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THE YELLOW PIGMENT IN THE FLOWER

PETALS OF BIRD'S FOOT TREFOIL

(*LOTUS CORNICULATUS* L.)

A thesis presented to the Department of Chemistry
of Union College, in partial fulfillment of the require-
ments for the degree of Bachelor of Science in Chemistry.

By David K. Blake, Jr.

Approved

by

Frederic C. Schmidt

April 29, 1942.

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THE YELLOW PIGMENT IN THE FLOWER
PETALS OF BIRD'S FOOT TREFOIL
(LOTUS CORNICULATUS L.)

BIRD'S foot trefoil is a low growing, bushy legume which is gaining popularity as a forage plant. Its small, sweet-pea-like blossoms are bright yellow, bearing a few thin scarlet lines along the standard (the topmost petal). The buds are scarlet-tipped, the scarlet disappearing as the flower matures.

For this investigation, the flowers were picked when mature, or nearly so, then were air-dried at 20 - 30° C. The petals were not separated from the calyx.

Due to limitations of time, experience, and material, I was unable to investigate adequately many points, some of them important ones, in this research. I shall endeavor to point out these deficiencies, and to suggest, where possible, their remedies.

I - Isolation.

The dry flowers (200 g.) were repeatedly boiled with 96% alcohol (in 1.5 liter portions - 12 liters in all) until the extract failed to give a precipitate with lead acetate solution. The extract was then concentrated (to 100 cc.) and water added (200 cc.). The resulting deep brown solution was decanted from a tarry precipitate of water-insoluble substances (chlorophyll, carotenoids, etc,) which gradually settled out.

This solution, which contained the coloring matter, gave, when treated with lead acetate solution, a copious orange precipitate; with dilute alkali, a deep reddish brown color; with 1% ferric chloride, an intense olive green color; with ferric chloride and alkali, an intense orange red color.

The vast majority of yellow flower pigments are either carotenoids (which are generally insoluble in water) or derivatives of 2- or 3-phenyl benzopyrone. The latter give intense red to green colors with ferric chloride; yellow to red precipitates with lead acetate; and deep yellow to brown colors with alkali. From the properties of the flower extract, it is evident that it contained some derivative of phenyl benzopyrone.

When this solution was boiled for three hours without the addition of acid, nothing separated on cooling. However, when it was boiled with 1% of its weight of sulfuric acid for a half hour, upon cooling a somewhat curdy orange yellow precipitate was obtained. From this behavior I conclude that the pigment is a glycoside (of which the precipitate was the non-sugar portion, or aglycon), since practically the only flower products which are easily hydrolyzed by dilute acid to give insoluble products are glycosides. Of course, the only real proof of this point would be the actual isolation and characterization of the glycoside. This was attempted, as follows: to a portion of the solution (45 cc.), lead acetate was added until precipitation ceased. The precipitate was not easily filterable, so it was washed several times with water by decantation. A suspension of the precipitate in water was reconverted to the glycoside by passing hydrogen sulfide through. The suspension was boiled and the lead sulfide filtered off. The filtrate was then evaporated to

a very small volume (1 cc.), when it became somewhat syrupy. No solid separated within a few minutes. Possibly some would have, had it been allowed to stand long enough, since very few glycosides are as soluble in water as this one would have to be if its solution were unsaturated. The addition of alcohol to this concentrate caused the formation of a brownish precipitate. Some of this was thrown out when ether was added. The precipitated glycoside was not readily recrystallized from alcohol or acetic acid. At this stage its investigation was abandoned. The possibility of purifying it by fractional precipitation from alcoholic solution with ether was clearly indicated, however.

The glycoside solution was hydrolyzed with 1% sulfuric acid, as explained above, and the orange yellow non-sugar portion of it which precipitated on cooling was filtered off. When air-dried, it weighed two grams. Since this was obtained from 200 grams of flowers, the amount of pigment is at least 1% of the dry weight of the flowers. The petals make up about half of the weight of the complete flower, so the amount of pigment in them is probably about 2%.

Purification of this material proved to be very difficult, as no solvent or solvent mixture could be found from which it could be crystallized. ^{directly.} Chromatographic adsorption on alumina was tried, but was unsatisfactory. Perhaps good results could be obtained by this method by a proper choice of adsorbent and solvent.

The best method I have been able to devise is the following: the 2 grams of pigment are pulverized and dissolved in 75 cc. of glacial acetic acid at room temperature. Insoluble material is filtered off. The solution is boiled down to 50 cc. and 25 cc. of boiling water are added. It is then slowly cooled to 10° and the precipitate collected and washed with ice cold 50% acetic

acid, and dried. It is again pulverized, placed on a filter paper in a funnel, and washed through with ether. A dark brown impurity is left on the paper. Most of the ether is boiled off from the solution, which is then transferred to a smaller container together with 25 cc. of glacial acetic acid. The remainder of the ether is then distilled off and water added at the boiling point until the solution is cloudy or until 50 cc. have been added. When this solution is allowed to cool, clumps of tiny, bright yellow needles should be obtained. If the material is not bright yellow, the ether treatment must be repeated. In any case, the next step is recrystallization from a mixture of benzene and glacial acetic acid. Analytically pure material should be obtainable by a sufficient number of alternate recrystallizations from water-acetic acid and benzene-acetic acid. In conducting these operations, contact of the hot solution with the air should be avoided as much as possible, as it is slightly susceptible to atmospheric oxidation.

II - Physical Properties

The pigment crystallizes from dilute acetic acid in tiny, bright yellow needles containing water of crystallization and melting at about 230° with considerable frothing and darkening. The anhydrous material probably melts within three degrees of 268° (corr.), although the best sample I obtained, after several recrystallizations from dilute acetic acid, shrunk at 230° , sintered and darkened at 245° , and melted at $261-264^{\circ}$ to a brownish black, viscous liquid. It was difficult to tell when melting occurred, due to the extensive darkening and shrinking and high viscosity of the material. In spite of the darkening, there

must have been relatively little decomposition, since the contents of the melting point tube, even after having been at the melting point for several minutes, dissolved completely in alcohol and gave the same characteristic reactions as a solution of unmelted material. The melted material also dissolved in hot water, precipitating out on cooling seemingly exactly as the unmelted material did when similarly treated.

As the impure material was successively recrystallized, the melting point showed a slow upward trend, and the sintering point approached the melting point. It is very probable, in view of the wide melting range, that even the best sample was not chemically pure. Unfortunately, there was not sufficient material to continue recrystallizing until the melting point was constant.

The pigment sublimes very slowly in a high vacuum at temperatures just below the melting point, more rapidly but with some decomposition at higher temperatures. There is probably too much decomposition, even at the lower temperatures, to warrant using vacuum sublimation as a means of purifying the substance, although this question should be further investigated.

The solubility relations of the compound are as follows:

It is soluble in: alcohol (about 25 g. per 100 cc. at room temperature), pyridine, acetone, ether, dioxane, glacial acetic acid, ethyl acetate, methanol, isopropanol; acids, alkalis.

It is sparingly soluble in: hot water (about 1 g. per liter), n-butyl ether.

It is insoluble in: cold water and non-polar solvents.

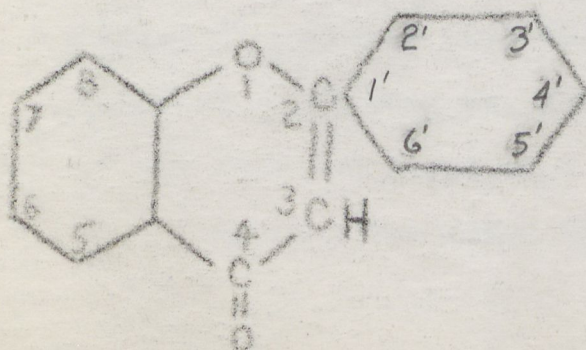
In all its solutions the compound is light yellow to deep orange red in color, depending on the concentration. The solutions in alkalis and concentrated acids are very much ~~more~~ deeper and redder than solutions of the same concentration in other solvents.

III - Chemical Reactions

The chief reactions the compound has been found to give are:

1. The dilute alcoholic solution (light yellow) gives an intense brown coloration with a very little dilute ferric chloride solution.
2. The same solution gives an orange red, flocculent precipitate with lead acetate solution.
3. The color of the alcoholic solution deepens to orange red when either dilute base or concentrated acid ~~is~~ is added. Also, the compound is soluble in both dilute base and concentrated acid.
4. Its dilute solution in concentrated sulfuric acid (light yellow) has a bluish green fluorescence; when the solution is made so dilute that the yellow color is barely perceptible, the fluorescence is pure blue.

The above reactions are highly characteristic of the large class of yellow plant pigments, derivatives of 2-phenyl or 3-phenyl benzopyrone, known as flavones and isoflavones, respectively.



2- phenyl benzopyrone
(flavone)

Hence it may be taken, not as proven, to be sure, but as highly probable, that the compound is a derivative of either flavone or isoflavone, probably the former, as flavone derivatives are much more abundant in nature than isoflavones.

5. When the alcoholic solution is made light yellow by dilution and a little concentrated hydrochloric acid and some metallic magnesium is added, a bright cherry red develops in a short time (about 1 minute).

6. When the alcoholic solution is maintained at 50° for three hours with 2% sodium amalgam, air being excluded, and the resulting solution poured into concentrated hydrochloric acid, no such cherry red color develops.

From these results, it is concluded that the compound is easily reducible with magnesium and hydrochloric acid and at least difficultly so with sodium amalgam.

With respect to their behavior toward these two reducing agents, flavone derivatives may be divided into four classes:

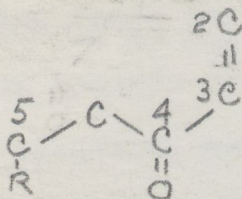
- a. Flavanones (2,3 dihydroflavones)
- b. Flavonols (3 hydroxyflavones)
- c. 3-substituted flavonols
- d. All other flavone derivatives.

According to Wolfrom (J.A.C.S. 62, 1485), it has been shown by Asahina and his co-workers that reducibility by magnesium and hydrochloric acid and not by sodium amalgam is characteristic of class (b) above and not of the other classes. Considering this, I think it probable that the compound under consideration is either an isoflavone or, more probably, a flavonol.

7. When two drops of a saturated solution of boric acid in acetone are added to .5 cc. of ~~the solution~~ an acetone

solution of the compound, so dilute that the yellow color is barely perceptible, a full yellow color rapidly develops.

According to Wilson (J.A.C.S. 61, 2303), this behavior indicates the presence of the grouping

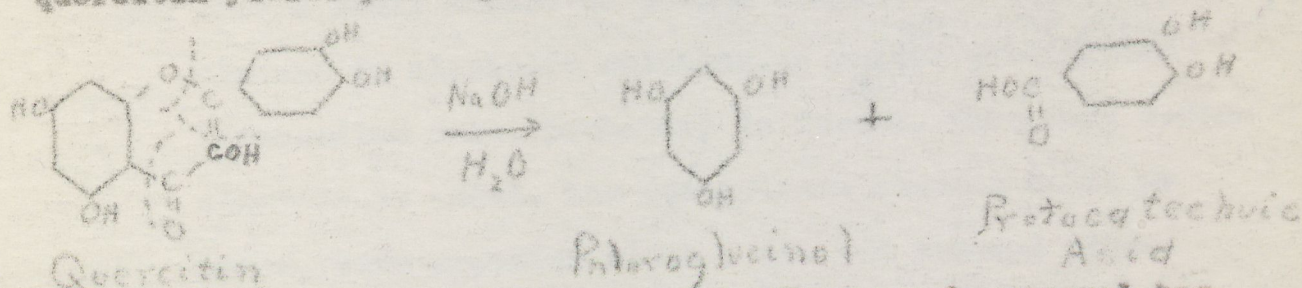


where R is an auxochromic group, which in this compound is very unlikely to be anything but OH or OCH₃. The numbers show how this residue fits into the flavone molecule. The implication of this test is that the compound possesses either a hydroxyl or a methoxyl group in position 5.

8. When the compound is fused with ten times its weight of potassium hydroxide and a very little water, the melt is at first blood red in color. If maintained at 210° for an hour, the color becomes dark brown, but there is little further change. However, if the temperature is raised to about 300°, there is a rapid and nearly complete decolorization. When the melt is maintained at 300° for about two minutes, then cooled and acidified with concentrated hydrochloric acid, extracted with ether, the ether extract in turn extracted with sodium bicarbonate solution, this solution acidified and in turn re-extracted with ether; this ether extract contains an acid (since it was extracted from ether with sodium bicarbonate) which, in aqueous solution gives an intense green color with a little 1% ferric chloride, changing to deep ~~pur~~ violet upon the addition of a very small quantity of sodium bicarbonate, and further changing to deep wine red with the further addition of sodium bicarbonate. This behavior is highly characteristic of protocatechuic acid, and not of any other polyhydroxy

benzoic acid.

When flavones and isoflavones are decomposed with alkali, the final result of the degradation is two products, one a polyhydroxy benzene from the fused benzene ring of the molecule, and the other a polyhydroxy benzoic acid, from the side ring. Thus, quercetin yields phloroglucinol and protocatechuic acid.



This behavior of flavones and isoflavones in general, has been well established. Since in the alkali decomposition of the compound here under consideration, protocatechuic acid was produced, it may be taken as virtually certain that it contains hydroxyl (or methoxyl; the alkali would demethylate these to hydroxyl groups at the high temperature) groups in the 3' and 4' positions.

The polyhydroxybenzene (the other of the two products to be expected) was not detected.

It is important that this alkali decomposition be much more thoroughly studied, especially the intermediate products, as it is only through them that the choice between a flavone or an isoflavone structure for the compound can be made, without resorting to synthesis.

9. A dilute alkaline solution (light yellow) of the pigment, when shaken with air and allowed to stand exposed to the atmosphere changes gradually to orange during a period of four days.

In this behavior it differs markedly from those flavone de-

~~derivatives~~ derivatives having three vicinal hydroxyl groups. Basic solutions of these compounds are rapidly oxidized by the atmosphere giving green, blue, or brown colors, depending on the pH. Hence it may be concluded that the compound probably does not have three vicinal hydroxyl groups.

10. When a small amount of sodium ethylate is added to the pigment in alcoholic solution, a yellow precipitate (monosodium salt, probably) is formed. When more sodium ethylate is added, the precipitate changes color to green, then greenish black (di- and perhaps polysodium salt). This, I think, is good evidence for the presence of at least two hydroxyl groups in the molecule.
11. When aluminum ion is added to a very dilute (very light yellow) solution of the pigment, a bright yellow color is produced. If stannic ion is added to the pigment solution, the color is golden yellow. Chromic ion does not seem to produce a color.
12. When the pigment is boiled with ~~acet~~ acetic anhydride and either sodium acetate or a little pyridine for an hour, and the solution cooled and shaken with water to hydrolyze the anhydride, a brownish solid is obtained. Attempts to crystallize this from alcohol, in which it is soluble, or from alcohol-water mixtures were unsuccessful, the material always separating as a tar. It is soluble in benzene, from which it can be precipitated with petroleum ether; and from which it can be adsorbed with alumina to give an orange yellow adsorbate, from which it is removed only with difficulty, thus rendering chromatographic adsorption on alumina a rather poor

method of purification. It seems likely that fractional precipitation with petroleum ether from benzene solution might work.

IV - Analysis

Due to the above mentioned uncertainty regarding the purity of the material obtained, the value of the analyses performed on it must be viewed with scepticism and the conclusions drawn from them must be of a tentative nature. It is obvious, then, that the data about to be presented are to be regarded as by no means final.

The most reliable of the determinations was that of the molecular weight. This was accomplished by the cryoscopic method with the Beckmann apparatus. Dioxane proved to be an excellent solvent for the purpose. Its freezing point constant was determined with pure naphthaline to be $4.85 \pm .03$ (mean of four determinations).

After each molecular weight determination, the dioxane was boiled off and the pigment recrystallized from dilute acetic acid. The successive determinations, then, may be assumed to have been performed on successively purer material. The results, in the order in which they were obtained, are:

$$M = 279, 277, 289, 282 \quad \text{Average } M = 282 \pm 4$$

Uncertainty due to freezing point constant is two parts in 300, so the average $M = 282 \pm 6$.

There seems to be a slight upward trend here, so it seems most likely that the molecular weight after complete purification would average about 290. It seems, from this, a hardly likely that the true molecular weight of the compound would be higher than 305 or lower than 275.

The percentage of hydroxyl group in the molecule was determined by the Zerewitinoff method. Two determinations, on samples

in slightly different states of purity were made. The results were

% OH 25% , 26%. Average % OH 26.5 %.

This average is, in all probability, within 7 % of the true value.

Making these liberal allowances for error in the molecular weight and per cent hydroxyl of the compound, then examining the corresponding constants of the various hydroxy- and methoxy-substituted flavones, one is struck by the fact that there are only two possibilities for this compound: it must be either a tetrahydroxy¹ or a pentahydroxy compound². It can have no methoxyl groups.

If the pigment is a pentahydroxy flavone, it is not the 3,5,7,3',4' compound (quercetin), for that has been shown definitely to have a melting point of 310°. The only other possibilities which are in harmony with the qualitative tests are 3,5,6,3',4' and 3,5,8,3',4' pentahydroxy flavone. If it is a tetrahydroxy flavone, it must be the 3,5,3',4' compound. None of the three latter compounds have been described in the literature.

An ultimate analysis of the compound (two samples of slightly different purity) was performed. The results were:

	Tetrahydroxy flavone requires	Pentahydroxyflavone requires
C: 57.2, 58.6 %	63.0%	59.6%
H: 3.5, 4.2%	3.52%	3.33%

These results are able to prove very little, due to their discordance, the seemingly impossibly high value for hydrogen of one of them, and the uncertainty as to the purity of the samples.

As mentioned above, the compound crystallizes from dilute acetic acid with water of crystallization. This is the dihydrate, as it loses two molecules of water when kept in a vacuum over phosphorus pentoxide, or when maintained at 110°.

- 1- Tetrahydroxy requires $M = 286$, % OH = 24 %
 2- Penta hydroxy requires $M = 302$, % OH = 28 %

Calculated for tetrahydroxyflavone dihydrate, 11.2% H_2O .

" " penta " " 10.7% "

Found 11.6, 10.8, 10.2%

When dried over Calcium chloride, 1 1/2 molecules of water are retained.

Calculated for tetrahydroxyflavone.1 1/2 H_2O , 8.6% H_2O .

" " penta " " 8.2% "

Found 8.25% "

V - Summary

The pigment of the flowers of bird's foot trefoil has been shown to be very likely to be a glycoside of either

- (a) most probably, 3,5,3',4' tetrahydroxy flavone or 3,5,6,3',4' or 3,5,8,3',4' pentahydroxy flavone, none of which have been described in the literature,

or,

- (b) possibly a tetra or pentahydroxy isoflavone, with hydroxyl groups in the 6 3',4', and 8 5 positions, and without three vicinal hydroxyl groups.