

# A NOTE ON CERTAIN USEFUL COLOUR REACTIONS OF ANTHOXANTHINS AND RELATED COMPOUNDS

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THAT the reduction of flavones and flavonols yields bright red products was known even before Willstatter and other workers discovered that pyrylium salts of the anthocyanidin type are thereby formed. This reaction which is complex and gives rise to various products, has been very useful in establishing the relation between quercetin and cyanidin and has been freely employed for detecting the presence of flavones, flavonols and flavanones in plant materials. The reduction has been effected in different ways. In acid medium, usually alcoholic hydrochloric acid, the anthoxanthin has been reduced by the action of magnesium powder or magnesium in conjunction with mercury. The vigorous evolution of hydrogen is quickly followed by the appearance of bright red or allied shades of colour. When sodium amalgam is used instead, the alcoholic solution is finally acidified in order to produce the coloured pyrylium salt. In regard to the scope of the reduction in acid medium, Shinoda<sup>1</sup> examined a number of hydroxyflavones and flavonols and came to the conclusion that the presence or absence of hydroxyl groups in the side phenyl nucleus determined the nature of the final colour formed. When there were no hydroxyl groups in this phenyl nucleus yellow colours were obtained, whereas with increasing number of hydroxyl groups deeper red shades resulted. Later Asahina *et al.*<sup>2</sup> claimed that by employing acid and alkaline reducing agents it is possible to differentiate between flavones, flavonols and flavanones. According to them flavanones yielded red pyrylium salts by reduction by both the methods, *i.e.*, (1) magnesium and hydrochloric acid and (2) sodium amalgam. Flavonols respond to reduction in acid medium only and flavones yield definite colour reaction only with the alkaline reducing agent, sodium amalgam. A similar behaviour was noted with the isoflavones, genistein, pseudobaptigenin and osajin which like flavones undergo reduction with sodium amalgam, and not with magnesium and hydrochloric acid, to form red-coloured products (see Walter,<sup>3</sup> Wolfrom *et al.*<sup>4, 5</sup>).

In the course of our work we had occasion to examine a number of naturally occurring flavones and flavonols along with a number of synthetic

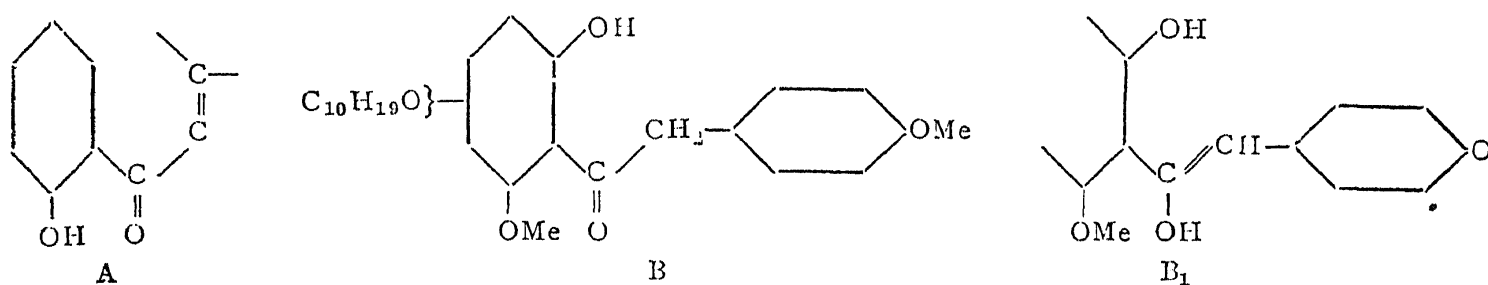
substances. Besides the hydroxylic compounds, completely methylated and sometimes partially methylated derivatives of these groups of compounds are found to occur in nature. Clear information of the behaviour of these methyl derivatives could not be obtained from the past literature. The clear-cut differences mentioned by Asahina *et al.* between flavonols and flavones, though valid in the simpler cases with a small number of hydroxyl groups, do not seem to hold good universally. For instance nobiletin<sup>6</sup> which is a flavone derivative gives a red colour when its alcoholic solution is treated with magnesium and hydrochloric acid. In regard to this reaction the results in general indicate that the pyrylium salt colours could range from golden yellow to deep red and purple, depending largely upon the number of hydroxyl groups present in the molecule. With less number of hydroxyl groups, say two, the colour is usually yellow or orange and with more heavily hydroxylated compounds it becomes deep red, brown red and purple. The methyl ethers seem to behave somewhat similar to the corresponding hydroxy compounds. The acetates, on the other hand, exhibit different properties; they give rise to practically no colour during the initial stages of the reduction and a colour develops only slowly, probably due to progressive hydrolysis by the alcoholic hydrochloric acid which gets hot during the course of the reaction. There does not seem to exist any sharp difference between flavones, flavanones and flavonols. It should, however, be noted that the first two groups due to the lack of a hydroxyl in the 3-position do not change into pseudo bases readily and hence do not require a high concentration of acid, whereas in carrying out the test for the flavonols sufficient amount of acid has to be added before the colour is seen. It may also be mentioned here that the addition of mercury does not seem to affect the course of the reduction with magnesium and hydrochloric acid to any appreciable extent.

With reference to reduction using sodium amalgam the same general considerations hold good. Though the difference pointed out by Asahina *et al.* between flavonols on the one hand and their methyl ethers and flavones on the other may be definite when pyrylium salts are prepared with larger quantities of materials, it is not quite dependable for qualitative tests using small quantities. In these tests even traces of products give colour and variations are introduced by the duration of the experiment, the concentrations of the solutions, etc. Using this method of reduction it is necessary to render the solution in the end strongly acid in order to obtain the colour. We have found that for this purpose sulphuric acid is more useful, since its concentration is high. Further, ordinary methylated spirit is preferable to anhydrous alcohol as solvent and reduction medium, since the latter solvent reacts only very slowly with the amalgam and hence the

reduction is too slow. From the typical cases mentioned later on, it is clear that flavonols also exhibit definite colour though compounds with no hydroxyl or with only a methoxyl in the 3-position form brighter red shades.

The boric acid test recently described by Wilson<sup>7</sup> is quite useful for detecting the presence of a 5-hydroxyl group in flavones and flavonols. Our results corroborate the structural features essential for a positive colour test as laid down by this author. A 5-methoxyl group serves equally well whereas an acetate group is unsuitable. In this connection it may be mentioned that similar conditions hold good even for the chalcones in which an *o*-hydroxyl or methoxyl gives rise to a positive colour. Incidentally it was noticed that when the simpler 3-hydroxyflavones without substitution in the 5-position are employed a marked blue fluorescence is generated, the solution remaining practically colourless. This suggests a possible method for the detection of small quantities of boric acid (compare Neelakantam and Row<sup>8</sup>) using these simple flavonols. The coming in of a 5-hydroxyl or methoxyl into the structure gives rise to deeply yellow solutions; no fluorescence could be detected in these cases.

From their work on osajin which is considered to be an iso-flavone derivative, Wolfrom *et al.* conclude that methylation of the hydroxyl in the system (A) leads to a negative boric acid test and that the compound (B) also gives a positive test probably due to enolisation as in (B<sub>1</sub>). According to Wilson's statement a methoxyl group in place of a hydroxyl in the ortho position satisfies the requirements for a positive colour test. Our work confirms this as far as the flavones, flavonols and chalcones are concerned. The question therefore requires careful verification in regard to the isoflavones using more examples.



As regards the enol structure suggested by Wolfrom *et al.* being capable of giving the boric acid colour we note that resacetophenone which can form some enol of the required type does not give a positive reaction. A more comparable case is that of  $\omega$ -phenylresacetophenone. When a pure sample of this is examined it does not give any colour in Wilson's boric acid test. Hence the claim of Wolfrom *et al.* that such compounds can

give a positive reaction by enolisation cannot be sustained. Obviously three requirements (1) a keto group, (2) an ortho hydroxyl or methoxyl and (3) an external double bond in conjugation with the C=O are essential for a positive boric acid reaction.

The results now obtained in the various tests are given below:—

#### I. *Reduction using magnesium and hydrochloric acid*

*Simple flavones and flavonols.*—7-Hydroxyflavone, 3-methoxy-7-hydroxyflavone, 3-hydroxy-7-methoxyflavone, 3:7-dihydroxyflavone and 2-methyl-3-methoxy-7-hydroxychromone—pale yellow; 3:7-dimethoxyflavone and 3-benzoyl-7-hydroxyflavone—golden yellow; 7-hydroxy-8-allylflavone and karanjin—deep orange yellow.

*Naturally occurring flavones and flavonols and their derivatives.*—Chrysin, luteolin and quercetagitritin—orange red; kempferol—feeble red; quercetin and patuletin—scarlet red; gossypetin, gossypitritin, herbacetin, populnetin and hibiscetin—brown red; quercetagetin—crimson; quercimeritritin, cannabiscetin, cannabiscitritin, hibiscitritin and isorhamnetin—red with pink tinge.

Pentamethylquercetin and pentamethylquercetagetin—orange red; hexamethylquercetagetin—deep crimson; 3, 7, 8, 3', 4'-pentamethylgossypetin—brown; 7-acetyl-3, 5, 8, 3', 4'-pentamethylgossypetin and hexamethylgossypetin—brown red.

Acetates of herbacetin, herbacitritin, gossypetin and patuletin—practically colourless for a few minutes during which the hydroxy compounds give a distinct and deep colour; slow development of the colour subsequently takes place due to hydrolysis.

*Flavanone derivatives.*—Naringin—bright pink; naringenin and hesperidin—reddish pink; butrin—brown pink; butin—deep bluish violet.

*Some typical compounds belonging to other groups.*—2, 6, 4'-trimethoxy-4-hydroxychalkone; 2:4-dihydroxy-4'-methoxychalkone, 7-hydroxycoumarin, 8-hydroxycoumarin, 5-hydroxy-4-methylcoumarin and 5:7-dihydroxy-4-methylcoumarin—no colour.

#### II. *Reduction with sodium amalgam*

A few typical cases are given. 7-Hydroxyflavone and pentamethylquercetin—deep red; 3-methoxy-7-hydroxyflavone, 3-hydroxy-7-methoxyflavone and 3:7-dihydroxyflavone—orange-red; chrysin—brown red; quercetin—orange brown.

#### III. *Boric acid colour reaction*

The test was carried out in the way described by Wilson. But in the case of the methyl ethers a higher concentration of citric acid was found

to be advantageous. Though it was not necessary to dry the acetone marked "extra pure" available in the market, it was noted that the addition of a drop of water discharged the colour. Hence access of water should be avoided. The following naturally occurring hydroxyflavones and flavonols gave a positive reaction: chrysin, luteolin, k mpferol, quercetin, quercetagenin, quercetagenin, quercimeritrin, cannabiscetin, cannabiscitrin, herbacetin, gossypetin, gossypitrin, hibiscetin, hibiscitrin and patuletin.

Positive tests were also obtained with the following methyl ethers of compounds of the above class: Pentamethylquercetin, hexamethylgossypetin, pentamethylgossypetin-7-acetate and hexamethylquercetagenin.

Acetates of the following pigments gave negative results: K mpferol, herbacetin, herbacitrin, hibiscetin, quercetagenin, gossypetin and gossypitrin.

2, 4-dihydroxy-4'-methoxychalcone, 2, 4, 4'-trihydroxy-3'-methoxychalcone, 2, 4, 3'-trihydroxy-4'-methoxychalcone and 2, 6, 4'-trimethoxy-4-hydroxychalcone gave positive results.

Among compounds that gave negative results in this test were the following: naringin, hesperidin, butrin, 7-hydroxyflavone, 3-benzoyl-7-hydroxyflavone, 2-methyl-3-methoxy-7-hydroxychromone, 7-acetoxyflavone, 7-allyloxy-8-allylflavone, 2-methyl-3-acetyl-7-hydroxychromone, 3-benzoyl-7-hydroxycoumarin, 3-benzoyl-5-methyl-7-hydroxycoumarin, 3-acetyl-7-methoxycoumarin, 3-acetyl-5-methyl-7-hydroxycoumarin, 4, 8-dimethyl-7-acetoxycoumarin, salicylic acid, phenyl salicylate, resacetophenone  $\omega$ -phenylresacetophenone and benzylidene-acetophenone.

3-Methoxy-7-hydroxyflavone, 3-hydroxy-7-methoxyflavone and 3:7-dihydroxyflavone gave with boric and citric acids a colourless solution with blue fluorescence. The fluorescence of the first was rather feeble whereas the other two gave bright blue of about the same intensity, indicating that the hydroxyl in the 3-position is primarily responsible for the reaction. The fluorescence was easily visible in diffused daylight even with very small quantities of boric acid. Since the dihydroxyflavone is more easily prepared it has been employed for detailed tests. 0.5 c.c. of a 10% solution of anhydrous citric acid in acetone and 0.25 c.c. of a 0.1% solution of 3:7-dihydroxyflavone in the same solvent were mixed in a tiny test tube (5/16" diameter) and treated with 1 drop (0.02 c.c.) of 0.05% solution of boric acid in acetone, corresponding to 0.01 mg. of boric acid. There was appreciable fluorescence which could be detected with care. With two drops of boric acid solution (0.02 mg.) the observation of the fluorescence was much easier and with five drops (0.05 mg.) quite easy. One drop of a 0.5% solution of boric acid (0.1 mg.) gave under the

same conditions a brilliant blue fluorescence. It may be mentioned here that it was found desirable to carry out a blank test also at the same time with only the flavonol and citric acid using a similar test tube made of the same glass; this was because the glass had a small effect on the observation of the colour phenomenon. Carried out as described here the sensitiveness of the test for boric acid is of the order of 1 in 100,000.

A similar use could be suggested for the yellow colour formed with other compounds giving positive Wilson's test. A suitable case is penta-methylquercetin which forms colourless solutions in acetone containing citric acid. The yellow colour developed on the addition of boric acid solution could be easily noted. But the test is not so sensitive as with the fluorescence, only one part in 30,000 being capable of detection.

#### Summary

Employing a large number of natural and synthetic flavones, flavonols, flavanones and certain related compounds the scope of the following colour reactions has been examined: (1) reduction with magnesium and alcoholic hydrochloric acid, (2) reduction with sodium amalgam and alcohol and (3) Wilson's boric acid test using boric and citric acid mixture in acetone solution. For the first two reactions the nature of the colour depends in general upon the number of hydroxyl or methoxyl groups in the molecule. In qualitative reactions it is not easily possible to effect minor distinctions between flavones, flavonols and flavanones. The boric acid test is very specific for 5-hydroxy- and 5-methoxyflavones and flavonols and *o*-hydroxy- and methoxy chalcones. It is not given by flavanones and simple aromatic ketones which do not satisfy the specific conditions. A combination of these three colour reactions gives a great deal of useful information.

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