Natural Colouring Matters and their Analogues*

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THE chemist has been attracted to the investigation of natural and artificial colouring matters for a variety of reasons, including not only colour-pleasure, the incentive of the knowledge that chlorophyll and hæmoglobin perform some of the most important functions in vital processes, and the industrial importance of dyestuffs and pigments, but also on account of the fact that visible colour more than any other property facilitates the experimental study of organic substances whether by analysis or synthesis. It furnishes a standard of homogeneity or a measure of concentration, it is an invaluable guide in the search for methods of separation and purification, and it at once indicates, by its appearance or disappearance, the occurrence of a chemical reaction. Small wonder that the successful outcome of the investigation of many colourless substances has awaited the discovery of some characteristic colour-reaction; a noteworthy example being vitamin A.

A catalogue of outstanding achievements in this field invites destructive criticism. I do not fear this, however, in recalling the researches of Laurent, Kekulé, Baeyer and Heumann on indigo; of Sir William Perkin, Hofmann, Otto and Emil Fischer, Meldola and many others on the basic dyes; of Griess and his host of followers on the azo-compounds; of Arthur Perkin and of Kostanecki on the flavones and flavonols; of Willstätter on the respiratory pigments and the anthocyanins; and, not least, of Hans Fischer on the synthesis of the prosthetic group of the blood pigment.

No attempt can be made to cover this vast field, but the mere mention of these topics serves to prove the immense theoretical and practical value of a study of organic colouring matters. The work proceeds, and a long chapter of great chemical and biological interest on the natural carotinoid pigments is even now being written by Karrer, Kuhn and others.

Occurrence of Anthocyanins and their Derivatives

The brilliant and pioneering researches of Richard Willstätter and his co-workers since 1914 have established the main features of the chemistry of the anthocyanins, which were recognised as saccharides, occasionally acylated, of the anthocyanidins. They exhibit amphoteric character, forming salts with both acids and bases. Thus the violet pigment cyanin, which can be isolated from blue cornflowers, red roses, deep red dahlias and other flowers, forms a blue sodium salt and a red hydrochloride. The hydrolysis of the latter by means of hot aqueous hydrochloric acid into cyanidin chloride and glucose is represented by the equation:

$$\underset{\text{cyanin chloride}}{C_{27}H_{31}O_{16}Cl} + 2H_2O = \underset{\text{cyanidin chloride}}{C_{15}H_{11}O_6Cl} + \underset{\text{glucose}}{2C_6H_{12}O_6}$$

The constitution of cyanidin chloride has been established by analysis and numerous syntheses; the first of these (Willstätter and Mallison) utilised the reduction of quercetin by means of magnesium in aqueous methyl alcoholic hydrochloric acid solution.

In this process a widely distributed anthoxanthin yields a widely distributed anthocyanidin, and the temptation to assume that similar reactions occur in the plant laboratory is very great. There is, however, very little justification for this view, and the experimental support brought forward in its favour will not survive careful scrutiny. The alleged crystalline anthocyans prepared by the reduction of natural flavones or plant extracts containing them are nothing but the said flavones with a small proportion of adsorbed colouring matter of anthocyanidin type. It seems much more probable that the flavones and anthocyanins are independently synthesised, although perhaps from a common starting point. The existence of genetic factors which control the occurrence of anthoxanthins independent of that of anthocyanins is strong evidence in favour of this view.

The anthocyanidins which have been isolated are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, malvidin and hirsutidin, represented as chlorides. All have been synthesised by unambiguous methods and the synthetic specimens have been carefully compared and identified with the natural products. Pelargonidin, cyanidin and delphinidin are the fundamental types, peonidin being a methyl ether of cyanidin and petunidin, malvidin and hirsutidin being, respectively, the mono-, di- and trimethyl ethers of delphinidin.

The greater number of the anthocyanins fall into a comparatively restricted number of categories, including: (*a*) the 3-monoglycosides and 3-monogalactosides, (*b*) the 3-rhamnoglycosides and other 3-pentoseglycosides, (*c*) the 3-biosides, (*d*) the 3 : 5-diglycosides, and (*e*) the acylated anthocyanins. It is unnecessary to recount the steps taken in reaching these conclusions, but they have been finally justified by synthesis in many instances.

In group (*a*) we find callistephin, the monoglycoside of pelargonidin occurring as one of the pigments of the aster and as the main pigment of scarlet carnations and many other flowers; the related galactoside, fragarin, is the colouring matter of the strawberry.

In the cyanidin series the corresponding pair is chrysanthemin and idæin, the former of wide

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distribution and the latter occurring in the skins of cranberries and in the leaves of the copper beech.

Peonidin 3-monoglycoside, termed oxycoccicyanin, is found in the skins of the larger American cranberries and œnin or malvidin 3-monoglycoside is the colouring matter of the skins of purple-black grapes, as well as of certain cyclamen and primulæ. The delphinidin representative undoubtedly occurs in bilberries in admixture with other pigments, and it has not yet been fully examined; the petunidin and hirsutidin representatives have not been isolated from natural sources, although there is reason to believe that the former occurs in the berries of the Darwin barberry and the latter has been synthesised.

In groups (*b*) and (*c*) we find large classes of anthocyanins of which only a few representatives have been closely studied. These include keracyanin (cyanidin 3-rhamnoglycoside), probably identical with antirrhinin (isolated by Miss R. Scott-Moncrieff), and mecocyanin, a pigment of red poppies which is now recognised by synthesis as cyanidin 3-gentiobioside. There is very little doubt that pelargonidin 3-rhamnoglycoside colours the scarlet gloxinia and that pelargonidin 3-biosides are of widespread occurrence, for example, in the ordinary orange-red nasturtium and in the flowers of the scarlet runner bean.

The anthocyanins of groups (*a*), (*b*) and (*c*), when derived from the same anthocyanidin, exhibit similar behaviour as indicators. Thus chrysanthemin, keracyanin and mecocyanin all give a violet solution in aqueous soda and this becomes blue on the addition of caustic alkali. On partial hydrolysis, meccocyanin and antirrhinin actually yield chrysanthemin.

The anthocyanins of class (*d*) are the most widely distributed and best-known members of this series of natural pigments; they include pelargonin, the colouring matter of the scarlet pelargonium and possibly the first anthocyanin to be obtained in a crystalline condition (Molisch's experiment), also cyanin, the isolation of which from the blue cornflower by Willstätter and Everest in 1914 was the first of an impressive series of investigations.

Peonin from the deep red peony and malvin from the wild mallow or from certain primulas, are the peonidin and malvidin representatives in this group, which is completed by petunin and hirsutin. Quite recently the delphinidin member has been isolated from *Salvia patens*.

The anthocyanins of group (*d*) differ from those of groups (*a*), (*b*) and (*c*) in their alkali colour reactions and in their marked instability to aqueous sodium hydroxide. Thus cyanin, which compares with mecocyanin in group (*c*), gives a pure blue solution in aqueous soda and the dilute solution becomes very quickly yellow on the addition of sodium hydroxide.

Pelargonin, cyanin, peonin, malvin and hirsutin have all been synthesised.

The anthocyanins can be characterised and qualitatively distinguished by their distribution between immiscible solvents, and in the case of disaccharides the use of *n*-butyl alcohol is convenient.

Acylated anthocyanins occur in all the anthocyanidin series; thus, on hydrolysis, delphinin, the pigment of species of delphinium, furnishes *p*-hydroxybenzoic acid as well as glucose and delphinidin.

Many other delphinidin derivatives are acylated by means of *p*-hydroxycinnamic acid, probably attached to the sugar hydroxyls, and pelargonin and cyanin also occur in acylated forms. These so-called complex anthocyanins are characterised by high distribution numbers; they are usually acylated 3 : 5-dimonosides, but in the delphinidin series, gentianin and violanin appear to be *p*-hydroxycinnamates of delphinidin monoglycoside and rhamnoglycoside respectively (Karrer). There is also some evidence of another type of depside anthocyanin in which the acyl group is directly attached to the anthocyanidin molecule and the glycoside group is borne by the hydroxyl of the acid residue.

Anthocyanins as Indicators and the Colours of Flowers

(With Mrs. G. M. Robinson)

The amphoteric character of the anthocyanins accounts for the exhibition of a wide variety of colours in a range of solutions of graded hydrogen ion concentration, and this method, using buffered solutions, can be employed for the characterisation of anthocyanidins and anthocyanins. Under the specified conditions the results are fully reproducible and the hydrogen ion concentration values have been controlled by electrical methods as well as by the use of indicators. Thus, if the *p*H of an acid cyanin solution is increased until the violet tone matches that of an alkaline cyanin solution, the *p*H of which is decreased in order to reach the same condition, then the *p*H of the violet solution will be found to be 7·0–9·0, depending on the shade of violet produced. Cyanin is red in solutions of *p*H 3·0 or less, violet at *p*H 8·5 and blue at *p*H 11·0. The red, violet and blue forms are the oxonium salt, the colour-base and the salt of the colour-base.

Now cyanin was isolated by Willstätter and his colleagues from the blue cornflower and from the red rose, and it seemed quite a simple step to assume that the cell-sap in the cornflower was alkaline and that in the rose acid, particularly in view of the fact that the absorption spectra of the coloured aqueous extracts correspond with these conditions.

It has indeed been generally assumed that the indicator colour of the anthocyanin will give a measure of the hydrogen ion concentration of the cell-sap, but unfortunately this method cannot be relied upon for several reasons. In the first place there is a glaring anomaly in the fact that direct measurement by electrical methods (glass

electrode as arranged by Mrs. Kerridge) shows that the cell-saps are all well on the acid side of the neutral point. Thus the conventional view for red flowers may well be correct, but some special circumstances must be invoked in the case of blue flowers.

Turning at once to the blue cornflower (the cultivated annual kind), a blue filtered extract made with distilled water was found to be sufficiently acid to turn blue litmus red. Using 3 gm. of petals in 14 c.c. of distilled water (*p*H 6·3 owing to dissolved carbon dioxide), the *p*H was 4·9. (These quantities were used throughout the experiments and the use of larger relative quantities of the petals did not alter the hydrogen ion concentration appreciably.) Addition of a buffered solution of *p*H 4·4 did not affect the colour, but the colour changed to violet when the B.D.H. Universal Buffer, *p*H 9·0, was added. It was at once apparent that the only simple explanation is that the cyanin anion is present in a complex form, giving a stable aggregate with a negative charge; in some way the strength of cyanin colour-base as an acid must be vastly increased.

Some form of colloidal solution was considered most likely to fulfil the necessary conditions, and Dr. Conmar Robinson, of the Chemistry Department, University College, London, kindly examined a filtered, distilled water extract of blue cornflowers and reported as follows:

"The solution contains ultramicrons easily visible in the slit ultramicroscope, but small enough to be in fairly rapid Brownian movement. Microcataphoresis showed them to be negatively charged. Without more quantitative work it is impossible to say if these particles can represent the bulk of the material present, but this seems probable if the solution is very dilute; the possibility of observing a colloidal impurity is always a trap. The visibility of the ultramicrons suggests a lyophobic colloid. It is, however, not precipitated even by 2*N* NaCl, which indicates that a protective colloid is also present."

Our next step was to attempt the production of blue cyanin sols stable in neutral or weakly acid solution, and some measure of success was achieved, although the solutions are by no means so stable as those from the blue cornflower.

If a little crystalline cyanin chloride is added to boiling tap-water (*p*H 8·0) then the usual violet solution results (see above), the colour being what we consider 'normal'. If, however, the cyanin is triturated in the cold for a minute with the water and gradually heated to boiling with shaking, then a beautiful blue solution results. The fact that the same materials can be used to produce two entirely different results shows that it can only be the state of aggregation of the cyanin which can have stabilised the anionic charge and hence produced a blue colour under the conditions that normally produce a violet solution. If very small quantities of cyanin chloride are employed, this phenomenon can be reproduced using distilled water. Willstätter and Everest found that their cornflower extracts contained xylan and other polysaccharides, and we have attempted to produce blue acid cyanin solutions in the presence of various polysaccharides. The addition of dispersed xylan and various kinds of starch, also agar-agar, makes the preparation of blue solutions of *p*H about 7·5 a very simple matter, but we have not yet found a way of imitating the cornflower solution in respect to its stability at *p*H 5·0.

Probably these colloid associations are much more readily formed at values of *p*H between 5·5 and 6·5, and on the whole the blue flowers have less acid cell-saps than the red flowers. The petals of the rose, in contrast with the cornflower, constitute an exception (*p*H 5·6).

It must be emphasised that variations of *p*H are quite insufficient in themselves to account for the colour changes and it is evident that the most important single factor for flower colour, given the nature of the anthocyanin, is the question of the condition of the pigment in solution, and it would appear that *all blue flowers are coloured by colloidal solutions of their respective pigments.*

Methods for the determination of the hydrogen ion concentration of the cell-sap of flowers depending on the use of the flower colours as indicators may be sound, but only if it can be guaranteed that the colloidal condition of the pigment solution is not altered by the extraction with the buffered solutions which are employed. In any event, the results bear no relation to the colours observed *in vitro* using isolated anthocyanins, and they cannot be transferred from flower to flower: the colour series depends almost as much on the other conditions in the cell-sap as on the hydrogen ion concentration and on the nature of the anthocyanin. Another aspect of *p*H of the cell-saps is that the higher values appear to be associated with the formation of delphinidin derivatives. The remarkable distribution in the tropæolum—Empress of India—is as follows: leaf, delphinidin diglycoside (*p*H 5·6); calyx, cyanidin 3-bioside (*p*H 5·0); flower, pelargonidin 3-bioside (*p*H 4·5). On the other hand, three scabious with anthocyanins based respectively on pelargonidin, cyanidin and delphinidin had all the same petal *p*H, 5·0.

We have already discussed elsewhere the influence of certain substances termed co-pigments on the colour of anthocyanin solutions; these effects are to be detected in strongly acid solution and the presence or absence of these substances is undoubtedly a factor to be taken into consideration. The extent to which the co-pigment effect is bound up with colloid phenomenon is a matter for future experiment and discussion, but it is convenient to maintain the term co-pigment for the present.

Dr. E. A. H. Roberts has observed the shift of the absorption bands of chrysanthemin and œnin chlorides on the addition of papaverine (strongly blueing effect) and narcotine (weak effect), and correlated this with a corresponding change

(lowering) of the distribution number of the anthocyanin using amyl alcohol.

It seems clear that papaverine salts and œnin salts combine in solution. The relation between the distribution number of œnin chloride and the concentration of the pigment seems to require the assumption that the molecules of the anthocyanin are associated (2 mols.) in aqueous solution and free in amyl alcohol. Chrysanthemin and idæin behave similarly, also malvidin 3-galactoside. This phenomenon appears to be related to that of co-pigmentation.

The naturally occurring co-pigments include the anthoxanthins (flavone and flavonol saccharides, etc.) and tannins and some efficient substances not yet identified.

The justification for assuming the operation of this factor can best be indicated by an example. Certain herbaceous phlox contain pelargonin, but have a much bluer-red colour than other flowers coloured by this anthocyanin. But the same observation applies to the extract in 1 per cent hydrochloric acid, and moreover the presence of much anthoxanthin is noted. Hence, all the circumstances point to co-pigmentation of the pelargonin salt in the flower petal.

Finally, we do not know whether or not traces of iron and other inorganic substances may affect flower colour. In this connexion the case of the blue hydrangea is always quoted, and we have observed that when the stalks of red hydrangea flowers are immersed in very dilute aqueous ferric chloride, the flowers slowly become blue. The ashes of many flowers contain 1–2 per cent Fe_2O_3, and the anthocyanin test for iron is one of the most delicate known.

Protection against Yellow Fever

THE discovery of a susceptible experimental animal for a human disease has rarely been followed by such a rapid development of practicable methods of control as in the case of yellow fever. The results of laboratory investigations since 1927, when Stokes, Bauer and Hudson found that certain species of Asiatic monkeys, *Macacus rhesus* and *M. sinicus*, could readily be infected with this disease, have elucidated most of the essential facts necessary for its control. Not the least of these discoveries has been the development of practicable methods of immunisation, and it is of interest to trace the successive stages leading up to this result.

The pioneer work of Hindle[1] showed that when yellow fever virus is exposed to the action of various agents such as formaldehyde, or phenol and glycerine, or simply kept exposed to air, it gradually loses its virulence and then passes through a phase when its inoculation into susceptible animals is followed by the development of immunity without any clinical signs of infection. Further exposure to the action of these agencies leads ultimately to the disappearance of all antigenic properties, and animals inoculated with such material failed to develop any immunity against the virus.

Although this method of protection gave very promising results with monkeys, and was used with success by Aragão in combatting the yellow fever epidemic in Rio de Janeiro in 1928, later experiments showed that there were considerable practical difficulties in estimating the exact degree of attenuation necessary to convert the virus into a vaccine. Moreover, at ordinary ice-box temperatures the vaccine soon lost its protective properties.

The persistence of a high degree of immunity after recovery from an attack of yellow fever is well known and serum from recovered patients has often been used in order to confer a passive immunity. The protection following such an injection, however, does not last more than a few weeks, and consequently is of little practical value except when it is necessary to protect anyone recently exposed to infection.

In marked contrast with the short duration of this passive immunity, is the strong and lasting protection following the injection of virus and immune serum, and this method has been recommended by Aragão, and Findlay and Hindle, as an alternative to the use of a vaccine. The method was used with success by Aragão in the protection of a few human volunteers, but the possible risks of fully virulent yellow fever virus precluded its use except in isolated cases.

The practical difficulties in the application of this method have been largely overcome by the important discovery (Theiler, 1930) that after a few passages in the brains of mice, yellow fever virus loses to a great extent its power of producing a general infection in man or monkeys, and acquires neurotropic properties. This so-called 'mouse virus', if inoculated subcutaneously in small doses of 1:1,000 to 1:10,000 of a mouse brain suspension, generally fails to produce any serious reaction, but the inoculation is followed by the development of immunity against the ordinary yellow fever virus approximating in intensity that found in recovered cases of the disease. Sellards and Laigret recommended this as a method of vaccination, but further observations have shown that the inoculation of mouse virus is not unattended with risk, and yet a further objection is the circulation of the virus in the blood, so that the patient would be a possible source of infection for susceptible mosquitoes. Sawyer, Kitchen and Lloyd[2], using a mixture of mouse virus and human immune serum, have elaborated a technique whereby the objections to the last two methods of protection have been largely overcome.