XXX.—*Some Constituents of Natural Indigo.*
Part I.

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It is well known that natural indigo contains, in addition to indigotin and indirubin, certain impurities such as indigo-brown, indigo-gluten, mineral matter, and frequently traces of kaempferol. Indigo-brown and indigo-gluten have been but little examined, for not only do they possess a somewhat uninviting nature, but their general reactions do not indicate any points of special interest. The present work has originated from a desire to improve the present methods of indigo analysis, and this it appeared could only be accomplished by the isolation and subsequent examination of the properties of these impurities. As a result, a new analytical method has been devised, termed the "tetrasulphonate" process, and a description of this has already been given by one of us (*J. Soc. Chem. Ind.*, 1906, 25, 735). In connexion with indigo-brown, the subject of this paper, it was important to determine if any relationship existed between this substance and indigotin itself, for should such be the case, an explanation could

thus be suggested as to the somewhat unsatisfactory yields of indigo which are obtained from the plant.

Literature on the subject of indigo-brown is extremely meagre, and appears to be confined to the communications of Chevreul (*Gmelin, Handbook of Chem.*, 1859, 13, 48) and Berzelius (*ibid.*). In order to isolate this product, the latter chemist digested powdered indigo with boiling dilute sulphuric acid to remove the gluten, and subsequently with potassium hydroxide solution to dissolve the brown. The alkaline liquid was neutralised with acetic acid, evaporated to dryness, the residue digested with boiling alcohol, and the solution evaporated. Thus obtained, the indigo-brown consisted of a dark coloured, transparent resin, sparingly soluble in alcohol, but readily so in alkaline solutions. It contained nitrogen, and on dry distillation evolved ammonia together with some quantity of an empyreumatic oil.

Schunck, during his well-known researches on indican, prepared from this substance by means of dilute acids (*Phil. Mag.*, 1855, [iv], 10, 74, and *ibid.*, 1858, 15, 127) various brown, amorphous products, for example, α -indifulvin, $C_{22}H_{20}O_3N_2$; β -indifulvin, $C_{44}H_{38}O_3N_4$; indifuscin, $C_{24}H_{20}O_3N_2$; indifuscone, $C_{22}H_{20}O_3N_2$; indiretin ($C = 66.04$; $H = 5.57$; $N = 3.83$); and indihumin, $C_{10}H_9O_3N$.

The latter compound Schunck considered was, perhaps, identical with indigo-brown, and described it as a brown powder insoluble in water and alcohol, but soluble in alkalis. With nitric acid it gave an orange-yellow, amorphous product.

Somewhat later, Schunck and Roemer (*Ber.*, 1879, 12, 2311) studied the action of hydrochloric acid on indican in the absence of air. Indigotin was not formed, but a brownish-yellow precipitate quickly separated, which was partly soluble in alkali. Experiments carried out with the object of converting this product into indigotin were unsuccessful.

EXPERIMENTAL.

Finely powdered crude Bengal indigo containing approximately 62 per cent. of indigotin was digested with boiling dilute hydrochloric acid to remove the gluten, during which operation it lost 21.5 per cent. in weight. Experiment showed that pyridine, probably the only suitable solvent for this purpose, readily extracted indigo-brown from the residue, and that for this object merely a short digestion at the boiling point was necessary. After cooling, the mixture was filtered and the residue washed with cold pyridine as long as the filtrate possessed a brownish-red colour, the final washings, which contained a trace of indigotin, being neglected. In this manner the indigo suffered a further loss of 18.03 per cent., and was then found to contain 91.1 per cent. of indigotin.

The pyridine filtrate was evaporated to a small bulk, and while hot was treated with twice its volume of alcohol, causing the separation of a brown precipitate, which was collected and repeatedly washed with boiling alcohol. As the final washings possessed a reddish tint, an indication of the presence of indirubin, the product was again dissolved in pyridine and reprecipitated with alcohol, but as this did not appear to effect a complete separation of the impurity it was extracted with boiling acetic acid until an almost colourless filtrate was produced. An analysis of the substance now revealed the presence of about 4 per cent. of mineral matter, and this could only be successfully removed by adding its solution in pyridine to hot dilute hydrochloric acid. The precipitate was collected, washed with dilute acid, then with water, and, finally, again extracted with acetic acid and dried at 100°. Before analysis it was heated to 160°, and in this manner frequently lost 10 per cent. by weight.

Found, C = 68·47, 68·53, 68·34, 68·46 ; H = 4·18, 4·13, 4·30, — ;
N = 9·95, 9·84.

$C_{16}H_{12}O_3N_2$ requires C = 68·57. H = 4·28 ; N = 10·0 per cent. .

In carrying out the nitrogen estimations of this and the other brown compounds referred to in this paper, it was found that the gas was evolved much more slowly than is usually the case, fully one hour longer being required for the operation.

This substance, which forms the main constituent of the pyridine extract, closely resembles in general properties the indigo-brown of Berzelius and the indihumin of Schunck (*loc. cit.*), and consists of a brown powder insoluble in alcohol, but soluble in alkaline solutions with a deep brown coloration. The figures required by Schunck's formula, $C_{10}H_9O_3N$ (C = 62·63 ; H = 4·71 ; N = 7·33), are not in agreement with those given above, and it thus appears that the two compounds cannot be identical. On the other hand, the fact that it is only completely dehydrated at 160° and the nitrogen difficulty referred to above may possibly account to some extent for this disparity. It is practically insoluble in all the usual solvents with the exception of hot pyridine, and its properties are of an uninteresting nature. On dry distillation, it gives off yellowish-brown vapours having an alkaline reaction, and which condense to form a small quantity of oily matter of the same colour, as noted by Berzelius, and its behaviour with nitric acid is in accordance with the statement of Schunck. It is readily reduced by zinc dust in alkaline solution with formation of a pale brown liquid, which, on neutralisation with acid, deposits a precipitate of the same colour, and this does not appear to reoxidise on standing in air. By means of acetic anhydride and sulphuric acid it is probably converted into an acetyl compound, which differs but little in property

from the original substance, except that it is less readily attacked by alkaline solutions. With acetic anhydride and zinc dust the acetyl derivative of its reduction product is certainly formed, and this consists of a pale brown, amorphous powder somewhat readily soluble in alcohol.

Action of Boiling Potassium Hydroxide Solution.—4.5 grams of the substance $C_{16}H_{12}O_3N_2$ were digested with 50 c.c. of a 50 per cent. potassium hydroxide solution for one hour at the boiling point in an open flask. The product dissolved with considerable difficulty, and as the liquid became more concentrated, a tarry precipitate, evidently consisting of a potassium salt, commenced to separate. The mixture, diluted with water, was carefully neutralised with sulphuric acid, and the resulting brown precipitate collected and washed. It weighed 2.85 grams.

The filtrate and washings were evaporated to dryness, the mass extracted with boiling alcohol, and the extract itself then evaporated. The crystalline residue was treated with water, the solution filtered from a trace of flocculent matter, neutralised with alkali and rendered faintly acid with acetic acid. Ether removed from this liquid a colourless acid, which was purified by crystallisation from benzene.

Found, $N = 10.22$. $C_7H_7O_2N$ requires $N = 10.21$ per cent.

It formed colourless prisms melting at $143-141^\circ$, and evidently consisted of anthranilic acid.

The brown residue, 2.85 grams, was again submitted to the action of the boiling alkali, by which means it was further reduced to 2.2 grams, a small quantity of anthranilic acid being thus produced. In a third treatment it was practically unattacked. These results are interesting and indicate that the substance $C_{16}H_{12}O_3N_2$ consists probably of a complex indoxyl derivative, which may be either derived from indoxyl alone or result from its condensation with some second substance derived from the indigo plant. With the hope of obtaining some further information on this point, it seemed desirable to determine the composition of the brown residue which was not attacked by the alkali.

For purification the product was dissolved in pyridine, filtered from a trace of insoluble matter, and the filtrate poured into dilute hydrochloric acid and thoroughly washed. For analysis it was dried at 160° .

Found, $C = 71.39, 71.29$; $H = 4.05, 4.23$; $N = 7.94, 7.92$ per cent.

It would not be of value to speculate as to the formula of this product, for which no simple expression appears to be available. It is interesting to note, however, that it contains a considerable quantity of nitrogen. It possesses a more olive tint than the sub-

stance $C_{16}H_{12}O_3N_2$, but its properties are of a similar nature. These call for no special note, and are of an uninteresting character.

Indirubin.

The alcoholic pyridine filtrates from the substance $C_{16}H_{12}O_3N_2$, on partial evaporation and standing, deposited crystals which were collected on the pump, washed with a mixture of pyridine and alcohol, and finally crystallised from nitrobenzene, employing animal charcoal.

Found, C = 72.94; H = 4.09; N = 10.72.

$C_{16}H_{10}O_2N_2$ requires C = 73.28; H = 3.82; N = 10.69 per cent.

It formed long, glistening needles soluble in boiling acetic acid with a violet-red colour, and had all the reactions assigned to indirubin. The identity of natural and artificial indirubins, about which there was formerly some controversy, is now fully established (Marchlewski and Radcliffe, *J. Soc. Chem. Ind.*, 1898, 17, 434), and no difference could be observed between the above-mentioned sample and one that had been synthetically prepared.

The amount present in the Bengal indigos examined was very small, and in one case only 5 grams were obtained from 5 lb. of the dye-stuff. As a result of experiments, no Bengal indigo has been found which, when analysed by the above method, did not contain traces of indirubin.

In Java indigos, as is well known, large quantities of this substance, are frequently present, and this is due to certain special processes which are employed during the manufacture of the colouring matter from the plant.

The more soluble Constituents of Indigo-Brown.

The mother liquor from which the indirubin had been removed on further treatment with alcohol deposited a small quantity of a brown, amorphous precipitate, which was collected, repeatedly washed with boiling alcohol and dried. It was now digested with boiling acetic acid, filtered from insoluble matter, evidently consisting of the above-described substance $C_{16}H_{12}O_3N_2$, the liquid allowed to stand overnight, and filtered again if necessary. The acetic acid solution was treated with boiling alcohol, causing the separation of a brown precipitate, which was removed and well washed with alcohol. Examination showed that it was contaminated with mineral matter, and it was therefore dissolved in pyridine, the solution poured into dilute hydrochloric

acid, and the product collected. Analysis of the substance, dried at 160° , gave :

Found, C = 66.13 ; H = 4.84 ; N = 9.85.

$C_{24}H_{22}O_5N_3$ requires C = 66.66 ; H = 5.09 ; N = 9.72 per cent.

It consisted of a brown powder identical in appearance with the substance $C_{16}H_{12}O_3N_2$, but differed from this compound in that it was soluble in acetic acid. In general properties, however, the two products are so similar that no detailed account is necessary in this case. The amount of this substance present in indigo-brown is so small that extended experiment was not possible, but sufficient was available to determine that on long digestion with boiling potassium hydrate solution, anthranilic acid is formed, and that the reaction appears to follow a similar course to that which was observed with the substance $C_{16}H_{12}O_3N_2$.

The alcoholic pyridine filtrate from the compound $C_{24}H_{22}O_5N_3$ deposited some crystals of indirubin, which were removed, and the filtrate was treated with alcohol. The precipitate thus formed was neglected, and the clear liquid diluted with its own volume of benzene and evaporated to a small bulk. On cooling, a brown, amorphous powder separated which was collected and washed with benzene. Examination showed that this product was contaminated with a red colouring matter, and to remove this it was dissolved in alcohol, the solution poured into ether, and the ethereal liquid extracted with dilute alkali. The brown-coloured extract was neutralised with hydrochloric acid, the precipitate collected, and very thoroughly washed. For analysis it was dried at 160° :

Found, C = 64.86 ; H = 4.81 ; N = 9.27.

$C_{16}H_{14}O_4N_2$ requires C = 62.43 ; H = 4.69 ; N = 9.39 per cent.

This compound, which is present in indigo-brown in but trifling amount, consists of a pale brown, amorphous powder, and is distinguished from the above-described brown substances by its ready solubility in alcohol, and the fact that when heated it gives a voluminous charcoal. Its general properties are, however, so similar to the other brown products as not to merit description.

The alcoholic benzene filtrate from which the above described compound had been isolated was evaporated to dryness, the residue dissolved in ether containing a trace of alcohol, and the solution extracted with dilute alkali in order to remove a small quantity of brown substance which was still present. The ethereal solution, on evaporation, yielded a crystalline residue which was washed two or three times with boiling light petroleum and purified by recrystallisation from nitrobenzene. This substance was indirubin, which is evidently, therefore, the only red compound present in the Bengal indigos examined during the

progress of this work. The statement by one of us (W. P. Bloxam, Trans., 1905, 87, 979) that indigo contains a non-nitrogenous red substance cannot thus be accurate, and arose from the contamination of the product with secondary compounds, which interfered with the satisfactory operation of the well-known qualitative method for the detection of nitrogen when applied to it.

Again, the statement of Bergtheil (*Report of the Indigo Research Station, Sirsiak*, 1906), "Preliminary examination . . . has shown decisively that there is more than one red body in most commercial indigos," is difficult to understand, at least as regards the Bengal variety, and his further results will be awaited with interest.

The light petroleum extracts from the crude indirubin isolated in the above-described manner were decolorised with animal charcoal and evaporated. An oily residue was thus obtained which became semi-solid on standing, and had the properties of a wax. The amount isolated was minute in respect of the quantity of indigo extracted. By exhausting the dried leaves of the *Indigofera arrecta* and *I. Sumatrana* with boiling alcohol, wax-like compounds were obtained of a very similar nature, which, however, did not appear to merit careful examination.

The investigation of a sample of Java indigo showed that this contained a brown product probably identical with that isolated from the Bengal variety, but in smaller amount. Some quantity of crystalline indirubin was also prepared from it.

Action of Boiling Dilute Acids on the Leaf Extract.

An aqueous extract of the air-dried leaf of *I. Sumatrana* (1000 grams) was evaporated to dryness, incorporated with sand, and extracted with alcohol. After removal of the alcohol, the residue was dissolved in water and digested at the boiling point with 10 c.c. of hydrochloric acid for one hour, and the mixture then allowed to stand overnight. 3.5 grams of a dark-coloured, resinous product had separated, which was collected and treated with alcohol, by which means a small quantity of crystalline indigotin (0.05 gram) remained undissolved. As an examination of the aqueous filtrate indicated the absence of indoxyl or other substances convertible into indigo by oxidation, this result is fully in harmony with the experiments of Schunck and Roemer (*loc. cit.*) on the action of hydrochloric acid on indican in the absence of air.

The alcoholic extract of the resinous substance on treatment with ether gave a reddish-brown precipitate, which was collected and well washed. For purification it was treated with cold alcohol, the residue dissolved in pyridine, and poured into dilute hydrochloric acid

with the object of removing a trace of mineral matter which adhered to it somewhat tenaciously. After being dried at 160° , it was found to contain $N=5.67$, and a second preparation gave $C=66.04$; $H=5.00$ per cent.

This product, though somewhat redder in appearance, had many properties in common with the indigo-brown substances described in this paper, but the low percentage of nitrogen it contains shows that it is more closely allied to the brown compounds that Schunck (*loc. cit.*) prepared from his indican.

On the other hand, as but a crude plant extract had been employed, it was quite possible that the brown compounds produced in this manner would be contaminated with non-nitrogenous impurities. The latter might, for instance, consist of the well-known reddish-brown anhydrides or phlobophanes which are obtained when solutions of catechol-tannins are boiled with dilute acids, and this was quite possible, as the work of Bergtheil (*Trans.*, 1904, **85**, 876) has indicated the presence of tannin in the leaf. To confirm this point, application was made to Prof. H. R. Procter, who was kind enough to allow analyses of the air-dried leaves of *Indigofera arrecta* and *Sumatrana* to be carried out in his laboratory, with the following result :

	<i>Indigofera</i> <i>arrecta</i> .	<i>Indigofera</i> <i>Sumatrana</i> .
Tannin matter absorbed by hide	2.6	1.6
Soluble non-tannin matters	30.4	38.4
Insoluble in water at 15°	55.5	52.1
Water	11.5	7.9

During these analyses a considerable amount of the indican present in the leaf was hydrolysed, presumably by its own ferment, and the separated indigotin was deposited amongst the hide-powder. Again, it is well known that hide-powder is apt, to some extent, to carry down substances other than tannins, so that it is evident that these varieties of *Indigofera* contain little if any tannin matter. On the other hand, if a compound capable of producing, as is anticipated, non-nitrogenous brown substances exists in the leaf, the presence of only a minute quantity is necessary to explain the point under discussion. During the "isatin" process which has been employed by Orchardson, Wood, and Bloxam (*loc. cit.*) for the analysis of the leaves, it was noted that in addition to indirubin certain brown products soluble in alkali are simultaneously formed, but it has not yet been possible to determine if these contain nitrogen.

When the compound $C_{16}H_{12}O_3N_2$ is dissolved in an alkaline hydro-sulphite solution, and the liquid allowed to stand at $50-60^{\circ}$ even for several days, no indigotin separates on oxidation, so that the frequently asserted superiority of the natural over the artificial variety of indigo, if correct, cannot be accounted for in this manner.

Summary.

The results of this investigation tend to strengthen the suspicion that the constituents of indigo-brown are derived from indoxyl during the manufacture of indigo from the plant. The fact that they are partially converted into anthranilic acid on boiling with strong alkali, and that their carbon percentage composition can be approximately represented as a multiple of C_8 , together with their high percentage of nitrogen, harmonises with this view. On the other hand, it suggests itself as possible that although they are derivatives of indoxyl, these compounds need not necessarily be produced during the manufacture, and may be formed from some substance other than indican present in the plant. That on boiling the leaf extract with dilute acid a somewhat similar brown product is formed, tends at first sight to confirm this view, but the question had then to be answered as to what becomes of the indoxyl during this operation, for little or no indigotin, owing to the absence of oxidising agents, is or can be produced. Schunck and Roemer (*loc. cit.*), who studied this reaction with indican in the cold, as previously stated, obtained an amorphous condensation product from this substance, though it is to be noted that some doubt now exists as to the purity of their compound in view of the isolation of crystalline indican by Hoogewerff and ter Meulen (*Proc. K. Akad. Wetensch. Amsterdam*, 1900, 2, 520). That indoxyl, however, does condense in this manner seems certain, for Hazewinkel (*Proc. K. Akad. Wetensch. Amsterdam*, 1900, 2, 152) states that when indoxyl is boiled with acid it changes into a red substance and emits a pungent odour. Rawson, again (*Report on the Cultivation and Manufacture of Indigo*, Mozufferpore, 1904, p. 86), says: "The blower . . . by quickly getting rid of CO_2 gas . . . prevents decomposition of a portion of the colouring matter into worthless brown substances, which takes place to a greater extent under other conditions." We have also noted that aqueous extracts of indigo leaf, on long standing, gradually exhibit a decrease in the quantity of indigotin produced by analysis, and this appears to be due to a secondary condensation of the character under discussion. Beyerinck (*Proc. Roy. Acad. Scien. Amsterdam*, 1899, 120), in a paper "on the formation of indigo from woad (*Isatis tinctoria*)," finds that the action of excess of air on a dying woad leaf causes a loss of indoxyl with formation of unknown colourless and brown substances. Again, he states that "strong acids, just as alkalis, though in a far less degree, favour the formation of indigo from indoxyl, but then part of this substance constantly changes into a brownish-black matter." Finally, "during the slow dying of the leaves of *Indigofera* plants in CO_2 containing air, a small quantity of a brown pigment is formed,"

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and ". . . many other indigo plants give in place of indigo a black or a brown pigment."

As regards the yield of indigo from the plant, Bergtheil (*loc. cit.*) considers that at least 87 per cent. of the theoretical yield of indigotin is accounted for in the manufacturing operation, including 5 per cent. remaining in the leaves after the steeping process, and 10 to 20 per cent. which is lost in the decantation of the supernatant liquid, or "seet" water. On the other hand, a difference of opinion exists as to the actual amount of indigotin which any given sample of the plant is capable of yielding. Very recently Rawson's ingenious persulphate process (*loc. cit.*) for the determination of indigotin from the leaf has been modified by Bergtheil (*J. Soc. Chem. Ind.*, 1906, 25, 735), and independently by Orchardson, Wood, and Bloxam (*ibid.*, 1907, 26, 4), with the result that higher values are now given by it. The latter three authors, however, employing the isatin process suggested to them by Prof. Beyerinck of Delft, by which means the indoxyl is estimated as indirubin, have obtained considerably enhanced figures over those given by the persulphate method, and these results, though given with some reserve, are exceedingly suggestive. In any case some loss remains to be accounted for, and this, we suggest, is probably to be found in the formation of indigo-brown.

The final answer to this and other questions connected with the manufacture of natural indigo can only be given with certainty by a study of the pure glucoside indican, and this we hope shortly to be in a position to communicate to the Society. The isolation of this substance from the leaf in the crystalline condition is by no means an easy matter, and at best a long and tedious process, and Hoogewerff and ter Meulen (*loc. cit.*), its discoverers, obtained but 5 grams from 17 kilos. of the raw material. Though we have been successful in preparing the pure compound from the leaves of *Indigofera arrecta*, the yield was also poor, and we are at present studying a method which we believe will result in the production of a much larger quantity of this exceedingly rare compound.

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