Botany. -- "*On the demonstration of carotinoids in plants.* Third communication: *The leaf of Urtica dioica L., the flower of Dendrobium thyrsiflorum Rchb. fil. and Haematococcus pluvialis Flot.*" Prof. By C. VAN WISSELINGH. (Communicated by Prof. J. W. MOLL).

(Communicated in the meeting of October 26, 1912).

In the first and second communications I have shown that carotinoids in plants present differences in the colour and shape of the crystals and in their behaviour towards reagents and solvents. It is obvious that the presence of different chemical bodies cannot be assumed when the crystals only differ in colour and shape or when only a slight difference can be observed on the addition of a reagent or a solvent. When, however, important differences in colour and shape accompany a very remarkable difference of behaviour towards reagents and solvents, then such a conclusion may be justified. I will show by means of a few striking examples that the latter case applies to the carotinoids found in plants.

Leaf of Urtica dioica L.

The substances accompanying the chlorophyll of the stinging-nettle have been accurately investigated chemically. We know from the investigations of WILLSTÄTTER and MIEG [1]) that two carotinoids are present in the leaves of the stinging-nettle, carotin identical with Daucus-carotin, and xanthophyll. These chemists found four times as much xanthophyll as carotin.

When leaves of Urtica dioica or leaf fragments are placed in MOLISCH's reagent and examined after a few days, there is found in each cell containing-chlorophyll an aggregate of red crystals resembling small parallelograms or needles and of orange-yellow plates, which are several times more long than broad and show more or less rounded ends; sometimes a few orange-yellow curved filamentous crystals project from the aggregate. We readily observe that the orange-yellow crystals form the greater part of the aggregates. If the crystals are investigated with reagents and solvents, differences become evident. When they are treated with sulphuric acid of 76 % they all finally become blue, but the orange-yellow ones are coloured first. The red ones often retain their own colour for a long time. The

[1]) RICHARD WILLSTÄTTER und WALTER MIEG, IV. Ueber die gelben Begleiter des Chlorophylls, JUSTUS LIEBIG's Annalen der Chemie, 355. Bd. 1907, p. 1.

different crystals are then distinguished very clearly with sulphuric acid of $66^1/_2$ %, only the orange-yellow crystals become blue.

If preparations with crystals separated by the potash method are placed on the slide in a solution of chloralhydrate (7 in 10) then after a time, for example, $1^1/_2$ hours, the aggregates appeared much changed; the orange-yellow crystals had been dissolved and the red needles or small parallelograms remained behind, sometimes still united. After 24 hours they had not wholly disappeared from the preparations.

With soap-spirit also (Pharm. Nederl. Ed. IV, without oil of lavender) a great difference in solubility was proved. After being one day in soap-spirit the orange-yellow crystals had disappeared from the preparations, whilst the red remained behind.

I obtained a still more striking result with a solution of phenol in glycerine (3 to 1). If this mixture is allowed to flow under the cover-slip, the orange-yellow crystals are seen to dissolve quickly whilst the red ones are unchanged even after 24 hours.

The investigation of the orange-yellow and red crystals can be facilitated in the following manner. Leaf fragments are placed for two hours in a 10 % solution of oxalic acid and then put into MOLISCH's reagent. In the tissue large red and yellow crystal-aggregates are thus formed, which can easily be studied.

On consulting the paper of WILLSTÄTTER and MIEG [1]) on carotin and xanthophyll, we must conclude that, of the crystals described, the red are carotin and the orange-yellow xanthophyll.

As in the leaves of Urtica dioica so in many other cases, in flowers, leaves and algae, I have been able to distinguish yellow, orange-yellow or orange-coloured crystals and red or orange-red ones, of different shape, which behave differently towards reagents and solvents and indeed in a more or less corresponding way. I do not doubt that in all these cases the plant contains different carotinoids side by side, in many cases probably a carotin together with a substance which belongs to the xanthophylls. I think it not improbable that the same carotin and the same xanthophyll are often found together, but I also consider that in a number of cases another carotin or xanthophyll is present. WILLSTÄTTER and ESCHER [2]) have already established the presence, in the fruit of Solanum Lycopersicum, of a carotin, lycopin, differing chemically from Daucus-carotin.

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[1]) l. c.

[2]) RICHARD WILLSTÄTTER und HEINR. H. ESCHER, Ueber den Farbstoff der Tomate, HOPPE-SEYLER's Zeitschr. für Physiol. Chemie, 64. Bd. 1910, p. 47.

The flower of Dendrobium thyrsiflorum. Rchb. fil.

The flower of Dendrobium thyrsiflorum Rchb. fil., is an example of an object containing a carotinoid, differing from those so far described. After treatment by the potash method, I found in the cells orange-yellow (KLINCKSIECK et VALETTE 151) much curved filamentous crystals, orange-yellow (151) whetstone-shaped plates and large and small aggregates of brightly coloured orange (101 though inclining towards orange-red 81) thin acicular crystals. The difference in colour is very striking. Some cells are more especially filled with one shape, others with the other shape. In this case I could observe no difference on using sulphuric acid of varying strength, nor with bromine water, but on the other hand iodine in potassium iodide solution brings it out. With the latter reagent the orange-yellow crystals at once become a fine green. The orange-yellow aggregates suffer no change of colour whatsoever, not even after 24 hours. The contrast in the colour of the crystals is very striking. So far as concerns their solubility in a solution of phenol in glycerine (3 to 1) the crystals also differ much. When the solvent is allowed to flow under the cover-slip, the orange-yellow crystals are at once seen to deliquesce, and form with the solvent yellow globules which dissolve entirely. On the other hand the orange-coloured aggregates do not at first show any signs of dissolving. Sometimes they are seen in the midst of the globules that are formed. Finally all the orange aggregates can be seen in the yellow solution of the orange-yellow crystals. Slowly the orange crystals dissolve also. If the preparations are placed for one day in the mixture of phenol and glycerine, the orange crystals also dissolve.

Because of the difference in colour, different behaviour towards iodine in potassium iodide solution and different solubility in phenol, I conclude that two different carotinoids occur in the flower of Dendrobium thyrsiflorum. The one of these which is to some extent reddish-orange in colour, is a carotinoid that is not common in plants. Such a carotinoid I have found only in Dendrobium thyrsiflorum. Its colour and other properties makes me inclined to think that it belongs to the xanthophylls rather than to the carotins.

Haematococcus pluvialis Flot.

The colouring matter of this interesting alga has been investigated by ZOPF[1]). The result of his enquiry was that it possesses not one but two

[1]) W. ZOPF, COHN's Hämatochrom ein Sammelbegriff, Biologisches Centralblatt, XV. Bd. 1895, p. 417.

colouring matters, which must be considered to be carotinoids. ZOPF found a yellow carotinoid such as is commonly found in green plants and a red one to which the alga is indebted for its frequently dark blood- or brown-red colour and for its name. ZOPF succeeded in separating the two colouring matters in the following way. The crude alcoholic extract of the alga was saponified with caustic soda. The chlorophyll was thus changed into a sodium compound, the fat into a soap and glycerine, the yellow carotinoid was set free and the red one converted into a sodium compound insoluble in water. When the saponification products after dilution with water, were treated with petroleum ether, the yellow carotinoid was removed, whilst the sodium compound of the red one separated out. After purifying the sodium compound the carotinoid was set free by means of dilute sulphuric acid, it was taken up in ether and investigated spectroscopically. The red carotinoid differs spectroscopically from the yellow, in colour and in the colour of its solutions, and also in its power of combining with alkalis and alkaline earths.

Haematococcus pluvialis has recently also been investigated by JACOBSEN [1]). By means of MOLISCH's potash method he obtained separation of crystals, but on the other hand he was unsuccessful with dilute acids and with TSWETT's resorcinol solution. The accuracy of ZOPF's results remained undecided.

Mr. JACOBSEN was kind enough to send me a culture of Haematococcus pluvialis on agar, and thus I was given an opportunity of studying this remarkable alga and of confirming the above mentioned conclusions of JACOBSEN. As is clearly shown by his beautiful plates, the aplanospores differ very much as regards colour; some have a green content, in consequence of the chlorophyll they contain, others are green at the periphery and red in the centre, whilst in others again the green of the chlorophyll is entirely masked and there seems to be only a red content. The red colouring matter is combined with a liquid fatty substance or, more accurately, is dissolved in it. This substance occurs in the form of globules in the cell-content.

As is to be expected, aplanospores which at first sight show differences, yield different results on investigation. In the green spores orange-yellow crystals were quickly separated out by MOLISCH's reagent; generally these are shaped like curved needles, which are often united into bundles; sometimes orange-yellow crystal plates were also observed. In addition to these plates, there were also a

[1]) H. C. JACOBSON. Die Kulturbedingungen von Haematococcus pluvialis, Folia Microbiologica, I, 1912, p. 24 et seq.

few small red plates, shaped like parallelograms. On the addition of sulphuric acid of 66$^1/_2$ or 76 % the orange-yellow crystals become blue without any deliquescence to globules or solution being observed. This takes place when sulphuric acid of 95 % is used. The small red crystals, shaped like parallelograms are not so quickly colcured blue as the orange-yellow ones or a more concentrated acid must be applied in order to colour them blue. The crystals also behave differently with respect to phenol glycerine. The orange-yellow quickly dissolve in it whilst the red remain undissolved. The orange-yellow crystals behave therefore like xanthophyll-crystals and the red ones like carotin-crystals. The investigation of the green aplanospores therefore gives no special result. Two carotinoids are found to accompany chlorophyll, an orange-yellow one and in small quantity a red one, as is usual in green plants.

In those aplanospores which are more or less red in colour there are found after treatment with MOLISCH's reagent reddish-violet crystal aggregates and, frequently, curved band shaped crystals. I now leave out of further consideration the small red crystals shaped like parallelograms. The crystals do not seem to be so easily separated out in the red aplanospores as in the green ones. It is advisable to allow MOLISCH's reagent to act for at least some days in order to decompose the fatty substance which tenaciously retains the colouring matter. If any of the fat remains behind, the investigation becomes more difficult in consequence.

By means of sulphuric acid of 66$^1/_2$ the reddish-violet crystals become blue, also with 76% sulphuric acid, but in this case the action is accompanied by partial solution, which sometimes is preceded by deliquescence. The surrounding medium becomes blue. The behaviour of the reddish-violet crystals towards sulphuric acid of varying strength is therefore different from that of the orange-yellow crystals. In a solution of phenol in glycerine (3 to 1) the reddish-violet crystals easily dissolve, and colour the solvent dark reddish-violet.

The crystals were not at one time of an orange-yellow colour, and at another time reddish-violet, but in many cases they oscillated between the two colours. Orange-yellow and reddish-violet crystals were never observed side by side in the same cell. These facts and the solution in 76 % sulphuric acid, as described, led me to suppose that the reddish-violet crystals were perhaps mixed crystals composed of two carotinoids. I then tried to separate them with solvents, and succeeded. The crystals often completely dissolve in acetone or absolute alcohol; the orange-yellow carotinoid remains in solution, but the other quickly separates out again in the cells in the form

of numerous small violet platelets. The experiment can be made in a test tube and also on a microscope slide. Under the microscope the process of solution, the yellow-coloration in and round the cells and the separation of the violet platelets can be seen.

The phenomena observed can be explained in the following way. The orange-yellow carotinoid is fairly easily soluble in acetone or absolute alcohol; the other one is practically insoluble, but its solubility is increased by the presence of the orange-yellow one, with which it forms mixed crystals. A solution of both is produced in the cells, and is quickly diluted, and this brings about that the carotinoid insoluble in acetone or in absolute alcohol separates out. I am confirmed in this opinion by an observation of ZOPF [1]). When he extracted the yellow carotinoid from the aqueous solution of the saponification products with petroleum-ether, the other separated out beneath the petroleum-ether.

I cannot distinguish any definite form in the violet platelets. They behave in the following way towards reagents and solvents. With sulphuric acid of 66½ % the colour is not modified or only slightly so, but with 76 % sulphuric acid the crystals quickly take a blue colour and this is speedily followed by dissolution. In a saturated zinc chloride solution in 25 % hydrochloric acid and in a saturated antimony trichloride solution in 25 % hydrochloric acid they become blue, then the crystals generally deliquesce to blue globules and dissolve. The solutions are bluish-violet or blue. With bromine water a very transitory bluish-green colour is observed. In a solution of phenol in glycerine (3 to 1) the crystals dissolve, whilst the solvent becomes bright reddish-violet.

If the reddish-violet crystals obtained from the red aplanospores by means of MOLISCH's reagent are compared with those separated out from alcohol and acetone and with the orange-yellow ones obtained from the green aplanospores by MOLISCH's reagent, then the first mentioned crystals, so far as their properties are concerned, must be placed between the other two, and this strengthens my belief that they are mixed crystals.

I must here remark that according to ZOPF [2]) the violet-red or blood-red carotinoid enter into combination with potassium hydroxide. On this account it should be assumed that the reddish-violet crystals, separated out with MOLISCH's reagent contain the potassium compound of the carotinoid and that the crystals obtained with acetone and alcohol consist of this compound. In the microchemical investigation

[1]) l. c. p. 419.
[2]) l. c. p. 419.

I have obtained no indication which points to this. When I treated the crystal platelets got from acetone or alcohol, with dilute sulphuric acid for 24 hours at the ordinary temperature I found them unchanged and moreover their solubility in various solvents remained the same. However this may be, ZOPF's results and mine obtained by different methods agree in this that in Haematococcus pluvialis more than one carotinoid occurs. According to ZOPF there are two, whilst I have succeeded in crystallising out three in the cells and in separating each from the other two.

Finally I must add a few experimental details. By cultivating Haematococcus pluvialis in various solutions, I obtained cultures with different aplanospores, both green and red. I cultivated the alga in the two following solutions: KNO_3 0.01, $(NH_4)_2HPO_4$ 0.01, $MgCl_2$ 0.01, Na_2SO_4 (hydrated) 0.01, H_2O 100 and NH_4NO_3 0.02, K_2HPO_4 0.02, $MgSO_4$ 0.02, H_2O 100 [1]). In the former solution most of the aplanospores had a green content, in the latter a red one, and this was an advantage in the investigation. I used a centrifuge for transferring Haematococcus from one solution to another and for washing out the material, which sank to the bottom on centrifuging so that the solution to be replaced could be poured off.

It results from this paper and the two previous ones, that my conclusions differ completely from those of TAMMES and of KOHL. The assumption, that only one carotinoid occurs in the vegetable kingdom, is not based on sufficient evidence. It was the result of microscopic and micro-chemical research. Nevertheless I believe that such investigation may contribute to our knowledge of carotinoids, provided that it be carefully carried out. I have found, for instance, that when different carotinoids occur in a plant or organ, it is in many cases at least possible, to distinguish them, that unknown ones can be detected (Dendrobium thyrsiflorum) and that sometimes a greater number can be demonstrated than has hitherto been possible by other means (Haematococcus pluvialis). The results I have obtained are in agreement with the macro-chemical investigation (Urtica dioica). When the quantity of material is insufficient for the application of other methods, a microscopical and micro-chemical inquiry is still practicable and moreover demands comparatively little time. The botanist who concerns himself with such work, should however consider, that it is impossible to solve by means of a few colour-reactions difficult chemical problems, such as, for example, the

[1]) H. C. JACOBSEN, l. c. p. 8.

identification of the carotinoids of different plants. As ZOPF [1]) justly says careful macro-chemical investigation alone can lead to decisive results in such cases.

Chemistry. — "*Equilibria in ternary systems I*". By Prof. F. A. H. SCHREINEMAKERS.

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On the equilibria occurring in ternary systems between liquid and vapour different theoretical [2]) and experimental [3]) investigations have already appeared previously. We will now discuss a few cases where, in addition to liquid and vapour, solid substances occur also.

The system F L G.

We choose for F a ternary compound and will assume that the three components occur in the vapour.

We now choose at a definite constant temperature T such a pressure P that no vapour can form. The isotherm can then consist only of the saturation line of the solid substance F. This saturation line is a closed curve surrounding the point F like the closed curve of Fig. 1, for instance.

On reduction of P a gas region appears somewhere and at the same time a heterogeneous region separating the gas region from the liquid region. In Fig. 1 the gas region is indicated by G, the liquid-region by L; the drawn line is the liquid-line, the dotted one the gas- or vapour line of the heterogeneous region. The straight lines drawn in this heterogeneous region unite the liquids with the vapours with which they can be in equilibrium.

We now have in Fig. 1 two homogeneous regions, namely the liquid region L and the gas region G; in addition we find two heterogeneous regions.

In one of them a mixture dissociates into L + G; we will call

[1]) W. ZOPF, Zur Kenntnis der Färbungsursachen niederer Organismen (Dritte Mitteilung), Beiträge zur Physiol. u. Morph. niederer Organismen, 1892, Erstes Heft, p. 36.

[2]) J. D. VAN DER WAALS. These Proc. Vol. IV p. 448, 539, 681; Vol. V p. 1, 121, 225. (1902).

F. A. H. SCHREINEMAKERS. Zeitschr. f. phys. Chem. **36** 257, 413, 710 (1901) **37** 129 (1901) **38** 227 (1901) **43** 671 (1903)

B. M. VAN DALFSEN. Dissertation, Amsterdam. (1906).

[3]) F. A. H. SCHREINEMAKERS. Zeitschr. f. Phys. Chem. **39** 485, **40**. 440, **41**. 331 (1902), **47** 445, **48** 257 (1904).

B. M. VAN DALFSEN. l.c.