with our results for the α-acid. The hexabromide numbers of the five preparations in Table V calculated to the basis of 100% purity are as follows:

Preparation	Purity, %	Hexabrom. no.	Hexabrom. no. calcd. to 100%
(1)	83.5	75.5	91.6
(2)	85.2	72.0	84.5
(3)	88.0	83.0	94.3
(4)	84.0	83.2	99.0
(5)	100.0	70–75	70–75*

In previous work on arachidonic acid[7] an equation was suggested for the determination of the amount of this acid present in fatty acid mixtures based on the polybromide numbers of pure arachidonic acid and of the fatty acid mixture. A similar equation was proposed for linoleic acid, based on tetrabromide numbers.[4b] We hesitate to apply this principle to linolenic acid other than as an approximation, because obviously, from the above data, the hexabromide number is dependent on the concentration of acids being brominated and especially because it varies in different preparations of linolenic acid. The average hexabromide number for the four crystallization specimens, calculated to 100% purity, is 92.3 or in round numbers 92. From this an approximate value for the content of linolenic acid in a mixture could be found from the equation

$$\% \text{ linolenic acid} = \frac{\text{H. N.} \times 100}{92}$$

where H. N. is the hexabromide number of the fatty acid mixture and 92 is the average hexabromide number for crystallization linolenic acid. In calculating the average, the H. N. for the α-acid is omitted for the reason of its obvious difference from the crystallization acids. It also should be pointed out that the hexabromide numbers calculated to 100% are slightly high because the percentage purities were calculated from the iodine numbers as a mixture of linoleic and linolenic acids. In spite of these objections to the method, we believe it to constitute a decided advance over any other now available both in accuracy and in specificity.

Summary

1. Low temperature crystallization procedures for the isolation of linolenic acid have been applied to the fatty acids of linseed and perilla oils.
2. By fractional crystallization of the fatty acids of these oils in acetone, 75% linolenic acid remained in the filtrate at −75°.
3. Crystallization of the 75% acid from petroleum ether resulted in preparations of 83.5 to 88% purity.
4. The constants of the crystallization acids are compared with those of α-linolenic acid, prepared by debromination.
5. The possibility of the multiple nature of linolenic acid is discussed; also the application of the hexabromide number as a method of estimating the amount of linolenic acid present in fatty acid mixtures.

COLUMBUS, OHIO RECEIVED AUGUST 15, 1938

[7] Ault and Brown, *J. Biol. Chem.*, **107**, 615 (1934).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF CORNELL UNIVERSITY]

The Colloid Chemistry of Leaf and Flower Pigments. I. The Precursors of the Anthocyanins[1]

BY WILDER D. BANCROFT AND JOHN E. RUTZLER, JR.

Introduction

Most of the research work on leaf and flower pigments has been done by organic chemists who were interested primarily in preparing and analyzing pure compounds, or by biologists who were interested primarily in genetics. It has seemed worth while to us to present to some extent the viewpoint of those colloid chemists who are interested especially in the changes of color with changing conditions.

Some points are much simpler when considered as colloid chemistry. Chlorophyll is soluble in benzene; but benzene will take scarcely any chlorophyll out of the green leaf.[2] Tswett[3] has shown that this is due to marked selective adsorption of the pigment of the leaf tissue. The phenomena of co-pigments or intensifying agents, studied by the Robinsons,[4] become more simple when considered as due to adsorption.

[1] Original manuscript received December 13, 1935.

[2] Willstätter and Hug, *Ann.*, **380**, 177 (1911).

[3] Tswett, *Ber. deutsch. bot. Ges.*, **24**, 318 (1906).

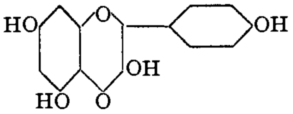
[4] Robinson and Robinson, *Biochem. J.*, **26**, 1663 (1932).

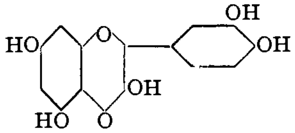
Since chlorophyll is the most important plant pigment and the one best known to the public, it might seem wise to begin with that. We shall get into a more controversial and to us a more interesting field if we start with the red autumn leaves and most of the red and blue flowers. These pigments are known as anthocyanin pigments and there is a wide difference of opinion as to the way they originated. The German chemist, Willstätter, believes that they are formed in the plant by reduction of flavones, while the English chemist, Robinson, believes that they are produced in the plant by hydrolysis and oxidation of what are known as leuco-anthocyanins. In this paper we plan to show how to differentiate between these two diametrically opposed views. Each is right in certain cases and each is wrong in others.

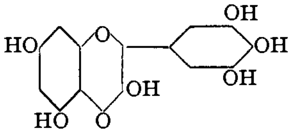
Anthocyanins and Anthocyanidins

In general the anthocyanins vary in color from red or purple through blue and green to yellow as the *p*H of the sap increases. The green is not a pigment color but is due to the combined effect of the yellow and blue. When one starts with a red flower and exposes it to ammonia vapor, the blue is apt not to be permanent in the absence of a stabilizer. If it changes fairly rapidly to yellow one gets no blue but only a green. If it changes very rapidly to yellow one gets neither blue nor green. The red leaves of the poinsettia are a case of this. It seems probable therefore that all blue flowers contain a color-stabilizer, but there apparently has been no systematic study of this point. Sodium chloride, sodium nitrate and alum solutions[5] are said to stabilize the blue in particular cases; but we do not know how general this is or what salts are found in blue flowers. The blue of *Salvia patens* extends over a *p*H range of 4–8.[6] Hirsutin, methyl malvin, is turned blue by a number of co-pigments:[7] tannin, 2-hydroxyxanthone glucoside, rutin,, quercetin, vanillin and quinaldine.

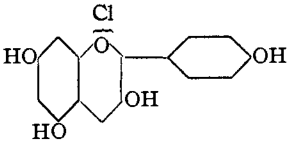
Pedagogically, most anthocyanins and anthocyanidins may be considered as reduction products of one of three flavonols or derivatives of them.[8] The three flavonols are kaempferol, quercetin and myricetin.

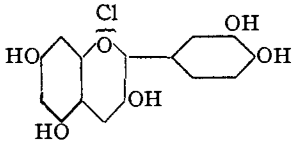

Kaempferol

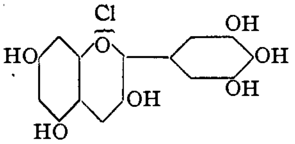

Quercetin


Myricetin

The corresponding anthocyanidin chlorides are pelargonidin chloride, cyanidin chloride and delphinidin chloride


Pelargonidin chloride


Cyanidin chloride


Delphinidin chloride

An anthocyanin differs from an anthocyanidin in having one or more glucose, galactose or rhamnose molecules attached to it. One or more of the hydroxyl groups may be methylated, giving a substituted anthocyanin if there are sugar molecules attached and a substituted anthocyanidin if the molecule is an aglucone.

Willstätter and Mallison[9] showed that cyanidin can be obtained by reduction of quercetin with a metal and an acid. Shibata, Nagai and Kishida[10] got quicker results with magnesium and mercury in acid solution. They consider the mercury as essential to a good result.

At Cornell we have obtained excellent data by

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[5] Willstätter and Everest, *Ann.*, **401**, 189 (1913); Molisch, *Bot. Ztg.*, **47**, 17 (1889); **55**, 49 (1897); Miyoshi, *Botan. Centr.*, **83**, 345 (1900).

[6] Buxton and Darbishire, *J. Genetics*, **21**, 71 (1929).

[7] Robinson and Todd, *J. Chem. Soc.*, 2299 (1932).

[8] Robinson, *Encyclopaedia Britannica*, **2**, 26 (1929).

[9] Willstätter and Mallison, *Ann.*, **408**, 147 (1915); *cf.* Schunck, *Proc. Roy. Soc.* (London), **42**, 187 (1884).

[10] Shibata, Nagai and Kishida, *J. Biol. Chem.*, **28**, 93 (1916–1917).

electrolysis in an acid solution, using a mercury cathode to give high over-voltage. Dr. T. S. Chapman[11] extracted shredded leaves cold with dilute formic acid (2–5%). A pink solution shows the presence of anthocyanins or anthocyanidins. If the pink color appears only after heating, leuco-anthocyanins are present. If a pink, yellowish or colorless solution is shaken with ether and if aqueous ammonia is added to the ether layer, the appearance of a distinctly deeper yellow going practically completely into the water layer indicates the presence of anthoxanthins. Not much deepening of the yellow, with much of the color staying in the ether, indicates the presence of "Tswett's water-soluble yellows."

To carry out the reduction, some of the formic acid extract was poured off the leaves and mixed with an equal volume of approximately molar sulfuric acid solution. Half of the mixture was poured into the reduction cell and half retained for comparison. On electrolysis with a platinum anode and a mercury cathode a change of color was usually noticeable within a few minutes and almost always within fifteen minutes; but it was customary to electrolyze for an hour to ensure a satisfactory reduction. It is wise to keep the solution cold to prevent hydrolysis, which would give a red color if leuco-anthocyanins were present.

Some sixty-five different kinds of leaves containing anthoxanthins were examined by Dr. Chapman and all but five gave satisfactory anthocyanin-like pigments as shown by the red color. The locust, the California privet, the hawthorn and the wayfaring tree yielded reduction products which were rather yellow or orange in comparison with the others. The reduction of the extract from the privet in particular gave a very yellow color to the solution.

There are marked differences in the ease of reduction. The flavones from the forsythia and the jonquil reduce much more readily than those from the sugar maple.

From these and other experiments it is clear that anthocyanins or anthocyanidins can be obtained from flavone or flavonol solutions by chemical or electrolytic reduction; but there is no proof that this happens in the living plant. Willstätter believes that anthocyanins are formed by reduction, but all that has been proved is that they might be so formed.

Many of the English chemists do not agree with Willstätter that anthocyanins and anthocyanidins are produced in the plant by direct reduction of flavones or flavonols. Keeble, Armstrong and Jones[12] came to the conclusion that the decolorization of anthocyanins by alcohol was due to the action of a reducing agent. Unfortunately, this paper was published before the one in which Willstätter showed that decolorization of anthocyanin by alcohol was not due to reduction. That eliminated Keeble's argument.

Robinson is the present leader of the movement in favor of the anthocyanin pigments being produced by oxidation. One reason for believing that flavones are not the immediate precursors of anthocyanins is that in many cases the anthocyanins do not correspond structurally with the flavones existing in the flower. This is not absolutely conclusive, because there might have been two sets of flavones originally and only one was reduced in the plant. Sands, Milner and Sherman[13] say: "We cannot assume that, because a certain flavonol is not found coexisting with its homologous anthocyanidin, the latter was formed independently rather than by way of the flavonol. As mentioned, the particular flavonol in question may have been converted fully into anthocyanidin." This may be true, but it has not yet been proved and we do know that anthocyanins can be made differently in the plant.

Leuco-anthocyanins and Leuco-anthocyanidins

Robinson and Robinson[14] believe that many and perhaps all anthocyanins are derived from leuco-anthocyanins[15] and leuco-anthocyanidins, substances which were not known as such to Willstätter.[16] Laborde found that unripe green or red grapes contain colorless chromogens which are converted into pigment on heating with dilute hydrochloric acid. Malvézin showed that the same chromogen developed color on heating with water at 85°, but only in the presence of air which may have caused oxidation to acid. Dezain, Tswett, Rosenheim, Jonesco and Kozlowski also have showed the formation of pigments of anthocyanin-like nature from colorless or pale yellow chromogens without the use of a reducing agent.

"Rosenheim[17] prepared a white amorphous pow-

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(11) Cornell University, Ph.D. Thesis, 1938.

(12) Keeble, Armstrong and Jones, *Proc. Roy. Soc.* (London), **86B**, 312 (1913).

(13) Sands, Milner and Sherman, *J. Biol. Chem.*, **109**, 203 (1935).

(14) Robinson and Robinson, *J. Chem. Soc.*, **744** (1935).

(15) *Cf.* Staats, *Ber.*, **28**, 2807 (1895).

(16) Willstätter, *Ann.*, **408**, 4 (1915).

(17) Rosenheim, *Biochem. J.*, **14**, 178 (1920).

der of glycosidic nature from the unripe berries of purple grapes or from ripe white grapes and stated that anthocyanidin could be obtained from it by the action of hydrochloric acid in the absence of oxygen. He proposed the expression leuco-anthocyanin for the new group of natural products."

Robinson and Robinson report that leuco-anthocyanins are of widespread occurrence in the vegetable kingdom and that a majority of them yield cyanidin on treatment with hot hydrochloric acid. A 10% sulfuric acid solution is even better because there is no difficulty about volatility. In 1933 Robinson and Robinson[18] said that it is probable that the leuco-anthocyanins and the anthocyanidins formed from them are in the same state of oxidation. In 1937 Mrs. Robinson[19] reported that the gum of ***Butea fondosa*** contains a substance which cannot be converted into cyanidin chloride by the action of hydrochloric acid alone; preliminary oxidation is necessary.

Robinson and Robinson[20] now consider that the state of oxidation of a leuco-anthocyanidin is that of a dihydroflavylium salt. If this is always true, hydrolysis and oxidation are necessary to produce an anthocyanin pigment from a leuco-anthocyanin. There seems to be nothing to show that an oxidation is always necessary. If Rosenheim is right there must be some cases not involving oxidation. Over-reduction of an anthocyanin or anthocyanidin gives a colorless system which turns red on oxidation,[21] but we do not yet know whether this ever happens in the plant.

Regardless of the question of oxidation it is clear that an anthocyanin pigment can be obtained in the laboratory from a leuco-anthocyanin; but we do not yet know definitely whether this occurs in the plant or, if so, in which plants. One reason for this general uncertainty is that much of the work has been done on flowers where there is usually no change in pigments and one cannot well analyze the flowers for flavones and leuco-anthocyanins before and after the change, though Hugo Fischer[22] says that the first appearance of color in the flower buds is generally two weeks before the time of blooming in the case of red and blue flowers. This is not necessarily true, because cases are known of flowers starting white under suitable conditions and then developing a color.

Variable White Flowers

The flowers of *Hibiscus* L. *mutabilis*[23] and of *Diervilla grandiflora*[24] open white and turn pink or red in the course of the first day. Quite a number of flowers are pale or white when grown in the dark.[25] Among them is the mountain laurel. A low temperature also makes some flowers come white.[25b] With some the reverse is true.[26]

We have not found in the literature any systematic study of the chemistry of these pigments of these variable white flowers. Shibata seems to have shown that the change of color of the white *Diervilla grandiflora* to red is due to a reduction of a flavone-like substance, but in 1916 he of course did not test for the presence of leuco-anthocyanin and consequently there is a possibility of error.

Red Autumn Leaves

If we shift from flowers to green leaves which turn red, it is a simple matter to analyze them before the red color appears. If we take a green leaf, moisten it with dilute sulfuric acid to ensure acidity and electrolyze it with a platinum anode and a mercury cathode, we shall get a red at the cathode if a flavone or flavonol is present because of the reduction and no red, or at most a red at the anode, if a leuco-anthocyanin is present because it does not go red on reduction. Of course precautions must be taken against an error due to hydrolysis. It may not always be easy to see small amounts of red pigment on the mercury; but rinsing it with normal butyl alcohol will concentrate the red color in the alcohol and make it visible. The presence of leuco-anthocyanin can be shown by shredding the green leaf and heating it with approximately 10% sulfuric acid solution until the solution becomes red or pink. Extracting the solution with normal butyl alcohol will make the red color more visible.

We have made tests on the green leaves of the sugar maple and the sumach in August, and on the

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(18) Robinson and Robinson, *Biochem. J.*, **27**, 206 (1933).

(19) Robinson, *J. Chem. Soc.*, 1157 (1937).

(20) Robinson and Robinson, *ibid.*, 747 (1937).

(21) Charlesworth, Chavan and Robinson, *ibid.*, 370 (1932).

(22) Fischer, *Flora*, **38**, 380 (1908).

(23) Kuyper, *Rec. trav. bot. néerl.*, **28**, 1 (1931).

(24) Shibata, Nagai and Kishida, *J. Biol. Chem.*, **28**, 93 (1916).

(25) (a) Hildebrand: "Die Farben der Blüthen," 1879, p. 49; (b) Klebs, *Sitzber. Akad. Wiss. Wien*, **42**, 155 (1905); ref. 22; (c) Sorby, *Proc. Roy. Soc.* (London), **21**, 479 (1873); (d) Kosaka, *Bot. Mag.*, **46**, 551 (1932).

(26) Onslow, "The Anthocyanin Pigments of Plants," 1925, p. 8.

green leaves of the flowering dogwood, the Virginia Creeper (*Parthenocissus quinquefolia*) and the Japanese Creeper (*Ampelopsis*) in September; also on the green skin of a Seckel pear.

The leaves of the sumach, the barberry and the flowering dogwood, contain flavones and no, or practically no, leuco-anthocyanins though there may be a mere trace of leuco-anthocyanin in the leaf of the dogwood. The leaves of the sugar maple and the Virginia Creeper and the skin of the Seckel pear contain leuco-anthocyanin and no, or practically no, flavone, while the leaves of the Japanese Creeper contain both flavone and leuco-anthocyanin. We can, therefore, say definitely that the red autumn leaves of the sumach, the dogwood and the barberry, are due to reduction of flavones, in accord with the view of Willstätter. The red autumn leaves of the sugar maple and of the Virginia Creeper and the red color of the ripened Seckel pear owe their color to the decomposition of leuco-anthocyanin in accord with the view of Robinson, while the red color of the leaves of the Japanese Creeper may be due either to flavones or to leuco-anthocyanins or to both simultaneously. We shall have to devise a new technique for this case or perhaps resort to quantitative analysis. It is now merely a question in most cases of collecting data on other leaves which turn red or which change from red to green.

DeVries[27] says that the leaves of *Drosera rotundifolia* are red when grown in strong light, but otherwise green. There should, therefore, be no difficulty in determining the precursors of the anthocyanins in this plant. Schanz[28] showed that when light less than 400 mμ was cut off by euphos glass, the leaves of red cabbages, young beets and begonias were green and not red. The colors returned in the sunlight. He concluded that the red was developed as a protection against the long ultraviolet. With oak seedlings grown in the open air, the young leaves were a beautiful yellowish-red; under ordinary glass the color was paler; under euphos glass and under red glass the yellow-red did not appear and the leaves were uniformly green.

After the red disappears from the young leaves of the copper beech in summer, leuco-anthocyanins are found to be present. On this statement of facts it seems probable that the red is developed from the leuco-anthocyanins. Abbott[29] has found that leaves of the copper beech are green in the spring when grown in the dark and turn red when exposed to light. He did not analyze these leaves for flavones or leuco-anthocyanins, and consequently one step in the proof is missing.

We do not yet know what factors determine the formation of flavones and leuco-anthocyanins, and consequently we do not know how the relative amounts of these two kinds of substances will vary with changing soil and climatic conditions. In Ithaca the leaves of a certain sugar maple contain flavones and no, or practically no, leuco-anthocyanins in May, and leuco-anthocyanins with practically no flavones in September. It is because of this that we do not yet dare to say definitely that the red of the copper beech is derived from leuco-anthocyanins.

To answer the question whether the conversion of leuco-anthocyanins into anthocyanidins necessarily involves an oxidation we have tried heating equal amounts of the leaves of the sugar maple with sulfuric acid in the presence and in the absence of hydroquinone. There was no marked difference in the amount of red color, and consequently there is no reason to assume that the leuco-anthocyanins must be considered as derivatives of dihydroflavylium salts. Since oxygen is apparently of very little importance in this case, we confirm the observations of Rosenheim. This does not in any way contradict the results obtained by Mrs. Robinson with the gum of *Butea fondosa*. There is no reason as yet why all leuco-anthocyanins should be derivatives of dihydroflavylium salts because some may be.

Enzyme Reactions

Whether in the formation of anthocyanin we are dealing with the reduction of flavone-like substances or with the hydrolysis of leuco-anthocyanins with or without accompanying oxidation, the reaction must apparently be an enzyme reaction because we know of no straight reducing agent that the plant could use which would carry a flavone to an anthocyanin, and because it seems improbable that there would ever be sufficient acidity to produce the hydrolysis of a leuco-anthocyanin without the presence of an enzyme. Miss Wheldale[30] says that "there is little doubt but that the formation of anthocyanin does in-

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(27) DeVries, *Bot. Ztg.*, **44**, 4 (1886).

(28) Schanz, *Pflüger's Arch. Physiol.*, **181**, 229 (1920).

(29) Abbott, *Nature*, **80**, 429 (1909).

(30) Wheldale, *J. Genetics*, **1**, 113 (1911).

volve a series of progressive reactions, each of which is controlled by a certain enzyme."

Enzymes can cause the reduction of sulfates,[31] a reaction which cannot be carried out chemically or electrochemically in the test-tube in the presence of water at ordinary temperatures.

If people can learn how to concentrate the enzymes and to inoculate leaves with them, it should be possible to make the leaves of the Norway maple red. That ought not be a very difficult task, because the stems of the leaves are apt to be red and once in a while one sees an occasional red leaf. When we know more about the enzyme reactions, we shall be able to explain why European forests do not show the brilliant coloring that we get in this country.

In front of the chemistry building at Cornell there is a sugar maple and a copper beech. Both sets of leaves contain leuco-anthocyanin but those in the copper beech do not turn red in the autumn, while those in the sugar maple do. There is no reason to attribute this behavior to differences in climatic or soil conditions and consequently there must be at least one other factor, apparently a genetic one. The green leaves of the Norway maple in Ithaca contain flavones and no leuco-anthocyanins. Since the stems of the leaves are usually red and the leaves only rarely so, there must be some reason why the proper enzyme is found only occasionally in the leaves. Flavones are present in the ginkgo; but we have never seen any red in Ithaca either on stems or leaves. The formic acid extracts of the leaves of the Norway maple and of the ginkgo turn red when electrolyzed with a mercury cathode. The yellowed leaf of the American elm will sometimes develop a little red when bitten by an insect. There is no experimental evidence that the insect causes a preliminary formation of leuco-anthocyanin and we must therefore attribute the red to flavones for the present.

Summary

1. Anthocyanin pigments may be obtained in the laboratory or in the plant by reduction of flavones or by decomposition of leuco-anthocyanins.

2. The red autumn pigments of the sumach, the dogwood and the barberry come from a flavone and those of the sugar maple, the Virginia Creeper and the Seckel pear from a leuco-anthocyanin. The Japanese Creeper contains both flavone and leuco-anthocyanin. We do not yet know which gives rise to the anthocyanin or whether both do.

3. On present data the red of the copper beech comes probably from a leuco-anthocyanin; but this is not yet proved.

4. There is no necessary contradiction between the experiments of Rosenheim and those of Mrs. Robinson. There are apparently some leuco-anthocyanins which require oxygen to develop the full red color and some which do not. On present data the sugar maple belongs in the second class or partly so.

5. The development of anthocyanins is always due to enzymes. If one could inactivate the enzymes without killing the leaves one would prevent the development of red. If one could supply the proper enzyme to the Norway maple, its leaves would turn red in the fall. The stems of the leaves usually turn red in the summer.

6. Since the leaves of the copper beech and of the sugar maple both contain leuco-anthocyanins but behave differently in the autumn, there is apparently a genetic factor to be considered in addition to the effects of soil and climate.

7. A method has been developed which will enable us to distinguish between the views of Willstätter and of Robinson in particular cases.

8. All blue flowers contain a stabilizing agent. A systematic study of this matter will lead to the production of blue roses.

9. There seem to be no satisfactory data on the reds which develop early and temporarily in the tropics.

10. The behavior of the sugar maple suggests the possibility of a flavone being the precursor of a leuco-anthocyanin; but there is no proof of this as yet.

11. There seem to be no data to show whether glucose as a nutrient or a suitable exposure to ultra-violet light will produce anthocyanins more rapidly from leaves containing flavones or from leaves containing leuco-anthocyanins.

ITHACA, N. Y. RECEIVED SEPTEMBER 30, 1938

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(31) Pozzi-Escot, *Am. Chem. J.*, **29**, 542 (1903).