and from (6) and (7)

$$\frac{A_2}{A} = \frac{bw}{a + bw} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (8)$$

In the subjoined table are given for a few capillary electrometers, examined without purposely inserted resistance the values of A_1 and A_2 in percentages of A.

TABLE VII.

Number of the capillary.	$100 \times \frac{A_1}{A}$	$100 \times \frac{A_2}{A}$
G. 103	91	9
B. 101	92	8
B. 102	93	7
B 103	96	4

In the course of these experiments valuable assistance has been given by Mr. H. W. BLÖTE and Mr. H. K. DE HAAS.

Botanics. — Prof. BEIJERINCK speaks: "*On the Formation of Indigo from the Woad (Isatis tinctoria)*" [1]).

Some years ago I wished to become acquainted with the so-called "indigo-fermentation", about which nearer particulars had been communicated by Mr. ALVAREZ. He examined *Indigofera* and says:[2]).

"If a decoction of the plant is prepared and sterilised atfer passing it into test-tubes or PASTEUR's-flasks, the reddish colour of

[1]) It was first my intention to treat „On the function of enzymes and bacteria in the formation of indigo." I have declined this plan for the moment, and give now only part of my experiments, because I see that also Mr. HAZEWINKEL, of the Experimentstation for Indigo at Klaten, Java, has obtained important results about that very subject, which results, for particular reasons, have however been imparted till now to a few experts only. Yet I cannot avoid mentioning some facts, found by me, the priority of which perhaps pertains to Mr. HAZEWINKEL, without my being able to acknowledge his claim. One indiscretion, however, I am obliged te commit: Mr. HAZEWINKEL has, already before me, established the fact, that by the action of the indigo-enzyme and of acids on indican, indoxyl is produced.

[2]) Comptes rendus T. 105, pag. 287, 1887.

the liquid remains many months unchanged without the appearance of indigo. But if some microbes of the surface-membrane of an ordinary indigo-fermentation are added, as also the special active bactery of it in an isolated condition, after some hours an abundant indigo-formation is observed."

I then tried to make from woad *(Isatis tinctoria)*, in which, according to the literature, indican, i. e the same indigo-producing substance as in the other indigo-plants should be present, a decoction with which I might repeat the experiment of ALVAREZ. But I could, neither by boiling, nor by extraction at low temperature, obtain from this plant a sap which remained unchanged at the air. Constantly, after a short time, indigo will separate out of it, without there being any question of the influence of bacteria or enzymes, so that the word "indigo-fermentation" would here be quite misplaced. Neither do purposely added bacteria or enzymes favour the indigo-formation from woad-decoction.

Later, however, I was enabled to convince myself that the statement of ALVAREZ is correct, as well with regard to the decoction of *Indigofera leptostachya* as to that of *Polygonum tinctorium* [1]), for which latter plant the same fact as described by ALVAREZ, has also been established by MOLISCH [2]).

So it was evident that the indigo-plants must belong to two physiologically different groups, and I subjected the concerned chromogenes to a further examination with the following results.

1. *The Chromogene of the Indigo-plants is Indoxyl or Indican.*

The chromogene of woad is not as is usually accepted indican, but the very instable indoxyl C^8H^7NO. *Indigofera leptostachya* and *Polygonum tinctorium*, on the contrary, contain the constant glucoside indican, the constituents of which are, in accordance with the supposition of MARCHLEWSKI and RADCLIFFE [3]), indoxyl and sugar,

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[1]) Much material of this *Indigofera*, as well full grown plants as seeds, I owe to the kindness of Mr. VAN LOOKEREN CAMPAGNE of Wageningen. This interesting plant, a native of Natal, has been cultivated, very rich in indican, in the open ground in the Laboratory-garden at Delft; at Wageningen several specimens had grown this summer to more than 1.5 M. height.

Polygonum tinctorium comes from China and is, as the woad, in the seed-commerce of VILMORIN in Paris.

[2]) Sitz.ber. d. Akad. d. Wiss. zu Wien. Math. Naturw. Klasse Bd 107 pg. 758, 1898.

[3]) Journ. Soc. for chem. Industry T. 87 pag. 430, 1898; Chem. Centralblatt Bd 65 pag 204, 1898. With thankfulness I remember the aid lent me by my chemical colleagues HOOGEWERFF and BEHRENS in the determination of indoxyl.

which has first been brought to certainty by Mr. HAZEWINKEL, and, without my knowing of his experiments, by myself. Woad, as an „indoxyl-plant" containing no indigo-glucoside, wants also an enzyme to decompose it. The two mentioned „indican-plants", on the other hand, do contain such an enzyme, which had already in 1893 been rendered probable by Mr. VAN LOOKEREN CAMPAGNE with regard to *Indigofera* [1]). I have prepared this enzyme, albeit in a very impure state, in rather great quantity and I hope afterwards to describe the experiments made with it.

The important difference between „indoxyl-" and „indican-plants" becomes particularly clear when comparing the different extraction methods. Thereof what follows.

If „indican-plants" are extracted with water below the temperature at which the indigo-enzyme becomes inactive, for instance below 40° C. or 50° C. („cold extraction"), and under careful exclusion of air, an indoxyl-solution is obtained. If, however, the same „indican-plants" are extracted by boiling („decoction"), the indigo-enzyme will be destroyed, and independently of removal or access of air, an indican-solution results, which can be kept perfectly unchanged when microbes are excluded, but either by the separately prepared indigo-enzyme, or by certain bacteria or yeasts, or also by boiling with acids, it can be converted into the constituents indoxyl and sugar. I have prepared from it the crude indican in a dry state, by evaporating to dryness the decoctions of both *Indigofera leptostachya* and *Polygonum tinctorium*. The brown matter, thus produced, resembles sealing-wax, is very brittle and can quite well be powdered.

Woad on the contrary, as an „indoxyl-plant", both by „cold extraction" and by „decoction" always gives the same produce i. e. an indoxyl-solution. Here, in both cases, the greatest care must be taken to exclude the air in order to prevent that the indoxyl, which is so easily oxidised, is converted already in the leaf itself, for then the indigo-blue is lost. Besides, access of air in a dying wood-leaf gives still in another way cause to loss of indoxyl under formation of unknown colourless and brown substances.

A sufficient removal of air during the preparation of the extracts is easily effected in the following way [2]). A well closing, wide-mouthed stoppered bottle is quite filled up with woad-leaves, hot

[1]) Verslag om'ient onderzoekingen over indigo, pag. 12, Samarang 1893.

[2]) The technical preparation of indigo from woad is described in GIOBERT, Traité sur le Pastel, Paris 1813, and in DE PUYMAURIN, Instruction sur l'art d'extraire l'Indigo du Pastel, Paris 1813.

water is poured in, the leaves are pressed together until all air is replaced, and the stopper is put on so as not to leave the smallest air-bubble. By the exclusion of the air, together with the high temperature, the leaves soon die and already after a few hours a clear, light yellow liquid can be decanted, which is rich in indoxyl. If some alkali is added and air blown through, the indigo-blue precipitates, the colour of which appears only pure after acidification. In a sufficient time of extraction there can be thus obtained from woad a liquid of which the proportion of indoxyl, according to REINWARDT [1]) who in 1812 applied the decoction-method on a rather large scale, answers to 0.3 pCt. "pure indigo" for the fresh leaves, which, as he remarks, might rise to the double amount in the South. If we consider that the indoxyl is especially concentrated in the youngest organs still in a state of cell-partition, that it diminishes considerably in full grown parts, and is almost or wholly absent in old leaves, we must conclude that the youngest organs may contain more than 0.3 pCt indigo. As the woad-leaves contain about 85 pCt water this would correspond to a little less than 2 pCt indoxyl in the dry matter [2]).

The indoxyl-containing sap, whether prepared by "cold extraction" from the indican-plants or by decoction from the indoxyl-containing woad, has the following characteristics. It is a light yellow, in cold greenish fluorescent fluid; at warming the fluorescence diminishes and comes back at cooling. The reaction is feebly but distinctly acid, of course not by the neutrally reacting indoxyl but by organic acids. At the air a copper-red film of indigo-blue is formed at the surface of the liquid.

$$2(C^8H^7NO) + O^2 = C^{16}H^{10}N^2O^2 + 2H^2O,$$

but this oxidation follows so slowly in the feebly acid solutions, that evaporating to dryness at the air is possible without too much loss of indoxyl. The indoxyl itself is soluble in water, ether, alcohol and chloroform, in the two last under slow decomposition when the air finds access.

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1) In a report of 6 December 1812 to the President of the Agricultural Comittee for the Department of the Zuiderzee, present as a manuscript in the library of the Academy of Sciences, Amsterdam.

2) But according to GEORGEVICS, Der Indigo, pag. 2 and 18, Wien 1892, the rate of indigo for woad would only amount to 0.03 pCt. In my laboratory Mr. VAN HASSELT found in three special cases 0.05 pCt., 0.07 pCt. and 0.09 pCt. indigo-blue in relation to the weight of the living leaves, which latter amount corresponds to c.a 0.6 pCt indoxyl with regard to the dry weight.

As soon as the liquid becomes alkaline, however feebly, the indoxyl oxidises at the air with much greater quickness to indigo-blue.

The statement of BRÉAUDAT [1]), that in the sap of *Isatis* there would be present an oxidase, by which this oxidation is effected, is not proved; in none of the three indigo-plants I have been able to find an oxidase producing indigo-blue from indoxyl. For, by preparing from the woad-leaves "crude enzyme" by finely rubbing them under, and extracting them with strong alcohol, whereby, after pressing and drying, a completely colourless powder is obtained in which all the enzymes must be present, it is found that the oxidising effect of this "crude enzyme" on an indoxyl-solution is very slight, ceases soon, and does not change by boiling, from which must be concluded that the oxidation cannot be attributed to oxidase, but is of a purely physical nature [2]).

The leaves of the indican-plants give quite the same result.

Though there originates during the slowly dying of woad-leaves at the air, a substance which gives rise to a total destruction of the indoxyl, yet about the nature of it I cannot express a supposition. If it might prove to belong to the group of the oxidases, it is surely in no other relation to the formation of indigo from indoxyl, than that it is very pernicious to it. For the indican-plants the same has been observed. In *Indigofera* this destructive influence is so strong that the „alcohol-experiment", of which later, wholly fails with this plant.

Hydrogen-superoxyd, too, causes the indoxyl gradually to vanish from the solutions, without any coloured products originating.

Strong acids, just as alkalis, (though in far less degree, favour the formation of indigo from indoxyl, but then part of this substance constantly changes into a brownish-black matter.

In feebly alkaline and in moderately acid solutions, indoxyl, warmed with isatine gives, in absence of air, a precipitate of indigo-red, which is isomeric with indigo-blue

$$C^8H^7NO + C^8H^5NO^2 = C^{16}H^{10}N^2O^2 + H^2O.$$

This precipitate separates quickly out of alkaline solutions as fine red, from acid ones as coarser dark crystal-needles and can easily be filtered. It is soluble in alcohol and so can be separated from

[1]) Comptes rendus T. 127, pag. 769, 1898 en T. 128, pag. 1478, 1898.

[2]) In a small porcelain vessel the menisc of the fluid furthers the oxidation of indoxyl to indigo-blue just in the same way as "crude-enzyme", strewed as a powder on the surface of the liquid.

the indigo-blue. On warming an indican solution with isatine and dilute hydrochloric acid, all the indoxyl which is set free precipitates as indigo-red, and I presume that a good quantitative indican determination may be based upon this reaction.

All the here mentioned characteristics of the indoxyl-containing plant-saps are also announced in the literature of the chemically prepared indoxyl, except the conduct towards isatine and hydrochloric acid which has perhaps not been examined.

Natural indigo prepared from woad, contains a small quantity of indigo-red; but whether this originates from the same indoxyl as the blue, or from an isomeric indoxyl, I cannot decide. Indigo-red I could also find in the indigo made from indican, whether chemically by boiling with acids, or by bacteria, or by enzymes. Consequently, if two indoxyls should exist, there should also exist two indicans.

2. *Demonstration of Indigo in the Indigo-plants themselves.*

For the demonstration of indigo in the plants themselves, Mr. Molisch described in 1893 his „alcohol-experiment" to which he afterwards repeatedly recurred [1]). In this experiment the parts of the plants to be examined are exposed, in a confined atmosphere, to alcohol- or chloroform-vapour, for instance by putting them into a glass-box, in which a small vessel with these substances is placed. Thus slowly dying all the indigo-plants become more or less blue, which is perceptible after the chlorophyll has been removed by extraction with alcohol. I found, however, that never all the present indoxyl or indican changes into indigo. The „alcohol-experiment" succeeds the best with *Polygonum tinctorium*, where at least most of the indoxyl changes into indigo. For woad the result is greatly dependent on the length of time which the experiment requires, even on the season, but invariably only a part, though it may be a great part, of the indoxyl passes into indigo. With *Indigofera* only a little indigo precipitates in the youngest leaflets and buds, while the older leaves become quite colourless by the alcohol-extraction though they are extremely rich in indican, so that, for this plant, the „alcohol-experiment" is without any value [2]).

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[1]) Sitz.ber. der k. Akad. d. Wiss. zu Wien Bd. 102, Abt. I, pag. 269, 1893; Bd. 107, pag. 758, 1898, and Berichte d. deutschen Botan. Gesellsch. Bd. 17, pag. 230, 1899

[2]) Quite wrongly Mr. Molisch declares: „Die pracisesten Resultate erhalt man bei *Indigofera* mit der „Alkoholprobe," and as wrong is his assurance „Durchwegs war zu bemerken, dass die in Europa gezogenen Pflanzen (von *Indigofera*) auffallend viel weniger Indigo lieferen wie die tropischen" (Berichte d. deutsch. Bot. Ges. Bd. 17, pag. 231, 1899).

For woad, as an indoxyl-plant, the alcohol-experiment can be improved by changing it into an „amoniac-experiment", by which the percentage of indigo is much heightened. If near the woad-leaves in the glass-box a vessel with ammoniac instead of alcohol is placed, death follows almost instantly. The leaves then first become of an intense yellow and afterwards, by the indoxyl-oxidation, of a deep blue colour. By subsequent extraction with alcohol the leaves become deeply blue as compared to the lightly coloured „alcohol-leaves". The „ammoniac-experiment" proves that all growing parts of the woad, even the roots, the rootbuds [1]), the cotyledons and the hypocotyl, contain indoxyl.

The explanation of the „alcohol-experiment" is, of course different for the different indigo-plants. This explanation must at the same time elucidate the following fact: Suddenly killed leaves, for instance leaves, which have been kept in vapour of 100° C., do not colour at the air, neither of woad, nor of *Polygonum*, nor of *Indigofera*, why then do they become blue when slowly dying off?

The answer for *Polygonum* and *Indigofera* lies partly at hand. By the temperature of the boiling-point, the indigo-enzyme has been killed, so the indican can no more be decomposed. If slowly dying, on the contrary, the indigo-enzyme can become active and indoxyl is formed [2]). But the explanation of the second part of the process, that is the transformation of indoxyl into indigo, — at the same time the only point which for woad, as an indoxyl-plant, requires our attention, — is less clear. I think that the course is as follows. In slowly dying leaves the indoxyl changes into indigo-blue, because, in this form of death of the cells, some alkali originates. In suddenly killed leaves, on the other hand, alkali-formation does not occur, they do not grow blue, and the indoxyl disappears in another way.

If in the leaves of indigo-plants the presence of an oxidase, acting on indoxyl, could be demonstrated, this would certainly explain quite well the action of higher and lower temperatures. But, as I said, I could not convince myself of its existence, so that I am necessarily led to the alkali-hypothesis.

The cause of the great lack of indigo-blue which, as above observed, diminishes the value of the „alcohol-experiment", lies in the

[1]) The production of leafbuds on the roots of the woad seems nowhere else mentioned. Other biennal Cruciferae produce also rootbuds, for exemple *Brassica oleracea*, *Sisymbrium alliaria* and *Lunaria biennis*.

[2]) Also a slow death of the leaves by drying or by frost renders the protoplasm permeable and the indigo-enzyme active.

fact, that during the slowly dying of the leaves *at the air*, a considerable quantity of indoxyl is lost in an unknown way. And in this circumstance I see one of the reasons why, in woad-leaves, there is produced so much more indigo by the „ammoniac-experiment" than by the „alcohol-experiment", because in the former the leaves die almost instantly, whilst the latter requires much more time.

With *Indigofera*, as said above, the „alcohol-experiment" produces hardly any indigo. I have therefore tried to substitute for it a better one, which is effected in the following way, and by which, also excellent results are to be obtained with *Polygonum*.

At the direct action of ammoniac, indican-plants form no indigo at all, for thereby not only the protoplasm is killed, but the indigo-enzyme, too, is so quickly destroyed that it cannot decompose the indican. But we can, before exposing to the alkaline vapour, decompose the indican and free the indoxyl, by making the plants die by complete exclusion of air, but which in this case should occur in such a way, that the indoxyl remains within the plant itself. Indican-plants turn then into „dead indoxyl-plants" and can in this condition, quite like the living woad, be subjected to the „ammoniac-experiment" with a very good result.

The simplest way by far to reach the double aim of killing the plants by exclusion of air and leaving the indoxyl in the cells, is by entirely plunging them into mercury, whereby asphixion follows with surprising quickness, the protoplasm becoming permeable and the indigo-enzyme and the indican mixing together. At a proper temperature [1]) the indican is then decomposed after a few hours and the freed indoxyl remains in the leaf, albeit not exclusively in the cells in which originally the indican was localised. The leaf is then taken out of the mercury, ammoniac-vapour is allowed to act upon it, and at last the chlorophyll is extracted by boiling with alcohol and some hydrochloric acid. Even old *Indigofera*-leaves, which by the "alcohol-experiment" become quite colourless, take a brilliant blue colour by this "mercury-ammoniac experiment."

Before I had worked out the mercury-method, I examined the results of killing the leaves by the asphixion in hydrogen, carbonic acid and the vacuum, in each case followed, in the same manner as in the mercury-method, by subsequent exposition to ammoniac-vapour and extraction of the chlorophyll with alcohol.

When the hydrogen was mixed with air a singular phenomenon

[1]) The influence of temperature on the action of the indigo-enzyme is interesting, I hope on another occasion to return to it.

was observed: the indoxyl disappeared so completely from the leaves, that, after the said treatment, they became quite colourless, whilst pure hydrogen produced intensely blue leaves.

In the carbonic-acid atmosphere there appeared, with the indigo, a small quantity of brown pigment, probably because the carbonic acid was not wholly free from air. The action of pure carbonic acid I have not yet examined.

The vacuum in a barometer-tube, above mercury, gives the same result as the submersion in mercury itself, but this method is, of course, more complicated.

3. *On the „coloured strip" in partly killed leaves.*

The following phenomenon is in near relation to the preceding. In many leaves, when partly dying off, a coloured matter will appear, just on the border between the living and the dead tissue; with woad and with *Polygonum tinctorium*, the chromogene of this coloured strip is indigo [1]). The experiment succeeds best if the leaf is partly killed by keeping it for a moment in the vapour of boiling water. The killed part remains green, although it may be a little more brownish than the living one.

As for woad I think the phenomenon should be explained as follows.

On the border between the dead and the living tissue, a strip of cells must occur which are in a condition of slowly dying. According to the preceding description, alkali will be formed in these cells and the indoxyl quickly oxidises to indigo-blue, nothing of it finding time for disappearing in another way. If the partly killed woad-leaf, immediately after death sets in, is exposed to ammoniac-vapour, it becomes, as might be expected, over its whole extent deeply blue. If it is, before the action of the ammoniac, left for some time to the influence of the air, then some indoxyl gets lost from the killed part which colours with ammoniac, a little less strongly than what remained living.

For *Polygonum tinctorium* the explanation is somewhat different, because the indoxyl must first be originated by the action of the indigo-enzyme. But this enzyme is destroyed by the hot vapour in the quickly dying part, whilst on the border between the living and the dead part there must be a number of cells in which the

[1]) With woad this experiment succeeds best with leaves from the rosettes of the first year in June; with *Polygonum* always equally well. In many other plants the "coloured strip" does not contain indigo but a black or a brown pigment.

protoplasm is killed or hurt, but in which the enzyme remains active. During the dying the protoplasm becomes permeable, indican and enzyme are mixed up, and indoxyl-formation is the result. But in the same cells there occurs, in consequence of the slowly succeeding death, an alkaline reaction, by which the indoxyl soon oxidises to indigo-blue, which therefore precipitates in these cells alone, and not in the quickly killed nor in the living cells. Put into ammoniac-vapour the living, as well as the dead part of the *Polygonum*-leaf remain uncoloured, in opposition to the woad-leaf, this, after the preceding, requires no further elucidation.

Of course, these phenomena would find a somewhat simpler explanation if they could be brought back to the action of an oxidase, present from the beginning. But an oxidase, producing indigo from indoxyl is, as said, not to be found.

To conclude I wish to observe, that some other phenomena, which are attributed to the effect of a „wound-irritation", for instance, the formation of starch and of red pigment, as also the development of warmth in hurt parts of plants, possibly repose also on alkali-formation in or near the damaged cells.

Physics. — Communication N°. 51 from the Physical Laboratory at Leiden by Prof. H. KAMERLINGH ONNES: "*Methods and apparatus used in the cryogenic laboratory*". I.

1. Last year the completion of the safety-arrangements, thought desirable for the cryogenic laboratory by the Privy Council, in accordance with the Report of the committee appointed by the Academy, enabled us again to take up the work. I intend now to publish, whenever the completion or the progress of researches allow, something about the methods and apparatus used in working at low temperatures and with liquefied gases.

In this way the short survey (Comm. N°. 14) of the arrangement of the cascade formed by the methylchloride-, ethylene- and oxygen-cycles will be continued or elaborated.

2. *Cryostat (boiling-glass and boiling-case) for measurements with liquefied gases (especially with liquid oxygen).*

In the above mentioned communication a method was described (§ 8) for using liquid gases in measurements. A sketch, shown on plate I of Communication N°. 27 [1]), may serve in some way to

[1]) Verslag der Vergad. Kon. Akad. 96/97. pg. 37. Comm. Leyden. N°. 27.