The Formation of the Anthocyan Pigments of Plants.—Part VI.

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In the previous communications of this series we have recorded the results of observations on the oxydases and chromogens concerned with the production of anthocyan pigments. The study of pigment formation is continued in the present communication, the sections of which deal with the following subjects:—

1. A pigment-producing glucoside of the wallflower (*Cheiranthus cheiri*).
2. The formation of pigment-producing substances from glucosides.
3. The biochemistry of Mendelian colour characters.

Section 1.—*A Pigment-producing Glucoside of the Wallflower.*

It is customary to divide the sap-pigments of plants into two series, the red, purple, and blue anthocyan pigments, and the yellow xanthein pigments. Miss Wheldale* has, however, suggested, on genetical grounds, that the anthocyan and xanthein pigments are related with one another. This author points out that most plants contain colourless or pale yellow substances which give a canary yellow colour with ammonia. When heated with dilute acid they assume a deep yellow colour and reduce Fehling's solution. Hence, they are to be regarded as glucosides.†

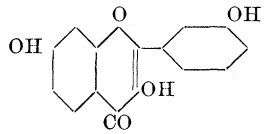
Miss Wheldale suggests that anthocyan is a compound of such a glucoside-like body with a "reddening" substance. In the absence of the latter and with the loss of a further substance (the sugar ?), the glucoside gives rise to a yellow xanthein pigment.

More recently,‡ Miss Wheldale states that the yellow pigments are largely present as glucosides, of which some, or possibly all, the hydroxyl groups are replaced by sugar. Specific hydrolysis may act on hydroxyl groups in certain positions, and when these groups are free from sugar, oxidation, and possibly condensation, may take place at these points. The residual hydroxyl groups in the anthocyan molecule would probably be replaced by sugar, and hence the anthocyans would occur as glucosides.

* 'Roy. Soc. Proc.,' 1909, B, vol. 81, p. 44.
† Compare Czapek, 'Biochem. d. Pflanzen,' vol. 1, p. 177.
‡ 'Biochem. Journ.,' 1913, vol. 7, p. 87.

It is to be remarked that this hypothesis postulates a larger number of glucose residues in the molecule of the yellow pigment than is the case in the varieties which have been studied. In none of these are more than two sugar residues present. Moreover, even in such cases, the sugar residues are not attached to different hydroxyl groups, but are united to one another to form a disaccharide. Further, the experience gained with amygdalin* suggests that such complex glucosides are only broken down by enzymes in one way, and hence, if this be the case, it is not probable that different enzymes act on the glucosides in such a manner as to set free different groups.

The yellow pigments are regarded by Miss Wheldale as belonging to the flavone or xanthone classes, and in this connection it may be pointed out that the constitution of the hydroxyflavone glucosides offers great possibilities of variation. Thus the sugar residue, which may be either glucose or rhamnose, is joined by an hydroxyl group to the flavone. The hydroxyl group may be attached to carbon in the oxygen ring or substituted in either of the phenol groups.



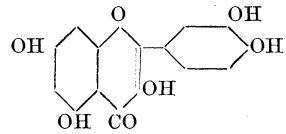
Such differences of constitution will correspond with differences in the properties of the several glucosides, and these different properties will be manifested particularly when the glucosides are acted on by enzymes or hydrolysed by acids.

A further possibility of the derivation of varied products from these glucosides follows from A. G. Perkin's observations (*vide infra*) to the effect that the glucosides of this class occur commonly in plants not singly but in association. In a valuable series of memoirs dating from 1895, A. G. Perkin has described a number of natural hydroxyflavone glucosides, and at the present time the structure of at least 12 of these bodies is known. The researches of Perkin have been supplemented by Kostanecki's synthetical work on the hydroxyflavone group.

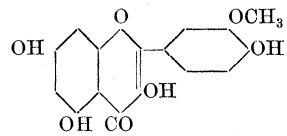
The outstanding fact revealed by Perkin's investigations is that, of the yellow pigments derived from many and diverse varieties of plants, all, without exception, are hydroxyflavone derivatives; indeed, with this evidence before us, it is not too much to assume that all the soluble yellow pigments of plants belong to this class. The observations described in this section

* Compare Armstrong, 'The Simple Carbohydrates,' 1912.

have reference to the soluble sap pigments of the wallflower (*Cheiranthus cheiri*). Of these pigments, A. G. Perkin* has already investigated the hydroxyflavones present in the deep yellow flower of the variety of wallflower known as "Cloth of Gold." He has shown that when the extract of sap pigment is hydrolysed by boiling with dilute sulphuric acid an olive yellow precipitate of the pigment separates on cooling. The precipitate is a mixture of quercetin



and its monomethyl ester iso-rhamnetin



Of these substances quercetin is distributed very widely in plants, but iso-rhamnetin has been met with only in the Indian dye, asbarg (from *Delphinium zalil*).†

The general characters and distribution of the pigments of the flowers of *Cheiranthus cheiri* are as follows :—

The petals contain both sap and plastid pigments. In the deep yellow varieties the colour is due to a plastid pigment which accompanies and masks a pale yellow (cream) sap pigment. Purple flowers contain a purple (anthocyan) pigment, together with a little plastid yellow. The same pigments occur in the brown wallflower, which owes its colour to the large amount of yellow which occurs, together with purple. Red flowers contain anthocyanin, together with xanthein, the yellow of which masks to some extent the blue constituent of the anthocyan pigment. In certain primrose-yellow varieties the colour changes with the age of the flower. The buds and newly opened blossoms are of a rich yellow, but as the flower grows older the yellow fades gradually till the petals are cream or almost white. The full yellow is due to the plastid pigment which occurs together with the pale primrose-coloured xanthein in the buds of young flowers. The plastid pigment disappears as the age of the flower increases, and the pale colour of the full blown flower is due to the persistence of the soluble sap or xanthein pigment.

* 'Chem. Soc. Trans.,' 1896, p. 1566.

† Perkin and Pilgrim, 'Chem. Soc. Trans.,' 1898, p. 267.

The waning of the yellow plastid pigment coincides with the disappearance from the petals of an inhibitor of oxydase. Thus, if petals of various stages are tested by means of benzidine and hydrogen peroxide, only the mature cream-white petals, from which the yellow pigment has disappeared, give an oxydase reaction. If, however, the petals are first treated for five minutes with absolute alcohol, and then with benzidine and hydrogen peroxide, they give a good oxydase reaction.

Inasmuch as we have shown already (Part IV) that absolute alcohol serves to remove the inhibitor of oxydase which occurs in the flowers of dominant white *Primula sinensis*, we conclude that there exists in this "primrose" strain of wallflower an oxydase inhibitor which is closely bound up with the yellow plastid pigment. As the latter is destroyed so is the former, and after their disappearance the oxydase contained in the cells in which they were present may be demonstrated by means of the ordinary oxydase reagents.

These observations, together with the fact that red and purple anthocyan only make their appearance in cells in which the plastids are degenerating, may, perhaps, offer a clue to the significance of the curious phenomenon of striping which is so common in wallflowers. In striped wallflowers the red or brown colour is broken by wider or narrower yellow bands. We believe that the phenomenon is to be interpreted in terms, first of the persistence of the plastids and of the inhibitor of oxydase, and, second, of failure of supply of chromogen.

That the former of these factors is concerned in the phenomenon is probable from the foregoing observations, and that lack of chromogen is also concerned is rendered probable by observations which we have made on the striped flowers of Honesty (*Lunaria annua*).

The garden varieties of this plant bear flowers showing all degrees of striping. In some the petals are self (uniformly) coloured; in others there is a small colourless area at the junction of limb and claw, and in others this white area may extend so far as to leave but a narrow border of magenta at the edge of the petal. The white regions of the flower are well supplied with peroxydase, and therefore their lack of colour is to be ascribed to lack of chromogen. That this is actually the case may be demonstrated by removing all the open flowers and some of the buds from a head of flowers. As a result of thus reducing the number of flowers of the inflorescence, those that are left assume in what should be the white region of the flowers a dark magenta colour, so dark as to look like a deep magenta splash on a pale ground. Into the physiological interpretation of this effect of the operation we need not enter now, but we may take it as demonstrating that streaking

or flaking of flowers is due, in some cases, to local inhibition of oxydase, and in others to a local defect of chromogen.

That the pale yellow or cream xanthein sap pigment of the wallflower is in some way related with the red and purple anthocyans is rendered probable from a study of the curious behaviour of a hybrid wallflower *Cheiranthus kewensis*, the issue of a cross between *Cheiranthus cheiri* and *C. mutabilis*. When the flowers of *C. kewensis* open they are of a pale yellow or cream colour, and of that colour they remain for a long time. Gradually, however, a faint reddish hue steals over them, deepens, and finally replaces the original colour. Experiments now to be described make it probable that what is witnessed here is a gradual formation of red anthocyan at the expense of the cream-yellow xanthein pigment.

If petals of the "primrose" race of the common wallflower, *C. cheiri*, or the soluble yellow extract therefrom be heated in aqueous alcoholic solution with a little concentrated hydrochloric acid and zinc dust a red pigment is formed. In the first phase of the reaction the xanthein glucoside is hydrolysed to a reducing sugar and to a yellow compound which is insoluble in water. For example, a solution of the pigment was divided into two parts, and the reducing power of the solutions was determined by the Bertrand method, in the one before and in the other after hydrolysis. The results were :—

Before hydrolysis = sugar equivalent to 6·5 c.c. of permanganate.
After „ „ „ 13·5 „ „

The glucoside is also hydrolysed slowly by the emulsin of almonds. After the yellow insoluble compound produced during hydrolysis has been dissolved in 50-per-cent. alcohol and reduced by zinc dust and an acid it undergoes reoxidation to an intense red pigment. The red pigment passes through green to yellow on the cautious addition of alkali. It is reduced to a colourless state by zinc dust and acetic acid or by zinc dust and ammonium chloride, the colour recovering on oxidation.

The solution of hydrolysed pigment separated by filtration from the yellow product gives no red colour when reduced. Thus we have chemical evidence that the yellow glucoside of the wallflower undergoes hydrolysis, and that its product is converted by reduction and oxidation into a red pigment. Since reduction and oxidation may take place in any plant cell we may infer that the pale yellow wallflower owes its lack of red or purple anthocyan to the absence of the agent for glucoside hydrolysis, and it may be predicted that the pale colour of the primrose (yellow-cream) race of wallflower is recessive to the anthocyan colour of red or purple races.

We have obtained by the means described already red pigments from the yellow and colourless flowers of many other plants, *e.g.*, yellow daffodil, yellow crocus, cream polyanthus, and chinese primrose. As a rule the colour is located at first especially in the veins, but it appears subsequently in the whole petal. The case of the daffodil is of particular interest, inasmuch as plant breeders have been at work for many years endeavouring to produce a red daffodil. This work has met already with some considerable success, and it may be predicted on biochemical grounds that the object will be achieved completely in the near future.

In the case of some flowers, *e.g.*, polyanthus, reduction by means of zinc dust is not necessary, the red coloration appearing after heating with acid and subsequent oxidation.

We have used the same reagents with the flowers of dominant and recessive white *Primula sinensis*, and find that, as is to be expected on theoretical grounds, dominant whites yield the red colour, and recessive whites, if they yield it at all, do so to an extremely slight extent. Thus we have biochemical evidence supplementary to that derived from plant-breeding experiments that dominant whites possess and recessive whites lack the prochromogen from which anthocyan pigments are derived.

We have described already (Part IV) the recovery of colour which takes place when a decolorised petal of the stock (*Matthiola incana*) is immersed in water, and we have stated our reasons for regarding the recovery of natural colour as the result of a process of oxidation. Subsequent experiments confirm this view.

Thus, by treating an aqueous solution of the purple pigment of stocks with zinc dust and acetic acid in the cold, the pigment becomes first pink, and is reduced subsequently to a colourless state. The colour returns rapidly on exposure to air, and still more quickly when the solution is warmed or treated with a drop of hydrogen peroxide. In each case the original purple is recovered. If zinc dust and ammonium chloride be used, reduction to a colourless state also takes place, but subsequent oxidation leads to the formation of first a pale green and then a blue colour.

Section 2.—*The Formation of Pigment-producing Substances from Glucosides.*

The present state of our knowledge does not allow of a perfectly satisfactory classification of the pigments which commonly occur in the flowers of plants. Nevertheless, these pigments may be grouped provisionally according to their mode of origin and chemical composition.

Classified according to their modes of origin, the flower pigments fall into two groups, those which are derived from the plastids, and those which are

formed independently of the plastids in special vacuoles of the cytoplasm. The former are called plast pigments and the latter sap pigments. In the mature cell the sap pigments occur in solution with the general cell sap, and hence they are referred to sometimes as the soluble sap pigments. They occur in two series, the soluble yellow pigments (see p. 115) and the so-called anthocyan pigments. Evidence has been given already (p. 117) to show that the soluble yellow pigments are to be regarded as flavone derivatives, and that they are related with the red and blue anthocyan pigments.

Of the plast pigments, two series also occur. One comprises the yellow and red carrotene pigments, together with the oxidised derivatives of the latter, the xanthophylls. The other series includes the chlorophyll pigments. Of these pigments, carrotene (a hydrocarbon) contains C and H, xanthophyll, the soluble yellow sap pigment, and the anthocyans contain C, H, O, and chlorophyll, like hæmoglobin and the animal melanins, contains N in addition to these elements.

It is known that the melanins of animal tissues are produced by the action of tyrosinase on chromogens. We show now that pigments containing nitrogen are formed readily by the action of plant enzymes on the glucosides which occur in plants. Hence it is probable that the formation of such nitrogen-containing pigments occurs normally in the living plant.

Two recently published researches of Chodat have important bearings on the mode of pigment formation in plants. In the one research, Chodat* proves that, when a vegetable oxydase acts on glycine, carbon dioxide, formaldehyde, and ammonia are produced. In the other, he demonstrates that, when *p*-cresol is oxidised by oxydase in the presence of an amino-compound, a series of coloured substances is produced, the colours of which depend on the nature of the amino-compound.

We have confirmed the accuracy of Chodat's conclusions and have applied them to an investigation of the behaviour of certain glucosides, arbutin, methyl arbutin, and others, when acted on by plant enzymes.

Arbutin and methyl arbutin are distributed widely among species of the genus Pyrus and also among the members of the Ericaceæ and certain other natural orders. When hydrolysed by emulsin, arbutin and methyl arbutin yield respectively quinol and its mono-methyl derivative. Inasmuch as emulsin usually contains a small quantity of oxydase, hydrolysis is accompanied by a darkening of the solution owing to the oxidation of the quinol. It has been suggested by Bourquelot and Fichtenholz that the natural blackening which takes place in the fallen leaves of many species of Pyrus is due to the oxidation of quinol.

* 'Arch. Sci. Phys. Nat.,' 1913, vol. 35, p. 140.

We now find that if a mixture of arbutin and glycine be hydrolysed by emulsin and then oxidised by means of the oxydase present in an aqueous solution of bran, an intense red coloration is produced. The red substance is evidently derived from the interaction of *p*-quinone with glycine, or its oxidation products formaldehyde and ammonia. A similar red colour is obtained when, instead of glycine, alanine, leucine, phenylalanine, tyrosine, or asparagine is used. The colour is also produced when arbutin is incubated at 37° with a little ground sweet almond and a drop of hydrogen peroxide. The almond contains emulsin, peroxydase and amino-acids, so that it allows of the whole series of changes.

The changes may be represented schematically as follows:—

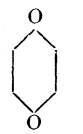
$$\text{Arbutin} + \text{water} \xrightarrow{\text{Emulsin.}} \text{quinol} + \text{glucose.}$$

$$\text{Quinol} + \text{oxygen} \xrightarrow{\text{Oxydase.}} \text{quinone.}$$

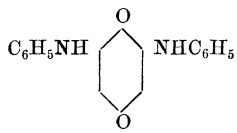
$$\text{Glycine} + \text{oxygen} \xrightarrow{\text{Oxydase.}} \text{formaldehyde, ammonia, and carbon dioxide.}$$

$$\text{Quinol} + \text{quinone} + NH_3 = \text{red compound.}$$

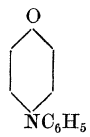
The production of a red colour from quinone and amino-acids was first observed by Wurster* and later by Raciborski,† but neither author offers any explanation of the phenomenon. The reaction has been studied recently by E. A. Cooper,‡ who holds that the colour results from the interaction of the $-NH_2$ groups of the amino-acid and the oxygen atoms of the quinone, and that the reaction is similar to that which takes place between an amine, such as aniline, and quinone. In the latter case the aniline residues may enter the ring in the 1, 3 positions, or, in addition, form an anil or dianil by replacing the quinone oxygen.



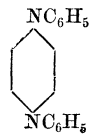
Quinone.



Dianilinoquinone.



Quinone anil.



Quinone dianil.

The abnormal behaviour of the amino-acids, under conditions when other compounds containing a primary amino-group form condensation products, was shown originally by Strecker.§ He proved that alloxan oxidises alanine

* 'Chem. Zentr.,' 1889, vol. 1, p. 392.
† 'Chem. Zentr.,' 1907, vol. 1, p. 1595.
‡ 'Biochem. Journ.,' 1913, vol. 7, p. 186.
§ 'Annalen,' 1863, vol. 123, p. 363.

to carbon dioxide, ammonia, and acetaldehyde. Hurtley and Wooton,* who have made a full study of the reaction, find that dimethylalloxan behaves in a similar manner. W. Traube† has found that benzoquinone and isatin have similar oxidising properties.

Glycine ethyl ester, $NH_2.CH_2.CO.OC_2H_5$,‡ behaves quite differently. With quinone in alcoholic solution it forms the di-ethyl ester of diglycinoquinone, together with hydroquinone; the amino-acid is not decomposed, since the ester group shields the carboxyl group from attack.

It is improbable, therefore, in view of these observations that the condensation between quinone and glycine takes place in the manner suggested by Cooper.

Ammonia by itself gives a brown coloration with quinone, but if formaldehyde be added the brown colour is converted into red. No coloration is given on mixing formaldehyde with quinone, but on the cautious addition of ammonia a red coloration is produced. The colour is very similar to that given by quinone and glycine; this last mixture gives the same reddish-brown shades when a slight excess of ammonia is added. Similarly, quinone gives no colour with benzaldehyde alone, but a red colour is produced on the addition of ammonia, the colour resembling the red obtained from phenylalanine and quinone. Salicylic aldehyde, quinone, and ammonia give rise at first to a red coloration and then to an insoluble brown substance.

Quinone forms a red coloration alike with glycine, alanine, leucine, tyrosine, phenylalanine, or asparagine. The colours are very similar to, not identical with, one another, and they are formed at much the same rate. The red is reduced immediately by zinc dust and acetic acid, and a colourless solution is obtained (see Section 1). The colour returns slowly on standing, more quickly on warming, and immediately on the addition of a drop of hydrogen peroxide.

Quinol is converted by an oxydase in presence of glycine into a red pigment; of its ethers dimethylquinol gives no coloration, and methylquinol a faint pink only, which is, perhaps, due to impurity or to its oxidation to quinone. Methyl arbutin should not, therefore, be capable of giving rise to this red pigment.

When the oxidation of arbutin at 37° is prolonged, action continues past the red stage. The solution becomes a chestnut brown, a brownish black precipitate is deposited, a little tarry matter appears on the surface, and a marked aromatic odour suggesting the smell of prunes is imparted to the liquid.

* 'Chem. Soc. Trans.,' 1911, vol. 99, p. 288.

† 'Ber.,' 1911, vol. 44, p. 3145.

‡ E. Fischer and Schrader, 'Ber.,' 1910, vol. 43, p. 525.

This conversion of a glucoside into substances which simulate both the colour and odour of ripe fruit is noteworthy, and suggests that in the natural process of ripening of fruits, not only are glucosides hydrolysed and certain of their products oxidised by oxydases, but also that amino-compounds, so common in plants, intervene in the reaction and determine the nature of the end products.

The reaction between quinone and the products of oxidation of the amino-acids is obviously very complicated, and we therefore content ourselves with a reference to two somewhat similar cases which have been emphasised as of biological importance :—

1. Strecker and Hurtley and Wooton have shown that by the interaction of glycine and alloxan, $CO<^{NH.CO}_{NH.CO}>C(OH)_2$, at the ordinary temperature, the amino-acid is oxidised to formaldehyde, carbon dioxide, and ammonia, whereas the alloxan is in part reduced to dialuric acid. The unchanged alloxan and dialuric acid combine to produce alloxantin, which with the ammonia forms murexide (ammonium purpurate). This substance has a characteristic purple colour. On warming the mixture, secondary changes take place with the formation of other coloured products, and in the case of tyrosine Hurtley and Wooton note that a " flowery " odour is produced.

2. Ruhemann has shown that triketohydrindene hydrate,

$$C_6H_4<^{CO}_{CO}>C(OH)_2,$$

which gives a deep blue coloration with amino-acids, behaves like alloxan. The hydrindene is now regarded as a valuable reagent for amino-acids, and it has already been of service in investigating problems of animal physiology. In this case also the amino-acid is oxidised to carbon dioxide, ammonia, and aldehyde, and the triketohydrindene is reduced. Hydrindanthin is produced and interacts with ammonia to form the blue ammonium salt of diketohydrindylidene-diketohydrindamine, $C_6H_4<^{CO}_{CO}>CH.N:C<^{CO}_{CO}>C_6H_4$, which is an analogue of murexide.

It is probable that similar changes take place in the case of quinone and amino-acids. Starting from arbutin, where the hydroquinone which is formed is oxidised in part to quinone, it is the ammonia from the amino-acid which is the essential factor in producing the red colour. The aldehyde derived from the amino-acid plays only an accessory part, for the reduced quinone is already present. In fact, the red is instantly produced when ammonia is added to the oxidised mixture. The addition of formaldehyde to hydrolysed and oxidised arbutin has no effect in producing colour, and this is in agree-

ment with the fact that the red colour is produced no matter what amino-acid be used. A series of similar experiments was made differing only in that different amino-acids in equivalent quantity were added in the several cases. The colours produced were compared with one another by means of the tintometer in order to obtain a rough indication of their relations with one another. The same series was again examined a few days later, when the solutions had become darker.

Amino-compound used.	Colour produced.
Glycine	value = 8 red + 13 yellow.
Alanine	" = 8 " + 11 "
Leucine	" = 7·5 " + 10 "
Phenylalanine	" = 5 " + 6 "
Tyrosine	" = 5·5 " + 6 "

The three aliphatic acids give similar colours, the two aromatic acids yield a somewhat different shade. Hence these pigments differ essentially from those obtained by Chodat from *p*-cresol, inasmuch as the colours of the latter depend on the nature of the amino-acid. Whatever be the explanation, the formation of pigment from arbutin and protein degradation-products is one which may well be of natural occurrence. In passing, it may be observed that quinone, like alloxan and triketohydrindene, may prove to be of use in the diagnosis of amino-compounds.

Substituted quinones such as 1 : 4-xyloquinone or 1 : 4-thymoquinone resemble quinone in giving a colour reaction with glycine on warming in aqueous-alcoholic solution; but in the case of these substances the reaction takes place much more slowly. Xyloquinone give rises to a claret red, thymoquinone to a tawny or brown red. There is apparently a difficulty in reducing the quinones, as neither of them gives a colour reaction with formaldehyde and ammonia.

We are investigating the behaviour of other glucosides and find that salicin, the glucoside of the willow and many other plants, gives an orange, passing to an orange-red, coloration when hydrolysed by emulsin and oxidised by an oxydase in presence of an amino-acid. Similar colours are obtained with glycine and with phenylalanine, the tintometer reading in a half-inch cell being in each instance 4·5 red + 1·8 yellow. Salicin incubated with ground sweet almond and a few drops of hydrogen peroxide gives a similar colour reaction.

Phloridzin, the glucoside present in the roots of many rosaceous trees, is composed of glucose and phloretin, a condensation product of *p*-hydroxy-hydratropic acid and phloroglucinol. When hydrolysed by emulsin in

presence of glycine it is converted into a yellow substance which becomes orange and finally orange red. A red insoluble deposit, which separates out, forms an orange-red solution in alcohol. In the tintometer we find for a $\frac{1}{8}$-inch cell: alcoholic extract, 3·5 red + 1·5 yellow; aqueous solution, 2·5 red + 5 yellow.

Finally, æsculin (from the horse-chestnut) gives a yellow precipitate, and aucubin (from the red berries of *Aucuba japonica*), a black precipitate under the conditions described.

The property of colour formation from a glucoside and an amino-acid seems to be a very general one, though we are unable to say whether the mechanism is in each case the same as we have postulated for arbutin, namely, oxidation of the phenol to a quinone, formation of a quinohydrone and interaction of this with ammonia to form a coloured salt.

In any case, Chodat's discovery of the resolution of amino-acid into formaldehyde and ammonia is obviously of fundamental importance. The ammonia may serve to provide the alkaline conditions so favourable for oxidation and it may react directly to form amino-compounds. The formaldehyde may take part in all manner of condensations leading to the production of complex substances.

Section 3.—*The Biochemistry of Mendelian Colour Characters.*

Of the various artificial chromogens which serve to determine the presence of oxydase in flowers, benzidine behaves most like the natural chromogens. For example, most artificial chromogens, α-naphthol, guaiacol, etc., serve well enough to indicate the presence of oxydase in the vascular tissues (veins), but they do not react as a rule with the oxydase contained in the epidermal cells, whereas benzidine gives uniformly good reactions with both epidermal and bundle oxydase (see Part I). Again, just as the reducing agents present in petals may reduce the anthocyan pigments to a colourless state, so these same agents reduce and decolorise the blue oxidation product of the interaction of plant oxydase and benzidine.

Inasmuch as benzidine has proved to be of considerable value for the investigation of plant oxydases, it may be useful to preface this section with a brief account of what is known of the oxidation products of benzidine, which are of an unusually complex character.

Willstätter and Kalb* have shown that the first oxidation product of benzidine ($NH_2C_6H_4.C_6H_4NH_2$) is probably the reddish-brown diphenoquinone di-imine $NH:C_6H_4:C_6H_4:NH$. On further more drastic oxidation, two molecules of this substance unite to form the yellowish-red diaminoazo-

* 'Ber.,' 1905, vol. 38, p. 1232.

diphenyl $NH_2.C_6H_4.C_6H_4.N{:}N.C_6H_4.C_6H_4.NH_2$. The blue- and violet-browns, so characteristic of the action of oxydases on benzidine, are due to complex, partially or *meri*-quinonoid salts of diphenoquinone di-imine with benzidine itself :—

$$\begin{array}{ll} NH{:}C_6H_4{:}C_6H_4{:}NH & = \text{Quinone diimine.} \\ \vdots \qquad\qquad \vdots & \\ NH_2.C_6H_4.C_6H_4.NH_2 & = \text{Benzidine.} \end{array}$$

The molecules are united through the partial valencies of the nitrogen atom. These compounds are *meri*-quinonoid, in that the quinone di-imine may be combined with several molecules of benzidine. For example, Willstätter and Piccard* describe a blue compound of the di-imine with four molecules of benzidine, and a brownish-violet compound with three molecules of the amine. Upon reduction, such *meri*-quinonoid compounds are converted into benzidine, whilst oxidation transforms them gradually into the quinone di-imine, as more and more of the benzidine is oxidised.

For the investigation of plant oxydase, and of inhibitors of oxydase, we find that it is convenient to use benzidine in two forms, viz., a $\frac{1}{2}$-per-cent. solution in 50-per-cent. alcohol, and a saturated solution in 1–2 per cent. of sodium chloride.† When rapidity of action is required, the latter solution is employed, but, when inhibition is under investigation, the alcoholic solution should be used side by side with the sodium chloride solution. In illustration of the rapidity of action of the sodium chloride benzidine solution, it may be mentioned that, if young seedlings of maize, etc., or mature roots of water plants such as *Hydrocharis morsus-ranæ* (frog bit) be immersed for a few minutes in this solution, the subsequent addition of a few drops of hydrogen peroxide causes almost instantaneously a bright blue coloration of their root-hair regions.

Again, if flowers known to contain an inhibitor of oxydase be treated with some agent, for example, absolute alcohol, which is known to remove the inhibitor (see Part IV), they fail to react with the alcoholic benzidine solution until the whole or greater part of the inhibitor has been removed, whereas such flower sgive a definite reaction with sodium chloride benzidine, even though the inhibitor has been only in part removed.

The subject with which we deal in this section is that of the cause of the range of flower-colour which occurs within a species. In illustration of the nature of this problem we may mention the facts known in the case of the flowers of the chinese primrose (*Primula sinensis*). In addition to white-

* 'Ber.,' 1908, vol. 41, pp. 1458, 3245.

† *Cf.* Madelung, 'Zeitsch. physiol. Chem.,' 1911, vol. 71, p. 204.

flowered races (dominant and recessive whites), the horticultural varieties of this species comprise races with blue, red, and magenta flowers, and our purpose is to put forward a biochemical hypothesis to account for the production of these distinct colours and for the genetical relations which obtain between them.

It has been suggested by Miss Wheldale* that each of the chief colours of such a series is determined by a special oxydase, but neither general considerations nor such observations as we have been able to make lend support to this view.

It is true that the flowers of different varieties of *P. sinensis* contain different amounts of oxydase, but we find no constant relation between amount of oxydase and type of coloration. Moreover, the recent researches of Bach† point definitely away from the hypothesis that oxydases are specific.

If hypothesis of specific oxydases be rejected, we must ascribe specific coloration either to the action of an oxydase on different chromogens or to the interaction, with a chromogen or an oxydase, of specific substances which modify decisively the colour produced in the course of the reaction.

Any discussion of these alternatives must take into account the observations of A. G. Perkin, that the hydroxyflavone glucosides of plants occur, as a rule, not singly but in groups. There is some ground for regarding these glucosides as constituting the prochromogens from which the anthocyan chromogens are derived, and it is therefore a matter of great significance to the student of genetics that the plant is, as it were, offered a choice of several pigment-forming materials on which its hydrolysing and oxidising enzymes may act.

Pending fuller investigation of the possibility that the colour of a variety may be determined by a selective action on one of a group of allied glucosides, we are inclined to adopt the latter of the two alternatives, and to suggest that the serial colours of flowers are due each to the intervention of specific substances in the reaction of oxydase on chromogen.

This hypothesis is rendered plausible by the following observations, first on the colours produced when a mixture of phenols is treated with oxydase and second, on the behaviour of our artificial chromogen benzidine when acted on by oxydase in the presence of various phenols.

When a mixture of phenols is treated with a plant oxydase a competition for oxygen ensues. For example, if oxydase be caused to act on guaiacol until the red colour is produced, the addition of other phenols brings about a more or less quick change of colour. Thus α-naphthol converts the red into mauve, and the ultimate colour which is produced is of a far deeper tint

* 'Prog. Rei Bot.,' 1910, p. 469.

† 'Arch. Sci. Phys. Nat.,' June, 1912, vol. 33.

than that which arises when α-naphthol and oxydase interact with one another. If *p*-cresol be added to the red solution produced by the action of oxydase on guaiacol a brown colour appears. Saligenin, on the other hand, does not modify the normal deep red colour given by guaiacol and oxydase.

Search in chemical literature brings to light a few records of similar observations. Thus Schoenbein, in 1856,* observes that guaiacum blue oxidises other oxidisable substances, and in doing so becomes reduced and decolorised. Kastle and Porch† find that the oxidation of *p*-phenylene diamine, guaiacum, and phenolphthalein, by means of an oxydase, is accelerated greatly by phenol, the cresols and β-naphthol. They recognise that these accelerators act probably as auxiliary oxygen carriers, and that they are themselves more or less completely oxidised in the process. Miss Wheldale‡ suggests that oxidised catechol acts as a peroxide.

p-Phenylene diamine (*p*-diamino-phenyl, $NH_2C_6H_4NH_2$) exhibits a somewhat different behaviour. Together with α-naphthol it constitutes the indophenol reaction for oxydase, which reaction is used largely by animal physiologists. In it the oxidised amine and phenol are coupled to an indophenol.

We find that phenylene diamine gives much the same violet-blue coloration with all phenols, including methyl quinol; but that benzidine and oxydase give with each phenol a distinct colour, similar to that produced when the phenol in question is oxidised by oxydase. It would appear, therefore, that nothing analogous with the indophenol reaction takes place when benzidine and phenols are acted on by oxydase.

It is to be noted that *p*-phenylene diamine is oxidised by atmospheric oxygen to a garnet red (tetra-aminodiphenyl-*p*-azophenylene), whereas it gives a dark brown product when oxidised by oxydase. The indophenol reagent is ill-adapted for the localisation of plant oxydases because of the readiness with which it oxidises spontaneously. Petals of recessive white *Primula sinensis* give a general purple reaction with it, and a brown with phenylene diamine, but solutions of these reagents become strongly coloured even without the addition of hydrogen peroxide, whereas this is not the case with benzidine and the other reagents which we use for the investigation of plant oxydases.

We have not used *as*-dimethyl-*p*-phenylene diamine ($NH_2C_6H_4NMe_2$), which substance is oxidised readily to quinone imine, the salts of which

* 'Journ. Prakt. Chem.,' vol. 57, p. 496.
† 'Journ. Biol. Chem.,' 1908, vol. 4, p. 301.
‡ 'Roy. Soc. Proc.,' 1911, B, vol. 84.

form red meri-quinoid compounds with the unchanged diamine—the so-called Wurster salts.

We have noted already that when hydroquinone is added to a mixture of benzidine and oxydase in which the blue colour has been allowed to develop the colour is discharged. It is not until all the hydroquinone has been oxidised that the blue colour begins to return, the limiting factor being the amount of hydrogen peroxide present. Most other phenols behave similarly to quinol, but since their oxidation products are generally coloured, the blue benzidine mixture becomes colourless for an instant only and then the solution assumes a lavender, green, red, or brown hue, according to the phenol chosen. This colour slowly changes, and as the benzidine blue returns it becomes masked, and finally overpowered by the blue.

The phenols experimented with include *p*-cresol, orcinol, guaiacol, α- and β-naphthol, thymol, pyrogallol, resorcinol, phloroglucinol, saligenin, phenol, methyl quinol, dimethyl quinol, etc.

The list comprises certain phenols which usually do not give a colour reaction with oxydase, *e.g.*, methyl quinol. Even with α-naphthol the normal lavender oxidation coloration is much more intense when produced in the presence of other phenols.

The behaviour of methyl quinol deserves special mention in that it affords the basis for our hypothesis as to the production of serial colours in flowers. With oxydase, methyl quinol gives no colour reactions; but if a little benzidine be added to the colourless solution the latter takes on a deep and persistent carmine colour. The blue benzidine pigment acts catalytically as an intermediary for the transmission of oxygen to the methyl quinol; that is, it may in this respect, and in this case, play the part of an organic peroxide, and thereby achieve the oxidation of a substance (methyl quinol) which resists the action of oxydase and hydrogen peroxide.

The power of benzidine to transmit oxygen to methyl quinol and other phenols may be illustrated by making use of the oxydase present in the flowers, or other parts of plants. For instance, if the flower of a recessive white *P. sinensis* be treated with benzidine and hydrogen peroxide, the petals assume the blue-brown colour characteristic of the benzidine-oxydase reaction. If, however, benzidine and methyl quinol be added together with hydrogen peroxide a carmine coloration is produced.

A similar oxygen transmitting power on the part of benzidine is exhibited in the behaviour of the white flowers of *Lychnis coronaria*. Treated with benzidine alone the petals become brown; with α-naphthol they take on—albeit with extreme slowness—a lilac or lavender colour. If, however, the petals be treated with benzidine and α-naphthol they assume immediately a

lilac colour which, taken in conjunction with the previous observations, indicates that benzidine facilitates the transference of oxygen from oxydase to α-naphthol.

In order to make clear the closeness of the analogy between the oxydase-benzidine and oxydase-benzidine-methyl quinol reactions on the one hand and those which lead to the production of the quinol colours—blue, red, and magenta—of such a plant as *P. sinensis*, it is necessary to give a brief account of the genetics of flower colour in this plant.

The flowers of *P. sinensis* stand in a definite and constant relation with one another. They form a series: recessive white, blue, red, magenta and dominant white. The biochemical nature of the whites has been described in an earlier communication (Part III).

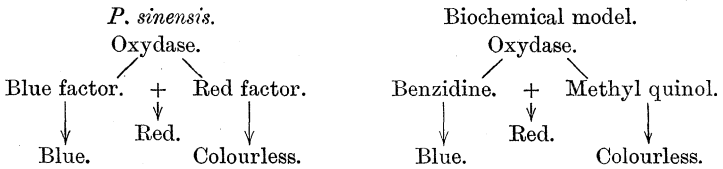
Of the coloured members of the series blue is recessive to both red and magenta, and red, which is dominant to blue, is recessive to magenta.

The Mendelian interpretation which fits the genetical facts is as follows:—

The character for blue flower depends on the presence of a single Mendelian factor. Red flowers also contain this factor and they contain in addition a factor for red which can produce its effect only in the presence of the "blue" factor. Similarly magenta flowered plants contain a magenta factor which when present together with the red and blue factors gives rise to the magenta character.

In the absence of the lower members of the series, colour is not produced and the colour of any flower is an indication that the series of factors is unbroken up to the factor for the colour character manifest in the flower.

We have thus a striking parallel between the colour series in *P. sinensis* and that which occurs with benzidine and methyl quinol. The closeness of the parallel is indicated thus:—



The peculiar behaviour of the red factor, first in failing to induce colour formation in the absence of the blue factor, and, second, in masking completely by a red pigment the activity of the blue factor, is to be accounted for thus:—The red factor determines the formation of a specific substance—perhaps of the nature of a phenol. That substance is not oxidised directly by the oxydase of the flower, but in the presence of the "blue" factor this specific substance receives oxygen from the blue pigment produced by the

agency of that factor, and, in consequence, the blue pigment is reduced to the state of a colourless chromogen. The observations recorded on p. 126 lend additional support to this hypothesis. It is there observed that various phenols intensify, though they may not change, the colour produced by the action of oxydase on artificial chromogens. On the practical side it is also known that intensifiers of pigment exist, that they possess the power of converting a pale into a deep shade, and that they behave each as a unit character. On our model it seems reasonable to assume that an intensifier is a phenolic or similar substance, and that the factor for an intensifier means the power of the cell to produce that substance.

Lastly, on the basis of this hypothesis we have a plausible explanation of the fact that many oxydase reagents, though they give good "bundle" reactions, fail to reveal the presence of oxydase in the epidermis. The vascular tissues contain considerable stores of oxydase and oxygen-carrier, and hence, through the agency of the carrier, oxygen is transferred to α-naphthol or similar "artificial chromogen." The epidermal tissue contains only a small quantity of the carrier of oxygen, and hence, in spite of the presence of oxydase, α-naphthol and similar artificial chromogens remain unoxidised in this tissue.

Conclusions.

1. The pale yellow sap colour of the petals of the wallflower is a mixture of hydroxyflavone glucosides. The glucoside mixture is hydrolysed readily by heating with mineral acids and more slowly by emulsin of almonds. The hydrolysed product if reduced and subsequently oxidised yields a red pigment.

2. The fact that flowers containing similar soluble yellow pigments may be caused, by suitable chemical treatment, to yield a red pigment, suggests that red mutations should be of possible occurrence in such species.

3. The formation of pigments, as the result of oxidation by oxydase of the hydrolysed products of glucosides, is determined by the presence of amino-compounds and is of very general occurrence. The behaviour of the glucoside arbutin (see p. 121) makes it probable that many of the pigments and odorous substances formed during the ripening of fruits arise as results of reactions of this type.

The pigments of plants may be classified provisionally as follows:—

I. Plast Pigments—

a. Chlorophyll pigments contain	C, H, O, N
b. Carrotene contains	C, H
c. Xanthophyll (oxidised carrotene) contains	C, H, O

II. Sap Pigments—

a. Yellow. Hydroxyflavone glucosides or derivatives thereof contain ..... C, H, O

b. Red, *e.g.*, of wallflower (see p. 117). Products of the action of oxydase on hydroxyflavone glucoside derivatives contain..... C, H, O

[Whether all anthocyan pigments are of this type is unknown.]

c. Red and brown, *e.g.*, of plum. Substances produced by the oxidation of phenols in the presence of amino-acids contain ..... C, H, O, N

d. As suggested in Section 3, the so-called anthocyan pigments (red and magenta) of flowers may arise as the result of the oxidation of phenol brought about by an organic oxygen carrier; contain ..... C, H, O

4. The benzidine-methylquinol-oxydase reaction (p. 128) provides an analogy with the II*d* type of pigment formation, and suggests the hypothesis that the higher members of a flower colour series (see p. 129) owe their origin to the presence with the lower members of specific substances which, acting as receivers of oxygen, reduce the pigments characteristic of the lower members of the colour series, accept oxygen, therefrom, and thereby become oxidised to pigments of specific colour.