had the kindness to determine the crystalline form which these salts, assume on solidifying.

The red TlI appeared to be regular, the yellow on the other hand biaxial. Thalliumnitrate also seems capable of crystallyzing from the melt in the regular system. This corresponds with the fact that the white as well as the red mixed crystals are regular.

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Botanics. — "*Further researches on the Formation of Indigo from the Woad (Isatis tinctoria)*". By Prof. M. W. BEIJERINCK.

Since my first communication on the chromogene of the woad [1]) I have found that the indoxyl does not exist in it in a free condition, as I then thought, but in a loose compound which I will call isatan, and which, by an enzyme, simultaneously present, the isatase, is easily decomposed with production of indoxyl.

1. *The research of* SCHUNCK.

As soon as I had come to this conclusion, the question arose, whether the matter prepared by SCHUNCK from the woad in 1855, and described [2]) under the name of "indican", can be either or not identic with isatan. That in many of his experiments he has indeed had isatan before him I consider as certain. But in carefully reading his essay I met with number of contradictions, which are only to be explained by SCHUNCK's working with two other substances besides, which he continually interchanges with each other and with isatan; these are indoxyl and a chromogene which colours intensely yellow by alkalies, occurs abundantly in the woad, precipitates, just like isatan, with basic lead acetate, but has nothing to do with indigo. If I well understand him he calls this substance „changed indican" and considers that it differs from it by containing one or two H^2O more, but this is a wholly unproved hypothesis.

Indoxyl was not known to SCHUNCK at all, but his second preparation method of the "indican" reposes on ether extraction of the dried plant. As isatan is not soluble in ether I suppose that

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[1]) On the Formation of Indigo from the Woad (*Isatis tinctoria*). Kon. Akad. van Wetenschappen, Amsterdam; Proceedings of the Meeting of 30th September 1899.

[2]) E. SCHUNCK. On the Formation of Indigo-blue. Part I. Philosophical Magazine (4) Vol. 10, pag. 74, 1855. For the indiglucine: Ibid. Vol. 15, pag. 127, 1858.

during the preparation small quantities of indoxyl originated from the isatan, which easily occurs under various influences, and for which ether is an excellent solvent.

However strange it may be, it was the matter colouring yellow by alkalies, and not the indigo chromogene itself, which SCHUNCK subjected to the three analyses on which reposes the well-known formula of the "woad-indican". Quite clear he is not, but so far as I conceive his meaning, the first and the third preparations, which he analyzed, contain no indican at all, yet he calls them the purest; the second he considers as less pure, and he seems to have subjected it to the analysis after having convinced himself that by precipitating it with alcohol, lead acetate and ammonia "it contained no longer unchanged indican", which consequently means, that he had before him the said matter turning yellow by alkalies and thus containing no more indigo chromogene.

Word for word he says the following, first concerning his analyses in general (l. c. Part I, pag. 89): "I have hitherto been unable, I regret to say, to ascertain the exact composition of indican by direct experiment. On account of the deliquescent nature, and its so readily undergoing change when heated, it was impossible to subject it to analysis in a free state and I was therefore obliged to have recourse to the lead-compound." Then follows the description of the three analyses themselves. Of the first he says (l. c. pag. 90): "Notwithstanding the care, however, which I took in the preparation of the specimen, I found that it did not contain unchanged indican, as a little of it, when tested with sulphuric acid, gave no indigo-blue. It is nevertheless the purest specimen of the lead-compound which I have analysed". Then he says of the second and third: "The next analysis which I shall give, places in a striking light the effect which alkalies exert on indican. I took some of the same solution of indican which I had employed for the preceding analysis, and which I found to give, when a little of it was boiled with acid, very pure indigo-blue; but instead of evaporating it, I added a large quantity of alcohol to it, and then precipitated with acetate of lead and ammonia. The precipitate no longer contained unchanged indican"... "The third analysis was performed with a lead-compound made in the same way as that of the first analysis." [1])

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[1]) Three analyses of such doubtful substances are the sole foundation upon which the well-known indican formula of SCHUNCK

$$C^{26}H^{31}NO^{17} + 2\,H^2O = C^8H^5NO + 3\,(C^6H^{10}O^5).$$

Indican — Indigo-blue — Indiglucine

All this is not quite clear, but I read from it that these analyses have nothing to do with the indigo chromogene itself, that is to say, with isatan, and I think that they relate to a mixture of the chromogene from the woad, which colours yellow by alkalies, and plant-slime ("indiglucine"). The explanation of this enormous fact should, I think, be sought in the following circumstances. SCHUNCK prepared the "indican" by alcohol extraction from carefully dried woad-leaves, which in itself is quite rational, because in this way relatively concentrated and rather pure solutions are obtained. But if the dried leaves are kept a little too long, for instance two days at 28° to 30° C., or if they grow a little moisty, the isatan vanishes completely from them. Though SCHUNCK evidently knew that the chromogene can easily disappear from the dry leaves, he does not mention the short time after which this occurs already, so that I think it very well possible that the chromogene has disappeared during his preparation without his having observed it. For it is to be kept in view that his method of demonstrating the indigo-blue qualitatively is highly deficient and consisted in decomposing the chromogene by "strong mineral acids", the very worst method to be followed, as strong acids are pernicious as well to isatan as to indoxyl.

My opinion that SCHUNCK at the moments when it was particularly important, had not to do with the indigo chromogene itself, but with another substance, is also based on several observations which he makes about the properties of the "pure indican". So we read on pag. 85 (l.c. Part I): "With caustic alkalies, baryta and lime-water the watery solution turns of a bright yellow." This reaction holds only good for the impurity which remains in the dried leaves after the isatan is destroyed in them. If in the preparations any isatan had been present the yellow colouring would have been immediately followed by the formation of indigo-blue, which then becomes much more distinctly visible than if the same preparation is decomposed by acids. Evidently he has examined different samples with acids and alkalies, and samples, free from isatan, only with the latter, else he would certainly have found that those preparations, which by acids produce indigo-blue, yield much more indigo if they are treated with an

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is based and which, since 1855, has been accepted, without criticism, in all great chemical manuals. Formerly I was inclined to write the formula thus:

$$C^{26}H^{27}NO^{17} + 2\,H^2O = C^8H^7NO + 3\,(C^6H^8O^6)$$
Indoxyl Glukoron

but now, having carefully studied SCHUNCK's essay, I think this interpretation also worthless.

alkali. Likewise the following statement of his preliminary researches is for the greater part unintelligible if it is admitted that SCHUNCK speaks of isatan. He says (l. c. pag. 81): "I was enabled to infer, with positive certainty, that the *Isatis tinctoria* contains a substance easily soluble in heat and cold water, alcohol and ether, which, by the action of strong mineral acids, yields indigo-blue; that the formation of the colouring matter from it can be effected without the intervention of oxygen or of alkalies; and that the latter, indeed, if allowed to act on it before the application of acid, entirely prevent the formation of colouring matter." In opposition to this, the fact must be stated, that the best method for demonstrating with certainty and quickness isatan or indoxyl in woad-sap, just consists in adding alkali to it, by which the isatan is decomposed and the indoxyl is quickly oxidized to indigo at the air; after this, the addition of acid may be desirable to decolour the yellow pigment formed by the alkali, by which the indigo-blue appears with greater purity.

The uncertainty of the whole research explains how it is possible, that SCHUNCK, when later becoming acquainted [1]) with *Polygonum tinctorium*, could think that the indican therein occurring, the composition of which, $C^{14} H^{17} NO^6 + 3 H^2 O$, has recently been determined by Messrs. HOOGEWERFF and TER MEULEN [2]), and which is entirely different from isatan, could be identic with his „woad-indican."

Consequently I believe that SCHUNCK cannot be considered as the discoverer of the isatan, though it is not to be doubted, that in his experiments, he has sometimes had this substance before him, and, basing on the above exposition I take his indican formula for not appliable to isatan.

2. *Preparation and properties of isatan.*

Indoxyl and isatan are very unstable and still at present most imperfectly known substances, which only in acid solutions can easily be distinguished from each other, in neutral solutions, without the use of isatase, with much more trouble, in alkaline solutions not at all, because in these isatan produces indoxyl.

The reason why at first I thought that the woad must contain free indoxyl and no compound of it, is the fact that in the extracts obtained

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[1]) On Indigo-blue from *Polygonum tinctorium*. The Chemical News. Vol. 39, pag. 119, 1879.

[2]) Kon. Akad. van Wetensch. te Amsterdam, 31 Maart 1900, pag. 598.

from young woad-leaves, rich in isatan, as well by decoction as by cold extraction, the isatan is decomposed and an indoxyl solution is obtained. Now I admitted in the beginning, that if in the woad, as was my leading theory, a glucoside was present, which, in analogy to the indican, must be decomposed by an enzyme, at the decoction no indoxyl but exclusively this glucoside would be obtained, because by boiling the enzyme is suddenly destroyed. In this view I was supported by the fact, that this indeed takes place with *Indigofera* and *Polygonum*, which by decoction yield indican, by cold extraction indoxyl.

But I began to doubt of the generality of this theory, when observing, that *Phajus grandiflorus*, which belongs to the indican plants, nevertheless [1]) produces indoxyl at decoction. So this seemed also possible with the woad, though it was clear that the properties of the „glucoside" ought in this case to be quite different from those of indican.

But I was only put on the right way, by the experience, that it is possible to obtain from the leaves of the woad, by the extraction with dilute acids a solution, which remains unchanged at the air, although it yields with alkalies much indigo-blue, while an equally acid indoxyl solution slowly oxidizes at the air to indigo. I then clearly saw why I had before obtained indoxyl from the woad. My experiments had been performed on a small scale; I had been able with care to select growing leaves and buds only; but they contain much isatan and so little acid, that the enzyme isatase can become active, so that by decoction, as well as by cold extraction with water, and even with alcohol, they produce indoxyl, though at the decoction and alcohol extraction mixed with much isatan, which fact I only observed later. If I had used older leaves which contain more acid, I should have found at once isatan quite free from indoxyl.

The relative constancy of isatan in feebly acid solutions, even at boiling temperature, can be utilized for its preparation.

Though the acidity during the extracting must be feeble yet it must be strong enough to prevent the decomposition of the isatan by the isatase. To this end an acidity of 1.6 to 3.2 cc. of normal oxalic acid per 100 cc. of the extraction liquid, (0.1 to 0.2 weight percentage) suffices, for the acidity of the older leaves themselves amounts to about 1,5 cc. normal per 100 cc. of the juice, and this is the very limit of acidity above which the isatan becomes inactive. If the extraction is effected by boiling, this degree of acidity should be

[1]) Indigofermentation. Kon. Akad. van Wetensch. Amsterdam, Proceedings of the Meeting of Mar 1900 pag. 573.

exactly observed. In cold extraction, with oxalic acid, the isatan is much less subject to decomposition, so that, below 50° C. solutions of 1 to 3 pCt. oxalic acid can safely be employed. But at these low temperatures the acid penetrates with less rapidity into the cells, in which accordingly the enzyme can become more or less active producing some indoxyl. Hence, in the acid extraction at low temperature, it is advisable to rub the leaves down in a mortar, immersed in the acid liquid.

In particular at boiling temperature and when using an extraction liquid of an acidity of 2 to 3 cc. of normal oxalic acid, it is easy to obtain a quite undecomposed isatan solution from the growing woad-leaves, even of the youngest still neutrally reacting meristemes. In consequence of the boiling temperature, aided by the perfect surrounding of the cells with the dilute acid, the isatase is destroyed simultaneously with the dying of the protoplasm, by which decomposition of isatan is quite excluded. As the extraction continues, there is an interchange between the feabler acidity within (0.5 cc. normal pCt.), and the stronger acidity without the young cell, and at the end of the experiment, a solution of isatan of 0.5 to 2 cc. of normal acid per 100 cc. of juice in obtained, when the weight of the leaves used, equals that of the extraction liquid.

More acid used in the boiling than the said percentage causes isatan decomposition, by which not only indoxyl but also brown products of decomposition originate.

Oxalic acid can be replaced by other acids and by acid salts. Thus I obtained good results with dilute sulphuric acid and phosphoric acid, and with a saturated solution of boric acid, at room temperature. Acetic acid causes a feebler decomposition than oxalic acid. When the appearance of brown products of decomposition during the boiling is taken as a criterion for the decomposition, I found that 12 cc. of normal acetic acid added to 100 cc. of juice (ca 0.8 weight percentage), is about proportioned to 5 cc. of normal oxalic acid (= 0.3 weight percentage). Acid salts act like acids. Kaliumbioxalate and biphosphate can only be used in strongly diluted solutions. With a cold saturated solution of kalium bitartrate the extracting may be operated at boiling temperature without decomposition; only by prolonged boiling a little indigo-blue is produced. I prefer, however, the extraction with oxalic acid. Therewith the solutions remain clear and of a light yellow and can very easily be filtered [1]); after filtering, the remaining leaf-matter is soft, but

[1]) If the woad-leaves are boiled with more acid than 2 to 3 cc. normal per 100 cc. of the juice, the decoction grows slimy and gives trouble in filtering.

by no means slimy, and can quite well be pressed dry, so that, in consequence of the high water percentage of the leaves, a quantity of extract is obtained nearly twice as much as the original volume of the oxalic-acid solution.

If with the thus obtained isatan solution enzyme experiments are to be performed, the acid must be removed, which is best done by boiling with chalk [1]). As the reaction of the chalk is slightly alkaline it should be very finely divided, as larger particles form a little indigo on their surface. After filtering off the oxalate and the superfluous chalk, a liquid results, somewhat brownish indeed, but not so much as to be hurtful to the enzyme experiments.

This liquid cannot be evaporated to dryness without being decomposed, even not at room temperature, because during the concentration the acidity increases. To neutralize the syrupic matter is troublesome.

The extraction of the isatan can also be effected with feebly acid alcohol, both in the cold and at boiling temperature. Fresh leaves are then to be preferred to dried ones, because in drying there always gets lost some, at last all isatan. The alcohol extract must be evaporated at low temperature and finally be neutralized with chalk. After boiling a brownish, almost neutral and very rich isatan solution is obtained, which can be purified with neutral lead acetate.

For further concentration the isatan can be precipitated with basic lead acetate, and the yellow precipitate be decomposed in the cold with oxalic acid. The lead oxalate separates freely from the isatan solution, and the excess of oxalic acid can be removed with chalk, the lead with sulphurated hydrogen. This solution can be kept without decomposition for some time, but after a few weeks the isatan vanishes.

In the decoction method with oxalic acid, followed by lead precipitation, the chlorophyll is removed from the very first and evaporation is excluded. More plant slime will then precipitate with the lead than by alcohol extraction, but on further purifying, this slime can be precipitated with ether-alcohol. I have as yet not been able to prepare dry isatan, as a powder, from these extracts, such as I before prepared the indican.

The most characteristic difference between indican and isatan consists in their behaviour to alkalies: indican is constant in concentrated alkaline solutions, isatan is decomposed by very feeble alkalies, even

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[1]) Neutralizing without endangering the subsequent enzyme action, can also be done with lead-, mangan-, magnesia-, or baryta-carbonate, but I prefer chalk.

in the cold. Concentrated solutions of dinatrium phosphate, phosphoric salt and ammonium carbonate produce indoxyl from isatan, already at room temperature. By acids, both indican and isatan are decomposed, but indican with much more difficulty, which is especially evident when using acid salts. So, isatan is already decomposed by boiling with dilute kalium bioxalate, in which indican is constant.

Both substances precipitate with basic lead acetate, producing yellow precipitates, which colour is probably proper to the substances themselves, and not to impurities.

Isatase, the specific enzyme from woad, does not act on indican; isatan on the other hand is not decomposed by the indigo-enzymes.

Isatan is not directly splitted by the common microbes; indirectly it may, of course, be decomposed by the alkali produced by microbes. Indican, on the other hand, as I have formerly shown, is directly decomposed by many microbes, either by ferment action of the protoplasm (katabolism), or by specific enzymes, proper to the microbes. This difference between isatan and indican is probably related to the nature of the substances set free in the decomposition beside the indoxyl. So the glucose, from the indican, is an excellent nutrient for many bacteria, whilst the very stability of the isatan in relation to microbes, seems to indicate that the matter, which besides indoxyl originates from it, is no glucose, perhaps no sugar at all.

3. *The isatase.*

The preparation of the woad-enzyme is effected in the same way as that of the indigo-enzymes. The related parts of the plant are rubbed down in living state under alcohol, and the alcohol is so often renewed until all the chlorophyll pigment is removed. After filtering and drying the crude isatase is obtained as a white, feebly acid powder in which, of course, all substances not soluble in alcohol are present, hence, all the other enzymes of the woad too. As the enzyme is quite insoluble in water it can be purified by extraction with destilled water, by which the other enzymes, at least those that are soluble, disappear. Solvents for the isatase itself I have not yet found.

As the woad, like the cabbages, is very rich in gypsum, the crude isatase contains so much of it that to remove it with destilled water is troublesome. I have therefore, in order to answer the question, whether in the action of isatase on isatan perhaps a sulphate is produced, as in the splitting of kalium myronate by

myrosine, prepared in the following way isatase free from gypsum. Woad leaves cut fine were rubbed down in destilled water, then pressed out, and the remaining matter extracted with water until the filtrate proved free from sulphuric acid. Then the chlorophyll pigment was removed by alcohol and the remaining matter dried and powdered.

Though the thus obtained preparation is poor in enzyme, because this is localized in the chlorophyll granules, which during the pressing of the leaves are for the greater part also pressed out, it is still sufficient to bring about a strong isatan decomposition. As was to be expected, sulphates were not thereby set free.

The isatase is spread through the whole woad-plant; it occurs as well in the growing parts as in full-grown roots, stems, leaves, and flowers. So the distribution is another than that of the isatan, which is wanting in all full-grown parts, and is the more accumulated in growing roots, stems, and leaves, the younger they are. Another distribution also than that of the indigo-enzymes in the indican plants, which are only found in the parts rich in indican.

On the other hand the distribution of the isatase within the cell itself, corresponds with that of the indigo-enzymes: both are localized in the chromatophores. The isatan has also, in the cell, a localisation corresponding with that of the indican, for in as much as can be inferred from micro-chemical experiments, both are found in the living protoplasm of epidermis, mesophyll and other parenchymatous tissues. For establishing the localisation of isatan and isatase in the cell, the same way can be followed which I formerly pointed out for detecting the indican and the indigo enzymes [1]).

As regards the isatan, for this end, not too thin microscopic sections of young, vigorously growing stems or leaves are put in a boiling mixture of hydrochloric acid and isatine; by the acid indoxyl is separated, which produces, with the isatine, red crystal needles of indigo-red, localized in the protoplasm. More difficult to observe, but still, I think, quite convincing is the precipitation of indigo-blue, as small granules, in the living protoplasm, when the sections, in a living state, are put in a mixture of boiling hydrochloric acid and ferrichlorid. Remarkable is the strong accumulation of isatan in the epidermis cells, and especially in the hairs found on the young leaves.

The localisation of isatase in the chromatophores can be demonstrated in two ways. Either little bits of the easily loosening epidermis of woad-leaves, or microscopic sections of stems or leaves, all in a

[1]) Indigofermentation p. 579.

living state, can be put in a neutrally reacting woad-decoction, rich in isatan, and heated to ca. 45° C. After some minutes already the chromatophores begin to colour blue; the intensity of colour increases some time, to reach its limit in an hour or so.

The blue-colouring of the colourless chromatophores of epidermis and stem-pith, is here distinctly to be observed, so that, particularly the fragments of the first, become very interesting preparations.

The localisation of the isatan in the protoplasm, of the isatase in the chromatophores, renders their inter-action in the living cell possible without any influence of the acid cell-sap. At the death of the cell, this state will suddenly change and the acidity of the cell-sap determines whether the isatase can act or not on the isatan.

In no other plant but the woad I have hitherto been able to detect isatase. I had expected its presence in some short-valved Cruciferae. So in *Capsella bursa pastoris*, where, in case the root-neck is much hurt, a trace of indoxyl can be pointed out, but here also the enzyme is wanting. Likewise it wants in the indican plants. Also all microbes examined are devoid of isatase.

4. *Action of isatase on isatan.*

The action of isatase on isatan is, as observed before, only possible in neutral or amphoteric and very feebly acid solutions. In alkaline solutions the observation becomes uncertain, because the alkali itself splits off indoxyl. If the acidity amounts to 1.5 cc. of normal acid per 100 cc. of the isatan solution, the action is much weakened, and at ca. 1.8 cc. of normal acid, there is no more decomposition of isatan at all, which is noteworthy as this percentage of acidity is reached in the cell-sap of older woad-leaves. This does not however exclude isatan-decomposition by the enzyme in the living cell, as the process can be limited to the protoplasm, in accordance with the localisation described.

As the action of the isatan is judged after the formation of indigo-blue, two chemical processes are involved in it, isatan-splitting and indoxyl-oxidation. If the experiment is performed with free access of air, for instance in a thin layer of the isatan solution, with the enzyme floating on it, the indoxyl changes directly into indigo; but if the isatan is decomposed with imperfect access of air, for instance, in the depth of an experiment tube, then it is necessary, during the experiment itself, to render the oxidation of the indoxyl as complete as possible by agitation with air, which does not however always succeed with sufficient quickness, and so limits

the accuracy of the experiment. Of course the liquid cannot be alkalized, because then not only the indoxyl formed by the isatase would become visible, but also the indoxyl set free by the alkali from the isatan not decomposed by the isatase. If the object is to observe the isatase action at a determined temperature, then the enzyme cannot be destroyed at the end of the experiment by heating, but this must be effected by some enzyme poison, as for instance sublimate.

Addition of acid to render the colour of the indigo-blue more pure must likewise be avoided, in order not to decompose isatan.

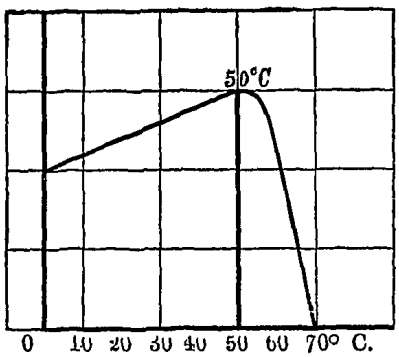
Accordingly it is necessary to perform the reaction in a very feebly acid solution, and to judge of the results without other precautions than a thorough aeration. I have not been able hitherto to answer the question after the nature of the matter, which at the isatan-splitting, most probably is set free beside the indoxyl. Pressed yeast, produces in woad-extract, heated with crude isatase at 30° C., more alcohol and carbonic acid, than in the same extract without isatase (in the proportion of 8 : 5), so that in the first there must certainly be formation of sugar capable of fermentation. But this sugar results, probably not from the isatan, but from the action of other enzymes, present in the crude isatase, on glucosides or carbohydrates, present in the isatan-solution, such as myrosine on myronates, and diastase on granulose.

The process of the decomposition cannot be studied with FEHLING's cupric solution, as the isatan is decomposed by the alkali.

That to SCHUNCK's "indiglucine" no value can be attached follows from § 1.

In order to state the influence of heating on the isatase action, the experiments were arranged as described elsewhere for the indigo-enzymes [1]), with the difference, that for the above reasons, alkalisation and subsequent acidification are here omitted. The very finely powdered enzyme is shaken in an experiment tube with the isatan-solution, and in a water bath, at determined temperature, heated a determined number of minutes. There are always performed two experiments at the same time, so that a colorimetrical comparison of the produced indigo is possible, e.g. at 48° C. and 50° C., or at 40° and 60°, 45° and 55°, etc. The best results were obtained with dilute isatan-solutions, which are brought, as exactly as possible, to an acidity of 0.5 cc. normal per 100 cc. of liquid, and with so little enzyme, that the complete conversion was very slowly accomplished and took about half an hour.

[1]) Indigofermentation pag. 586.



Action of isatase on isatan.

The optimum for the action was found at 48° to 50° C., but could not be determined more accurately as differences of two 2° C. produce no distinct colorimetrical difference. At 70° C. the enzyme is completely destroyed. The minimumlimit is low, far below 0° C., as is seen in the figure. Noteworthy is the slowness with which the intensity of action decreases at decrease of temperature, and the quickness with which it takes place when the temperature rises. So the action at 10° and at 0° C. respectively is as strong as at 60° and 60.5° C.

On other substances but isatan isatase seems not to act; it has certainly no action on indican, neither could I decompose with isatase the potassium indoxyl-sulphate in horse urine.

When judging of these experiments it must be kept in view that other enzymes are present in the crude isatase, which may produce substances not indifferent for the isatase action. So, mention was made above of the presence of myrosine and diastase in the crude isatase preparations, and below I will refer to the presence of peroxydase.

5. *Extraction of indoxyl from the woad-leaves.*

Once acquainted with the chief proporties of isatase and isatan, it is possible at will to extract isatan or indoxyl from the woad. Though in my former communication I spoke already of the indoxyl extraction, my being unacquainted with isatase prevented me from doing this with perfect clearness.

As alkalies produce indoxyl from isatan the extraction of woad-leaves therewith will at every temperature produce indoxyl. But by the presence of alkalies the indoxyl becomes so very oxidisable and then passes at the air so quickly into indigo, that the air, ever present in the leaves, causes a great portion of the indoxyl to get lost. On the other hand, neutral, or feebly acid solutions oxidize much more slowly; it is true that also in these finally all the indoxyl passes into indigo, but such solutions keep unchanged for hours at room temperature and are fit for studying the properties of the indoxyl.

The chief point for obtaining such neutral or feebly acid indoxyl solutions from woad-leaves, is during the extraction to further the isatase-action, consequently to do the very thing which I formerly

indicated as essential for the indoxyl extraction from indican plants, where all depends on the action of the indigo-enzymes. With woad this can best be effected by keeping the extraction temperature between 45° and 50° C, and by addition of chalk or of a salt of feebly alkaline reaction, partly to neutralize the acid of the leaves. Thus a good result is obtained by entirely filling a wide-mouthed stoppered bottle with young woad-leaves, and pouring over them a $^1/_2$ pCt. dinatrium-phosphate solution ($Na^2 H PO^4 + 12 H^2 O$), heated at about 50° C., removing the air as much as possible, closing the bottle and allow it to stand at 40° C. for 24 hours. By decantation and pressing the leaf matter, boiling and filtering, all the indoxyl is obtained in an amphoteric solution, which is somewhat brownish, but is excellent for indoxyl experiments. The presence or absence of undecomposed isatan is observed by precipitation with lead acetate, whereby the indoxyl remains dissolved. The indoxyl can also be shaken out with ether and in the remaining liquid sought with isatase for isatan. Not decomposed isatan remains also in the filtrate, when the indoxyl is allowed to oxidize at the air and the indigo-blue is filtered off.

The ether solution of the indoxyl, obtained by shaking it out of the extract, can be evaporated at low temperature at the air, by which the indoxyl is left behind as a liquid soluble in water, which can be coloured by different impurities. Though the watery solution of this "purified indoxyl" is inconstant at the air, its oxidation to indigo-blue proceeds slowly enough for studying the influence which different substances exert on this process.

Various circumstances have induced me to put anew the question, whether in this oxidation an oxidizing enzyme is active [1]). After much doubt I have finally, as before, come to the conclusion that such is not the case. My primitive uncertainty was caused by the very unequal acceleration of the oxidation of indoxyl solutions by different powders spread on the surface. So the oxidation is somewhat furthered by the crude enzyme of woad, and very strongly, by that of *Indigofera leptostachya*, but by boiling, the crude enzymes are by no means deprived of this property. By a minute comparison of the behaviour of crude indoxyl solutions prepared from isatan and indican, with "purified" ones [2]), I ascertained

[1]) Mr. BRÉAUDAT erroneously asserts (Compt. rendus T. 127, p. 769, 1898 and T. 128, p. 1478, 1898) that in the extracts of *Isatis* indigo-white occurs, which, by an oxydase is turned into indigo-blue.

[2]) Besides from woad I prepared indoxyl by decomposing in a closed bottle a 4 pCt. indican solution with indigo enzyme at 60° C. Moreover Mr. H. TER MEULEN had the kindness to prepare for me in the Chemical Laboratory of the Polytechnical School indoxyl solutions in chemical way. The "purified" indoxyl was always obtained by ether extraction.

that, both in the crude enzymes and in the crude indoxyl solutions, there are present soluble and insoluble chemical compounds, which influence the quickness of the indoxyl oxidation, but which are not destroyed by enzyme poisons and by heating, and which accordingly have not the nature of enzymes.

Crude isatase has neither an oxidizing action on pyrogallol, hydrochinon, and guajac emulsion.

Though thus oxydase is wanting in the crude isatase, there is present in it, as in all such like powders, prepared at random from higher plants, peroxydase ("leptomine" of RACIBORSKI) [1]), that is the enzyme which, in the presence of hydrogen peroxyd, colours guajac emulsion blue. But indoxyl is by no means oxidized by it to indigo.

6. *Nekrosis and Nekrobiosis.*

Living tissues can die off in two ways: by necrosis, that is the dying of the protoplasm with simultaneous destruction of the enzymes, and by necrobiosis, in which the protoplasm dies, but the enzymes remain active. The phenomenon, formerly described by me as the "blue stripe" in partly killed woad-leaves, on the confine of the living and the dead portions, which both retain their green colour, reposes accordingly on necrobiosis. The action of isatase on isatan explains this phenomenon satisfactorily and renders my former hypothesis of alkali formation at the dying of the protoplasm superfluous.

The simplest way to perform the experiment is to kill the tip of a young woad-leaf in a BUNSEN flame, or in the vapour of boiling water, then to allow the leaf to remain at ordinary temperature, by which in the said part alone indigo precipitates. If the chlorophyll pigment is extracted with alcohol, then both the "living" and the "dead" parts become colourless, the portion between them blue. The phenomenon is best distinguished in young woad-leaves; in older leaves, with a higher acid percentage, it is hardly to be observed because the acid renders the isatase inactive.

In various other plants, too, nekrobiosis causes formation of pigments. If these pigments are brown or black, and if the experiment is performed in the usual way with the leaves of these plants, then the coloured stripe may become still much more marked than in the woad. Particularly fit for this demonstration are the leaves of *Pyrus communis*, *Trollius*, *Aconitum*, *Asarum.*, *Salix purpurea*, *Populus nigra* and several other species, which at necrobiosis turn of a jet

[1]) Berichte der Deutsch. Botan. Gesellschaft. Bd. 16, pag. 52, 119, 1898.

black and at necrosis remain green. Pear-leaves especially are recommendable for the experiment; the enzyme in them is tyrosinase, the nature of the chromogene is unknown, tyrosine it is not. Hence, when preparing a herbarium, the chief thing to keep such plants uncoloured, is to prevent necrobiosis. This frequently happens of itself, as the acid cellsap is so much concentrated in drying, that enzyme action cannot occur; so in the drying of woad-leaves, where the highly sensitive isatase remains inactive. In other cases, to obtain this end, it will be necessary to destroy the enzyme, either by boiling water, or by poisonous vapours.

Sometimes necrobiosis gives rise to aromatic or stimulant matters, which are present in the plant itself as glucosides, from which they are set free by specific enzymes at the dying of the cells. This fact is well-known regarding the myronates and the myrosine of the Cruciferae, the amygdaline and emulsine of the Amygdaleae, the spiraeine, gaultherine and gaultherase of *Spiraea*. But it holds good, too, for the cumarine of *Asperula odorata*, which appears not in it as such, but as a glucoside, which by necrosis continues unchanged and hence can be removed from the plant by boiling, while there is besides in this plant a specific enzyme, which by necrobiosis produces from the glucoside cumarine. This enzyme is not identic with emulsine and differs likewise from gaultherase. In a quite corresponding way the aromas originate from the fruit of the vanilla and the roots of *Geum urbanum*.

The comparative study of necrosis and necrobiosis in plants shows the way for the detection of a number of new chromogenes or glucosides and specific enzymes.

Conclusions.

Indoxyl occurs not, as I formerly thought, in a free state in the woad but as a loose compound, called by me isatan.

Isatan is only constant in feebly acid solutions, and is obtained by extracting the woad therewith. It is decomposed, under formation of indoxyl, by alkalies and stronger acids, and in solutions, less acid than 1.5 cc. of normal acid per 100 cc., by an enzyme, isatase, which acts the most vigorously at 50° C., and occurs in all parts of the woad-plant.

Isatan is not decomposed by the indigo-enzymes nor by microbes in as much as the latter do not form alkali. Isatase does not act on indican.

Isatase is localized in the chromatophores, isatan in the protoplasm,

which is in accordance with the formerly described localisation of the indigo-enzymes and of indican.

If woad is extracted without acid, so that the isatase can act, or with dilute alkalies, e.g. $^1/_2$ pCt. solution of dinatrium phosphate, indoxyl is produced.

The necrobiotic stripe in partly killed woad-leaves results from the action of isatase on isatan.

Geology. — "*The Amount of the Circulation of the Carbonate of Lime and the Age of the Earth*". II. By Prof. EUG. DUBOIS. (Communicated by Prof. J. M. VAN BEMMELEN.)

In my first communication on this subject I have quoted a number of reliable data from which it follows that the waters of those rivers in whose drainage areas much limestone occurs, as is mostly the case with the larger rivers, are more than saturated with carbonate of lime, when reaching the ocean.

In consequence of their being polluted, to an extraordinary high degree, with organic matter, the quantity of carbonate of lime in the waters of many rivers of that kind, whose drainage areas are very thickly populated, in Europe and partly too in other parts of the world, is larger than in the primitive condition, before man existed in large number, thus during almost the whole past of the earth. In this respect I draw attention to the relatively higher quantity of carbonate of lime in such rivers as the Thames and the Seine, and also of the difference in that quantity between small and large rivers and lakes, as well as of some other facts showing the influence of the pollution of the water by organic matter on the relative quantity of dissolved carbonate of lime. The drainage water of soils, rich in humus, holds, for instance, considerably more carbonate of lime in solution than would correspond with saturation under the only influence of the atmospheric carbonic acid. But down the course of the rivers the last influence becomes by far the more preponderant.

Taking into consideration that in general the quantity of carbonic acid, produced by the decomposition of organic matter, increases somewhat at the mouths of the rivers, where much of that matter settles, and starting from the existing analyses, it seems to me that an average quantity of 95 mgrms. carbonate of lime per litre of water would represent, on the whole, with approximative accuracy, the primitive condition at the mouths of those rivers which have been so largely in contact with limestone that their waters could be saturated with carbonate of lime.