[CONTRIBUTION FROM THE DIVISION OF FOOD SCIENCE AND TECHNOLOGY, NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, CORNELL UNIVERSITY]

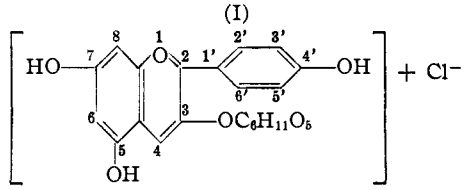
# The Anthocyanin of Strawberries[1]

BY ERNEST SONDHEIMER AND Z. I. KERTESZ

In connection with the work proceeding in this Laboratory on the color deterioration of strawberry products, it became desirable to isolate and identify the red anthocyanin pigment of strawberries. Robinson and Robinson,[2] using qualitative tests, have identified a pelargonidin 3-glucoside in strawberries and Nair and Robinson[3] found pelargonidin 3-galactoside in wild strawberries (*Fragaria vesca*). However, we were unable to find a method for the isolation of anthocyanin from strawberries in the literature. Since the strawberry is a comparatively poor source of anthocyanin, containing only 300–400 mg. per kg. of berries,[4] it is not surprising that the direct precipitation as the picrate was unsuccessful. We have used preliminary extraction of the pigment with 1-butanol[5] but other solvents, particularly 4-methylcyclohexanol may also be used to good advantage. After evaporation of the solvent, the picrate can be precipitated and converted into the chloride. Using such methods, a crystalline anthocyanin has been isolated from cultivated strawberry varieties (*Fragaria chiloensis Duch. var. Ananassa, Bailey*). The pigment can be readily purified by repeated conversion of the picrate to the chloride.

Examination of the hydrolytic products revealed the compound to be a mono-glucoside of pelargonidin. The hydrogen peroxide degradation of the anthocyanidin yielded *p*-hydroxybenzoic acid. With sodium hydroxide hydrolysis at 100° a fraction was obtained which gave several qualitative tests indicating the presence of phloroglucinol. The callestephin chloride isolated by Willstätter and Burdick[6] from asters (*Aster chinensis L.*) and the synthetic pelargonidin 3-monoglucoside chloride prepared by Robertson and Robinson[7] seem in every respect identical with the anthocyanin isolated by us from strawberries. Fortunately, a number of physical properties and color reactions of the 5-substituted glucosides unequivocably differentiates these from the 3-substituted compound (I). The evidence for 3-substitution is further strengthened by observations of Karrer and Helfenstein[8] that ferric chloride attacks anthocyanidins readily on the free 3-hydroxyl group. It is significant to note that the isolated glucoside is relatively stable to ferric chloride while the aglucone, in dilute solutions, is rapidly decolorized by the addition of this reagent.

(I)




3,5,7,4′-Tetrahydroxyflavylium chloride 3-β-glucoside

## Experimental

**Preparation of Pigment Concentrate.**—Capped, frozen strawberries were pressed after they had sufficiently thawed at room temperature. The 5 l. of clear juice thus obtained was saturated with sodium chloride and extracted twice with 1.25 l. of 1-butanol. This usually removed 75–80% of the materials absorbing at 500 mμ. It was advantageous to centrifuge the juice after most of the alcohol had been siphoned off. The filtered extract was concentrated in vacuum below 50° in a nitrogen atmosphere. Colorless impurities were removed by filtering the solution during distillation. When the volume had been reduced to about 50 ml., the distillation was stopped and the flask drained. The adhering material was removed by dissolving it in about 25 ml. of ethanol. These washings were combined with the butanol concentrates and the crude pigment was precipitated by the addition of 10 volumes of anhydrous ether. After standing for about an hour the mixture was centrifuged and the supernatant liquid discarded. The precipitate was washed several times with 150-ml. portions of anhydrous ether. The yield of crude concentrate was 5 g.

**Preparation of the Anthocyanin Picrate.**—The above granular pigment preparation, which still gave a very strong color reaction with ferric chloride, was dissolved in 200 ml. of 0.01% hydrochloric acid at 40°, filtered, saturated with finely powdered picric acid and stored at 0° for forty-eight hours. Washing with 1% hydrochloric acid followed by ether and drying yielded 1 g. crude picrate. The ferric chloride test is best made by adding 1 drop of 1% ferric chloride solution to a few ml. of an alcoholic solution of a small quantity of the pigment preparation. A mixture containing one drop of 1% hydrochloric acid and the alcoholic pigment solution should be used for comparison. A brown coloration with ferric chloride indicates impurities.

**Preparation and Purification of the Anthocyanin Chloride.**—The crude picrate preparation was completely soluble in 15 ml. of ethanol containing 5% hydrochloric acid (made by adding concentrated hydrochloric acid to absolute alcohol). The addition of 225 ml. of anhydrous ether precipitated flocculent anthocyanin chloride. After standing two hours at 0°, the mixture was filtered through a sintered-glass funnel and thoroughly washed with ether. This preparation was still impure since it gave a positive ferric chloride reaction, and was therefore reconverted to the picrate by treating with 200 ml. of 0.01% hydrochloric acid at 40°, filtering off the insoluble material, and saturating the filtrate with picric acid. After standing for forty-eight hours at 0° the mixture was filtered and the precipitate washed and dried. The beautiful microscopic prisms obtained were orange colored and showed a bronze luster. The 70% yield obtained in this operation could be improved by either storing the above

(1) Journal Article No. 758, New York State Agricultural Experiment Station. These investigations were in part supported by a grant from the National Preservers Association.

(2) Robinson and Robinson, *Biochem. J.*, **26**, 1650 (1932).

(3) Nair and Robinson, *Ber.*, **67A**, 98 (1934).

(4) Sondheimer and Kertesz, *Anal. Chem.*, **20**, 245 (1948).

(5) Rosenheim, *Biochem. J.*, **14**, 73 (1920).

(6) Willstätter and Burdick, *Ann.*, **412**, 149 (1917).

(7) Robertson and Robinson, *J. Chem. Soc.*, 1460 (1928).

(8) Karrer and Helfenstein, in "Annual Review of Biochemistry," Vol. I, 551 (1932).

filtrate for additional periods of two to three days after saturating it again with picric acid or by concentrating the solution in vacuum. The 700 mg. of picrate used yielded 405 mg. of purified anthocyanin chloride. Sometimes material which had been converted to the picrate twice still gave a weak ferric chloride test and since further purification did not entail more than a 15–20% loss, it was deemed safer to go through one more conversion. The final yield of non-crystalline chloride was 350 mg., or about 20% of the total anthocyanin contained by the strawberries used.

Using Willstätter and Burdick's method for the crystallization of callestephin chloride,[6] the pigment was transformed into hair-like microscopic orange-red crystals. Non-crystalline anthocyanin chloride (230 mg.) was dissolved in 2 ml. of methanol and filtered through a micro sintered-glass funnel. The beaker and funnel were washed with 1 ml. of methanol and to the combined solutions 0.75 ml. of 12% hydrochloric acid was added. This solution was allowed to evaporate partially at room temperature. After twenty-four hours the crystals were filtered off and washed with a little 12% hydrochloric acid. A recrystallization yielded 147 mg. of air-dried material.

On drying *in vacuo* at 105°, the glucoside lost 8.8 and 8.4% of its weight. Previous values found were 7.1 and 7.2% for the synthetic glucoside and 8.05% for the isolated material, 7.1% being equivalent to 2.0 and 8.8% to 2.5 moles of water of crystallization. The physical properties and color reactions of the anthocyanin were found to be similar to those reported in the literature for pelargonidin 3-glucoside.[6,7]

This outlined procedure gave similar results when repeated with different batches of several varieties of strawberries.

**Identification of the Anthocyanidin and Carbohydrate Components.**—In order to identify the aglucone and carbohydrate constituents, the anthocyanin was hydrolyzed by dissolving it in a small volume of water, adding an equal quantity of hydrochloric acid and boiling the solution for three minutes. The mixture was allowed to cool to room temperature and filtered. The homogeneous, crystalline precipitate was washed with cold 1% hydrochloric acid, then with ether, and air dried. The filtrate was then extracted with amyl alcohol to remove the small quantity of anthocyanidin which remained in the solution and the resulting practically colorless liquid was neutralized with sodium bicarbonate. After concentrating, this solution was used to establish the identity of the carbohydrate component.

**(A) Anthocyanidin.**—The anthocyanidin chloride precipitated from the hot hydrochloric acid solution in the form of yellow-brown microscopic platelets. These could be recrystallized from hot dilute hydrochloric acid in the form of short red tetragonal prisms. The anthocyanidin picrate was formed when a warm solution of the chloride, containing a trace of hydrochloric acid, was saturated with picric acid. On cooling red needles separated. When the picrate was reconverted to the chloride by treatment with hydrochloric acid in ethanol and addition of ether, the compound precipitated mainly in the form of swallowtailed twin prisms. The iodide was obtained from boiling hydrochloric acid and phenol in the shape of elongated orange prismatic needles, appearing deep red under dark field illumination.

With minor exceptions, typical color reactions of pelargonidin chloride were obtained. Robertson, Robinson and Sugiura[9] described its aqueous sodium carbonate solution as having a blue color which appears violet when viewed under a metal filament lamp. We noted a similar behavior in the anthocyanidin purified by conversion to the picrate. However, the material which was not thus purified but was nevertheless crystalline and apparently homogeneous, had a much stronger dichroism appearing blue with transmitted light and deep violet with reflected light. Similar effects were noted in alcoholic solutions and with sodium hydroxide. With sodium bicarbonate a violet color was obtained. A tendency for the violet color base to precipitate from distilled water, sodium acetate solution, and aqueous alcohol was observed. Negative results in the methoxy determinations for the glucoside and the aglucone extends the methoxylated anthocyanidins. Following the procedure for the hydrogen peroxide oxidation outlined by Karrer[10] and using 50–100 mg. of anthocyanidin, a crystalline substance was obtained which could be further purified by sublimation. That the compound was a carboxylic acid having a para hydroxy group was indicated by a positive Millon spot test and negative nitrous acid test for phenols.[11] A positive hydroxamic acid test could be obtained only after the compound was treated with thionyl chloride. The resublimed compound melted at 210–212° and the mixed m. p. with *p*-hydroxybenzoic acid was 211–213°. Melting points were determined with a Fisher Scientific Co. melting point block.

When the anthocyanidin was hydrolyzed with 10% sodium hydroxide at 100° in an atmosphere of hydrogen for one hour, or when heated with 60% potassium hydroxide for two to three minutes, several qualitative tests indicated the presence of phloroglucinol. The reaction mixture was neutralized with hydrochloric acid and extracted with ether. The *p*-hydroxybenzoic acid was removed by shaking with saturated sodium bicarbonate solution. An aqueous solution of the ether residue gave the following tests, confirming the presence of phloroglucinol: A pine splint, moistened with hydrochloric acid, and dipped into the solution turned violet-red. A red precipitate was obtained with aniline nitrate and sodium nitrite. With ammoniacal potassium ferricyanide a golden-yellow colored solution was obtained. Dry ammonia gas precipitated from the ether solution a yellowish solid which decomposed on heating. Addition of vanillin in hydrochloric acid gave a red color.

The anthocyanidin chloride lost 5.39% of its weight on heating in a vacuum oven at 100°. The calculated loss for 1 mole water of crystallization is 5.55%.

*Anal.* Calcd. for $C_{15}H_{11}O_5Cl \cdot H_2O$: C, 55.48; H, 4.04. Found: C, 55.37; H, 3.88.

**(B) Carbohydrate.**—Oxidation of the solution containing the sugar residue with nitric acid gave a water soluble substance which was identified as the potassium acid saccharate by its microscopic appearance. Fructose was excluded by the negative potassium hydroxide reaction[12] and mannose by the negative diphenylamine tests. The recrystallized phenylosazone derivative melted at 208–210° and the mixed m. p. with glucosazone was 208–210°, establishing that D-glucose is the carbohydrate component of the anthocyanin.

**Quantitative Hydrolysis of the Anthocyanin Chloride.**—The hydrolysis was carried out as above, using 118.4 mg. of anthocyanin dissolved in 11 ml. of water plus 9 ml. of concentrated hydrochloric acid. To minimize the loss of glucose, the amyl alcohol and ether used to extract the filtrate were washed with 10 ml. water which was added to the sugar containing solution and the volume adjusted to 50 ml. The glucose was determined by Bertrand's method using 20-ml. aliquots. Calculated yields for the mono-glucoside of pelargonidin chloride (anhydrous) are 65.2% pelargonidin chloride (anhydrous) and 34.8% glucose. The values found were 67.2% aglucone and 32.1% glucose. The slightly high value for the aglucone is not unexpected since under the conditions of isolation and crystallization partial hydrolysis may have taken place. For this same reason no carbon and hydrogen determinations were made on the anthocyanin.

**Absorption Curves.**—The absorption curves, shown in Fig. 1, were obtained with a Beckman spectrophotometer, model DU, using 1-cm. quartz cells. The molecular extinction coefficient $\epsilon$ was calculated from the optical den-

(9) Robertson, Robinson and Sugiura, *J. Chem. Soc.*, 1533 (1928).

(10) Karrer, in Klein's "Handbuch der Pflanzenanalyse," Julius Springer, Vienna, Vol. III, 2, 1932, p. 959.

(11) Feigl, "Qualitative Analysis by Spot Tests," Elsevier Publishing Co., Inc., New York, N. Y., 1946, p. 329.

(12) Ekkert, *Ber. ungar. pharm. Ges.*, **5**, 17 (1929).

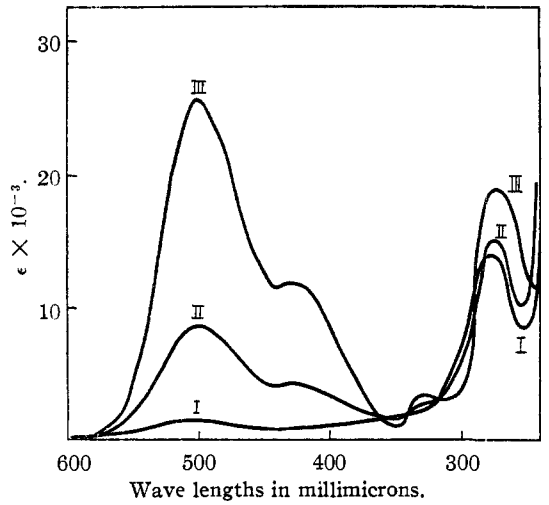



Fig. 1.—Molecular extinction curves of pelargonidin 3-monoglucoside isolated from strawberries, in aqueous solutions in distilled water (I), in Sörensen's sodium citrate–hydrochloric acid buffer of *p*H 3.40 (II) and in similar buffer of *p*H 2.00.

sity $\log_{10} I_0/I$. In the case of the anthocyanin no allowance was made for the possible presence of a small proportion of anthocyanidin in the molecular weight calculation.

## Discussion

In the absence of authentic samples, "typical" color reactions, physical properties, and crystalline structure are very difficult to evaluate. It is possible that some of the divergencies noted, as tendency toward color base formation, crystalline structure of the iodide and pronounced dichroism in alkaline solutions, are due to isomers or other closely related compounds.

Pratt and Robinson[13] have synthesized two isomers of pelargonidin, namely, 3,5,7,2′-tetrahydroxyflavylium chloride and 3,5,7,3′-tetrahydroxyflavylium chloride, and found several differences in their behavior. Most significant of these is their color in alkali and the ease with which they form pseudo salts. Although the possible presence of the two above isomers in the anthocyanidin isolated from strawberries would seem to explain its observed behavior, this assumption could not be proved since tests for *o*- and *m*-hydroxybenzoic acids in the hydrogen peroxide degraded material were negative. However, these latter tests were of necessity performed on very small samples.

Limited experiments were also conducted with red carnations, which Robinson and Robinson[14] list as a good source of pelargonidin 3-monoglucoside. The purified material (non-crystalline) from this source gave color reactions in alkali similar to the anthocyanin and anthocyanidin isolated from strawberries.

In Fig. 1 and 2 the absorption spectra of the glucoside and aglucone in aqueous and alcoholic

(13) Pratt and Robinson, *J. Chem. Soc.*, **127**, 1182 (1925).
(14) Robinson and Robinson, *Biochem. J.*, **25**, 1687 (1931).

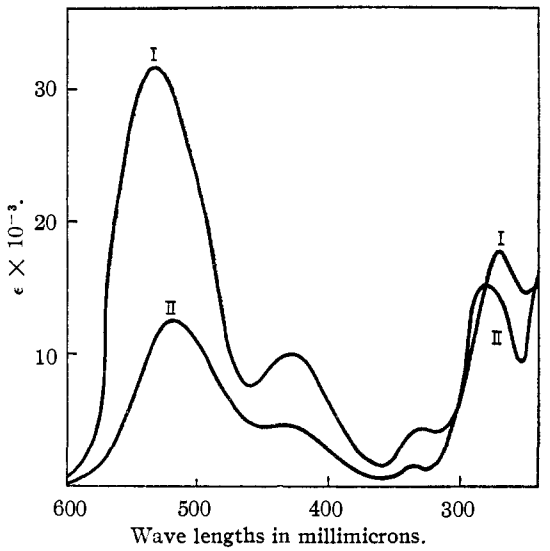



Fig. 2.—Molecular extinction curves of pelargonidin chloride (I) and pelargonidin 3-monoglucoside chloride (II), obtained from strawberries, in ethanol containing 0.001 *M* hydrochloric acid.

medium are given. These results are in agreement with observations that aqueous solutions of the glucoside are not as violet in tone as alcoholic solutions, and that the aglucone in alcohol has a more violet tinge than the glucoside. The intensity of absorption at equal molecular concentrations in alcoholic solutions of equal hydrochloric acid content is lower for the glucoside than the anthocyanidin. It is interesting to note that changes in *p*H produce much greater alterations in the visible part of the spectrum than in the ultraviolet region. The anthocyanin represented in Curve I in Fig. 1 is most likely mainly in the form of the pseudo salt. No changes are produced in the position of the peak at 500 millimicron with respect to wave length on lowering the *p*H below 4.0.

Of two published absorption curves for pelargonidin chloride[15,16] only the one reported by Hayashi appears to be similar to the curve presented here. The extent of agreement between the general shape and position of the absorption peaks of this curve and the one obtained by us is noteworthy. The curve obtained by Schou differs greatly from the latter. He found peaks at 504.5, 454.0, 400.5, 331.0 and 267.0 mμ. As far as can be ascertained these differences are not due to solvent effects. The absorption curves for purified, but non-crystalline, anthocyanin and anthocyanidin obtained from red carnations are very similar to our curves for the pigment obtained from strawberries.

## Summary

1. A method for the isolation of an anthocyanin from strawberries is described.

(15) Schou, *Helv. Chim. Acta*, **10**, 907 (1927).
(16) Hayashi, *Acta Phytochim. (Japan)*, **9**, 1 (1936).

2. The pigment, which is responsible for all or most of the red color of strawberries, is believed to be pelargonidin 3-monoglucoside. The possible presence of two isomers of pelargonidin in the isolated material is discussed.

3. Absorption curves for the glucoside and the aglucone are presented.

GENEVA, N. Y. RECEIVED MAY 26, 1948

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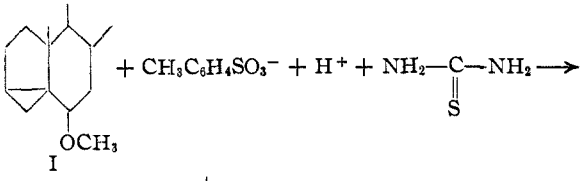
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

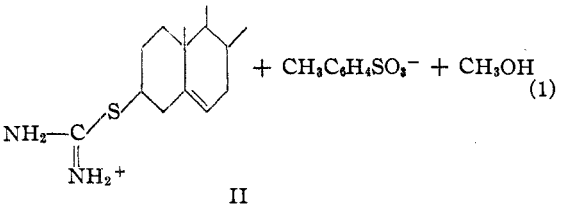
# Mechanism of Formation of Cholesterylisothiuronium Salts from *i*-Cholesteryl Methyl Ether[1a]

BY R. G. PEARSON, LEE A. SUBLUSKEY AND L. CARROLL KING

In recent papers from this Laboratory[1b,1c] it was shown that *i*-cholesteryl methyl ether (I) reacts with thiourea and *p*-toluenesulfonic acid in alcoholic solution to give cholesterylisothiuronium tosylate (II).



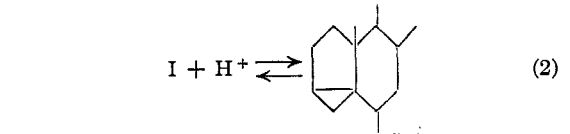
I



II

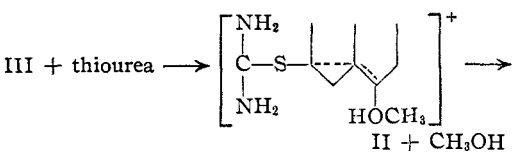
(1)

Since there is a change in the nature of the ions present during the course of the reaction it is possible to make a kinetic study by measuring the corresponding change in the electrical conductivity. The results of such a kinetic study indicate that the reaction is first order with respect to each reactant. Two possible mechanisms are consistent with the experimental data: (a) reaction between I and hydrogen ion resulting in an oxonium complex (III),[1,2] which then reacts with nucleophilic thiourea in an abnormal type of displacement (3); or (b) formation of III followed by reversible ionization into methanol and a carbonium ion (IV) which can then react with thiourea at the 3 position as shown in (5).

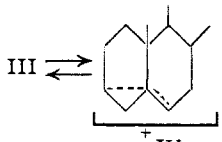


III

(2)



II + $CH_3OH$ (3)

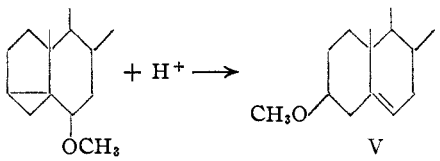


IV

(4)

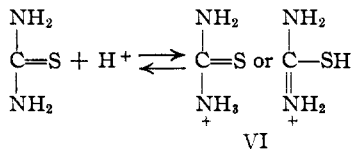
IV + thiourea ⟶ II (5)

The kinetics are complicated by the simultaneous acid catalyzed rearrangement of the isomeric ether (I) to cholesteryl methyl ether (V), which will not react further to give the product (II), and by the formation of a thiuronium ion (VI) from thiourea and hydrogen ion.



V

(6)



VI

(7)

The rate of reaction (6) was determined directly by measurement of the change in optical rotation in the absence of thiourea. In the presence of sufficient excess of thiourea this side reaction is of minor importance.

The concentration equilibrium constant for reaction (7) in dry methanol was determined by independent means and will be reported elsewhere.[3] Its numerical value of *ca.* 15 offers evidence that in the presence of excess thiourea much of the hydrogen ion is tied up as thiuronium ion (VI).

## Experimental

**Materials.**—*i*-Cholesteryl methyl ether was prepared from cholesteryl *p*-toluenesulfonate by the method of Stoll,[4] m. p. 78–79°, $[\alpha]^{23}D$ 51.2°. *p*-Toluenesulfonic acid monohydrate was purified by several recrystallizations from concentrated hydrochloric acid, m. p. 104–105°. Thiourea was purified from a C.P. grade by recrystallizing

(1a) Presented before the Organic Division of the American Chemical Society, Chicago meeting, April, 1948; (b) King, Dodson and Subluskey, THIS JOURNAL, **70**, 1176 (1948); (c) King, *ibid.*, **70**, 2685 (1948).

(2) Meyer, Ph.D. Thesis, Northwestern University, 1943.

(3) Pearson and Tucker, forthcoming publication.

(4) W. Stoll, *Z. physiol. Chem.*, **207**, 147 (1932).