[CONTRIBUTIONS FROM THE SHEFFIELD LABORATORY OF YALE UNIVERSITY.]

STUDIES ON NITRATED PROTEINS. IV. THE IDENTIFICATION OF 3-NITROTYROSINE AMONG THE PRODUCTS OF HYDROLYSIS OF NITRATED FIBROIN.

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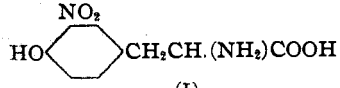
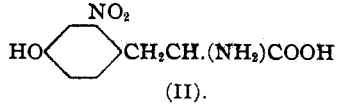
In 1912, K. Inouye[1] made the interesting observation that, if silk fibroin is first nitrated under certain conditions and then subjected to hydrolysis with sulfuric acid, *nitrotyrosine* is one of the products of decomposition. The acid was separated from the other products of hydrolysis by precipitation with phosphotungstic acid, and melted, according to his statement, at 215–216°. He found by analysis 12.13 % of nitrogen, while the calculated value for an amino acid of this constitution is 12.38 %. From the acid filtrate left after precipitation of this hydrolytic product he was able to separate a second aminoacid (?) having a higher melting point. From 50 g. of fibroin 0.3 g. of this substance was obtained which melted at 233–234°. This product gave on analysis 12.26% nitrogen. Inouye believed that his first product melting at 215–216° was *mononitrotyrosine* and wrote as follows regarding the second acid:

"..... wich hingegen bezüglich des Schmelzpunktes von dem oben erwähnten Präparat ab und war auch bedewtend leichter löslich. Es ist hiernach wahrscheinlich, dass es sich um unreines Mononitrotyrosin handelte."

Inouye was unable to assign a definite structural formula to his nitroacid because the structure of Strecker's nitrotyrosine,[2] which is prepared by the action of nitric acid on tyrosine, was not known at the time of publication of his paper. The acid might be assigned one of two possible formulas (I and II), and it was not improbable that he was actually dealing with both modifications.



(I).



(II).

Since the publication of this paper of Inouye's the constitution of Strecker's acid has been established by Johnson and Kohmann[3] and it was therefore of the greatest importance to obtain again Inouye's nitroacid from nitrated fibroin and compare it with the synthetical product. This has now been done, and in this paper evidence will be presented which has led to the conclusion that Inouye's acid is an ortho-nitrophenol combination corresponding to Formula I.

[1] *Z. physiol. Chem.*, **81**, 82 (1912).

[2] *Ann.*, **73**, 70 (1850).

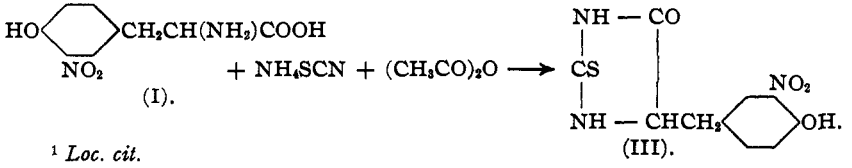
[3] THIS JOURNAL, **37**, 1863 (1915).

Johnson and Kohmann[1] have shown that tyrosine undergoes nitration, under the conditions recommended by Strecker[1] and also Städeler,[2] forming a mixture of the two possible isomers represented by Formulas I and II. The *ortho*-nitro compound is the chief product of the reaction. The presence of the two acids was shown by conversion into their corresponding thiohydantoins, which we succeeded in separating by fractional crystallization. The *o*-acid was observed to melt at 231° with decomposition. Its thiohydantoin derivative and that of the *m*-acid melted at 238–242° and 270°+, respectively. The *m*-acid (II) has not been synthesized.

I have now repeated the work of Inouye and find, as he has already shown, that nitrotyrosine is formed by hydrolysis of a nitrated fibroin with sulfuric acid. I first followed his directions and confirmed his observation that the acid is precipitated with phosphotungstic acid. I also examined the filtrates after this precipitation and succeeded in identifying nitrotyrosine. Both of these products were carefully examined and it is my conclusion that they are identical. In other words, Inouye only partially precipitated his nitrotyrosine with phosphotungstic acid. I examined very carefully the acids from both sources and found them to be identical with respect to crystalline habit, solubility, and their melting points. Inouye's work was repeated with slight modifications in details of manipulation, and here again only one nitroacid was obtained and it was proven by analysis to be a *mono*-nitrotyrosine. I obtained no evidence of the formation of more than one modification, nor could I detect any evidence suggesting that a *dinitrotyrosine*[3] was present among the hydrolytic products.

The structure of the *nitrotyrosine* obtained by hydrolysis of Inouye's nitrated fibroin is to be represented by Formula I. It is identical with Johnson and Kohmann's *ortho*-nitroacid prepared from tyrosine and forms the same characteristic hydrochloride.

Its structure was also established in the following manner: It interacted with ammonium thiocyanate, in the presence of acetic anhydride, forming a hydantoin combination which was converted into 2-thio-4-(3-nitro-4-hydroxybenzyl)-hydantoin (III) by hydrolysis with hydrochloric acid. This hydantoin was identical with that already described by Johnson and Kohmann.[1] Both compounds melted at 238–240° and a mixture of the two melted at exactly the same temperature.



(I). (III).

[1] *Loc. cit.*

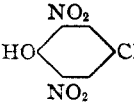
[2] *Ann.*, **116**, 77 (1860).

[3] Johnson and Kohmann, THIS JOURNAL, **37**, 2166 (1915).

Inouye states that his nitroacid, which was precipitated by phosphotungstic acid, melted at 215–216° and that the second acid which was isolated from the filtrates melted at 233–234°. The first melting point is too low and the latter nearly identical with that of the synthetical acid. Our acid melted at 231°+ with decomposition. This point of decomposition, however, is dependent upon the rate of heating and it was my experience that the melting point could be raised to 233–236° if the sulfuric acid bath was heated rapidly. According to our data Inouye's second product (melting at 233–234°) was his purest acid, and his analytical results confirm such a conclusion.

Inouye's observation was of biochemical importance as being the first experimental evidence in support of the assumption, that the yellow color produced in the *Xanthoproteic Reaction* for proteins is dependent in part on the formation of a *mono*-nitrotyrosine combination. From the results of this investigation we are therefore able to ascribe a definite structural formula to this acid. *The aminoacid tyrosine and tyrosine linked in the protein (fibroin) are attacked in a similar manner when exposed to the action of nitric acid, and substitution of the nitro group takes place in a position ortho to the phenol group.*

Whether the second *ortho* position will be substituted by more vigorous treatment of fibroin, forming a dinitroprotein, remains to be established. Such a combination should give on hydrolysis 3,5-dinitrotyrosine (IV) which has been described in a previous paper by Johnson and Kohmann.[1]



(IV).

The study of nitrated proteins will be continued.

Experimental Part.

Nitration of Fibroin.—In this investigation I used the purest skein silk. It was nitrated according to the directions given by Inouye[1] and thoroughly washed with water and dried at 100° to a constant weight.

Hydrolysis of Nitrated Fibroin with Sulfuric Acid.—Fifty grams were hydrolyzed for 10 hours by digesting with sulfuric acid (100 g. concentrated acid diluted with 180 g. of water), and the sulfuric acid then exactly precipitated by addition of barium hydroxide. After filtering off the barium sulfate we obtained an orange-colored solution. This was then concentrated, by evaporation on a steam bath, to a volume of 175 cc. and the latter then examined for nitrotyrosine.

This solution was acidified with sulfuric acid, sufficient reagent being used to produce approximately a 5% solution. To this was added an excess of phosphotungstic acid, when a gummy, dirty brown precipitate was

[1] *Loc. cit.*

obtained. Part of this assumed a granulated condition while the remainder adhered to the sides of the precipitation jar. (This solution was saved.) The precipitate was washed with 5 % sulfuric acid and finally decomposed by digesting with barium hydroxide. The barium tungstate and barium sulfate were filtered off and the filtrate freed from barium by precipitation as barium carbonate. The salt was then removed by filtration and the solution concentrated to a small volume and the last traces of barium removed by adding the required amount of sulfuric acid. After filtering from barium sulfate this solution was concentrated to a small volume and allowed to stand for several days in a desiccator over concentrated sulfuric acid. I obtained about 0.75 g. of material here which crystallized in the form of burrs or rosets of small crystals. This gave Millon's reaction, when applied under special conditions,[1] and possessed all the properties of *mono*-nitrotyrosine.

The acid filtrate left behind after precipitation with phosphotungstic acid (see above) was combined with barium hydroxide to precipitate all the sulfuric acid and excess of phosphotungstic acid. The barium sulfate and barium phosphotungstate were then filtered off and, after saturation with carbon dioxide to precipitate barium as barium carbonate, the resulting solution finally concentrated to a volume of about 100 cc. This was orange-red in color and still contained barium. It was diluted with water and the barium precipitated as the sulfate by adding exactly the required amount of sulfuric acid. The solution was then filtered and allowed to evaporate spontaneously in a vacuum over concentrated sulfuric acid. After concentration to a volume of about 15 cc. crystals began to deposit. About 1.5 g. of material separated here. When first crystallized from boiling water the compound separated as corpuscular crystals which were colored brown. On repeated crystallization, however, the material grew lighter in color and finally deposited in beautiful lemon-yellow rosets or burrs of small needles (or slender prisms). These crystals began to contract at 221°, when heated in a capillary tube, and decomposed at 231° with violent effervescence, giving a black residue. This decomposition point could be raised to 233–236° by rapid heating. Millon's test was positive if carefully applied and the compound dissolved in alkaline solutions with an intense red color. It was dried for analysis at 100°. The substance was identified as *nitrotyrosine*, $(NO_2)^3(OH)^4$-$C_6H_3.CH_2CH(NH_2)COOH$.

Calc. for $C_9H_{10}O_5N_2$: N, 12.3. Found: N, 12.14.

Nitration of Fibroin for a Second Hydrolysis Experiment.—One hundred and sixty-six grams of the purest skein silk were used in this experiment. This was suspended in 4 liters of nitric acid of density 1.12,

[1] Johnson and Kohmann, *Loc. cit.*

and the reaction allowed to continue at ordinary temperature for 48 hours. The silk had then turned bright yellow. It was washed repeatedly with water to remove every trace of nitric acid and finally with 95% alcohol. After drying thoroughly at 80–90° the weight was 155.0 g. Therefore the loss by the treatment applied was 6.6%. If silk noils had been used for the nitration instead of the skein silk the loss in weight would have been much greater.[1]

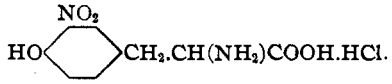
Hydrolysis of the Nitrated Fibroin.—Four hundred and sixty-two grams of concentrated sulfuric acid were diluted with 792 cc. of water, and the nitrated silk hydrolyzed by heating with this diluted acid at 125° for 11 continuous hours. On cooling the resulting solution a black, insoluble substance was obtained. This was separated, but the amount obtained was so small that it was impossible to carefully study its behavior. It was insoluble in water, but dissolved at once in aqueous ammonia and cold sodium hydroxide solution, forming black solutions which were not decolorized by digestion with bone-coal. On adding acids to the alkaline solutions the melanine-like substance was reprecipitated again in an amorphous condition. It possessed no definite melting point. It is our intention to take up again the study of this peculiar substance.

The filtered solution was treated as usual with barium hydroxide to remove all the sulfuric acid. As long as the solution was acid it was practically colorless, but just as soon as the acid was completely precipitated and barium hydroxide was added in excess the solution assumed a red color due to the formation of the barium salt of nitrotyrosine. This change was very characteristic. After filtering from barium sulfate the solution was concentrated to a volume of 145 cc. and having the consistency of a syrup. This was then allowed to stand for one week when a thick magma of crystalline material was obtained. This substance was separated from the syrupy mother liquor by filtration and then dried at 80–90°. The weight was 80 g.

This material (80 g.) was dissolved in 700 cc. of water and 40 cc. of concentrated sulfuric acid added to the solution. This acid solution was then treated with an excess of phosphotungstic acid when we obtained a heavy precipitate of the phosphotungstate of nitrotyrosine. This was separated by filtration and finally decomposed in the usual manner by digesting with a solution of barium hydroxide. The precipitate was filtered off and the excess of barium then removed by adding the calculated amount of sulfuric acid. The filtrate from the barium sulfate was nearly colorless, but turned intensely red when made alkaline. This was concentrated to a volume of about 20 cc. and the syrupy solution mixed with 2–3 times its volume of cold, concentrated hydrochloric acid. The hydrochloride of nitrotyrosine separated at once as yellow

[1] Johnson, Hill and O'Hara, THIS JOURNAL, 37, 2170 (1915).

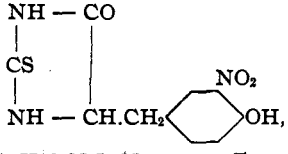
needles. The last dissolved at once in water but was reprecipitated by saturating the solution with hydrochloric acid. This salt began to change color at about 220–221° and then decomposed with violent effervescence at 233–235°. It was identified as the hydrochloride of *3-nitrotyrosine.*[1]



A mixture of this salt with that prepared from pure nitrotyrosine melted at exactly the same temperature. The salt was dried for analysis by heating at 100°.

Calc. for $C_9H_{11}O_5N_2Cl$: N, 10.66. Found: N, 10.66.

Conversion of 3-Nitrotyrosine from Nitrated Fibroin into Its 2-Thiohydantoin—2-Thio-4-(3-nitro-4-hydroxybenzyl)-hydantoin.[1] — Five-tenths of a gram of the above hydrochloride was dissolved in dilute ammonia and the solution evaporated to dryness to decompose the hydrochloride. The dry residue was then dissolved in 10 cc. of acetic anhydride with 0.7 g. of anhydrous ammonium thiocyanate and the mixture heated at 100° for one hour. The solution was finally heated for a few minutes to its boiling point. After this treatment, an excess of dilute hydrochloric acid was added and the solution evaporated to dryness at 100°. The residue obtained was dissolved in fresh hydrochloric acid and the evaporation repeated. On triturating the residue to remove ammonium chloride I obtained the thiohydantoin insoluble in water. It was also insoluble in alcohol but crystallized from boiling glacial acetic acid in the form of diamond-shaped prisms. The substance melted at 238–239° with effervescence, turning red before decomposing. Johnson and Kohmann[2] observed that the thiohydantoin of 3-nitrotyrosine (synthetical material) can be heated to 242° before it will decompose. This, however, happens when the sulfuric acid bath is heated rapidly. I have observed variations of 5–6° and under normal conditions it usually will melt from 238–240°. A mixture of Johnson and Kohmann's thiohydantoin with the hydantoin prepared from the nitrotyrosine from silk melted at 238–239°. The two compounds therefore were identical and the structure of my hydantoin is to be represented by the following formula:



Calc. for $C_{10}H_9O_4N_3S$: N, 15.73. Found: N, 15.6.

The investigation of nitrated proteins will be continued.

NEW HAVEN, CONN.

[1] Johnson and Kohmann, *Loc. cit.*

[2] *Loc. cit.*