On intravital precipitates.

by

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The precipitates caused by basic substances in living plant cells have long attracted the attention of investigators and the literature on this subject is already voluminous. Charles Darwin was the first to investigate these precipitates. He [1]) first mentions the phenomenon in his work on insectivorous plants, and calls it aggregation. As de Vries [2]) has pointed out, Darwin includes two different phenomena under this name: in the first place, the movements which he discovered in the protoplasm of the cells of the glands of *Drosera rotundifolia* and other insectivorous plants, movements which occur whenever stimulation causes an increased secretion, and in the second place the precipitates which occur in the protoplasm when ammonium carbonate is used as a stimulus.

As Ch. Darwin [3]) has shown, precipitates with ammonium carbonate and with ammonia are also formed in many other cases in living plant cells. He stated that the precipitates no longer occur when the preparations are heated in water for 2 to 3 minutes to the boiling point and on this account he was inclined to consider the

[1]) Charles Darwin, Insectivorous plants. 1875, p. 38. Chapter III.

[2]) Hugo de Vries, Ueber die Aggregation im Protoplasma von *Drosera rotundifolia*. Bot. Zeit. 44. Jahrg. 1886, p. 1.

[3]) Charles Darwin, The Action of Carbonate of Ammonia on the Roots of certain Plants. The Journal of the Linnean Society. Botany. Vol. XIX. 1882, p. 239.

reaction as a vital one. With regard to the chemical nature and physiological significance of the substance of which the precipitates are composed, Darwin expressed himself very cautiously. He supposed that they consist of protein and considered that we have to deal with an excretion product. He concluded his last-mentioned paper as follows: "But I hope that some one, better fitted than I am, from possessing much more chemical and histological knowledge, may be induced to investigate the whole subject". From this it follows that Darwin may have thought that another explanation of the phenomenon he had discovered was also possible.

Fr. Darwin [1]) defends his father's views, as far as the chemical nature of the precipitate is concerned, which ammonium carbonate produces in the tentacles of *Drosera rotundifolia*. De Vries [2]) is also of the opinion that the precipitate belongs to the group of the proteins, as far as its behaviour towards reagents is concerned.

The precipitates caused by ammonium carbonate in the cell-sap of *Spirogyra* and of other plants, have also been investigated by Pfeffer [3]). In his opinion they are composed of protein tannate and give reactions both with protein- and with tannin-reagents.

Loew and Bokorny [4]) have written numerous papers

[1]) Francis Darwin, The process of aggregation in the tentacles of *Drosera rotundifolia*. Quarterly journal of microsc. science. Vol. XVI. 1876, p. 309.

[2]) l. c. p. 42 ff. and 57 ff.

[3]) W. Pfeffer, Ueber Aufnahme von Anilinfarben in lebenden Zellen. Untersuchungen aus dem botan. Institut zu Tübingen. 2. Bd. 1886—1888. p. 239 ff.

[4]) O. Loew und Th. Bokorny, Ueber das Vorkommen von activem Albumin im Zellsaft und dessen Ausscheidung in Körnchen durch Basen. Bot. Zeit. 45. Jahrg. 1887. p. 849. — Ueber das Verhalten von Pflanzenzellen zu stark verdünnter alkalischer Silberlösung. Bot. Centralblatt. 10. Jahrg. 1889. XXXVIII. Bd. p. 581 and 614. XXXIX. Bd.

on the subject of precipitation in living plant cells by various basic substances. In these publications, the same points generally have been stated, so that they can here be dealt with together.

In the opinion of these two investigators the precipitates which have been caused in the cells by ammonium carbonate, antipyrine and caffeine consist of active protein. The bodies of which the precipitates are composed, called by these writers proteosomes, can be formed both in the protoplasm and in the cell-sap. According to Loew and Bokorny the formation of proteosomes is a real vital reaction. When the cells have been killed, the reagents mentioned cannot any longer bring about the phenomenon, because the active protein has been changed into passive protein.

The two authors describe peculiarities of the precipitates and mention positive results which they obtained with various protein reagents. The precipitates are stated to be composed either exclusively of active protein or they contain also other substances, such as tannin, but it is emphatically declared in this connection, that the admixture of other substances is "unwesentlich".

p. 369. XL. Bd. p. 161 and 194. — Versuche über aktives Eiweiss für Vorlesung und Praktikum. Biologisches Centralblatt. 1891. XI. p. 5. — Zur Chemie der Proteosomen. Flora. 1892. Ergänzungsb. p. 117. — Aktives Eiweiss und Tannin in Pflanzenzellen. Flora. Cl. 1911. p. 113—116. Autoreferat. Botan. Centralblatt. 32. Jahrg. 1911. I. Halbjahr. Bd. 116. 1911. p. 361.

Th. Bokorny, Neue Untersuchungen über den Vorgang der Silberabscheidung durch actives Albumin. Jahrb. f. wiss. Bot. XVIII. Bd. 1887. p. 194. — Ueber die Einwirkung basischer Stoffe auf das lebende Protoplasma. l. c. Bd. XIX. 1888. p. 206—220. — Ueber Aggregation, l. c. Bd. XX. 1889. p. 427. — Zur Kenntniss des Cytoplasmas. Ber. d. d. bot. Gesellsch. Bd. VIII. 1890. p. 101. — Zur Proteosomenbildung in den Blättern der *Crassulaceen*. l. c. Bd. X. 1892. p. 619. — Ueber das Vorkommen des Gerbstoffes im Pflanzenreiche und seine Beziehung zum activen Albumin. Chemiker-Zeit. 1896. No. 103. p. 1022

The views of Loew and Bokorny that precipitates caused in living plant-cells by ammonium carbonate, ammonia, antipyrine, caffeine and other basic substances are protein precipitates have been contested by Af Klercker[1]), Klemm[2]) and Czapek[3]). All these consider that the precipitates are in reality tannin precipitates. On treating these and the cell-sap with protein reagents they always obtained negative results, while on the other hand tannin reagents gave positive ones.

It is worthy of notice that Klemm in connection with his experiments with methylene-blue regards tannin as of secondary importance in the case of *Spirogyra*. Here another as yet unknown substance might cause the precipitate.

Czapek states that the precipitates may sometimes take up other substances, such as colouring-matter from the cell-sap and lipoids. Also, in spite of the negative results of experimental investigation, he thinks that the precipitates sometimes may contain protein substances, because the latter occurs in the cells.

There is a divergence of opinion between the last-mentioned investigators as to the place where the precipitates occur. Af Klercker holds that they occur in the cell-sap. Klemm thinks that detailed study will probably show more and more, that they are formed exclusively in the cell-sap and not in the protoplasm or in both, as

[1]) J. E. F. Af Klercker, Studien über die Gerbstoffvakuolen. Inaug. Diss. Tübingen 1888.

[2]) P. Klemm, Beitrag zur Erforschung der Aggregationsvorgänge in lebenden Pflanzenzellen. Flora 1892, p. 395. — Ueber die Aggregationsvorgänge in *Crassulaceen*zellen. Berichte d. d. bot. Gesellsch. Bd. X. 1892, p. 237.

[3]) F. Czapek, Ueber Fällungsreaktionen in lebenden Pflanzenzellen und einige Anwendungen derselben. Ber. d.d. bot. Gesellsch. Bd. XXVIII. 1910. Heft V. p. 147.

Bokorny wrongly asserts for the *Crassulaceae*. On the other hand Czapek believes, that they can occur in the cell-sap and in the cytoplasm as, inter alia, may be the case in the leaf of *Echeveria*.

In 1897 an interesting investigation by Overton [1]) was published. He experimented on *Spirogyra* with ammonia, amines, caffeine, pyridine, quinoline, piperidine, and alkaloids. He has no doubt at all that the precipitates which are found in the cell-sap are compounds of tannin with the above substances. He describes in detail the phenomena which are brought about by solutions of caffeine of different strength, namely, when successively stronger or weaker solutions are added. In explanation it is said that the compound of tannin and caffeine are in a condition of hydrolytic dissociation.

Shortly before the appearance of Czapek's publication quoted above I [2]) made a preliminary communication on the demonstration of tannin in the living plant and on its physiological significance. While searching for a method of studying the physiological significance of tannin in Spirogyra my attention was also drawn to antipyrine and caffeine, substances which had not then been used for that purpose.

Like Overton I described the precipitates as tannin precipitates and have never for a moment thought of regarding them as protein precipitates. All the results were in agreement with the view that they were tannin

[1]) E. Overton, Ueber die osmotischen Eigenschaften der Zellen in ihrer Bedeutung für die Toxikologie und Pharmakologie. Zeitschr. f. Physikal Chemie XXII. Bd. 1897, p. 189.

[2]) C. van Wisselingh, Over het aantoonen van looistof in de levende plant en over hare physiologische beteekenis. Verslagen der Koninkl. Akad. van Wetenschappen te Amsterdam, Maart 1910. On the tests for tannin in the living plant and on the physiological significance of tannin. These Proc. XII, p. 685.

precipitates. In the paper referred to above I drew attention to the fact that they were earlier described erroneously by Loew and Bokorny as protein precipitates. To this these authors [1]) soon replied.

In connection with the various views on the chemical nature of intravital precipitates, I have further considered whether protein might occur in them and subsequently performed some experiments on *Spirogyra maxima (Hass.)* Wittr. which in my opinion render much more certain the view that the precipitates contain no protein, than was already the case. It follows moreover from these experiments that the precipitates occur in the cell-sap and not in the cytoplasm. I will first explain this point.

Bokorny [2]) assumes that in *Spirogyra* proteosomes are formed in the cytoplasm as well as in the cell-sap. He thinks he has furnished proof of this by combining the formation of proteosomes with abnormal plasmolysis.

He placed *Spirogyra* in a mixture of equal parts of a 10 % solution of potassium nitrate and a 0.1 % solution of caffeine. After the action proteosomes were observed in the cytoplasm as well as in the contracted vacuole. Klemm [3]) agrees with Bokorny with respect to the localisation of the precipitate in *Spirogyra*. Klemm first allowed the precipitate to occur and then plasmolysed.

When Bokorny [4]) first brought about abnormal plasmolysis with a 10 % solution of potassium nitrate and subsequently allowed basic substances to act, he found only

[1]) O. Loew and Th. Bokorny, Aktives Eiweiss und Tannin in Pflanzenzellen. l.c.

[2]) Th. Bokorny, Neue Untersuchungen über den Vorgang der Silberabscheidung durch actives Albumin. l. c. p. 206

[3]) P. Klemm, Beitrag zur Erforschung der Aggregationsvorgänge in lebenden Pflanzenzellen. l. c. p. 407.

[4]) Th. Bokorny, Ueber die Einwirkung basischer Stoffe auf das lebende Protoplasma. l. c. p. 209.

proteosomes in the contracted vacuole and explains this by assuming that on the death of the protoplasm the active protein is changed into passive and that then no more proteosomes can be formed, so that a vital reaction in given no longer.

Without considering this explanation for the present, I content myself with pointing out that, when the above experiments are repeated, careful observation already shows that so far as the localisation of the precipitate is concerned, Bokorny's view, accepted by Klemm, is incorrect.

When first abnormal phasmolysis is produced with a 10 % solution of potassium nitrate and this is followed by application of a 10 % solution of potassium nitrate which contains in addition 1 % antipyrine or 0.1 % caffeine or if a rod with ammonia is then held above the preparation, precipitation takes place exclusively in the contracted vacuole. If the reagents are allowed to act simultaneously or in reverse order, i.e. if the precipitation is first produced by the antipyrine or caffeine solution and followed by abnormal plasmolysis, then it is seen that the contraction of the vacuole is accompanied by continued expulsion of the precipitate which is surrounded by cytoplasm. If the whole process is not followed under the microscope, but if the final result alone is observed, then it is easy to imagine that precipitation has also taken place in the cytoplasm and thus to draw an erroneous conclusion, as did Bokorny.

As already mentioned, some investigators have obtained all possible protein reactions with the intravital precipitates, whilst others have only got negative results. I may remark that protein reactions at our disposal are in general not sensitive as microchemical reactions. When these reactions, namely, the test with sugar and sulphuric acid, the biuret test, Millon's test and the nitric acid test, are tried on minute pieces of coagulated egg-white, the various colorations can indeed be easily seen, but yet it is noticed that

most of the reactions can have no great value for microscopic investigation. With Millon's reaction, and the nitric acid and biuret tests the colour with very thin pieces of egg-white is very faint.

With a minute object such as the protoplast of *Spirogyra* which in addition to protein contains also other substances, little is to be expected from the three last-mentioned reactions. In accordance with this I did not obtain favourable results, but the reaction with sugar and sulphuric acid yielded better ones. The objects were left in a sugar solution for some time and then sulphuric acid was allowed to flow in. I used a mixture of 9 parts by weight of concentrated sulphuric acid and one part by weight of water, therefore sulphuric acid of $85^1/_2\,\%$. This mixture has a much smaller carbonising action on the sugar than concentrated sulphuric acid and is therefore to be preferred. With small pieces of egg-white the reaction is very striking. At first the colour is red (compare Klincksieck et Valette, Code des Coleurs, 1908, N°. 16 and 21), sometimes with a very weak violet tint, then pure red (Kl. et V. N°. 41) and afterwards orange-red (Kl. et V. N°. 51). With very thin pieces the colour is still observable. The reaction is also very suitable for microchemical use. In *Spirogyra* the protoplasts are coloured a distinct light red, the nucleus with the nucleolus and the pyrenoids are darker.

At this point I mention a reaction which is indeed not a real protein reaction, but which may sometimes serve for the indirect microchemical demonstration of protein, namely, the test with tannin and iodine in potassium iodide solution. In botanical papers I have found it stated that iodine in potassium iodide solution gives a precipitate with a tannin solution and can be used to demonstrate tannin microscopically. I have not been able to confirm this and it is moreover in conflict with what is generally stated in chemical handbooks, namely, that a tannin solution is

coloured violet by means of an iodine solution such as iodine in potassium iodide. Of course care must be taken that the violet colour is not masked by the addition of much iodine. In chemical books I have found no mention of a precipitate.

When hide-powder or pieces of egg-white are brought into contact with a tannin solution, washed with water after some time and then treated with iodine in potassium iodide solution, they usually show a dirty brown colour; after repeated washing with water a fine violet colour (Kl. et V. 591, 596) appears, however.

This reaction can also be applied to *Spirogyra*, but in this case the tannin solution is unnecessary, because *Spirogyra* itself contains tannin in solution in its cell-sap. The filaments of *Spirogyra* are warmed to 60° in water. They are then killed, the tannin leaves the vacuole and partly combines with the protein of the protoplast. If the filaments are now treated with iodine in potassium iodide solution and afterwards washed with distilled water until the iodine reaction of the starch disappears, it is then found that those parts of the protoplast which are rich in protein, are coloured violet. The nuclei with the nucleoli are finely coloured, the pyrenoids more faintly.

I have been no more able to find protein in the intravital precipitates with caffeine, antipyrine and ammonium carbonate than were Af Klercker, Klemm and Czapek; neither when te precipitates with caffeine and antipyrine had been treated according to Bokorny's [1]) method with $^1/_{10}$ % ammonia and had thus become insoluble.

Nor have I been able to obtain a protein reaction when the precipitates were some weeks old and had become insoluble. *Spirogyra* can, it should be noted, remain alive for several weeks in a 1 % antipyrine-solution and in a

[1]) Th. Bokorny, Zur Kenntnis des Cytoplasmas. l.c. p. 106.

0.1 % caffeine-solution. At first the precipitates aggregate and form globules; gradually their solubility diminishes. When the filaments are then transferred into water, the globules leave vesicles behind, which have disappeared after some days. After a few weeks the globules seem altogether insoluble. In dead cells brown globules are found, which are also insoluble in water. Neither the globules nor their insoluble residues gave even a protein reaction with sugar and sulphuric acid, whilst the protoplast became distinctly coloured red. On the other hand the globules gave tannin reactions.

It is remarkable that Loew and Bokorny [1]), who have repeatedly insisted on the protein nature of the precipitates, assert in one of their latest publications that the colour-reactions for protein substances, such as that of Millon and the biuret reaction, are not the most important protein tests, although they formerly relied on these. Now they prefer coagulation by rise of temperature, by alcohol and by acids.

I treated *Spirogyra*-filaments, with precipitates produced by 1 % solution of caffeine, by Bokorny's method with a saturated caffeine solution containing 20 % alcohol or I exposed the filaments for a short time to the action of 10 % nitric acid or warmed them to 60° in a 1 % solution of caffeine. In the first two cases I observed solution, in the last case coalescence. The results by no means proved the protein nature, as is especially evident from the following experiments.

When I mixed 1 % solutions of gallnut- or of *Spirogyra*-tannin with an equal quantity of a 1 % caffeine-solution and heated the mixture to 60° or added 10 % nitric acid, the precipitate which was formed underwent a modification.

[1]) O. Loew und Th. Bokorny. Aktives Eiweiss und Tannin in Pflanzenzellen. l.c.

It agglutinated more or less and a portion had clearly become much less soluble in water, so that after some days in an excess of water there was still a considerable resinous residue undissolved. It is possible that Loew and Bokorny succeeded by heating and by the action of nitric acid to transform part of the precipitate in the cells into an insoluble modification, but this is by no means a proof of its protein nature.

Loew and Bokorny [1]) declare the formation of protosomes with ammonium carbonate, antipyrine and caffeine to be a true vital reaction. They say that when the cells are dead, formation of protosomes can no longer take place, because the active protein has become passive. I shall proceed to show how, starting from dead material, precipitates can be produced with antipyrine, caffeine and other basic substances, which completely agree with those observed in living material.

That in dead cells of *Spirogyra* no precipitates occur with the above basic substances, is simply due to the fact that the dead protoplast and the cell-wall allow the tannin to escape. A portion of the tannin gets outside the cell and another portion enters into combination with the protein-substances present in the cell. It is specially fixed in the nuclei and the pyrenoids. Now antipyrine, caffeine and other basic substances can obviously no longer cause any precipitate in the vacuole.

It can be proved as follows that in dead *Spirogyra* part of the tannin passes out. Pieces of *Spirogyra*-filaments are placed between slide and cover-slip in a 1 % solution of egg-white or in a $^1/_2$ % gelatin or glue solution. These colloids do not penetrate into the cells and cannot therefore form any precipitate with the tannin of the cell-sap. When

[1]) O. Loew and Th. Bokorny, Ueber das Verhalten von Pflanzenzellen zu stark verdünnter alkalischer Silberlösung. Bot. Centralbl. Bd. XXXVIII. p. 614.

carefully heated above a micro-flame, the cells are successively killed. The tannin passes through the protoplasmic layer and cell-wall and forms a precipitate in the egg-white-, gelatin- or glue-solution. On careful heating the precipitate lies immediately against the *Spirogyra*-filament. The cells which are still alive are not surrounded by a precipitate. It can be established by using solutions of ferric salts, and other tannin reagents, that the precipitate formed outside the filament is a tannin precipitate.

When *Spirogyra* has been slowly heated in water to 60° in a test-tube placed in a water-bath, it dies. In this case much tannin usually combines with the protein present in the protoplast and only a little leaves the cell. When a large quantity of *Spirogyra* was heated to 60° in very little water, the liquid sometimes gave after filtration only a very weak tannin reaction with ferric salts, whilst the nuclei and pyrenoids always gave a distinct reaction. The nuclei and pyrenoids also gave a distinct tannin reaction with iodine in potassium iodide solution. When sufficiently washed out with water they show a fine red violet coloration.

When starting with dead material, it is desired to produce with antipyrine, caffeine and other basic substances precipitates which agree with those occurring in living cells, the following method may be adopted. A number of *Spirogyra*-filaments are taken, washed out with distilled water, which is allowed to drip off as much as possible and then they are heated to 60°, dried as well as possible by means of gentle pressure between filter-paper, and extracted 2 or 3 times with a mixture of 4 parts of ether and 1 part of alcohol, such as is used in the extraction of tannin from gallnuts; the fluid obtained is filtered and evaporated in a vacuum. The residue, which resembles gallnut-tannin, is dissolved in a little distilled water and filtered. We thus obtain a solution, which gives all the possible tannin reactions, with ferric salts, potassium bichro-

mate, egg-white and gelatin solutions, caffeine, antipyrine etc.

The precipitates with antipyrine and caffeine solutions, with pyridine and quinoline-vapour, and other basic substances completely resemble those occurring in living cells: little spheres or globules which show Brownian movement and gradually aggregate to larger masses, which on the addition of water dissolve and behave towards reagents as tannin precipitates, all of which completely resembles what we observe in living cells.

From the above experiments it is evident that what Loew and Bokorny take to be reactions of active protein are in reality none other than reactions of tannin and the proteosomes none other than precipitates of different basic substances with tannin. It is further evident that after death these precipitates can be as distinctly produced as in living cells and can therefore hardly be called vital reactions.

The question what substances the precipitates can contain in addition to tannin-compounds is more difficult to answer than it was to demonstrate the tannin character of the precipitates in living cells. That other substances may be present in the precipitates, is already clear from observations on cells containing red colouring matter as well as tannin in solution in the cell-sap. The precipitates take up the red colouring-matter and large red-coloured spheres finally arise through the aggregation of many globules.

The question whether the intravital precipitates can contain protein will now be dealt with. As already stated Pfeffer [1]) assumes that the precipitate which is produced in *Spirogyra* by ammonium carbonate, consists of protein and tannin, which, according to him, both occur in solution in the cell-sap. The acids present in the cell-sap are

[1]) l. c. p. 239.

supposed to prevent the precipitation of the protein by the tannin. When these acids are neutralised a protein-tannin precipitate is produced according to Pfeffer.

Pfeffer thinks that the formation of the precipitate in *Spirogyra* must be explained otherwise than the precipitation of tannin by ammonium carbonate, because in *Spirogyra* filaments a precipitate occurs with ammonium-carbonate at greater dilution than in solutions of tannin. Af Klercker [1]) has erroneously considered this observation incorrect. I have indeed found it to be correct and I have also come to the conclusion that organic acids can entirely or partly prevent the precipitation of protein and gelatin by tannin.

On the other hand, in order to explain his observations Pfeffer assumes various factors, without proving their existence, whilst he takes no account of other existing factors. In the first place Pfeffer ought to have considered whether the tannin in *Spirogyra* is really identical with gallnut-tannin. It is quite possible that the tannin in *Spirogyra* is a different chemical body from gallnut-tannin and behaves rather differently towards ammonium-carbonate. Then Pfeffer has failed to demonstrate the presence of organic acids in the cell-sap. Also he has not proved the presence of protein in the precipitate and moreover he has not investigated whether the formation of the precipitate may be influenced by other substances.

As to the first point, I have found that gallnut-tannin and *Spirogyra*-tannin in general behave similarly towards reagents and solvents. Also a solution of ammonium-carbonate must be more concentrated in order to produce in a solution of Spirogyra-tannin a precipitate than is necessary to produce it in the living cells of *Spirogyra*. The first point may therefore be left.

It is otherwise with the presence of acids in the cell-sap.

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[1]) l. c. p. 37 ff.

When *Spirogyra* is washed out and then disintegrated, the mass has a faint acid reaction to litmus paper but a solution of gallnut-tannin and of *Spirogyra*-tannin are likewise acid. A suitable microchemical method for demonstrating free acids in the cell-sap, does not appear to exist. No value can be attached to Loew and Bokorny's[1]) method. They lay filaments of *Spirogyra* in a potassium iodide solution and seeing that no iodine is set free, they infer the absence of free acid in the cell-sap. The liberation of iodine by free acid cannot be explained chemically, for although dilute acids might set free hydriodic acid from potassium iodide, they cannot liberate iodine.

I attempted to demonstrate free acid in the living cells of *Spirogyra* as follows. I placed *Spirogyra* in a solution of potassium iodide (0.1 %) and of potassium iodate (0.025 %), but no separation of iodine by free acid was indicated ($5KI + KIO_3 + 6HCl \rightarrow 6KCl + 6I + 3H_2O$).

On heating *Spirogyra* for some time in a 0.1 % solution of citric acid, before placing it in the solution of potassium iodide and iodate a very faint blue colour in the starch and faint violet coloration of the nuclei was to be seen; the latter had taken up tannin from the cell-sap, for in the meantime the cells had perished. This result points to light absorption of citric acid and separation of iodine by this acid. The method seems to yield useful results and probably in the first experiment iodine would also have been liberated, in case *Spirogyra* contained free acid.

It should be noted that *Spirogyra* is very sensitive to dilute solutions of organic acids. In a 0.1 % solution of citric acid, tartaric acid, malic acid, quinic acid, it quickly dies.

On these grounds it is very improbable that *Spirogyra*

[1]) O. Loew and Th. Bokorny, Ueber das Vorkommen von activem Albumin im Zellsaft und dessen Ausscheidung in Körnchen durch Basen. l. c.

contains so much acid that protein and tannin should be able to appear together in soluble form in the cell-sap. The experiments which I am about to describe, also show that Pfeffer has incorrectly interpreted his observations.

Whilst with many reagents it is quite easy to demonstrate tannin in the cell-sap of *Spirogyra* because the cell-wall and protoplasm are permeable to these reagents, the most important tannin-reagents, namely, those which belong to the protein group cannot permeate. For this reason I heated *Spirogyra* in egg-white-, gelatin- or glue-solutions.

On the death of the protoplasts the tannin passes through the protoplasmic layer and the cell-wall and a precipitate is formed outside the cell. If, instead of allowing the tannin to pass out, a little protein solution could be introduced into the cell-sap which contains the tannin and if we could investigate the result, this would go a long way in my opinion towards solving the problem of whether in the cell-sap protein exists in solution as well as tannin. Should the cell-sap remain clear, one might be able to assume that the cell-sap was of such composition as to contain dissolved tannin and protein side by side. If, on the other hand, a small amount of protein-solution produced a precipitate, then this might be taken to exclude the simultaneous presence of the two substances.

I will proceed to explain how I succeeded in introducing a protein-solution into the cell-sap, causing a precipitate which on closer investigation was found to be a compound of tannin and protein.

As I [1]) have previously described, the cytoplasm in *Spirogyra* possesses an alveolar structure. The hyaloplasm forms the walls of the alveoli, which are filled with a watery solution. By the action of reagents the structure

[1]) C. van Wisselingh, Zur Physiologie der *Spirogyra*zelle. Beitr. zum Botan. Centralblatt. Bd. XXIV (1908). Abt. I. S. 190 ff.

is destroyed without the immediate onset of death. Often the hyaloplasm is seen to form a wall, which separates different portions of the contents. If abnormal plasmolysis is produced with, for example, 10 % potassium-nitrate solution then the hyaloplasm forms a wall round the contracted vacuole.

As I [1]) have previously stated, it may not be assumed that this wall is a special organ and accurately represents that part of the protoplast which in the cell constitutes the lining of the vacuole.

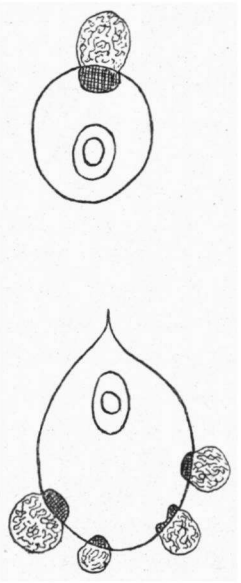
If dilute chloral-hydrate or phenol solutions act on the living cells, other phenomena are again observed [2]). Cytoplasm collects round the nucleus and, taking up water, forms a vesicle whose wall again consists of hyaloplasm and whose content except for the nucleus is chiefly an aqueous solution. Smaller vesicles are formed on the suspensory threads.

If instead of the last mentioned solutions a 5 % solution of ether (5 parts by weight of ether and 95 parts by weight of distilled water or ditch water) is used, then the death of the protoplasts is accompanied by the following phenomena. Cytoplasm flows towards the nucleus and collects there; the suspensory-threads are detached and are taken up by the protoplasmic mass, which has a granular appearance; round the nucleus a vesicle forms, which lies quite free in the cell-sap. The wall of the vesicle is again composed of a hyaloplasmic layer; the nucleus is seen lying inside the vesicle and between the protoplasmic wall of the vesicle and the nucleus there is an aqueous solution, in which some granules can be distinguished. The protoplasmic wall is at first fluid and

[1]) l. c. p. 185 ff and 192 ff.

[2]) C. van Wisselingh, Untersuchungen über *Spirogyra*. Botan. Zeiting. 1902. Heft VI. S. 121 ff.

stretched. When the protoplast dies, this changes; the protoplasmic-wall becomes rigid and often acquires folds and creases. The nuclear-wall also, which is stretched as long as the protoplast lives, contracts irregularly. By the walls different fluids are at first separated; this also is changed by death. When the nuclear wall contracts, we may assume that its content comes into contact with that of the vesicle, but this is not accompanied by any noticeable phenomenon. It is otherwise when the content of the vesicle and the cell-sap come into contact. This takes place at one or more points on the circumference of the visicle. At these points precipitates are produced, but it cannot be seen whether at first small openings or tears occur in the vesicle. It is often possible to distinguish two parts in the precipitates: the one is compact and seems to lie within the vesicle; the other is looser and occurs outside the wall of the latter.



Vesicles round the nucleus with precipitates of protein and tannin.

When the precipitates are investigated with reagents, they are found to consist of protein and tannin. With sugar-solution and $85^1/_2\,^0/_0$ sulphuric acid they become very distinctly red, especially the more compact portion; after treatment with iodine in potassium iodide solution and washing out with water they show a reddish violet colour. With ferric acetate they become blue-black, with potassium bichromate brownish-red.

From these results I think the following conclusions may be deduced. The vesicle contains a solution of protein, which is derived from the cytoplasm and probably occurs

there in soluble condition in the alveolar fluid. When the protein-solution and the cell-sap containing tannin come into contact with each other, the above mentioned precipitates are formed, from which it follows, in my opinion, that in addition to tannin protein in solution cannot be present in the cell-sap. They would at once form an insoluble compound with each other. It is thus impossible that, as Loew and Bokorny assume, the precipitates, which are formed in the cell-sap by basic substances, are protein-precipitates or, as Pfeffer assumes, precipitates of protein and tannin.

In reality they are tannin precipitates. Although the possibility is not excluded that other substances are sometimes present in small quantity, experimental investigation yields the proof, that there can be absolutely no thought of protein-substances in the first place.

Tannin and protein are separated in the living cells in a remarkable manner. Tannin in solution occurs in the cell-sap; proteins can be demonstrated in the nucleus, the chromatophores and the cytoplasm. They are either solid, as for example, the pyrenoids of the chromatophores or dissolved, as in the cytoplasm. The nucleoli which contain a viscous substance, in which the two nucleolus-threads lie [1]) give specially clear protein-reactions.

There still remains the question why a solution of ammonium-carbonate which causes a precipitate in the cell-sap of *Spirogyra*, may be much more dilute than that which produces a precipitate in a solution of gallnut-tannin or of Spirogyra-tannin.

It is obvious that in the water in which *Spirogyra* grows and also in the cell-sap salts are present and I have on this account traced the influence of various salts

[1]) C. van Wisselingh, Ueber den Nucleolus von *Spirogyra*. Bot. Zeit. 1898, p. 202. — Ueber abnormale Kernteilung, l. c. 1903, p. 217.

on the precipitation of gallnut- and *Spirogyra*-tannin by ammonium carbonate. I found that precipitation is favoured by salts; expecially is this the case with calcium salts. The formation of a precipitate in the cell-sap at greater dilution of ammonium carbonate is therefore readily explicable.

Intravital precipitates can in many case also be brought about by aniline dyes. Pfeffer [1]) has described this in detail. In particular he recommended methylene-blue which gradually produces a precipitate in the living cells of *Spirogyra* with a very dilute solution.

In Pfeffer's [2]) opinion the tannin is completely precipitated as a methylene-blue compound. The precipitate is also supposed to contain protein. When the solution of methylene-blue is sufficiently dilute, the precipitation is regarded as innocuous to the vital processes. The explanation which Pfeffer gives of the phenomenon he has observed is incorrect, whilst he greatly overestimates the value of the results obtainable by his method.

Pfeffer [3]) writes: "In allen Fällen werden also Methylenblau und andere Farbstoffe wertvolle Reagentien sein, mit deren Hülfe, ohne Schädigung, Aufschlüsse über Vorkommen und Verteilung gewisser Körper in der Zelle zu erhalten sind. Mit solcher vielseitig ausnutzbaren Methode lässt sich unter richtiger Erwägung nach vielen Richtungen hin eine Kontrole des jeweiligen Zustandes des Zellsaftes und der Veränderungen dieses im Laufe der Entwicklung erreichen."

Pfeffer frequently writes of the harmlessness of his method to life. As a proof of this he cites for instance

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[1]) l. c.

[2]) l. c. p. 183 and 218.

[3]) l. c. p. 191.

the growth of *Spirogyra*-filaments. In two cases this amounted in four days to 12 and 26 %. I must here remark that Pfeffer has made no comparative experiments. If the rate of growth of *Spirogyra* cells in ditch water is studied, it is seen to be much greater. After two days the increase in length in 14 cases was found to be 25 to 75 % and after four days in 18 other cases 40 to 75 %. From Pfeffer's results it is therefore clear that dilute solutions of methylene-blue also are harmful.

My own experiments on *Spirogyra* maxima with methylene-blue (methylene-blue pro usu interno, the hydrochloride), indicated that it was very harmful. In a solution of 1 part in 10000 parts of ditch-water all the cells perished in one day. In solution of 1 part in 500.000 parts of ditchwater or Knopp's fluid many dead cells were seen after one day and in a solution prepared with distilled water of the same strength the number of dead cells was still greater. No growth was observed. The poisonous action of methylene-blue is the reason why there can be no question of "Kontrole des jeweiligen Zustandes des Zellsaftes und der Veränderungen dieses im Laufe der Entwicklung", as Pfeffer imagines.

It has been already demonstrated above that the cell-sap of *Spirogyra* contains no dissolved protein. The precipitate with methylene-blue cannot therefore as Pfeffer believes contain protein. In his opinion the precipitate is actually a compound of tannin with methylene-blue, which cannot be brought into agreement with the fact that solutions of methylene-blue, even stronger than those used by Pfeffer remain clear with solutions of gallnut- and *Spirogyra*-tannin. This is not explained by Pfeffer.

It is noteworthy that when *Spirogyra* is placed in a dilute methylene-blue solution (1 in 500,000) there is no gradual formation of a precipitate which is coloured blue from the beginning, but there is first a colourless or al-

most colourless precipitate and that this is then gradually coloured a deeper and deeper blue. Of this Pfeffer makes no mention.

On examination of the precipitate with reagents tannin reactions could be obtained, for example, the black coloration with ferric acetate. It may therefore be assumed that tannin is precipitated. The quantity of the precipitate even in *Spirogyras* with much tannin was however, small compared with other tannin precipitates.

Hence I doubted whether the tannin is completely precipitated. After one day I could not, indeed, demonstrate any tannin in the cell-sap in addition to the precipitate, but it seems that the cells may lose tannin by exosmosis. For when, for example, pieces of *Spirogyra*-filaments were placed in a dilute solution of methylene-blue, containing $^1/_2$ % gelatin, a precipitate was formed outside the cells and between the layers of the cell-wall which separated from each other. The precipitate was a compound of gelatin with tannin and became coloured black with ferric acetate. I cannot therefore venture to assume with Pfeffer, that a complete precipitation of tannin takes place in the cell-sap.

It seems to me that various factors play their part in the production of the precipitate. In the first place the harmful action of the methylene-blue, of causing great modifications in the organism. Further the presence of salts appear to assist the formation of precipitate. In a solution of one part of methylene-blue in 500.000 parts of distilled water the phenomenon was not so clear as in a solution of the same strength made with ditch-water or Knopp's fluid. A number of experiments in test tubes with methylene-blue, salts, gallnut- and *Spirogyra*-tannin led to the conclusion that the appearance of a precipitate is not only affected bij the presence of salts but that also atmospheric oxygen comes into play and finally, that me-

thylene-blue itself has no precipitating action, but that in one way or another a tannin precipitate is formed which gradually takes up more and more of the dye. How the precipitate is produced I cannot definitely say, but its formation does certainly not depend on a simple precipitation of tannin by methylene-blue, as Pfeffer assumes.