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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE WEST VIRGINIA AGRICULTURAL EXPERIMENT STATION]

The Anthocyanin Pigment of the Winesap Apple[1]

BY IRA J. DUNCAN AND R. B. DUSTMAN

It is known that the coloration of fruits is affected by a number of factors including light, temperature, supply of nitrogen and moisture from the soil, concentration of carbohydrates and organic acids in the cells and perhaps by other factors. Due to the commercial importance of apple-growing in West Virginia and due to the desirability of high color on apples in the market, it was deemed advisable to undertake an investigation of the red pigment of the apple at this station. The first logical step in an investigation of this type involves a determination of the nature of the substance in question. The present work deals with the isolation, purification, identification and determination of the chemical nature of this pigment.

Historical

During the past fifty years considerable work has been done on the chemistry of the natural coloring matter of flowers, fruits and berries. Willstätter and his co-workers were pioneers in these investigations. In their work on the cornflower Willstätter and Everest[1a] cleared up many experimental difficulties encountered in the investigation of plant pigments. They also introduced a rapid test to distinguish glucoside from non-glucoside pigments. The work of Robinson,[2] Karrer,[3] Anderson[4] and many others during recent years has contributed much information to our knowledge of anthocyanin pigments.

These pigments occur in plants as glucosides, accompanied in a few instances by a small amount of sugar-free pigment. In the substances so far reported the sugar occurring in combination with the non-glucoside fraction is glucose, galactose or rhamnose, but by far the most common is glucose.

Robinson and Robinson[2] developed a rapid method for testing qualitatively the anthocyanins occurring in leaves, flowers and fruits. In this method the pigments are characterized by observing their color reactions with alkalies and ferric chloride, and considerable use is made of their distribution between immiscible solvents. The work of these investigators indicated that the skins of the Winesap and Jonathan apples contain the pigment cyanidin 3-monoside. The work of these and other investigators indicates that in the monoglucosides the sugar molecule is attached to the number three carbon atom.

Experimental

From time to time during the fall of 1933 and 1934 quantities of Winesap apples ranging from 7 to 25 bushels were procured and efforts were made to isolate the red pigment

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[1] Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 162.

[1a] Willstätter and Everest, *Ann.*, **401**, 189 (1913).

[2] (a) MacDowell, Robinson, and Todd, *J. Chem. Soc.*, 806 (1934); (b) Murakami, Robertson and Robinson, *ibid.*, 2665 (1931); (c) Robinson, *Chem. & Ind.*, 737 (1933); (d) Robinson and Robinson, *Biochem. J.*, **25**, 1687 (1931).

[3] (a) Klein, "Handbuch der Pflanzenanalyse" (Anthocyane Von P. Karrer) 111/2, Julius Springer, Wien, 1932, p. 941; (b) Karrer and Meuron, *Helv. Chim. Acta*, **15**, 1212 (1932); rev. in *C. A.* **27**, 302 (1933).

[4] (a) Anderson, New York Agr. Expt. Sta., Tech. Bull. 96; (b) Anderson and Nebenhauer, *ibid.*, Tech. Bull., 146; (c) Shriver and Anderson, *ibid.*, Tech. Bull., 152.

from the skins by using various methods[4b,5] described for the preparation of the pigment of the grape, cranberry and certain flowers. These methods failed to give satisfactory yields of apple pigment. The difficulty was probably due to the relatively low percentage of red pigment in the skins as they were removed. Extracting a given weight of skins even when a minimum volume of extracting liquid is employed results in a relatively dilute solution of the pigment and a high concentration of sugars, waxes, gums and other impurities.

Finally, the method of Willstätter and Burdick[6] used for the isolation of asterin and callistephin from the flowers of the purple-red aster, with some modifications, was found to give fair results. Consequently the skins from fifteen bushels of apples were placed in two-gallon, wide-mouthed jars, and the red pigment was extracted with ethyl alcohol containing 0.1% hydrochloric acid. After twenty-four hours the alcoholic solution was filtered and the coloring matter in the filtrate was precipitated with neutral lead acetate solution. After standing for twelve hours the green gelatinous precipitate of the lead salt was filtered, washed with cold water and allowed to remain on the filter until practically air-dry. The precipitate on the filter was then treated with glacial acetic acid. This dissolved the coloring matter completely and left many colorless impurities undissolved. The pigment was precipitated from the dark-red acetic acid filtrate with two volumes of ether. The lead salt thus obtained was filtered and decomposed on the filter with a mixture of propyl alcohol and methyl alcohol containing 25% hydrochloric acid in the ratio of 10 to 1 by volume. This process left still other impurities undissolved. On treating the alcoholic filtrate with 3 or 4 volumes of ether the coloring matter was precipitated as the chloride salt. This precipitate was filtered, washed with ether and then dissolved in ethyl alcohol containing 0.1% hydrochloric acid. The lead salt was again formed by treating the alcoholic solution with neutral lead acetate solution. After filtering, washing and drying the entire purification process was repeated by resolution in acetic acid, precipitation with ether, decomposition of this precipitate with the propyl alcohol–methyl alcoholic hydrochloric acid mixture, and precipitation finally of the chloride salt with ether. The chloride salt was converted to the picrate by dissolving in warm water and treating the solution with warm picric acid solution. The picrate was again converted to the chloride by solution in methyl alcohol containing 5% hydrochloric acid and treating the acid alcoholic solution with 4 or 5 volumes of dry ether. The chloride precipitated as a red amorphous powder. Crystallization by slow evaporation of the alcohol from a mixture of aqueous hydrochloric acid and ethyl alcohol yielded long needle crystals having a bronze metallic luster. Five additional crystallizations yielded a product free from amorphous material and apparently pure.

A small amount of the chloride salt was again converted to the picrate and the picrate recrystallized from warm water. Fine brownish-red needles were formed. The picrate gave a cherry-red solution in ethyl alcohol and an orange-red solution in water. When a drop of the latter solution was placed on a filter paper it produced a spot with a violet center and yellow exterior. This is the result of hydrolytic dissociation according to Willstätter and Mallison.[5] The picrate is partially decomposed when an aqueous solution is heated above 70°.

The anthocyanin chloride is very soluble in water giving a brownish-red solution which assumes a bluish tinge when greatly diluted. The color does not fade entirely on standing, and a faint red color remains even after boiling. The salt is soluble in ethyl and methyl alcohols producing bluish-red solutions; it is soluble also in very dilute hydrochloric acid but in 7% hydrochloric acid it is almost insoluble. A number of color reactions with inorganic salts were observed. An aqueous solution of the glucoside, previously acidified slightly to prevent decolorization, gave a purplish-red color with a solution of sodium acetate, while sodium carbonate gave a violet color. Sodium or potassium hydroxide gave at first a blue color which changed to green and then to yellow. The red color was not restored on acidification, showing decomposition had taken place with these alkalies. The pigment is precipitated by neutral lead acetate from both alcoholic and aqueous solution, giving blue and purple lead salts, respectively. One drop of very dilute ferric chloride solution gave a deep purple color when added to an aqueous solution of the pigment and a blue color when added to an alcoholic solution.

The bronze luster of the air-dried chloride salt changed to a green color when the salt was dried over sulfuric acid but the bronze color returned on exposure to the atmosphere for a short time. The dry salt melted with decomposition at 210–212°. Apparently the glucoside loses all of its water of crystallization when dried over sulfuric acid at room temperature in a vacuum desiccator at one mm. pressure for forty-eight hours, since no further loss occurred when it was heated at 108° for an additional twenty-four hours in a vacuum of one to five mm. pressure.

Analyses of the air-dried chloride salt of the pigment gave C, 47.31; H, 4.88; Cl, 6.69; O, 41.12 (by difference); and H_2O, 8.58, respectively. Calculation of the empirical formula gave $C_{21}H_{21}O_{11}Cl \cdot 2.5H_2O$, which requires C, 47.58; H, 4.95; Cl, 6.70; O, 40.78 and H_2O, 8.51, respectively. No methoxy groups are present in the molecule. C, H, Cl and methoxy groups were determined by Pregl's micro methods.[7] The analytical values given are averages of triplicate determinations throughout the paper.

Calculation of the percentage composition of the water-free glucoside from the experimental values gave: C, 51.75; H, 4.30; Cl, 7.33; and O, 36.62 (by difference). $C_{21}H_{21}O_{11}Cl$ requires C, 52.01; H, 4.33; Cl, 7.32; and O, 36.44.

The analyses, the physical and chemical properties and the color reactions of the pigment of the Winesap apple agree closely with those of idaein chloride isolated from the cranberry by Willstätter and Mallison.[5]

Quantitative Hydrolysis of the Glucoside

The glucoside pigment, presumably idaein chloride, 0.2591 g., was hydrolyzed by dissolving in 10 cc. of water, adding 10 cc. of concentrated hydrochloric acid and boiling

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(5) (a) Willstätter and Mallison, *Ann.*, **408**, 15 (1915); (b) Willstätter and Miegs, *ibid.*, **408**, 122 (1915).

(6) Willstätter and Burdick, *ibid.*, **412**, 149 (1916).

(7) (a) Pregl, "Quantitative Organic Microanalysis" (Translated by Ernest Fyleman from 2d ed., 1924), pp. 15–72, 102–127; (b) *ibid.*, pp. 150–158.

the acid solution for two and one-half minutes. Crystals of the anthocyanidin chloride began to separate after the solution had boiled for one minute. At the end of two and one-half minutes the solution was immediately cooled and placed in the refrigerator for several hours. The non-glucoside salt separated from the solution as long red-brown needles with a metallic luster. These were collected in a weighed, fritted-glass bottom crucible, washed with a little cold 7% hydrochloric acid and dried over sulfuric acid. Recovery of the air-dried anthocyanidin chloride amounted to 64.09% of the original weight. $C_{21}H_{21}O_{11}Cl{\cdot}2.5\ H_2O$ theoretically yields 64.24% of anthocyanidin chloride.

The sugar in the filtrate from the hydrolysis of the glucoside was identified in the following manner: the slightly red acid solution was neutralized with solid lead carbonate, the resulting 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 neutralized with solid sodium bicarbonate and the solution was concentrated to a small volume by evaporation at 40–45° before an electric fan. The yellow solution was decolorized with Norit and the colorless sugar solution in a test-tube was converted to the osazone by adding to it 0.160 g. of pure phenylhydrazine hydrochloride and 0.240 g. of sodium acetate and heating in a boiling water-bath. The osazone separated after several minutes as clusters of orange-yellow needles. After thirty minutes in the boiling water-bath the contents of the tube were allowed to cool. The osazone was then filtered, washed with cold water and twice recrystallized from hot 10% aqueous pyridine solution. The purified osazone melted at 195–196° with decomposition. The melting point corresponds to that of galactosazone. A mixture of galactosazone, prepared from pure galactose, and the unknown osazone mixed in about equal proportions also melted at 195–196° with decomposition. The identification of the sugar as galactose was further confirmed by saccharimetric readings and copper oxide reduction values on a solution of the sugar. The specific rotation of the unknown sugar calculated from the saccharimeter readings was +81.0° at 20°. The specific rotation of galactose is recorded as +80.2° at 20° when determined on a 1% solution.[8] Likewise, the copper oxide values for the unknown sugar averaged 4.3% higher than the corresponding values for an equivalent weight of pure galactose when the latter was subjected to the same acid treatment as that used to hydrolyze the glucoside.

The anthocyanidin chloride obtained by the hydrolysis of the glucoside was recrystallized by dissolving in ethyl alcohol, adding one-half its volume of 7% hydrochloric acid, and allowing the alcohol to evaporate slowly from a loosely covered beaker. After twenty-four hours the crystals were filtered and three times recrystallized by the same procedure. The final crystallization yielded a product free from amorphous material and apparently pure. The anthocyanidin chloride is soluble in methyl and ethyl alcohols giving beautiful violet-red solutions. It is much less soluble in water and only slightly soluble in dilute hydrochloric acid. The crystals failed to melt when heated to 350°. Dilute solutions of inorganic reagents gave the following results: ferric chloride changed the color of an aqueous solution of the pigment from red to violet; lead acetate gave a violet precipitate of the lead salt; sodium or potassium hydroxide gave a blue color which on standing turned green and then brown; sodium carbonate when added to an aqueous or alcoholic solution of the pigment gave first a violet then a blue color; ferric chloride when added to an alcoholic solution gave a pure blue color.

The non-glucoside salt loses its water of crystallization when heated to 108° for twenty-four hours at one to five mm. pressure.

Analyses of the air-dried sample of the anthocyanidin chloride gave C, 52.64; H, 3.95; Cl, 10.43; O, 32.98 (by difference); and H_2O, 5.55, respectively. Calculation of the empirical formula gave $C_{15}H_{11}O_6Cl{\cdot}H_2O$, which requires C, 52.85; H, 3.85; Cl, 10.41; O, 32.89; and H_2O, 5.29, respectively. Calculation of the percentage composition of the water-free salt gave C, 55.73; H, 3.53; Cl, 11.04; O, 29.70 (by difference). $C_{15}H_{11}O_6Cl$ requires C, 55.80; H, 3.44; Cl, 10.99; O, 29.77.

Here also the analyses, the physical and chemical properties and the color reactions of the unknown anthocyanidin chloride correspond very closely to cyanidin chloride. The identity with cyanidin chloride was further confirmed by the products formed when it was fused with alkali. The material was heated with potassium hydroxide and a small amount of water in a crucible at 210–220°. Decomposition took place in about one minute. After five minutes the temperature was raised rapidly to 250° for just a moment. After the melt had cooled it was dissolved in water, the solution was neutralized with hydrochloric acid and the slightly acid solution was shaken out with ether. After evaporation of the ethereal solution the residue was dissolved in warm water, made slightly alkaline with sodium bicarbonate and again shaken with ether. The sodium bicarbonate solution, from which the phenol was removed, was then acidified weakly and shaken with ether to remove the acid portion. The two ether solutions were evaporated to dryness and the residues dissolved in warm water. The aqueous solutions were decolorized with Norit, filtered and the filtrates were evaporated until crystals began to separate. Solid compounds separated on cooling. The phenolic constituent was shown to be phloroglucinol by the intense red color imparted to a pine shaving when immersed in concentrated hydrochloric acid. Also, the crystalline form appeared identical with that of pure phloroglucinol when both were crystallized under similar conditions. The acid constituent was probably protocatechuic acid as characterized by the greenish color produced with ferric chloride and by the sublimate it formed when heated. The sublimate gave the green color test for catechol when treated with ferric chloride. The identity of the acid portion could not be fully confirmed because of the small amount of material available. No other constituents were found. Since the alkali decomposition products of cyanidin chloride are phloroglucinol and protocatechuic acid, the identity of the anthocyanidin chloride obtained from the apple is further confirmed as cyanidin chloride.

Summary

The anthocyanin pigment in the skins of Winesap apples has been isolated in pure form and

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(8) "International Critical Tables," Vol. II, p. 351.

identified as idaein chloride, a pigment which Willstätter and Mallison[5] isolated from the cranberry. The glucoside was isolated as the lead salt. By the use of differential solvents it was partially purified and was then converted to the picrate. The pigment was eventually obtained in pure form as the chloride salt. The anthocyanin chloride on hydrolysis yields molecular proportions of cyanidin chloride and galactose.

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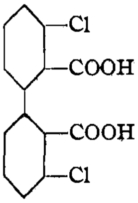
The Constitution of the Isomeric Dichlorofluorenone Carboxylic Acids Formed by the Action of Sulfuric Acid on 3,3′-Dichlorodiphenic Acid

BY ERNEST H. HUNTRESS AND EDWARD R. ATKINSON

Earlier work in this Laboratory[1] has shown that when 3,3′-dichlorodiphenic acid (I) is heated with concentrated sulfuric acid at 170° there is formed a ketocarboxylic acid isomeric with, but different from, the 1,6-dichlorofluorenone-5-carboxylic acid (II) quantitatively obtained at 125°. This isomeric acid (designated in the cited reference as "Acid X") has now been identified as 1,6-dichlorofluorenone-4-carboxylic acid (VII) and is identical with the product obtained by the action of sulfuric acid upon 5,5′-dichlorodiphenic acid (VI). Experiments undertaken to elucidate such an unexpected result have now led to the further discovery that 1,6-dichlorofluorenone (XI) and 1,8-dichlorofluorenone (XII) are converted to 3,6-dichlorofluorenone (XIII) on heating in concentrated sulfuric acid. The rearrangement of fluorenones with other substituents is being examined further.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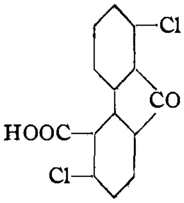
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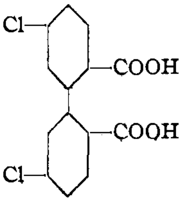
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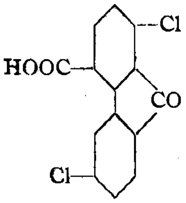
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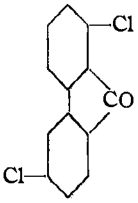
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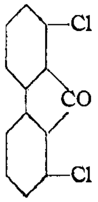
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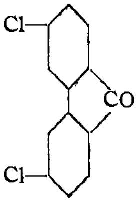
XIII

In carrying out the preparation of the isomeric ketocarboxylic acids from 3,3′-dichlorodiphenic acid (I), it is now evident that our previous isolation of 1,6-dichlorofluorenone-5-carboxylic acid (II) after heating the 3,3′-dichlorodiphenic acid in sulfuric acid at 125° for one hour was fortuitous, since if the treatment be continued longer (*e. g.*, for five hours) the product is 1,6-dichlorofluorenone-4-carboxylic acid (VII) ("Acid X"). A brief study of the temperature at which this isomerization occurs is given in the Experimental Part.

This change does not occur when phosphoric acid is used instead of sulfuric acid. With phosphoric acid at 215°, 3,3′-dichlorodiphenic acid yields only 1,6-dichlorofluorenone-5-carboxylic acid (II); 5,5′-dichlorodiphenic acid (VI) similarly treated gives only 1,6-dichlorofluorenone-4-carboxylic acid (VII). Thus, although phosphoric acid does not effect rearrangement, it does cause ring closure.

The Evidence for the Structure of 1,6-Dichlorofluorenone-5-carboxylic Acid (II)

The original data on this acid[2] have been confirmed and extended. The acid gives correct analysis for halogen and the expected neutralization equivalent. On treatment with thionyl chloride it yields the corresponding acid chloride (m. p. 180–181°, uncorr.) and this with ammonium hydroxide gives an amide (m. p. 281°, uncorr.). The latter yields[1] (p. 4268) an amine (m. p. 257°, uncorr.). These three derivatives are all distinctly different from the corresponding products of the "Acid X" series, and the depression of corresponding mixed melting points has been confirmed. Furthermore, this keto acid on heating gave 1,6-dichlorofluorenone (XI). In addition to the above results, we have also been able to effect the deamination of the aminodichlorofluo-

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(1) Huntress, Cliff and Atkinson, THIS JOURNAL, **55**, 4262–4271 (1933).

(2) Huntress and Cliff, *ibid.*, **55**, 2559–2567 (1933).