

CHEMISTRY OF THE COLOUR TESTS FOR CHALKONES, FLAVANONES AND AURONES

H. G. KRISHNAMURTY AND T. R. SESHADRI

Department of Chemistry, University of Delhi, Delhi-7

THE commonly used reagents for colour tests of anthoxanthins are the following: (A) Mg-HCl; (B) Na, Hg-HCl; (C) Zn-HCl; (D) NaBH₄-HCl; (E) Ac₂O-H₂SO₄; (F) SbCl₅ in CCl₄; the tests (D) and (E) are of more recent introduction. The colourless or yellow solutions of anthoxanthins develop colours ranging from orange to purple, which fade on keeping. A useful account of the mode of carrying out the tests (A), (B), (C) and (F) and their specificities is given by Geissman.¹ The present paper is intended to present some observations on the tests given by flavanones, chalkones and aurones.

Tests (A) and (B) for Flavanones.—In these tests, substituted flavanones give bright red to purple colours. Geissman and Clinton³ made a detailed study to isolate the possible reduction products and to identify the substances responsible for the colour. Their experimental data led them to the conclusion that the colours are not due to flavylum salts as originally thought but arise by the action of acid on the reduction products of flavanones such as flavan-4-ols, flavpinacols and of reduction products of 2'-hydroxy chalkones arising from flavanone ring opening. An important observation made by them was that 5, 7, 4'-trimethoxyflavan-4-ol (prepared by catalytic reduction of 5, 7, 4'-trimethoxy flavanone) produced a deep carmine colour with acid and this was identical with the colour produced in Na, Hg-HCl reaction. They formulated the sequence of steps (Chart I) leading to the formation of the substance responsible for colour which was considered to be the carbonium ion (4). Similar ions derivable from flavpinacols were also considered to contribute to the colour. Certain relevant experiments have now been done with the reagent D with the object of verifying these formulations and they are presented here.

Test (D): (1) Flavanones.—This test⁶ consists in reducing the anthoxanthin with sodium borohydride followed by acidification with conc. hydrochloric acid. Among the flavonoids only flavanones were reported to give a positive reaction. Rowell and Winter¹² have used this test for the estimation (spectrophotometric method) of flavanones present in citrus fruits. Reduction of flavanone with sodium borohydride is a straightforward reaction leading to the

formation of flavan-4-ol as the only product. The formation of deep colour in this reduction procedure is undoubtedly due to the action of acid on a flavan-4-ol.

(ii) **Chalkones.**—Contrary to what was recorded by Horowitz,¹¹ chalkones could be selectively reduced at the carbonyl function using sodium borohydride in methanol-ethanol solution. In this manner, the chalkone (5) (m.p. 160°) gave a colourless crystalline substance (m.p. 104°) whose properties (chemical, U.V. and I.R.) are in agreement with the structure (6). It produced purple colour when treated with a strong acid. This colour and the one obtained with 5, 7, 3', 4'-tetramethoxyflavan-4-ol¹¹ (2) (m.p. 117°) are spectrally identical (λ_{\max} 565 m μ). A noteworthy observation is that while the compound (6) produced colour immediately, the flavan-4-ol gave colour of maximum intensity after a small time interval. This difference is understandable since for the formation of the quinonoid chromophore (4a or 4b) the heterocyclic ring in the flavan-4-ol has to open out under the influence of acid. These observations provide evidence to demonstrate that carbonium ion of the type (4) arising in accordance with Geissman's scheme is involved in the colour test.

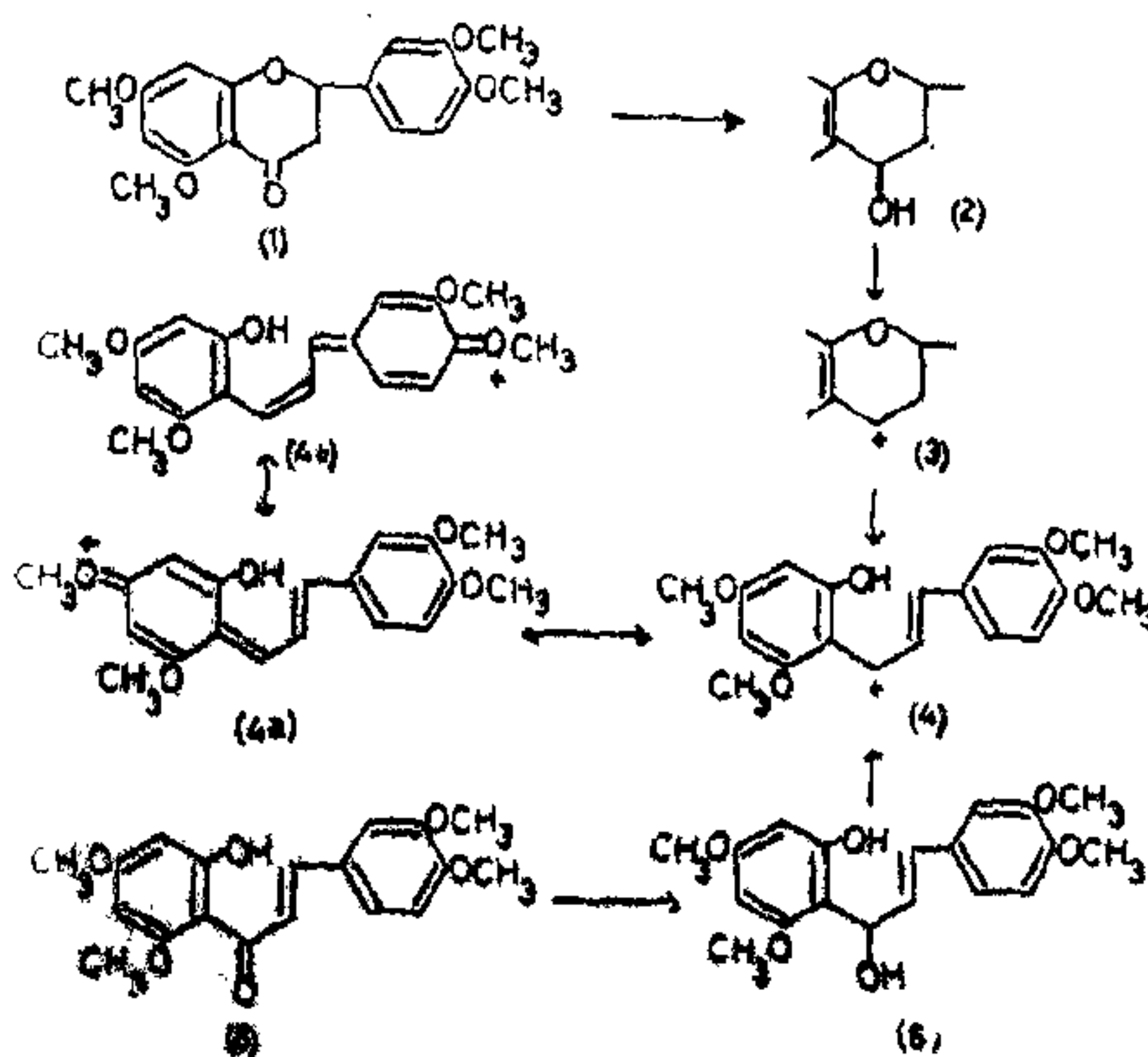


CHART I

A second and more satisfying evidence in support of the above scheme is provided by the following experiments. Selective reduction of 4-methoxy chalcone (7) with sodium borohydride gave a colourless unsaturated alcohol (8) (m.p. 48° ; lit.² 52°). Its I.R. spectrum showed the presence of hydroxyl and no carbonyl. It is different from the propanol (9) prepared from the chalcone (7) by Pt, H_2 reduction and it also gives the propanol by further catalytic reduction. It is very sensitive to acid and gives an immediate red colour with ethanolic HCl while the propanol (9) showed no change.

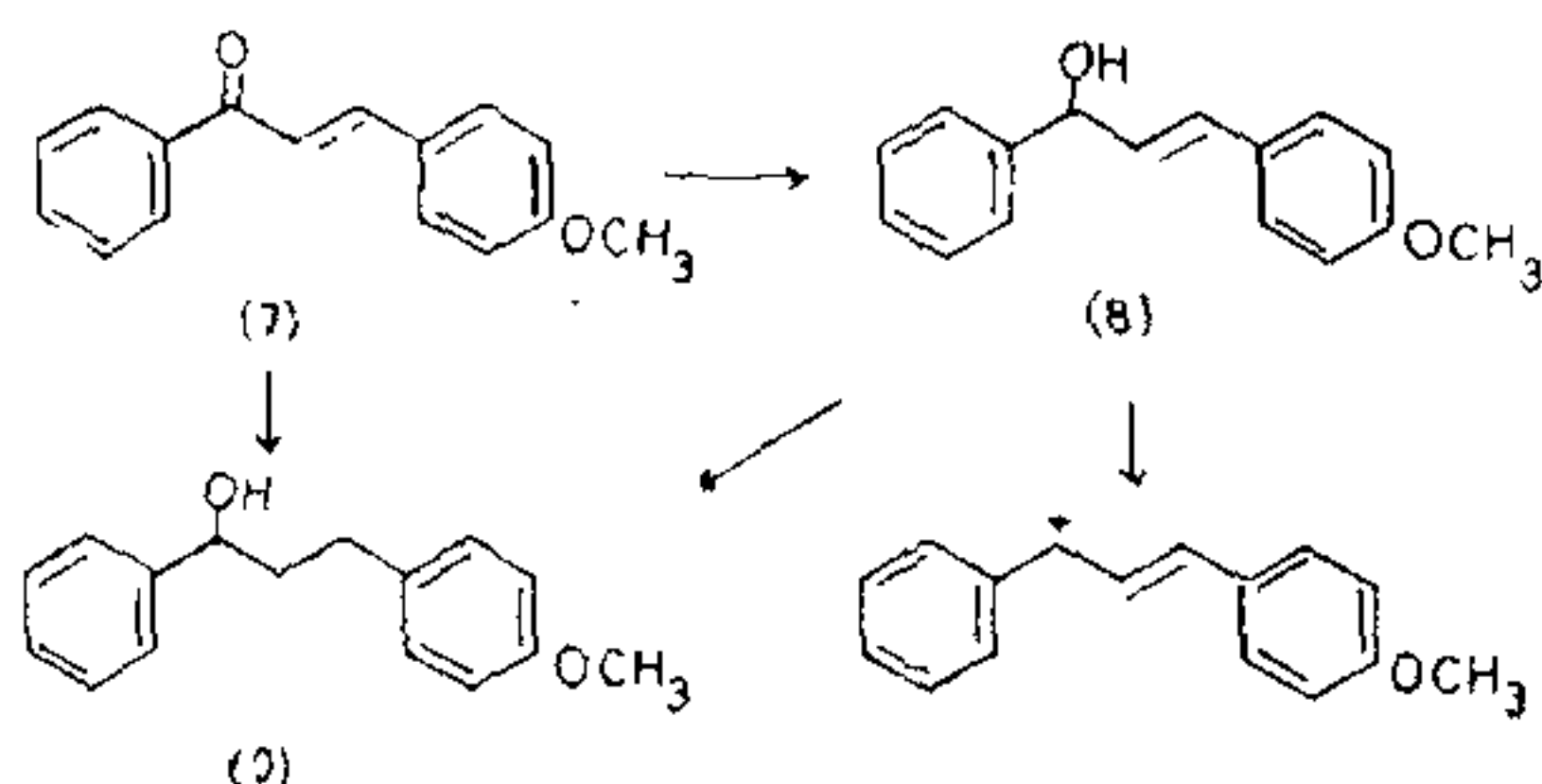


CHART II

Further evidence for the structure of the chromogenic compound is provided as follows: The diol (10) obtained⁹ by the reduction of the corresponding dibenzoyl methane gave on treatment with mineral acids in the cold a red purple colour spectrally identical with the colour produced by 5, 7, 4'-trimethoxy flavan-4-ol. The possible course of the reactions is shown in Chart III.

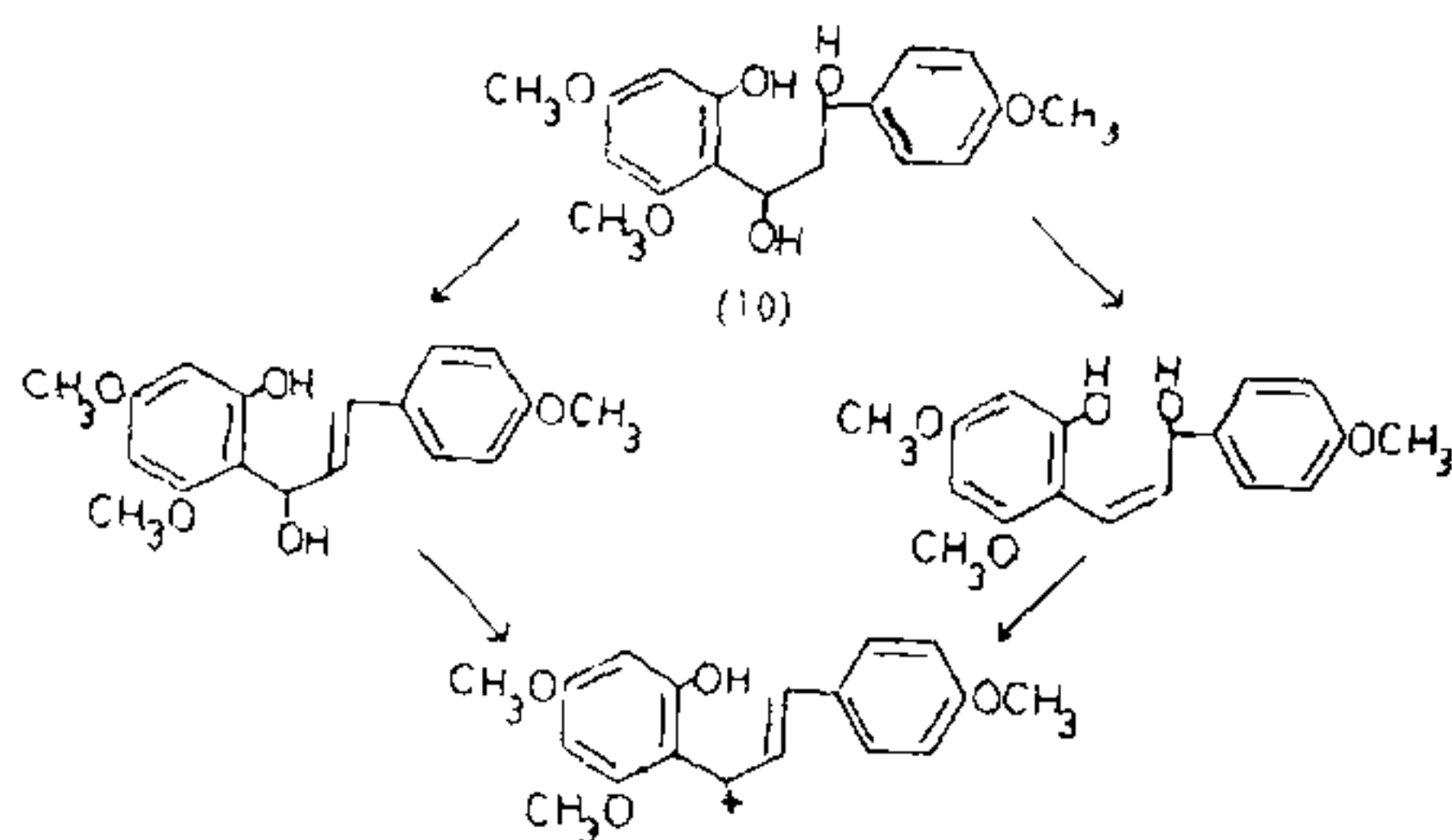


CHART III

The expectation that flavpinacols can also contribute to the colour in tests (A) and (B) has now been confirmed. 5, 7, 4'-trimethoxyflavanone by Al, Hg-alcohol reduction provides the flavpinacol¹⁰ as the chief product. It gives deep red-violet colour with mineral acids and this behaviour is similar to that of 5, 7, 4'-trimethoxyflavan-4-ol. It may be pointed out that substituted (hydroxy or methoxy) flavpina-

cols cannot be isolated under the condition of test (A) due to their sensitiveness to acids.

The new results summarised above fully substantiate Geissman and Clinton's scheme and it is clear that their observations pertain to the deep colour (initial colour) that develops rapidly in the tests (A) and (B). They contended that flavylum salts may not be present in the final acid solution even as minor components. We described⁹ in an earlier paper the conversion of flavan-4-ols and the reduced dibenzoyl methanes into flavylum chlorides. As already mentioned the deep colour developed in the cold by the flavan-4-ol (2), and the alcohols (6) and (10) is not stable; on standing for some time it is lost. On the other hand if the original solution is heated there is gradual change to yield finally a stable orange or red solution. Taking as an example 5, 7, 4'-trimethoxyflavan 4-ol or the diol (10) the initial deep colour had $\lambda_{max.}$ $545 m\mu$ (ethanol-conc. HCl) and after boiling for 45 min. the orange red solution had $\lambda_{max.}$ $475 m\mu$ and this value remained constant. From the final red solution apigeninidin trimethyl ether was isolated. The substance (6) showed similar colour changes and we have established by chromatography and absorption spectrum the presence of 5, 7, 3', 4'-tetramethoxyflavylum chloride in the final acid solution. These changes mean that the carbonium ion is undergoing oxidative (aerial) cyclisation to give flavylum salt. The poor yield (about 8%) of the flavylum salt in all of these cases could be explained on the basis that a predominant competitive reaction involves polymerisation to yield phlobaphenes. A formal representation of this conversion is shown in Chart IV (cf. flavan 3:4-diol-anthocyanidin change¹⁴).

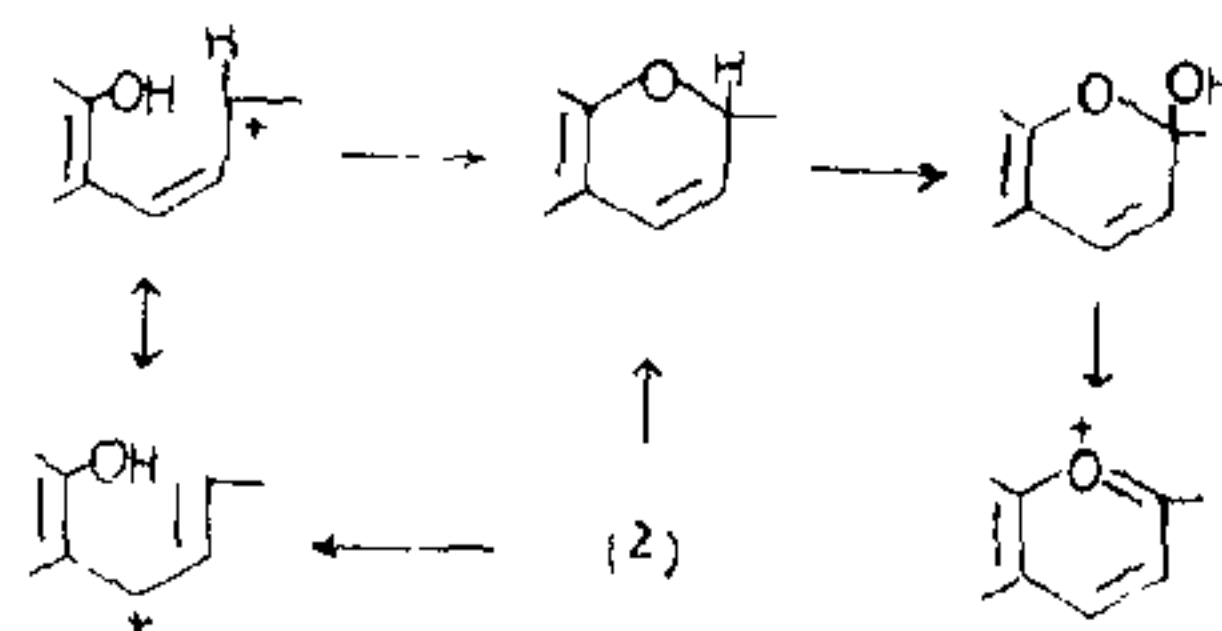


CHART IV

It may be mentioned here that flavan 3, 4-diol (11) derivatives (natural and synthetic) unlike flavan 4-ols do not give such intense colours in the cold. They give, however, red pink colours due to 3-hydroxy flavylum salt along with phlobaphenes in warm acid solution. This difference from flavan 4-ols could be explained on the following basis. Even after

ring opening to give enol (12) the extended conjugation as in (4) is not possible on account of its tendency to go into the keto form (12a).

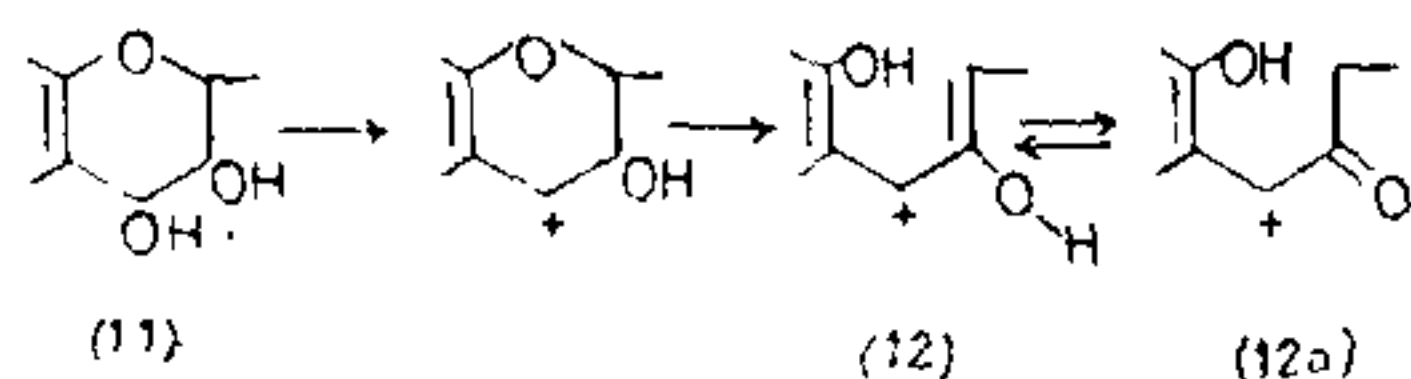


CHART V

(iii) *Reduction of Aurones.*—One can expect that the carbonium ion (15) (Chart VI) derived from an aurone should also exhibit colour. In order to establish this point 4, 6, 4'-trimethoxy aurone (13) was subjected to reduction using NaBH_4 . The properties of the product clearly showed that the aurone carbonyl underwent reduction. The product (14) produced a purple colour with conc. HCl in ethanol solution and the colour is stable since the structure cannot undergo further change.

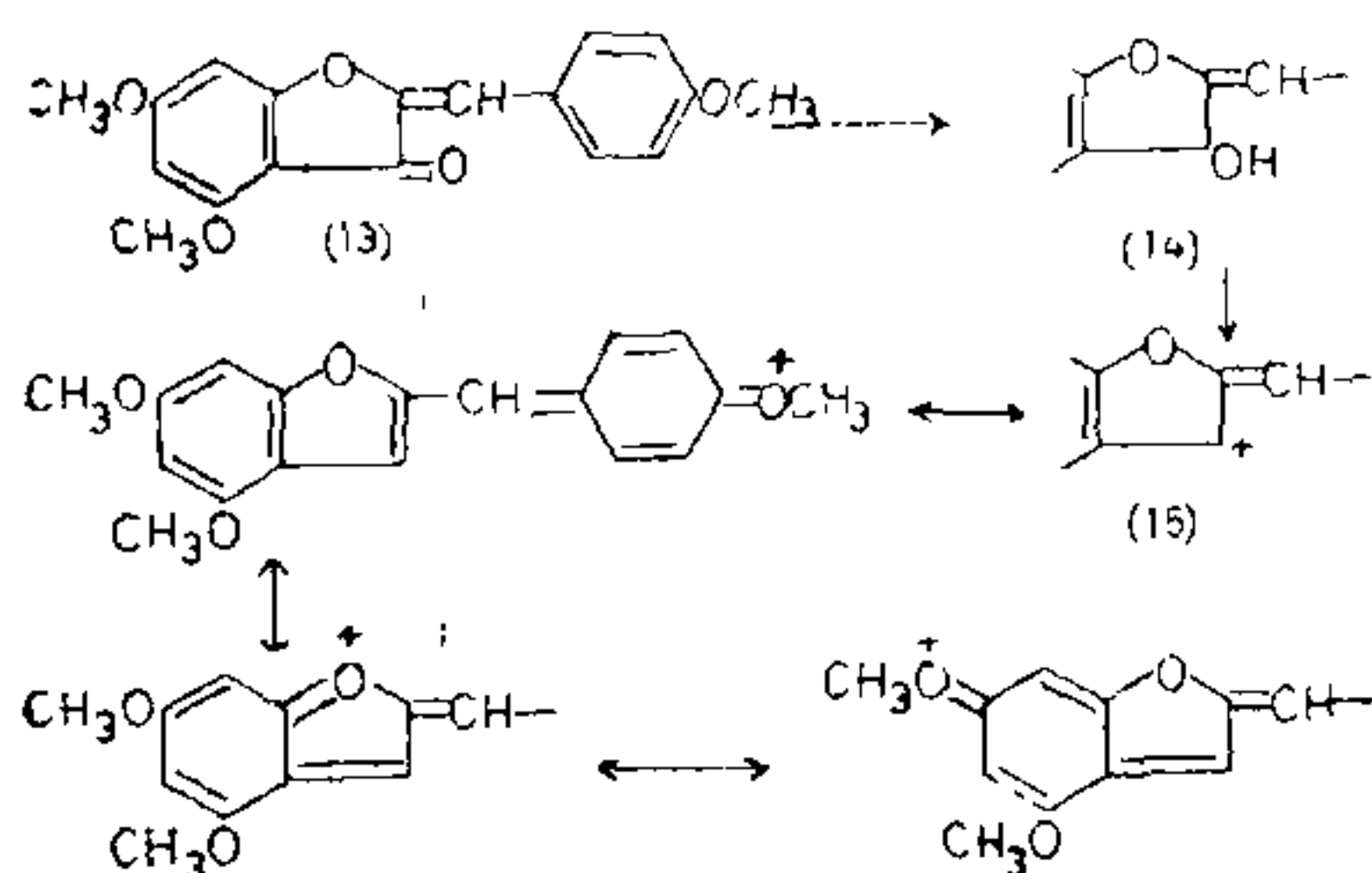


CHART VI

Test (E).—King and White⁵ found that substituted (hydroxy, methoxy) chalkones, flavanones, aurones and 2-hydroxy-2-benzyl coumaran-3-ones in acetic anhydride solution produce deep colours (orange to purple) when treated with a drop or two of concentrated sulphuric acid. The test is negative with other flavonoids. The mechanism of this reaction has not been explained. The following observations throw light on it.

Chalkones and Flavanones.—That chalkones and flavanones dissolve in concentrated sulphuric acid and produce vivid colours is well known.¹ In the first case an acid-base interaction leading to the carbonium ions (18) is believed¹ to be involved. A large bathochromic shift in the visible colour occurs when acetic anhydride is employed as the solvent. The cause of colour in the acetic anhydride-sulphuric medium on chalkones (16) appeared to be due to changes summarised in the formulas 16-19 (Chart VII). The consequence of the last acetylation step

(18→19) is to favour the retention of the charge on the carbon and thus shift the equilibrium largely to the carbonium ion side. In line with this reasoning we find agents like HClO_4 and ZnCl_2 (Lewis acid) which are capable of generating acetylum ions¹ from Ac_2O serve equally well in the place of sulphuric acid.⁵

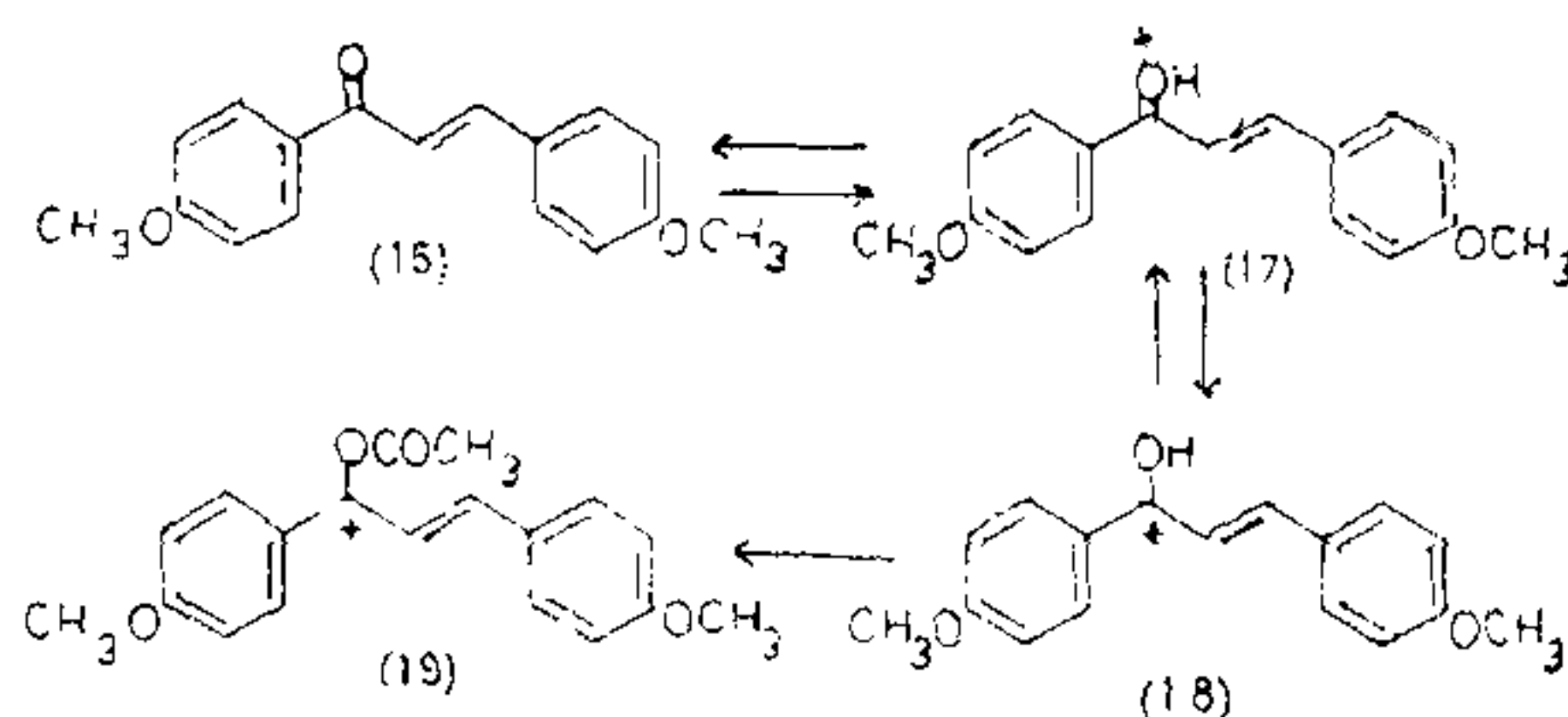


CHART VII

The essential chromogen is evidently $-\text{CO}-\text{CH}=\text{CH}-$ flanked on either side by aryl groups. Flavanones, which lack this system still produce colour. We were originally inclined to interpret the process as involving acid catalysed ring opening to 2'-acetoxy chalkone which gives the colour.

To test this possibility the action of $\text{Ac}_2\text{O}-\text{H}_2\text{SO}_4$ on 2'-hydroxy-3, 4, 4'-trimethoxy chalkone and the isomeric 7, 3', 4'-trimethoxyflavanone was studied. Firstly, the colour in both the cases was found to be spectrally identical. The chalkone gave intense colour (λ_{max} 542 m μ , $E_{1\text{cm}}^{1\%}$ 1406) instantaneously while the flavanone showed only weak absorption (λ_{max} 542 $E_{1\text{cm}}^{1\%}$ 132) to start with but showed increase in intensity with time (30 sec. — 132; 2 min. — 142; 10 min. 162; 20 min. 216; 30 min. 216), reaching a constant value after 25 min. In a search for the products of the reaction, the chalkone and the flavanone were separately kept in Ac_2O solution (0.2% w/v) containing two drops of con. H_2SO_4 at 10–15°. The reaction time was varied from 10 min. to 60 min. The deeply coloured solution was then poured into ice-cold water. The colour disappeared immediately. The chalkone gave nearly quantitative yield of its 2'-acetoxy derivative (m.p. 90°) when the reaction time was 10 min., increase in time seriously brought down the yield. The flavanone was recovered after reaction time of 10 min. and 2'-acetoxy chalkone was present in only small amount and seemed to undergo other changes. When the reaction time was increased by 20 min. the recovery of the flavanone was poor. Longer reaction always led to coloured products and deeply coloured aqueous solution.

The 2'-acetoxy chalcone also behaves similarly on long treatment. Owing to these side reactions it has not been possible to demonstrate the conversion of flavanone into 2'-acetoxy chalcone under the conditions of test (E). The spectrophotometric measurements are definite about the identity of the chromogen. These observations led us to the conclusion that the positive response given by flavanone is due to the formation of traces of chalkones formed during the reaction as a result of ring opening and their immediate conversion into coloured products; the chalkones are indeed extremely sensitive in this test. The flavanone samples used in our work were spectrally and chromatographically pure and gave no detectable ferric colour and therefore our samples were free from any chalkone contaminant (King and White⁵ expressed the possibility that their flavanone samples were probably impure).

Reaction with Acetylium Perchlorate Reagent.—Support for the suggestion that in test (E), the effective reagent in the acetylium ion is provided by the use of acetylium perchlorate ($\text{CH}_3\text{CO}^+\text{ClO}_4^-$) prepared¹³ from acetyl chloride and anhydrous sodium perchlorate in glacial acetic acid solution. Solution of chalkone (5) and flavanone (1) were used in two solvents. (a) acetic anhydride, (b) carbon tetrachloride or chloroform. The observations are as follows: Chalkone (5) in solvent (a) gave purple colour and in (b) deep red, the colour development in both cases being instantaneous. Obviously there is direct electrophilic attack by acetylium ion at the carbonyl. The colour in (a) is the same as that shown in test (E). The flavanone (1) gave in (a) a very faint red purple only after about five minutes and the colour deepened on standing, whereas in (b) it gave no red colour and even with excess reagent the solution turned orange yellow on long standing. Thus positive and rapid response is shown by chalkones and not flavanones and they could therefore be distinguished. The lack of reaction of flavanones in non-polar solvent (b) shows that there is need for the preliminary formation of chalkone which does not take place in (b), but does occur in polar solvent (a). The above observations explain why in test (E) using acetic anhydride and sulphuric acid flavanones also give colour.

Colours of Chalkones in the Tests (D) and (E).—The colours produced by some typical chalkones in tests (D) and (E) are given in Table I: from them the following important points could be noted with regard to test (D): (1) Unsubstituted chalkone and the 2'-hydroxy compound

give no colour; at best a transient and feeble red is produced. (2) Hydroxyl or methoxyl in the 4-position alone is sufficient to give red colour while a hydroxyl or methoxyl at the 4'-position produces only orange-red. (3) A combination of substituents at the 4' and 4 positions is favourable and produces deep colours. (4) The presence of 4-amino or 4-dimethylamino groups confer stability to the colour. The reduction products from these amino chalkones behave like acid-base indicators.

TABLE I

Name of the chalkone	Test (D) ^a $\lambda_{\text{max.}}$ (m μ)	Test (E) ^b
1 Chalkone	No colour	Yellow
2 2'-Hydroxy	"	"
3 4'-Hydroxy	480-485	"
4 4'-Methoxy	(Unstable red)	Orange
5 4-Methoxy	550	Red
6 4:4'-Dimethoxy	565	Purple
7 2'-Hydroxy-3, 4, 4' tri-methoxy	(Unstable blue)	Blue changing to red
8 2'-Hydroxy-3, 4, 4' tri-methoxy	570	Purple blue
9 2'-Hydroxy-4:4':6' tri-methoxy	540	Deep pink
10 2'-Hydroxy 3:4:4':6'-tetramethoxy	560	Purple
11 4-Dimethylamino	495	Wine red
12 2'-Hydroxy 4-dimethylamino	550	Red

^a About 2 mg. of the chalkone in ethanol (30 c.c.) was reduced with NaBH_4 and then treated with conc. HCl (8 c.c.). Measurements were made as rapidly as possible because of the tendency of the colours to fade.

^b Spectrophotometric measurements of the colours showed broad maxima. The colours described here were observed in about 0.01% solution in Ac_2O (10 c.c.) and one drop of H_2SO_4 .

Test (E) for Aurones and 2-Hydroxy 2-Benzyl Coumaran-3-ones.—The colour development is rapid with these compounds. In the case of 2-hydroxy-2-benzyl coumaran-3-ones dehydration to give aurones (20 to 14) is an easy process and constitute the first step⁵ in the colour test and subsequent changes (14 \rightarrow 21 \rightarrow 21a, Chart VIII) run parallel to those of chalkones.

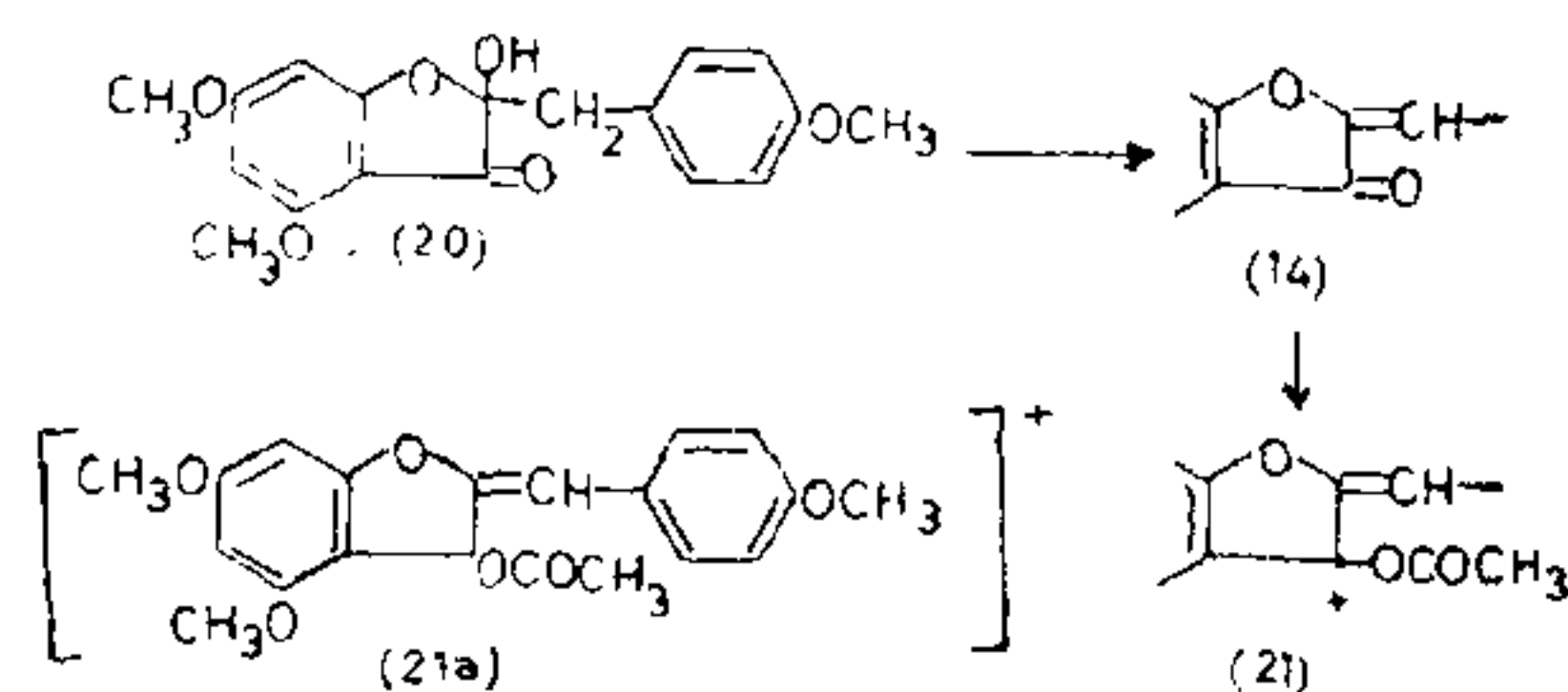


CHART VIII

Summary.—A study has been made of the sodium borohydride-hydrochloric acid reaction of flavanones. This reaction is given by chalcones and aurones also. These results provide support for the explanation offered by Geissman and Clinton for the colour reactions of flavanones with magnesium-hydrochloric acid and sodium amalgam-hydrochloric acid. In all these cases the same type of chromophore $\text{Ar}-\overset{+}{\text{C}}\text{H}-\text{CH}=\text{CH}-$ is formed. Borohydride reduction products of flavanones and 2'-hydroxy chalcones when heated in alcoholic hydrochloric acid produce flavylum salts. A study of the acetic anhydride sulphuric acid test for chalcones, flavanones and aurones shows that the chromogen involved is $\text{Ar}-\text{CO}-\text{CH}=\text{CH}-\text{Ar}$ and that the flavanone ring should open in order to develop colour. The reaction involves the attack by acetylium ions at the carbonyl of the above chromogen giving $-\overset{+}{\text{C}}(\text{OAc})-\text{CH}=\text{CH}-$

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THE INFLUENCE OF SOME AGRICULTURAL PRACTICES ON THE ENZYME ACTIVITY OF COCONUT WATER

M. NAGARAJAN AND K. M. PANDALAI

Chemistry Department, Central Coconut Research Station, Kasaragod

IT is now well established that a particular plant itself is the best indicator of the nature and quantity of nutrients it can absorb and utilise from the soil. Thus, the use of the plant tissue analysis, particularly foliar composition studies for diagnosing mineral deficiencies of crop plants such as rubber, oil palms, groundnuts, tea, cocoa, etc., has now become very popular. Suitable methods have been standardised to overcome the usual difficulties experienced in the case of perennial plants in the matter of leaf sampling from inaccessible heights from the ground level, availability of samples of comparable physiological age, possible nutrient level fluctuations on account of climatic changes and so on. In his studies on the potash content of nut water as a guide to the manuring of coconut palm, Salgado¹ found that potash content of nut water affords a relatively simple method of assessing potash availability in the soil in relation to the needs of the palm. He claimed superiority for this method over the conventional methods of soil

analysis assuming coconut water to be analogous to plant sap in indicating the physiological status of the palm and of the soil conditions on which it grows. We examined the pattern of enzyme activity in the nut water in relation to cultural and manurial practices received by the palm to see whether this could be more useful and reliable for diagnostic purposes. Indeed the determination of enzyme activity in plants has been shown to provide information about the effect fertilizers have on plant by Hofmann² who concluded that the amount and the activity of enzyme depends on the nature of the soil, on the fertilizers used and the method of cultivation during the vegetative period. He also stressed that enzyme activity orientates crop quality, stability on storage, germinating power and certain other properties and that the determination of enzyme activity of plants is also simple to carry out and exact and comparable values are usually obtained. The activity of the enzymes catalase and saccharase in estimating the biological activity