[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF CORNELL UNIVERSITY]

Colloid Chemistry of Leaf and Flower Pigments. II. Qualitative Analysis of Leaf Pigments[1]

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In spite of all the work that has been done on plant pigments, nobody seems to have worked out any quick and easy method of identifying qualitatively the types of pigments occurring in leaves and flowers. The late Mrs. Onslow[2] says that no such thing is possible.

Even if Mrs. Onslow's pessimistic view were right—which we do not admit—we believe that a poor system of qualitative analysis would be better than none at all. Our experience convinces us that such a system will bring to light many interesting facts that have been overlooked in the past. Nobody knew, for instance, that leuco-anthocyanins are present in the leaves of the sugar maple in July but not in May. A careful study of this system will show whether flavone-like substances are or are not the precursors of leuco-anthocyanins in this plant. We[3] have been using our system of qualitative analysis successfully in determining the precursors of anthocyanins in specific cases.

It is for these and other reasons that we are presenting a preliminary system of qualitative analysis of leaf pigments. We do not know the limits of accuracy and there are cases in which the proposed scheme does not function well owing to excessive adsorption. It has worked well for us so far, and we are presenting it for consideration and criticism. Most of the data were available in the literature and we have merely arranged and checked them. We have been using the method for over three years and have found it helpful.

Unless otherwise stated all leaves are used fresh and are shredded by hand, although the scheme of analysis also works when dry leaves are employed. Such terms as carotenes, xanthophylls, flavones, anthocyanins, anthocyanidins and leuco-anthocyanins, are used generically and refer to any or all of the pigments grouped under any one head and not necessarily to any single pigment. It would probably be wiser to extract in some cases in an oxygen-free atmosphere and in the dark, but these precautions were not taken and may be considered as future refinements, which may be of importance when studying the flavones and leuco-anthocyanins.

Recommended Method of Qualitative Analysis

1. **Tests for flavones, leuco-anthocyanins, anthocyanins and anthocyanidins** are made by treating the shredded leaves with a 2–10% solution of formic acid, extracting by heating in boiling water if no pink or red color appears in the cold extract after from six to twenty-four hours. The solution may be colorless, pale yellow, red or pink. If colorless or pale yellow, no anthocyanins or anthocyanidins are present. Addition of aqueous ammonia or aqueous caustic soda causes a marked deepening of the yellow color if flavones are present. If the stronger formic acid causes a visible decomposition of the chlorophyll, this must be taken into account.

In a few cases with yellow autumn leaves, notably with those of the dogwood before it turns pink, the formic acid solution is yellow and the color does not deepen appreciably on adding ammonia. If enough ether is added to make a second liquid layer, the yellow goes to a considerable extent, sometimes completely, into the ether layer. This means that flavones are present at most in small amounts. The yellow color is due chiefly to what Tswett[4] calls "water-soluble yellow pigments of unknown constitution." Since the organic chemists have not yet determined how many of these there are or what their special properties are, it is impossible at present to differentiate them.

A pink or red color in the formic acid solution denotes anthocyanins, anthocyanidins or both, either accompanied or unaccompanied by flavones. If the solution is colorless with cold extraction and pink, red or brownish-red when extracted hot, practically all of the pink or red pigment was present in the leaf as leuco-anthocyanins. In such a case it is more satisfactory to heat the shredded leaves on the water-bath in a 5–10% sulfuric acid solution. With a strong acid, hydrolysis takes place rapidly and it is only necessary to heat for fifteen minutes to an hour to get a good test. In cases of doubt it is desirable to add butyl alcohol to the cooled solution. The red pigment concentrates in the butyl alcohol layer and becomes conspicuous.

To determine the presence of anthocyanins and anthocyanidins in the pink formic acid solution, add isoamyl alcohol to the pink solution and dilute gradually with water. If the isoamyl alcohol extracts practically all the pink color from dilute acid solution, the pigments are anthocyanidins and not anthocyanins. This case occurs rarely except when one has broken down a leuco-anthocyanin. If the isoamyl alcohol extracts practically no pink color, the pigments are anthocyanins. If both layers become pink, continued extraction with isoamyl alcohol may be done until one or the other of the layers becomes practically colorless. Willstätter and Everest[5] recommend

(1) Original manuscript received December 13, 1935.

(2) Onslow, "The Anthocyanin Pigments of Plants," 1925, p. 49.

(3) Bancroft and Rutzler, THIS JOURNAL, **60**, 2738 (1938).

(4) Palmer, "Carotinoids and Related Pigments," 1922, p. 63.

(5) Willstätter and Everest, *Ann.*, **401**, 207 (1913).

washing the amyl alcohol layer with dilute sulfuric acid.

To determine flavones in the presence of anthocyanins, shake the pink formic acid solution with ether. Remove the practically colorless ether layer and add aqueous ammonia or caustic soda to it. A yellow color in the water layer denotes the presence of flavones. Anthocyanidins do not interfere with this test, and leuco-anthocyanins do not go into the ether layer. If the ether layer is yellow and the color goes only partly into the water layer on adding aqueous ammonia or caustic soda, Tswett's "water-soluble yellow pigments of unknown constitution" must be present along with flavones.

If the amyl alcohol test for anthocyanidins is negative, the amyl alcohol layer may be removed and tested for flavones with aqueous ammonia or aqueous caustic soda. Anthocyanidins interfere with this test.

Anthocyanins are glycosides and anthocyanidins are the corresponding sugar-free pigments. So far as we now know, anthocyanidins occur only very rarely as such in plants. A test for them is desirable, partly because it is not uncommon for chemists accidentally to convert anthocyanins into anthocyanidins in the laboratory, and partly because anthocyanidins may occur more often than we now realize in autumn leaves whose red color is due to decomposition of leuco-anthocyanins.

2. **Tests for carotenes and xanthophylls** can be made more rapidly if the leaves are air-dried or vacuum-dried. It is safer, but not necessary, to use the leaves that have been extracted with the formic acid solution, because that eliminates any possible confusion over flavones and water-soluble yellows. Treat air-dried leaves with a low-boiling petroleum ether or with a so-called heptane, 90–100°. A little chlorophyll dissolves, so a greenish-yellow denotes presence of carotenes or xanthophylls or both. The green can be removed, if desired, by letting the solution stand in contact with aqueous hydrochloric acid. Add 90–95% methyl alcohol to the petroleum ether or heptane solution. Xanthophylls go into the methyl alcohol layer and carotenes stay in the upper layer. If both are present, the extraction should be continued until one or the other layer becomes practically colorless.

3. **Tests for chlorophyll** in red, yellow and brown leaves can be made by treating them, after or before extraction with formic acid and with heptane, with a methyl alcohol solution or with a 50–50 mixture of methyl and isoamyl alcohols. A green color, which disappears on adding aqueous hydrochloric acid, denotes presence of chlorophyll. The disappearance of the chlorophyll is a time reaction and does not occur instantaneously. Since chlorophyll goes readily into benzene and anthocyanin does not, the two can be separated qualitatively by shaking with benzene.

Simplified Method of Analysis

A quicker, simpler and less accurate method of qualitative analysis is to extract the leaves with methyl alcohol which takes out practically everything. Make up an ether–water system such that the two liquid layers are about equal in volume. Add some of the methyl alcohol extract. A green color in the ether layer denotes chlorophyll. This green will disappear gradually on adding hydrochloric acid, more slowly after adding ammonia. It is safer not to draw final conclusions in regard to yellow pigments until the chlorophyll has disappeared pretty completely. To the ether–water mixture containing some of the methyl alcohol extract, add aqueous hydrochloric acid solution. A pink color in the water layer indicates anthocyanins or anthocyanidins. Remove the ether layer and add aqueous ammonia to it. A yellow color in the water layer shows the presence of flavones. A yellow color exclusively or chiefly in the ether layer indicates the so-called water-soluble yellow pigments.

Remove the ether layer, add "heptane" to it and evaporate off the ether. Add 90–95% methyl alcohol. A yellow color in the heptane denotes carotenes and a yellow color in the water layer shows xanthophylls, subject always to possible errors due to the presence of water-soluble yellow pigments.

Positive results by this method are reliable. Negative results should be checked if there is any reason to doubt them. Methyl alcohol usually will extract anthocyanins readily, but we have seen a red cactus blossom which was scarcely touched by methyl alcohol and gave up its pigment readily to a formic acid solution. Acetic acid has very little effect on the flavones in the jonquil. Since methyl alcohol increases the mutual solubility of ether and water, a large amount of methyl alcohol may introduce complications, while smaller amounts of methyl alcohol may mean correspondingly smaller amounts of pigment to be tested.

Discussion

Both these methods work with from a fraction of a leaf upward. Any group of pigments that cannot be determined in an extract from six leaves can be ignored for all ordinary purposes, though there may be especial reasons for determining such pigments as flavins. For substances like these, special methods must be and have been developed.

By using an approximately constant weight of leaves in approximately the same state of dryness and a constant amount of solution, the methods can be made semi-precise. It was possible to show one year that leuco-anthocyanins were present in the leaves of the sugar maple in Ithaca in July and not in the leaves of the Norway maple. It was also shown that the green leaves of the copper beech, which had been red earlier in the season, contained relatively large amounts of leuco-anthocyanins in July, small amounts early in September and an almost negligible amount toward the end of September. A more exhaustive study of this system ought to give some definite information as to the precursors of leuco-anthocyanins. We do not know now whether flavones are or are not possible precursors of the leuco-anthocyanins and we do not know anything about the reds that appear in young leaves in tropical forests.

Summary

It is possible to identify many leaf and flower pigments by suitable treatment with methyl alcohol, butyl alcohol, amyl alcohol and ether in acid and alkaline solutions.

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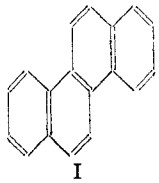
[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

A New Synthesis of Chrysene Derivatives

BY MELVIN S. NEWMAN

The desirability of preparing methyl derivatives of all of the five polycyclic aromatic hydrocarbons of the formula $C_{18}H_{12}$ has been pointed out previously.[1] Of these, derivatives of 1,2-benzanthracene have received the greatest attention. With the recent synthesis of 8-methyl-1,2-benzanthracene[2] all of the monomethyl derivatives of 1,2-benzanthracene have been made available for the study of the effect of structure on cancer-producing activity.[3] Of the other members of this class of hydrocarbons chrysene, I, offered a most promising field for research not only because of the similarity of its carbon skeleton to that of the steroids but also because the active carcinogenic agent in coal tar, 3,4-benzpyrene, II,[4] may be considered as a chrysene substituted in positions 4 and 5.



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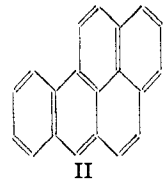

II

Of the known methods for the synthesis of chrysene and its derivatives[5] none seemed adequate for the preparation of various methyl- and dimethylchrysenes desired for biological testing. The work herein reported was undertaken in an effort to find a general method suitable for the preparation of variously substituted derivatives of chrysene.

The successful synthesis proceeds from benzalacetophenone by well-known reactions to α,γ-diphenylbutyric acid.

$$C_6H_5COCH{=}CHC_6H_5 + HCN \longrightarrow$$

$$C_6H_5COCH_2CH(CN)C_6H_5 \xrightarrow[H_2SO_4]{CH_3OH}$$

$$C_6H_5COCH_2CH(COOCH_3)C_6H_5 \xrightarrow{H_2O}$$

$$C_6H_5COCH_2CH(COOH)C_6H_5 \xrightarrow[HCl]{Zn—Hg}$$

$$C_6H_5CH_2CH_2CH(COOH)C_6H_5$$

The acid chloride of α,γ-diphenylbutyric acid was cyclized in benzene solution by aluminum chloride to 1-keto-2-phenyl-1,2,3,4-tetrahydronaphthalene III (87% yield) and the remaining steps in the synthesis are indicated below. This part of the synthesis consists essentially of an extension of the phenanthrene synthesis of Cook.[6]

Although one might expect the ketone III to be somewhat hindered, the Reformatsky reaction proceeded vigorously and the yield of the acid IV varied from 60 to 68%. The double bond is placed in the ring instead of the alternate position of conjugation with the carboxyl group because of the failure to isolate III from the products of ozonization. Surprisingly, low pressure catalytic reduction of this acid, its sodium salt, or its methyl ester using palladium or platinum catalysts failed almost completely. Invariably the acid was recovered for the most part unchanged. Reduction in excellent yield was finally effected by the action of 2% sodium amalgam on an aqueous alcoholic solution of the sodium salt, *using acid which had been recovered from unsuccessful attempts at catalytic hydrogenation.* When freshly prepared pure acid was used, *no reduction took place.* While an exhaustive search into the reason for this phenomenon was not made, the effect was noted in several different runs. If an alcoholic solution of the pure acid were shaken with Adams platinum catalyst and hydrogen and then recovered, this acid could be reduced by sodium amalgam. It may be that

(1) Newman and Joshel, THIS JOURNAL, **60**, 485 (1938).

(2) Cook and Robinson, *J. Chem. Soc.*, 505 (1938).

(3) For the most recent report on the activity of methyl-1,2-benzanthracenes see Shear, *Am. J. Cancer*, **33**, 499 (1938); Fieser, *ibid.*, **34**, 37 (1938).

(4) Cook, Hewett and Hieger, *J. Chem. Soc.*, 395 (1933).

(5) Fieser, "The Chemistry of Natural Products Related to Phenanthrene," Rheinhold Publishing Corporation, New York, N. Y., 1936 or 1937, p. 22.

(6) Cook, Hewett and Lawrence, *J. Chem. Soc.*, 71 (1936).