X. OBSERVATIONS ON THE ACTION OF CHLORINE ON LIGNIFIED TISSUES.

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(Received November 28th, 1933.)

DURING a study of the chemical nature of the cell membrane in plant tissues use was made of the chloramine reaction as a means of detecting protein in the cell-wall. Plant tissue sections were submitted to the prolonged action of chlorine gas and subsequently treated with aqueous solutions of sodium hydrogen phosphate and potassium iodide. Under these conditions proteins, if present, produce chloramines which liberate iodine from potassium iodide, resulting in a yellow coloration of the cell-wall [Wood, 1926].

This reaction led to interesting results with lignified tissues apart from its original application. A pink coloration was developed in these tissues [Wood, 1927], and while investigating the origin of this it was found necessary to devise some means of detecting vanillin in the cell-wall and of distinguishing this compound from coniferin, since it was thought possible that the coloration might be due to a compound of vanillin or coniferin, or their derivatives, with chlorine. Other possibilities were that it might be due to a compound of lignin with chlorine or to the nature of the lignin present. It has been shown by the author that this coloration is not due to oxidation of the cellulose base, but to the presence of the halogen [Wood, 1927].

Vanillin has been shown to occur in linden bark, in the summer only, by Bräutigam [1900, 1, 2]; it was found in a board by von Lippmann [1904], is stated to predominate in the oldest lignified tissues by Ellram [1896] and has been shown to occur in decayed oak-wood by Sullivan [1914].

It is well known that the sulphite and soda liquor from the manufacture of wood-pulp contains vanillin.

Coniferin occurs frequently in plant material. Kubel [1866] found it in the cambial sap of all conifers. It is converted by hydrolysis into coniferyl alcohol, and by subsequent or direct oxidation into vanillin [Tiemann and Harmann, 1874]. Hence it was necessary to perform distinguishing tests for these substances.

The possibility of the production of vanillin from coniferin, or on further oxidation vanillic acid by the chlorine-phosphate-iodide treatment to which the plant sections were submitted, must be taken into account, for Tiemann and Mendelsohn [1875] record the reverse process.

Should vanillin be detected after chlorination, its origin is a matter of some importance.

As the pink coloration only appeared in the presence of the halogen, and evidently a definite compound was formed, the problem was to show whether it was due to:

(a) a compound of chlorine with coniferin, or coniferyl alcohol which may be produced by the hydrolysis of coniferin;

(b) oxidation of coniferin to vanillin and the subsequent formation of a chlorovanillin;

(c) the formation of chlorovanillin from vanillin naturally present in the cell wall;

(d) the prolonged treatment with chlorine resulting in the formation of vanillic acid or possibly the production of a chlorovanillic acid.

No information could be obtained as to the existence of any compound of coniferin or coniferyl alcohol with chlorine, so that this part of the work was concerned with proving the presence or absence of the coniferyl nucleus before and after chlorination.

Menke and Bentley [1898] describe the formation of a chlorovanillin when vanillin in solution in chloroform is treated with chlorine for some time. Hann [1925] records the preparation of chlorovanillin, probably identical with the above, when vanillin dissolved in glacial acetic acid is treated with chlorine in the presence of fused sodium acetate.

But these chlorovanillins are colourless or yellow substances and the compound under investigation is pink, hence if chlorovanillin is produced, there must be some further reaction, resulting in a coloured compound.

Hann *et al.* [1929] describe the preparation of 5-chlorovanillal-3-nitro-*p*-toluidine in the form of iridescent brick-red granules. It should be noted that this possesses a deep colour because it is a nitro-anil; the colour is not associated with the presence of the chlorine.

They prepared also iodo-derivatives of substituted vanillins with the isomeric tolylrhodanic acids, and found that as a general rule all the iodo-derivatives are red, brown or deep yellow, whereas the analogous chlorine compounds are yellow. All these compounds were found to be insoluble in water, slightly soluble in alcohol and organic solvents, and to dissolve in concentrated sulphuric acid with the production of brilliant red colours. Hence there are two further possibilities of the origin of the observed pink colour; *viz.* it may be due to:

(a) the production of chlorovanillin and its subsequent union with an amine derived from the protoplasmic constituents of the cell, or from the cell-sap, to produce a chlorovanillal derivative which then reacts with the potassium iodide;

(b) to the formation of an iodo-derivative of the substituted vanillin on treatment with potassium iodide.

EXPERIMENTAL.

The experimental work was carried out on plant tissues, in the form of transverse sections of stems, and where possible similar experiments were performed on the chemical substances suspected. The twenty-three species examined included those which had been found already to give an intense pink coloration, those which gave a faint coloration and some which gave negative results. They included Gymnosperms, which all gave an intense coloration, and Angiosperms which gave a variety of results.

Owing to the comparatively small proportion of any section of the plant tissue giving the pink coloration in the chloramine reaction, no method has been devised so far for the extraction of the substance responsible, hence most of the work had to be based on colour reactions, although wherever possible these represented a definite chemical reaction and were not unexplained colour effects.

When bromine is used instead of chlorine a similarly coloured body is produced, though this element is not to be preferred to chlorine for use in this test [Wood, 1931].

The remote possibility of the metallic ion used having some effect also had to be considered.

A similar coloration to that produced when potassium iodide was used was obtained with sodium iodide and with magnesium iodide, it was therefore inferred that the nature of the metallic ion was without influence on the coloration. With potassium chloride, no pink coloration was obtained, and with potassium bromide, an orange-red coloration, which did not resemble the usual pink colour associated with this test, but was somewhat like that obtained when bromine was used instead of chlorine.

These results appear to give additional proof that the effect is connected with the presence of iodine.

In practice all results obtained were more satisfactory if the material, which was kept in alcohol, were placed in water for 12 to 24 hours before being submitted to the action of chlorine. The chlorinated material was then kept in alcohol (as otherwise, in spite of the presence of chlorine, fungi sometimes attack the sections) being treated with water again before completing the test.

Certain products of the chlorination of lignin dissolve in alcohol, *e.g.* lignone chloride [Cross and Bevan, 1895]. Although this substance is light yellow in colour, it was thought possible that it might react with iodine to produce a substance of a different colour. By the use of alcohol the lignone chloride is partially removed before proceeding with the rest of the test.

Schorger [1926] states that the yellow colour produced in lignified tissues by the action of chlorine is due to a combination of the chlorine with the lignin, and that if a dilute solution of sodium sulphite is applied, as in the Cross and Bevan method of determining cellulose, the liquid is coloured pink to purple-red [Bevan and Cross, 1880; Webster, 1883]. By this method the chlorinated products are stated to be removed from wood. Hence the next step was to remove such products of chlorination from the sections. This was effected by boiling them with 2 % sodium sulphite solution after chlorination, then treating them with sodium hydrogen phosphate and potassium iodide and comparing the colour reactions with those produced in sections which had not received the sodium sulphite treatment.

Transverse sections of the stems of fifteen species were examined in this way, chlorination being effected by chlorine gas and by chlorine dissolved in carbon tetrachloride. The pink colour appeared in the same way as if sodium sulphite had not been used, and there was only extremely slight coloration of the liquid.

Sections extracted with glacial acetic acid, which also dissolves lignone chloride, failed to give the test.

The last two series of experiments also show that the more satisfactory results obtained when material that has been preserved in alcohol is treated with water before and after chlorination should not be ascribed to the partial removal of lignone chloride, since the presence of this substance apparently has no effect upon the development of this pink coloration, although a certain amount of this substance is probably formed.

It was next necessary to show whether the pink colour was associated with the lignin complex or not; in investigating this point a large number of plants were examined. The reactions employed for lignin were those produced with diamine blue, brilliant Congo blue, orcinol, phloroglucinol, *o*-, *m*- and *p*-xylylene, and *m*-xylylene acetate.

In the majority of these reactions the pink colour was found to be associated with a positive reaction for lignin in primary and secondary xylem, and in many

cases in stelar sclerenchyma. The results obtained with cortical and stelar sclerenchyma were indefinite, pointing to considerable variation in the chemical composition of these tissues.

Since a connection had been established between the reaction and the lignin complex, the next step was to determine what parts or products of this complex were concerned. It was evident from the amount of the coloration, which in no case was so intense as to render the cell-wall opaque to light, that a portion only of the bulk of the cell-wall was concerned.

There are two types of lignin described by Paloheimo [1929], *ortholignin*, which is resistant to the action of concentrated hydrochloric acid and is scarcely attacked by boiling with dilute alkali, and other kinds of lignin, called hydrolysable lignins, which are readily removed by strong acids and dilute alkalis.

Sections of the stems of seven species were treated separately with caustic soda and with hydrochloric acid and the aniline sulphate and Maule tests used to give the characteristic lignin reactions. The results were compared with those obtained from similar tests made on untreated material, the depth of colour indicating how much lignin had been removed.

All the above tests were performed both before and after chlorination and correlated with the chloramine test made on other sections of the same material. The pink colour is not influenced to any marked degree by the presence or absence of either *ortholignin* or hydrolysable lignin.

The next possibility seemed to be that the colour was due to some compound either produced in small quantity from the lignin as a whole, or loosely associated with the lignin, or perhaps to some encrusting substance. As indicated earlier, two such substances are coniferin and vanillin. Differential experiments were made for the detection of coniferin and vanillin in plant-tissues, and the results obtained were correlated with those of similar experiments made with vanillin and coniferin themselves. These compounds were submitted to the action of chlorine gas, or to that of chlorine dissolved in carbon tetrachloride, and in every case to subsequent treatment with sodium hydrogen phosphate and potassium iodide. The colorations produced were mainly yellow to orange or brown with coniferin, and orange, red or reddish brown with vanillin, indicating that the latter was more likely to be associated with the coloration produced in plant tissues.

In general, the xylem formed in the autumn gave a stronger vanillin reaction than the spring xylem, which often indicated traces of coniferin. The older lignified tissues gave a stronger reaction for vanillin than the younger tissues, thus confirming Ellram's work [1896], and the younger tissues sometimes gave only a very slight reaction. This suggests that vanillin may be produced from coniferin as the tissues become older. Where there was a positive chloramine reaction, the colour reactions for vanillin were always more intense, particularly in two of the species examined, *viz. Laurus nobilis* and *Bambusa nigra*, and where there was a negative chloramine result, the reactions for vanillin were either negative or extremely slight.

As might be expected from the earlier work the reactions varied with the sclerenchymatous tissue involved [Wood, 1927]. The variation was mainly in the shade of the coloration indicating either vanillin or coniferin, the general result being that vanillin in varying amounts was indicated. The reactions for vanillin were generally stronger in cortical sclerenchyma than in stelar sclerenchyma.

The results obtained with all these tissues show that vanillin, and not coniferin, has some connection with the pink colour of the chloramine reaction.

This eliminates the possibility of it being due to a compound of chlorine with coniferin or coniferyl alcohol.

Since it was possible that coniferin, present in the cell-contents, or the cell-wall, might have undergone oxidation by the prolonged chlorine treatment, ultimately producing vanillin, the results of the previous experiments on material, both before and after chlorination, were carefully examined. Sections of six species were again submitted to the codeine test for vanillin [van Itallie and Steenhauer, 1927, 1, 2] both before and after chlorination. In most cases the vanillin appeared to be present before as well as after chlorination, showing that it was naturally present in the material and was not an oxidation product of chlorination.

The possibility of some of the reactions attributed to coniferin and vanillin being also given by hemicelluloses could not be ignored, particularly as these substances may be transition products in the change from pectic substance to lignin, when lignification of the cell-wall takes place [O'Dwyer, 1928].

Schultze defines hemicelluloses as the carbohydrates occurring in the plant-cell that are dissolved by dilute alkalis, readily hydrolysed by hot, dilute mineral acids and destroyed by Schulze's reagent [Schulze, 1890; 1891; 1892]. Schorger [1926] in addition considers that they should be soluble in boiling water.

Hemicelluloses, such as wood gum or xylan, may be present in the cell wall. In order to determine whether wood gum or xylan were present in the original material used, the following experiments were carried out with sections of stems of eight species. Some sections were treated with 5 % aqueous caustic soda in the cold and the usual phloroglucinol test applied, both in the cold and on warming.

The latter test was also made on untreated material both in the cold and on warming. The xylan is removed by caustic soda. Xylan is coloured by phloroglucinol on warming, while lignin is coloured in the cold [Wheeler and Tollens, 1889]. If a positive reaction is produced on the untreated material in the cold this is due to lignin alone, but the coloration produced on warming is due to the combined action of lignin and xylan. Naturally if xylan is removed with caustic soda, any coloration is due to lignin alone. Maximum effect was produced if the sections, which had been treated with caustic soda, were left in water for some days before being treated with phloroglucinol.

The colour produced with phloroglucinol was even more brilliant when the sections were treated with caustic soda, which seems to show that in these specimens there was not any appreciable amount of xylan and certainly not enough to have any marked effect in the tests with which this paper is concerned.

The colour produced with phloroglucinol was examined carefully in every case for any reddish-violet tinge, as this indicates the presence of a compound of *iso*-eugenol with oxygen [Herzog and Hillmer, 1929]. Dilute alkalis produce *iso*-eugenol from eugenol, and this on oxidation gives vanillin.

Only in two of the Gymnosperms, *Cycas revoluta* and *Larix europaea* was any purplish tinge perceptible in untreated material in the cold and on warming. In *Larix europaea* when the material had been treated with caustic soda this coloration persisted, but only when the phloroglucinol test was made in the cold. This shows that *iso*-eugenol was not usually present in the material used in these tests.

Caustic soda removes any coniferyl alcohol present, so that none of the above results, after soda treatment, can be attributed to this substance.

The possibility of the connection of vanillin and chlorine with the development of the pink colour obtained in the chloramine reaction having been

established the nature of the compound present was next investigated. Further confirmation of the responsibility of the halogen was afforded by the analogous results which were obtained when bromine was substituted for chlorine [Wood, 1931]. Chlorovanillin, at first sight, appears to be an obvious solution, but this substance is colourless or light yellow in colour, so, should the suspected connection be true, the compound responsible for the coloration must be a product of the reaction of chlorovanillin with either (a) the other reagents used, (b) some substance occurring in the cell or cell-wall, or (c) with both the former and the latter.

Chlorovanillin was prepared according to Hann's method [1925], and experiments were made with it in the endeavour to clear up these points. In the first place, the reagents used, *viz.* sodium hydrogen phosphate and potassium iodide, were investigated.

Chlorovanillin was used (a) dissolved in alcohol, (b) in hot water, alone and in the presence of acetic acid, and separate experiments were carried out with each of these four solutions, using:

- (1) sodium hydrogen phosphate and potassium iodide;
- (2) sodium hydrogen phosphate;
- (3) potassium iodide in which iodine had been dissolved, to obtain the maximum effect of the iodine which may be set free by the chloramine produced from any protein present in the cell-contents.

In no case was there any indication of a pink coloration, hence chlorovanillin and the reagents used are not alone responsible for this pink colour.

Since 5-chlorovanillin can form vanillal derivatives with amines (see above), the next experiments involved the examination of the effect of sodium hydrogen phosphate and potassium iodide upon:

- (a) chlorovanillin and an amine;
- (b) vanillin and a chloramine;
- (c) chlorovanillin and a chloramine.

The slight acidity due to the sodium hydrogen phosphate must be taken into account when considering the results obtained, hence a similar series of experiments was conducted in which the phosphate was omitted.

Further, since the possibility of the effect being due to a substituted compound of vanillin had to be considered, the whole of this part of the work was repeated substituting vanillic acid for vanillin and vanillic acid treated with chlorine for chlorovanillin.

The experiments were conducted using:

- (a) sodium hydrogen phosphate and potassium iodide;
- (b) sodium hydrogen phosphate alone;
- (c) potassium iodide alone, on all the substances enumerated above. All the solutions used were alcoholic, ethyl alcohol being employed. Sodium hydrogen phosphate and potassium iodide were used in the solid form.

The compounds containing chlorine and an amine group used were chloramine-T, *p*-chloroaniline, *o*-chloroaniline, and the compounds containing the amine group alone were dimethylaniline, dimethylaniline hydrochloride, aniline hydrochloride and xyldine acetate. In the first series of experiments the method of procedure was to add the amine to the vanillin in a test-tube, boil and then to add a little solid sodium hydrogen phosphate and potassium iodide, then a few drops of water, just sufficient to effect the solution of these substances, and to boil again. The resulting liquid was then put into small porcelain dishes and a

strip of filter-paper immersed in it, with one end out of the liquid, to observe the effect of the mixture upon cellulose, the object being to simulate an encrusting substance upon a cellulose base as might occur in the plant cell-wall.

Slow evaporation to dryness in the air was allowed to take place and the effect on the filter-paper carefully watched. As one end of the filter-paper was arranged to be out of the liquid, the colour effect produced could be observed as the liquid crept up the paper and then slowly evaporated. By arranging the amount of liquid in each dish carefully, the free end of the filter-paper was kept dry at the top. This was necessary, as many of the colour effects produced appeared as the paper dried just where the wet paper met the dry paper. The second and third series of experiments were conducted in a similar way, except that in the second series the potassium iodide was omitted, and in the third series no sodium hydrogen phosphate was used.

The three series of experiments were repeated using a strip of superfine "Tarantulle" instead of the filter-paper.

Finally, the whole of the experiments were repeated, as a confirmation of the results obtained.

In the latter series of experiments in addition to the filter-paper and superfine "Tarantulle", strips of purified wood-pulp were used.

Vanillin produced a pink colour on these materials when used with *p*-chloroaniline and *o*-chloroaniline in the presence of potassium iodide, the most brilliant effect being produced when both the sodium hydrogen phosphate and the potassium iodide were employed. Chlorovanillin produced a similar pink colour with *p*-chloroaniline and *o*-chloroaniline when potassium iodide was present alone and also when sodium hydrogen phosphate was used as well.

Vanillic acid gave a slight pink coloration in the presence of *o*- and *p*-chloroaniline respectively when the iodide was present. It is possible that this may be due to minute traces of vanillin in the vanillic acid.

Vanillic acid treated with chlorine gave indefinite results, owing to the liberation of iodine, which obscured any other colorations that might have occurred.

There was some indication of a faint pink coloration with both *o*- and *p*-chloroaniline, possibly due as indicated above to minute traces of vanillin.

In the last series of experiments it was found that frequently a considerable time had to elapse, often as much as twelve hours, before the colour developed, so that in every case the strips of material were examined the following day, in addition to the preliminary examination. This was probably due partly to variation in the atmospheric saturation and partly to variations in temperature which altered the rate at which evaporation of the liquid took place. This evaporation could not be increased by gentle warming as then either the colour did not appear at all, or it appeared only momentarily and rapidly disappeared.

In no case was it a permanent coloration, fading even at the ordinary temperature in the course of a few days.

Similar effects have been observed in sections of plant material which developed a pink coloration in the xylem after being submitted to the chloramine test which has already been described. In these cases also this coloration takes a certain time to develop and fades in the course of a few hours.

As additional proof that chlorine is an element essential to the reaction, and as a control to the other work, another series of experiments was made, using strips of filter-paper, "Tarantulle" and wood-pulp as before, and employing vanillin and vanillic acid respectively, with dimethylaniline hydrochloride and aniline hydrochloride and so having no chlorine present either substituted in the

vanillin nucleus or in the amino compound. These experiments were entirely negative.

Hence the results indicate that:

- (1) potassium iodide (*i.e.* iodine) is essential to the reaction;
- (2) the presence of sodium hydrogen phosphate is without effect upon the reaction;
- (3) an amine and chlorine must be present.

The fact that an amine is essential to produce this coloration eliminates the possibility of its being due to an iodo-derivative of the substituted vanillin.

The conclusion that must be drawn is that in these experiments most probably some kind of vanillal derivative is produced from the vanillin, chlorine and amine, similar to those prepared by Hann *et al.* [1929] and that it reacts with the iodine to produce a pink-coloured substance, though whether the compound is an additive or substituted one remains to be determined. The method of experiment introduces many variables including atmospheric oxidation of the amines, but it was employed because it was to similar conditions that plant sections were exposed during the experiments made upon them.

To elucidate the reactions that take place in plant material is a difficult matter, but with reference to the pink coloration produced in the chloramine reaction when employed with such material the following facts have been proved:

- (a) it is associated with vanillin and chlorine;
- (b) it only appears when iodine is subsequently present.

Although the cell-wall does not contain any appreciable amount of protein [Wood, 1926], its close association with the cell-contents, which are rich in amino-compounds, renders these available for possible reactions. The experiments with chemical substances indicate that colorations are produced, when the conditions are such that a vanillal derivative might be formed, which, reacting with iodine, produces a substance resembling in colour and properties that appearing in the lignified tissues of some plants when treated with chlorine and subsequently sodium hydrogen phosphate and potassium iodide.

The inference drawn is that most probably in this reaction chlorine acts upon the vanillin in the cell-wall, forming chlorovanillin which reacts with a chloramine or mixture of chloramines, produced from protein within the cell to produce a vanillal, which gives a coloured substance when iodine is present.

Should the chlorine not affect the vanillin to any marked degree, probably vanillin itself may react with the chloramines from the cell-contents and still produce the coloration.

It is possible that both series of reactions take place simultaneously.

Having found some indication of the kind of substance producing this coloration, and the chemical experiments with filter-paper, "Tarantulle" and wood-pulp showing that it is possible that the vanillin responsible is absorbed or at least very loosely united with some form of lignin as a base, experiments are now being carried out to investigate these points and the chemical nature of the base.

Since it has been found that there is more vanillin in older than in younger tissues, and more in autumn than in spring xylem, the author suggests that this substance may be present as an excretory product, which is set free when the function of the tissues becomes more largely that of mechanical support. This upholds the theory that has been advanced that the vanillin present is either adsorbed upon the lignin base or only loosely associated with this base.

SUMMARY.

1. When investigating the origin of a pink colour produced in lignified tissues when the chloramine reaction is employed, differential tests for coniferin and vanillin were made.

2. Experimental work is described in which it is shown that the coloration is not due to lignone chloride or to the products of chlorination removable by sodium sulphite, that it is closely associated with the presence of iodine and also of lignin, but that the nature of the lignin itself is not concerned, so that the latter association is either very loose or the substance is encrusted on the lignin. It is shown that vanillin is present when the coloration is produced, and that there is more vanillin in older than younger tissues, in autumn than spring wood, in cortical than in stelar sclerenchyma, and that it is naturally present in the cell-wall. Hemicelluloses and coniferyl alcohol are not responsible for the observed effect.

3. Under similar conditions to those used with plant sections experiments were made with vanillin, chlorovanillin, vanillic acid, vanillic acid treated with chlorine, and amines or chloramines, in which filter-paper, "Tarantulle" and wood-pulp were used upon which to "encrust" the compounds concerned. Indications were obtained that a vanillal derivative was formed which reacted with iodine to produce a similarly coloured substance to that obtained in plant tissues.

4. The conclusion drawn is that vanillin, in loose association with some form of lignocellulose, is acted upon by chlorine producing a chlorovanillin, which with the chloramines produced from protein within the cell, forms a vanillal derivative which gives a pink-coloured substance in the presence of iodine.

5. The possibility that vanillin is an excretory product, produced when the function of the tissues concerned has become chiefly that of mechanical support, is discussed. This theory is upheld by the fact that vanillin predominates in older tissues and in autumn wood.

These investigations were carried out in the Botanical department of Birkbeck College, University of London, and have been assisted by a grant from the Royal Society and another from the Dixon Fund of the University of London. The author desires to express her thanks to Prof. Dame Helen Gwynne Vaughan for valuable help and advice during the progress of the work and to Prof. Sugden for many helpful criticisms and suggestions.

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