Botany. - *"The physiological significance of certain glucosides."* By Dr. TH. WEEVERS. (Communicated by Prof. F. A. F. C. WENT).

In the Proceedings of the ordinary meeting of the Mathematical and Physical Section of September 27th 1902 there appeared an account of some investigations on glucosides in relation to plant metabolism, and of this the present paper is to be considered a continuation.

It was shown at the time that both salicin from Salix purpurea L. and the glucosides from the seeds of Aesculus Hippocastanum L. are reserve materials; the former substance is used in the opening of the buds, the latter in germination.

At the opening of the buds of Salix purpurea saligenol[1]) appears, which points to a hydrolysis of salicin; the quantity found was, however, so small, that the actual aromatic product must be another substance, and as such I regarded catechol, which can be demonstrated in all parts of the plant. When the salicin diminishes, the catechol increases and in various cases the change in the amount was found to be roughly proportional to the molecular weights. These quantitative experiments induced me to suppose that catechol, after hydrolysis of the salicin and transport to other parts of the glucose thus formed, remains localised in the cell and again combines to form salicin with glucose, which is brought from elsewhere or has been formed on the spot by assimilation. Thus PFEFFER's hypothesis would be confirmed, that the compounds of benzene derivatives with carbohydrates serve to form substances not easily diffusible; in the present case catechol would form with the transport substance, glucose, the reserve substance salicin.

In the later investigation the validity of this supposition was tested on another glucoside, namely arbutin, which accompanied by small quantities of methylarbutin, occurs in numerous Ericaceae. Similarly the occurrence of fairly large quantities of hydroquinone, the aromatic product of arbutinhydrolysis is recorded for these Ericaceae. Experiments with Vaccinium vitis idaea L. showed, however, that the latter statement is not quite correct; generally the amount of free hydroquinone is very small, and in autumn it is even zero; it increases, however, considerably during the formation of the young shoots in spring.

Arbutin is rapidly hydrolysed by an enzyme, which I obtained from Vaccinium vitis idaea, so that only those quantitative deter-

[1]) Saligenol = salicylalcohol; catechol = orthodioxybenzene.

minations give good results, in which the parts have been killed by boiling water or better by boiling alcohol.

The quantitative estimation takes place as follows: the parts of the plants are killed by boiling alcohol, and are then extracted with warm water; the extract is treated with lead acetate, of which the excess is removed with sodium phosphate. The free hydroquinone is obtained in almost colourless crystals by extracting the liquid with ether. After extraction the aqueous solution is boiled for one hour with dilute hydrochloric acid, to hydrolyse the arbutin completely, and the hydroquinone formed is then again extracted by shaking with ether.

In case the amount of hydroquinone was small, the estimation was carried out in the same way as was indicated for catechol in the former paper, viz. by comparing the sublimates with those of a standard solution. If the quantity of hydroquinone was more than a few milligrams, the estimation was carried out by titration with FEHLING's solution according to ALLIHN's method, after a table of the amounts of copper, reduced by pure weighed-out hydroquinone, had been prepared as a basis of calculation.

The isolation of methylhydroquinone was carried out by extraction of the ether residue with benzene, in which methylhydroquinone is soluble, but not hydroquinone itself. The amount of methylarbutin and of methylhydroquinone, however, proved to be small, so that no considerable error was made in calculating everything as arbutin and hydroquinone. The arbutin content showed considerable individual variation and as one specimen was too small for an experiment, the experiments were carried out with a large number of plants from the same spot. The leaves are small, so that it was impossible to trace the variations between day and night by the method of cutting the leaves in half; I therefore only determined the changes during the opening of the buds.

Arbutin plays the part of a reserve material, which in Vaccinium vitis idaea, an evergreen shrub, is principally deposited in the leaves, and there increases in amount during the winter until the opening of the buds in May. Then the amount varies from 2—3 % for plants in the shade, to about 5 % for plants in the sun [1]).

Before the opening of the buds no hydroquinone, or merely a trace, is present in the leaves, and the same applies to the stems and roots, which latter at this period also contain but traces of arbutin. With the development of the young shoots from the buds

[1]) All values calculated for dry weight.

the amount of arbutin diminishes progressively, and that of hydroquinone first increases, but afterwards quickly diminishes when assimilation begins in the young shoots. On comparison of the amount of arbutin in a number of plants (May 3rd) before the opening of the buds, with that after the opening (May 24th), taking old and young parts together, 22—32 % of the total was found to have disappeared.

This glucoside is therefore also used in the opening of the buds and undergoes before use a fermentative hydrolysis, as a result of which hydroquinone shows itself in the tissues. The amount of hydroquinone is, however, much smaller than one might expect from the amount of arbutin which has disappeared (for 100 milligrams of arbutin, for instance, 6 milligrams of hydroquinone). Thus a part of the hydroquinone appears here to be directly worked up in metabolism, and the hydroquinone, which is present in the opening buds, also disappears again rapidly as soon as assimilation becomes vigorous. I therefore found only traces of arbutin in the second budding of some plants in August, which is nevertheless also accompanied by arbutinhydrolysis.

During the opening of the buds I often found that no arbutin occurs in the stem, a further proof that the glucoside is not transported as such.

In the buds of the pear-tree Rivière and Bailhache [1]) have demonstrated hydroquinone, and this induced me to search for a hydroquinone-glucoside in this case also. Such a glucoside was indeed found to exist and the amount of combined hydroquinone was again much greater than that in the free state. The enzyme, which here I was also able to prepare from the young shoots, rapidly brings about the hydrolysis of the hydroquinone-glucoside [2]), so that it is necessary to kill the parts by boiling alcohol. Then one finds in the adult leaves 0.01—0.03 %, in the young shoots 0.3 % of hydroquinone. In this case methylhydroquinone and methylarbutin are absent.

The glucoside is found in the buds, leaves, wood and bark of the branches and also in the roots, but in the bark only. The amount in the root-bark is very small (0.05 %); that in the wood of the

[1]) G. Rivière et G. Bailhache. De la présence de l'hydroquinone dans le Poirier. C. R. de l'Acad. de Sc. Paris CXXXIX. 1904.

[2]) I could not obtain the glucoside in a crystalline form, so that I could not determine with certainty from the physical constants, whether it is identical with arbutin; the products of hydrolysis, hydroquinone and glucose, which I found, make the identity very probable, however.

branches is less than that in the bark. The quantitative determinations, which took place in the same way as in Vaccinium, were carried out on a single tree of the variety Bonne Louise d'Avranches.

The glucoside is formed in the leaves, and the formation begins in the young shoots as soon as they themselves begin to assimilate.

On comparison of the amounts in the halves of a leaf in the morning and in the evening, the glucoside is found to increase during the day and to diminish during the night. This diminution during the night is again accompanied by an increase of hydroquinone and the amounts are in the proportion of 100 : 18 [1]), whereas the molecular weights are in the proportion of 100 : 40. A portion of the hydroquinone, which has been liberated in the nocturnal hydrolysis thus appears to be directly transformed in metabolism, or else we must assume, that a part of the arbutin is transported as such.

During the summer the amount of arbutin increases in the bark, from 1.5 % to 4.5 %, so that we may safely infer, that the arbutin is deposited there in order to be used up in the formation of new shoots in the spring. At that season the amount in the bark diminishes greatly (about 70 % of the total) and increases less markedly in the young shoots, so that the total diminution from March 30th to May 2nd amounted to about 27 %. During this process the amount of free hydroquinone is greatly increased by fermentative arbutin hydrolysis and from April 26th to May 2nd, for instance, the decrease of arbutin was to the increase of hydroquinone in the proportion of 100 : 40, exactly that of the molecular weights; in other words the whole of the aromatic product remains localized in the cells [2]). Afterwards, when assimilation has begun in May, hydroquinone is rapidly worked up and arbutin is formed at its expense. Hence we find PFEFFER's hypothesis once more confirmed: hydroquinone combines with the transport substance glucose, with formation of the reserve material arbutin.

In the case of Salix purpurea I found in 1902 that by slow dessication 25 % of the salicin disappeared from the bark. This pointed to the presence of a salicin-splitting enzyme, but at the time I was not able to demonstrate it. On continuing the experiments

[1]) In Salix purpurea also the relation of salicin decrease to catechol increase in day and night experiments differed more from the theoretical than in the case of the opening of buds of branches placed in water.

[2]) Most of the hydroquinone formed, was in the young shoots; this might therefore be interpreted as an indication, that a transport of arbutin takes place in this case.

I soon obtained better results: bark from the stem was placed in 96 % alcohol immediately after the pealing of the branches; the alcohol was allowed to evaporate slowly in the air and after some days the liquid which was only feebly alcoholic, was filtered. On the addition of salicin 9 % was hydrolysed after 24 hours, after 48 hours 17 %, after 96 hours again only 9 %, measured by the glucose formed.

Young shoots were also ground in a mortar and pressed through fine plancton gauze, the juice was treated with an excess of 96 % alcohol; a white precipitate was thus formed, which was filtered off, washed with alcohol and finally dissolved in water. The colourless slightly opalescent liquid, thus obtained, did not reduce FEHLING's solution, did not contain saligenol or catechol and hydrolysed on the addition of salicin after 24 hours 8%, after 48 hours at 40° 28%, after 72 hours 46%. The extent of the hydrolysis was measured by estimating the glucose formed; in the ethereal extract saligenol could be demonstrated [1]). From the young shoots a mixture of enzymes had thus been obtained, among which was an enzyme which splits salicin into glucose and saligenol [2]). I obtained the same enzyme from Populus canadensis Mchs.

The salicase is not identical with emulsin, nor with amygdalase, for it does not bring about the decomposition of amygdalin.

The above mentioned experiment with the weakly alcoholic extract of the bark, pointed to a reversible action of the enzyme, as did also some other experiments. For when I dissolved 200 mgs. of glucose and 100 mgs. of saligenol in 2 cc. of water and added salicase (with thymol as antiseptic), the reducing power was diminished by 5% after 2 days at 40°, which diminution might be explained by the possible formation of salicin [3]). I had not, however, sufficient material to make this out with certainty.

The discovery of this salicase induced me to investigate once more the occurrence of saligenol in Salix purpurea, as formerly I had only been able to find minute traces. For this purpose I compared in the

[1]) Recognized by allowing the sublimate of the ethereal extract to crystallize from aqueous solution, and by the reactions with cupric acetate and with ferric chloride.

[2]) When I had already completed my investigation, my attention was drawn to a paper by W. SIGMUND (Sitz. ber. Akad. d. Wiss. Wien. CXVII. Bd IX 1909) who obtained from other species of Salix and of Populus an enzyme, called by him salicase, which hydrolyses salicin, and from Calluna vulgaris and Vaccinium myrtillus an arbutase, which hydrolyses arbutin.

[3]) After boiling with dilute hydrochloric acid the reducing power was again as great as before.

same shrubs at different dates the quantities of catechol and of saligenol in the young shoots. The quantitative estimation of the latter substance was carried out in the same way as that of hydroquinone and of catechol, by comparison of the sublimates with that of a standard solution; the separation of the two benzene derivatives was achieved by complete precipitation of the catechol by lead acetate.

Per 200 young shoots I found	23	April	34	mgr.	catechol	— saligenol
	28	„	26	„	„	— „
	2	May	9	„	„	— „
	5	„	1	„	„	— „
	8	„	2	„	„	7 mgr. „
	16	„	1	„	„	24 „ „
	28	„	35	„	„	— „ „

Neither at this time, nor at any other time saligenol can be detected in the bark, and in the young shoots it only occurs for a few days exactly as in the case of the methylsalicylate during the opening of the buds of the beech [1]).

This absence of saligenol is not due to a lack of salicase, for in the bark of the branches, where the salicin diminishes greatly, and in the young shoots, where salicin is used, this enzyme was always present. The saligenol must therefore be directly transformed, and as I have shown before catechol is the probable end-product of this transformation.

The possibility naturally suggested itself of obtaining from Salix purpurea an enzym which should convert saligenol into catechol.

The above mentioned mixture of enzymes readily splits off molecular oxygen from hydrogen peroxide, and therefore contains catalase [2]), but in addition it contains two other oxydases, which differ from laccase and from the system hydrogenperoxide-peroxydase and tyrosine, both on account of their behaviour towards the cresols and because

[1]) Compare Onderzoekingen over Glukosiden: Diss. Amsterdam 1902. Th. Weevers.

[2]) An attempted separation of catalase and catecholase by heating was unsuccessful, and it might be argued that it is here unnecessary to postulate the presence of a separate catalase; hitherto the splitting off of molecular oxygen, however, has always been regarded as characteristic of catalase and conversely the known vegetable catalases do not give the reactions with catechol and saligenol; for the above physiological considerations this is, moreover, of no importance.

The crude enzyme from Salix purpurea contains no manganese and acts on catechol both in a feebly alkaline and a feebly acidic medium (litmus and rosolic acid as indicators). By heating to 100° C. the enzyme is at once destroyed. The views of Dony Hénault Bull. Ac. Roy. Belgique 1909 are therefore not applicable to this case, nor those of Euler Bolin, Zeitschr. phys. Chem. 1908.

of the absence of the guiacum-blue coloration after addition of H_2O_2. They were named after their typical reactions saligenolase and catecholase; both gave an almost colourless, somewhat turbid solution.

They may be separated by heat, for after heating to 85° C., the saligenolase had been destroyed, but not the catecholase. Catecholase oxydises catechol: a catechol solution which by itself remains unchanged for days, changes at once after the addition of catecholase through green to black and after 5 minutes there is a definite black precipitate; saligenol is not changed by catecholase.

Both oxydases together evidently form from saligenol, although more slowly, the same product as the catecholase forms from catechol. The most obvious hypothesis is therefore to assume that *saligenolase forms catechol from saligenol.*

In any case this oxidation of saligenol (salicyl-alcohol) is quite different from that in the laboratory, where salicylic acid is always the final product [1]).

In the living plant this black substance [2]) never appears, but it only occurs in necrobiosis; therefore the most attractive hypothesis seems to me that catecholase and catechol are separated from each other in the cells but that this is not so with saligenolase and saligenol, so that the latter enzyme can act and can form catechol, which then cannot be decomposed by catecholase, as is the case in necrobiosis and in the above mentioned experiments.

In the former paper I investigated the changes of salicin and catechol in branches, with buds opening, when placed in water in the dark, and now I have done the same with branches budding while still attached to the plant. The objection in this case is, that the branches do not form a separate whole, so that influx and exit is possible, while assimilation very soon sets in. For all these reasons one may expect the observed values for the relation of the salicin disappeared to the catechol formed to differ more from those calculated by theory. I thus found, for instance, an increase of 94 mg. catechol accompanied by a decrease of 457 mg. salicin, i.e. a proportion of 21:100, while the molecular weights are in the proportion 38:100. I also found however, that in the opening of the buds the amount of populin increases at the expense of salicin, so that the diminution in the glucoside which furnishes catechol on hydrolysis was placed at too high a value.

I also repeated on a larger scale the earlier experiments with

[1]) Neither catecholase nor saligenolase have any action on salicylic acid.

[2]) Perhaps this black colouringmatter is the same as that formed from homogentisic acid under the influence of alkali.

etiolated budding branches and with bisected leaves (the halves of the leaf being compared in the evening and in the morning). In etiolated budding the catechol increase and salicin decrease were in the ratio 36 : 100; in leaves the nocturnal increase of catechol was to the decrease of salicin in the ratio 31 : 100 (for 2000 leaves), which agrees sufficiently well with the hypothesis.

It is not until the salicin content of the bark becomes great (6 à 7 %) and equals that of the leaves, that the salicin ceases to diminish in the leaves overnight and the catechol no longer increases, the glucose transport of the hydrolysed salicin then seems to have stopped. Whether the salicin concentration influences the transport, or whether the concentration gradient of the glucose does so, remains for further investigation.

In order to determine also the populin present in the bark of Salix purpurea I used a press-juice obtained from Aspergillus niger, which was precipitated by an excess of alcohol. The precipitate contained a mixture of various enzymes, among which is one which completely hydrolyses populin, as was shown by experiments on the pure glucoside.

In addition, the mixture of enzymes also contained emulsin, invertin and maltase; in order to utilize this mixture for the quantitative determination of populin the increase of reducing sugar after the action of the Aspergillus enzyme was to be diminished with that, obtained after the action of emulsin, invertin [1]) and maltase [2]).

As might be imagined the method only gives useful results, when the populin is present in large quantity, for in the glucose-values found for populin all the errors of the other determinations accumulate, and these errors can never be completely excluded in the case of hydrolysis by enzymes.

The method cannot therefore be applied to Salix purpurea, where the populin is quantitatively unimportant. It was found, however, that populin is formed in large quantity in the normal young shoots but is, on the other hand, wholly absent from etiolated shoots; this is the reason why in the etiolated budding the ratio of salicin to catechol agrees so much more closely with that which might be expected theoretically.

I also attempted to determine populin quantitatively in Populus species, but did not obtain good results, because the populin content is too small in the species I have hitherto examined, viz: P. alba L.,

[1]) Salix purpurea contains small quantities of saccharose in the leaves and bark.

[2]) The extract had been obtained from the parts with warm water and had been treated with lead acetate, so that it did not contain any starch or dextrins.

P. canescens Sm., P. monilifera Ait. and P. tremula L. I found here, as in the case of Salix that the young etiolated shoots contain no populin and the normal ones a fair amount. In all the species investigated, salicin is present and also catechol; for one of these plants, P. monilifera Ait. the changes of the two substances during the formation of the young shoots were studied.

In the opening of buds on the tree as well as in etiolated budding of branches placed in water, salicin decreases, catechol increases; the quantities are in the ratio 100 : 66 and 100 : 64. The increase in catechol is therefore greater than might be expected from the decrease in the salicin, and this I think must be ascribed to the diminution in the populin (benzoylsalicin).

I obtained from this Populus, in the manner described above, an enzyme, populase, which splits off benzoic acid from populin, so that the formation of catechol as end-product of populinhydrolysis is indeed very probable [1]).

In Populus monilifera and also in P. tremula salicin increases in the leaves during the day and decreases during the night, just as in the case of Salix; a quantitative determination of the catechol was however impossible on account of the resin present.

Catalase is present in the young shoots and also catecholase; the demonstration of the presence of saligenolase proved difficult as the enzyme mixture is not so pure and becomes darker fairly rapidly when exposed to air.

In the species of Populus examined I found considerable quantities of saccharose, which there plays the part of a reserve material and is present in the leaves, bark and wood (Populus monilifera Ait. and P. tremula L.). In the opening of the buds a large portion is used and the amount in the bark than falls from 1 % to 1 %; the experiments with bisected leaves also point to a behaviour similar to that observed by Brown and Morris [2]) in Tropaeolum. During the night 32% and 35% of the total disappeared from the leaves. Like so many other plant organs the young shoots accordingly also contain invertin. For further details reference may be made to the publication about to appear in the Recueil des Trav. Bot. Néerl.

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[1]) With the mixture of enzymes from Salix purpurea I could not obtain any hydrolysis of populin.

[2]) Brown and Morris. Journ. of the Chem. Society. 1893.