrate from the hydrogen sulfide precipitate was concentrated on the water-bath, the concentrated solution was transferred to a volumetric flask, then was neutralized with sodium bicarbonate and was made to volume. The sugar was determined by the method of Shaffer and Somogyi[14] and calculated as glucose. Found, 38.99% glucose in the air-dry salt. The sugar determined with a saccharimeter amounted to 40.22%. Twenty ml. of the sugar solution with 3 g. of sodium acetate and 2 g. of pure phenylhydrazine hydrochloride in a test-tube were heated in a boiling water-bath. Eight minutes after immersion the osazone separated as yellow needles. After thirty minutes in the boiling water-bath, the tube with contents was removed and cooled. The osazone was filtered, washed with cold water and recrystallized from hot 50% alcohol. The purified osazone melted at 208–210° (cor.), which was not depressed by admixture with glucosazone. The specific rotation of the sugar, calculated from the saccharimeter readings, was +55.4° at 20°. $C_{28}H_{33}O_{17}Cl\cdot 2.5H_2O$ requires for two molecules of glucose, 49.90%; found, 38.99 and 40.22%. Willstätter and Burdick[9] found glucose on hydrolysis of petunin to be several per cent. below the theoretical value.

The anthocyanidin chloride obtained on hydrolysis was not quite pure, so it was dissolved in hot 0.5% aqueous hydrochloric acid, filtered from some insoluble amorphous product and the hydrochloric acid concentration increased to about 10.5%. The pigment solution was placed in the refrigerator for twenty hours. The spindle-shaped rounded leaflets were filtered on a sintered-glass bottom crucible and washed with a little 10% hydrochloric acid; air-dry weight 0.2012 g.

Anal. Calcd. for $C_{16}H_{13}O_7Cl\cdot 2.5H_2O$: C, 48.31; H, 4.56; Cl, 8.91; OCH_3, 7.80; H_2O, 11.32. Found: C, 48.36, 48.13; H, 4.70, 4.74; Cl, 8.66, 8.52; OCH_3, 7.44, 7.55; H_2O, 12.18.

Loss in weight on drying over phosphorus pentoxide at 100° at a pressure of 3 mm. for three and one-half hours was 13.20%. Here the anthocyanidin chloride also lost hydrogen chloride in addition to water. The dried anthocyanidin chloride on analysis gave for Cl, 7.60%, based on the air-dry weight. The difference in Cl content is equivalent to 1.02% hydrogen chloride; therefore, the water of crystallization was considered to be 12.18%. Reynolds, Robinson and Scott-Moncrieff[15] reported that the high loss on drying of delphin chloride indicated that water may have been removed from the molecule.

The anthocyanidin gave all the qualitative reactions of petunidin chloride. It was extracted by amyl alcohol from 1% aqueous hydrochloric acid. When the amyl alcohol was shaken with sodium acetate, it became violet-blue, especially at the interface, and on addition of two drops of dilute ferric chloride, the color changed to pure blue. The color reactions of the pigment in the buffered solutions of graded *p*H were practically identical with those found for 3′-O-methyldelphinidin chloride.[10] The pigment was not extracted by the "cyanidin reagent," but on shaking with the "delphinidin reagent," it was gradually extracted by successive extractions.[8] Almost all of the pigment was destroyed in the "oxidation" test. The anthocyanidin chloride reduces Fehling solution in the cold.

The anthocyanidin chloride was decomposed according to the method of Karrer and Widmer[16] by boiling with 20% sodium hydroxide, and later with 10% barium hydroxide, in a stream of hydrogen. The phenolic and acidic decomposition products were separated with sodium bicarbonate in the usual manner. The phenol was identified as phloroglucinol by its transient violet color with ferric chloride, red color with a pine shaving and concentrated hydrochloric acid, and the formation of a cinnabar-red diazobenzene compound. The acid gave a green color with ferric chloride. Although the acid could not be fully identified because of the small amount of material available, since the pigment contains a methoxyl group and the ferric reaction indicates the presence of neighboring free hydroxyl groups, the acid constituent is probably 3-methylgallic acid.

Summary

The skins of the dark purple Hunt muscadine grape contain an anthocyanin pigment which has been named *muscadinin*.

Muscadinin has been isolated as the chloride and its properties indicate that it is probably 3,5-diglucosidyl-3′-O-methyldelphinidin chloride.

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(14) Shaffer and Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).

(15) Reynolds, Robinson and Scott-Moncrieff, *J. Chem. Soc.*, 1235 (1934).

(16) Karrer and Widmer, *Helv. Chim. Acta*, **10**, 28 (1927).