VI. NOTE ON THE CHARACTERISATION OF THE ANTHOCYANINS AND ANTHOCYANIDINS BY MEANS OF THEIR COLOUR REACTIONS IN ALKALINE SOLUTIONS.

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FEAR and NIERENSTEIN [1928] have pointed out that the colour reactions of the anthocyanins, which are indicators, should be examined in solutions of definite p_H and their contention is undoubtedly correct. It would be a mistake, however, to conclude that the earlier observations are entirely lacking in significance simply because this refinement has not hitherto been adopted, and, for example, the effect of elimination or modification of the hydroxyl in position 3 in the pyrylium ring on the colour reaction is very marked. Thus both luteolinidin chloride [Pratt and Robinson, 1925] and chrysanthemin chloride [Willstätter and Bolton, 1916] give violet solutions in aqueous sodium carbonate and these become blue on the addition of sodium hydroxide; cyanin chloride [Willstätter and Everest, 1913] gives a blue solution in aqueous sodium carbonate.

It was inferred that chrysanthemin chloride should be the 3-glucoside of cyanidin chloride and this has since been proved to be correct by the synthesis of the anthocyanin.

The examination of the reactions of the anthocyanidins in a range of buffered solutions has shown that this method is by far the most reliable for purposes of comparison and characterisation; it is of even greater value in this connection than a study of the absorption spectrum.

On the one hand various properties—*pseudo*-base formation and colour-base precipitation, ease of oxidation and so forth, are incidentally revealed and on the other, different anthocyanidins, as for instance, peonidin and malvidin, may exhibit identical absorption spectra [Schou, 1927].

A few examples have been selected to illustrate the method and it is hoped that the data will be found useful for the identification of anthocyanidins derived from natural sources.

Fear and Nierenstein state that cyanidin chloride from cyanin behaves differently from synthetic 3:5:7:3':4'-pentahydroxyflavylium chloride and it is evident that by some misfortune they have not succeeded in obtaining a homogeneous specimen of the latter substance.

We repeat an emphatic affirmation that pure synthetic 3 : 5 : 7 : 3' : 4'-pentahydroxyflavylium chloride, best prepared by hydrolysis of its benzoyl derivative, exhibits no divergences from cyanidin chloride and this is true, to the minutest detail, in regard to the tests described below.

Indeed the constitution of cyanidin chloride in its main structural outlines (carbon and oxygen skeleton) is established as firmly as that of any other compound and no attempt [Malkin and Nierenstein, 1928] to reconcile the results of Nierenstein [1921] in the field of catechin chemistry with the conflicting results of Freudenberg and Purman [1924], by suggesting a modified cyanidin formula, can succeed. The explanation of the discrepancies must be sought elsewhere.

EXPERIMENTAL.

The buffered solutions were prepared from the tubes of "Universal Buffer Mixture," supplied by the British Drug Houses Limited. This mixture is stated to be compounded in accordance with a formula of Prideaux and Ward [1924] but the exact composition has not been disclosed.

It may happen that this excellent commercial product may be unobtainable in some quarters or at some future time and as it is in any case necessary to guarantee the reproducibility of our results we think it well to mention that a mixture of 0.04 g. mol. each of phenylacetic acid, boric acid and potassium dihydrogen phosphate appeared to be the equivalent of the contents of one of the tubes supplied. Phenylacetic acid, boric acid and potassium dihydrogen phosphate (0.02 g. mol. each), together with n cc. of 0.2 N sodium hydroxide, were dissolved in water and made up to 1000 cc. The solutions used and the approximate p_H of each, as found by means of indicators, were the following: (1) $n = 0$; p_H , 3.2; (2) $n = 25$; p_H , 3.8; (3) $n = 50$; p_H , 4.4; (4) $n = 75$; p_H , 5.0; (5) $n = 100$; p_H , 5.6; (6) $n = 125$; p_H , 6.2; (7) $n = 150$; p_H , 6.8; (8) $n = 175$; p_H , 7.4; (9) $n = 200$; p_H , 8.0; (10) $n = 225$; p_H , 8.6; (11) $n = 250$; p_H , 9.2; (12) $n = 275$; p_H , 9.8; (13) $n = 300$; p_H , 10.4; (14) $n = 325$; p_H , 11.0; (15) $n = 350$; (16) $n = 400$; (17) $n = 450$.

The anthocyanidin chloride (25.00 mg.) was dissolved in 98 % alcohol (100 cc.) and 1 cc. of the solution added from a burette to 10 cc. of the buffered solutions or of other solutions mentioned below.

Apigeninidin chloride. The alcoholic solution was orange; 1 % hydrochloric acid, bright canary yellow unchanged after 24 hours; 20 % hydrochloric acid, bright canary yellow and after 24 hours almost the whole of the salt had separated in clusters of microscopic prisms; (1) more orange yellow than the solution in 1 % hydrochloric acid, unchanged after 24 hours; (2) more intense yellowish orange, slight fading after 24 hours; (3) reddish orange fading slightly during 24 hours; (4) orange red, after 24 hours, long, slender crystals of colour base deposited; (5) darker orange red, after 24 hours colour base crystallised in slender needles; (6) brownish red, after 24 hours slight fading; (7) brownish red, a little bluer, same as (6) on keeping; (8) still a little

bluer brownish red, same as (6) on keeping; (9) bluish red, same as (6) on keeping; (10) cherry red, bluer than (9) and became like (6) on keeping; (11), (12), (13), (14), (15) same as (10); (16) same as (10) but became reddish orange after 24 hours; (17) same as (10) but became greenish yellow after 24 hours.

Pelargonidin chloride. 1 % and 20 % hydrochloric acid, orange red with bluish tinge in thin layers; (1) identical with hydrochloric acid solutions, colour faded but more slowly than in (2), (3) and (4), in 2 hours very faintly coloured, in $3\frac{1}{2}$ hours colourless and remained so; (2) a little browner orange red, almost all colour disappeared in 17 minutes and colourless in 30 minutes; (3) bluish red fading quickly to pink and became colourless a little more rapidly than (2); (4) cherry red fading rapidly, still more so than (3); (5) bluish red, after 15 minutes rose coloured, after $3\frac{1}{2}$ hours colourless (it should be noted that observations at all intermediate times could not be made and, for example, this solution may have been colourless after 2 hours); (6) violet red, after $3\frac{1}{2}$ hours pale pink and after 24 hours still very weak red colour; (7) slightly bluer violet red, subsequent changes like (6); (8) like (7), after $3\frac{1}{2}$ hours faded brownish pink, more intense than (7); (9) deeper violet, after 25 minutes reddish violet, after 24 hours weak reddish brown whereas (8) at this stage had a much weaker red colour; (10) violet, blue in thin layers, in 5 minutes pale violet, in 25 minutes reddish violet, after 24 hours weak reddish violet; (11) a little bluer than (10), after 25 minutes reddish violet, after 24 hours weak reddish violet; (12) a little bluer than (11) and dichroism less pronounced, after 10 minutes violet, after 25 minutes red violet, after 24 hours weak reddish violet; (13) again bluer, after 10 minutes violet, after 25 minutes red violet and after 24 hours very weak reddish brown; (14) similar, thin layers are greener blue, after 10 minutes violet, after 25 minutes violet, after $3\frac{1}{2}$ hours weak reddish violet, after 24 hours very faint orange; (15), (16), (17) similar to (14) on mixing and after 24 hours, but became pale grey blue in 3 hours.

Cyanidin chlorides. 1 % and 20 % hydrochloric acid, eosin red, much bluer than pelargonidin; (1) red, slightly bluer than 1 % hydrochloric acid; the colour faded very slowly, after $18\frac{1}{2}$ hours colourless; (2) bluish red, after $2\frac{1}{2}$ minutes colour distinctly less intense, after 10 minutes considerable fading, after 45 minutes colourless; (3) reddish violet, after 10 minutes very weak, after 25 minutes colourless whereas at this stage both (2) and (4) retained weak colour; (4) bluer reddish violet, after 10 minutes colour much faded, after 45 minutes still weak violet, after $18\frac{1}{2}$ hours colourless; (5) reddish violet, still bluer than (4), 15 seconds after mixing the colour-base separated giving opalescent solution; after $18\frac{1}{2}$ hours precipitate of violet colour-base and colourless supernatant liquid; (6) bluer reddish violet of increased tinctorial intensity, no precipitate, after $18\frac{1}{2}$ hours almost colourless—very faint pink; (7) violet tinged with red, after $18\frac{1}{2}$ hours very faint pink; (8) very like (7); (9) again similar but a little bluer and already blue in very thin layers, after

8 minutes became weak grey violet, after $18\frac{1}{2}$ hours almost colourless; (10) much bluer violet and pure blue in thin layers, after $18\frac{1}{2}$ hours extremely weak grey; (11) similar to (10) but less dichroic, not so red violet in thicker layers, after $18\frac{1}{2}$ hours same as (10); (12) same as (11); (13) same as (11), after $18\frac{1}{2}$ hours pale orange yellow; (14) less dichroic than (11), after $18\frac{1}{2}$ hours more intensely yellow than (13); (15) still less dichroic and still deeper yellow on keeping $18\frac{1}{2}$ hours; (16) greenish blue, after 30 minutes colour largely disappeared, after $18\frac{1}{2}$ hours deeper yellow than (13); (17) bluish green, after 8 minutes almost colourless, after 30 minutes orange yellow, after $18\frac{1}{2}$ hours deeper yellow than (13).

5-O-Benzoylcyanidin chloride. The alcoholic solution was the colour of an aqueous permanganate solution. 1 % hydrochloric acid, bluish red of no great tinctorial intensity, after 30 minutes faint pink, after $1\frac{1}{2}$ hours colourless; 20 % hydrochloric acid, the salt was precipitated leaving a colourless solution; (1) bluish red rapidly fading and colourless after 1 minute; (2) bluish red, after 12 minutes colourless; (3) similar but still slower fading, after 12 minutes faint pink, after 30 minutes colourless; (4) similar, a little slower fading; (5) red violet, after 12 minutes weak violet pink, after 30 minutes pale reddish violet, after 24 hours colourless; (6) more intense violet fading somewhat rapidly, after 12 minutes very pale violet, after 24 hours still a violet tinge; (7) very similar to (6) but faded a little less throughout; (8) similar to (7) and fading a little less throughout; (9) blue violet, did not fade very rapidly but became more violet; (10) blue with violet tinge becoming more violet, after 12 minutes violet blue, after 30 minutes violet, after 24 hours reddish yellow; (11) blue with slight violet tinge, subsequent changes like (10); (12) pure blue in thin layers, after 6 minutes violet blue, after 25 minutes blue violet, after 24 hours yellow; (13) same as (12) but became violet a little more slowly; (14) pure blue, after 20 minutes green, after 24 hours yellow; (15) pure blue, after 5 minutes green, after 20 minutes greenish yellow, after 24 hours yellow; (16) greenish blue changing through green to yellow more quickly than (15); (17) similar to (16).

Peonidin chloride. 1 % and 20 % hydrochloric acid, eosin red, same shade as cyanidin, unchanged after $18\frac{1}{2}$ hours; (1) eosin red a little bluer than hydrochloric acid solutions, after 15 minutes faded and after 24 hours colourless; (2) cherry red, fading is most rapid at this p_H , after 30 minutes colourless; (3) bluer red, after 2 minutes browner red fading rapidly, after 15 minutes the colour was weak, after $11\frac{1}{2}$ hours colourless; (4) bluish red which quickly faded and became browner, after $18\frac{1}{2}$ hours some colour-base had separated; (5) very much like (4); (6) reddish violet which did not fade so quickly as (5), (4), (3) and (2), after $18\frac{1}{2}$ hours some colour-base had separated; (7) violet with reddish tinge, bluer than (6), fading comparatively slowly, after $18\frac{1}{2}$ hours faint pink and no precipitate; (8) violet, after $18\frac{1}{2}$ hours faint reddish violet; (9) bluish violet, blue in thin layers, after $18\frac{1}{2}$ hours weak violet; (10) blue, violet in thick layers, after $18\frac{1}{2}$ hours bluish grey; (11) a little greener blue

becoming still greener in 1 minute, after $18\frac{1}{2}$ hours bluish grey; (12) like (11), after $18\frac{1}{2}$ hours greenish grey; (13) greenish blue, after $18\frac{1}{2}$ hours weak yellow; (14), (15), (16), (17) almost same as (13) but curiously the fading in (16) and (17) was less rapid than in (14) and (15); this suggests that both oxidation and *pseudo*-base formation are involved.

Malvidin chloride. 1 % and 20 % hydrochloric acid bluish red, unchanged after 24 hours; (1) a little bluer red, weak pink after 20 minutes, colourless after 50 minutes; (2) similar to (1) but fading more rapidly, almost colourless after 20 minutes; (3) bluish red at once changing to pale brownish cherry red, after 5 minutes pale rose, almost colourless after 20 minutes; (4) similar to (3); (5) similar to (3) and (4) at first but fading is most rapid and in less than 15 minutes there was a brown violet red precipitate in a colourless solution, after 3 hours there was still a flocculent precipitate but after 5 hours all the solid had passed into solution; (6) violet fading rapidly with a slight precipitation; almost colourless after 3 hours; (7) pure violet solution blue in thin layers and no precipitate, after a few minutes faded violet blue, after 3 hours pale blue, after 24 hours almost colourless; (8) blue, violet in thick layers and rapidly fading, after 10 minutes the series (8), (9), (10), (11), (12) had all faded similarly and there was little divergence in the colours which ranged from weak violet blue to weak greenish blue, after $2\frac{1}{2}$ hours the series (8) to (12) were very pale blue to greenish blue, after 24 hours (10), (11) and (12) were pale orange; (9) pale blue fading rapidly; (10) greenish blue fading rapidly; (11) similar to (10); (12) similar to (10); (13) greenish blue becoming greener as it faded, bluish green after 30 minutes, the change to green occurred more and more rapidly in the ensuing series (13) to (17), on keeping changes like (10); (14) similar, yellowish green after $1\frac{1}{2}$ hours; (15) similar, greenish yellow after $1\frac{1}{2}$ hours; (16) similar, brownish yellow after $1\frac{1}{2}$ hours; (17) similar.

Cyanin chloride. In the case of anthocyanins, 25.00 mg. were dissolved in 100 cc. of methyl alcoholic hydrochloric acid (1 cc. aqueous hydrochloric acid, sp. gr. 1.16, made up to 1000 cc. with methyl alcohol); the acid thus introduced causes a reduction of 0.1 in the p_H of all the solutions. The specimen of cyanin chloride employed was derived from deep-red dahlia flowers and was kindly sent to us by Professor R. Willstätter, to whom we are greatly indebted.

Solutions in 1 % and 20 % hydrochloric acid were salmon red; (1) pink, much less intense owing to *pseudo*-base formation; (2) a little bluer than (1) and rapidly faded; (3) bluish red fading rapidly; (4) permanganate-coloured fading rapidly; (5) similar, fading less quickly; (6) similar but colour more intense and fading quite slowly; (7), (8), (9) similar to (6); (10) reddish violet; (11) violet; (12) violet; (13) bluer violet; (14) violet blue fading quickly; (15) blue fading quickly; (16) greener blue fading rapidly; (17) similar.

After 15 to 20 minutes (1) was pink and the colour diminished in the series to (5) which was colourless; (6) had faded but was intense in comparison with (1); (7), (8), (9), (10), (11) and (12) had faded somewhat and formed

a series ranging from violet red to violet; (13) was weak violet; (14) weak violet blue and (15), (16) and (17) were almost colourless (grey).

After 5 hours (1) was feebly pink and the colour diminished to (5) which was colourless, then gradually increasing reddish violet to (10) which was however very pale coloured; (11) was similar but not so clear toned; (12) to (17) inclusive were pale yellow.

After 20 hours the solutions in 1 % and 20 % hydrochloric acid were unchanged; (1) was pink and the colour in the range diminished to (5) which was colourless, then increased to (9) which was weak pink; (10) was weak brownish pink and (11) and the remainder were pale yellow.

Malvin chloride. We are greatly indebted to Professor P. Karrer for the provision of a specimen of this diglucosidic anthocyanin. The solutions in 1 % and 20 % hydrochloric acid were bluish red; (1) bluish red becoming pale rose in a few seconds; (2) quickly decolorised; (3) same as (2); (4) bluish red fading in the course of 2 minutes; (5) reddish violet fading slowly; (6) violet fading slowly; (7) bluish violet; (8) violet blue; (9) blue; (10), (11) and (12) similar to (9); (13) blue fading to greenish blue; (14), (15), (16), (17) blue fading with increasing rapidity to pale green.

After 20 minutes (1) was pink, (2), (3), (4) and (5) were colourless, (6) was weak reddish violet, (7) was a little deeper violet; (8) was bluish violet; (9), (10), (11) and (12) were blue; (13) was greenish blue; (14) was bluish green; (15) was yellowish green; (16) was greenish yellow, and (17) was yellow.

After 5 hours (1) to (8) inclusive were almost colourless ((1) was tinged pink, (7) reddish violet, and (8) pale blue); (9) was weak violet blue; (10) was weak pure blue; (11) was pale greenish blue; (12) was pale green, and (13) to (17) inclusive were yellow.

After 20 hours (1) was faint pink; (2) to (6) inclusive were colourless; (7) was very faint pink; (8) was very faint blue; (9) was pale blue; (10) was blue; (11) was bluish green, and (12) to (17) were yellow. The solutions in 1 % and 20 % hydrochloric acid were unchanged.

Other anthocyanins have been similarly characterised and the results will be subsequently recorded in connection with synthetical experiments.

It should be stated that all the tests were carried out in the summer and that the room temperature was 20–25°; no precautions were taken to exclude air.

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