a partial hydrolysis of the pyrimidine. The crystals, which were yellow, were filtered off, washed with ether and dried in a vacuum over sulfuric acid. They melted at 136°. A nitrogen determination showed that we were dealing with a hydriodic acid salt of the chloropyrimidine. The compound also gave tests for chlorine and iodine.

Calc. for $C_{11}H_9N_2SCl.HI$: N, 7.68. Found: N, 7.3, 7.26.

This salt could not be crystallized from the common solvents without dissociation. It is insoluble in ether, carbon tetrachloride and benzene, slightly soluble in acetone and very soluble in absolute alcohol. It was easily hydrolyzed by water, giving an oil which finally solidified and melted without further purification at 45°. This was identified as the original chloropyrimidine. When this was mixed with some of the chloro compound there was no lowering of the melting point. The yield of the salt was 12 grams of 84% of the theoretical.

NEW HAVEN, CONN.

[CONTRIBUTIONS FROM THE SHEFFIELD CHEMICAL LABORATORY OF YALE UNIVERSITY.]

STUDIES ON NITRATED PROTEINS. V. THE HYDROLYSIS OF NITRO-FIBROIN WITH HYDROCHLORIC ACID.

BY TREAT B. JOHNSON AND ARTHUR J. HILL.

Received May 20, 1916.

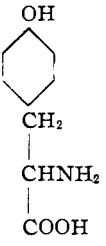
The term "Nitro-Fibroin," as we shall apply it in this paper, is the name of a modified protein which is obtained by treatment of pure fibroin, under definite conditions, with nitric acid of specific gravity 1.12. This sulfur-free protein is very easily attacked by strong nitric acid, but the reaction is not productive of consistent results unless applied under carefully regulated conditions. We have found that, if the protein be exposed to the action of acid of the above strength for a long time and at ordinary temperature (18–25°), a stage in the transformation is finally reached where practically no further action on the protein takes place. In other words, it has been our experience that a nitrated protein of quite definite constitution is formed, which is optically active and does not respond to Millon's test for tyrosine. This substance is obtained in the form of an orange-colored powder and the yield corresponds to about 70% of the weight of the original protein. The method of preparing this interesting product (nitro-fibroin) has already been described in a previous paper from this laboratory.[1]

Inouye[2] prepared a nitrated fibroin by the action of cold nitric acid (sp. gr. 1.12) on silk fibroin and obtained after 48 hours' treatment a yield of nitroprotein corresponding to 85–90% of the weight of fibroin taken. This product was subjected to hydrolysis with dilute sulfuric

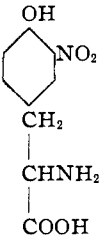
[1] Johnson, Hill and O'Hara, THIS JOURNAL, 37, 2170 (1915).

[2] *Z. physiol. Chem.*, 81, 80 (1912).

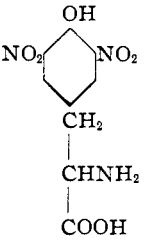
acid, when he made the interesting observation that a nitrotyrosine of unknown constitution was one of the products of decomposition. Johnson[1] repeated this work of Inouye's and isolated, after hydrolysis, the same nitroamino acid. He showed that it is a definite combination formed by the action of nitric acid on the tyrosine in the protein and is to be represented structurally as an *o*-nitro derivative of this α-amino acid as represented by Formula II.[2]



(I).



(II).



(III).

In our third paper on nitrated proteins[3] we called attention to the interesting observation that the nitration reaction—fibroin + nitric acid—can be followed qualitatively by Millon's test for only a few hours. Generally, after about 24 hours' treatment at ordinary temperature, the protein fails to respond to this characteristic reaction, indicating a fundamental change in the tyrosine phenol nucleus, and possibly the end of the first phase of the reaction. The isolation of *o*-nitrotyrosine (II) from the products of hydrolysis of Inouye's nitrated fibroin was the first definite evidence produced showing the true nature of this change. Neither Inouye nor Johnson obtained any evidence of the presence of a dinitrotyrosine (III) among the hydrolytic products of this nitrated protein.

In the light of these results it was therefore of especial interest to investigate the character of the hydrolytic products formed by complete hydrolysis of "nitro-fibroin" prepared according to the method of Johnson, Hill and O'Hara.[4] It was important to determine whether the tyrosine in fibroin undergoes further changes by long exposure to nitric acid (sp. gr. 1.12) with formation of 3,5-dinitrotyrosine (III), which has recently been described by Johnson and Kohmann.[5] Furthermore, it was also important to determine the nature of the other α-amino acids which are constituents of nitro-fibroin. The complete hydrolysis of this protein has now been accomplished, and in this paper we record the results of our investigation.

[1] THIS JOURNAL, 37, 2598 (1915).
[2] Johnson and Kohmann, THIS JOURNAL, 37, 1863 (1915).
[3] Johnson, Hill and O'Hara, *Loc. cit.*
[4] *Loc. cit.*
[5] THIS JOURNAL, 37, 2164 (1915).

Experimental.

The Preparation of Nitro-Fibroin in Quantity.—The nitro-fibroin was prepared according to directions already given in the literature,[1] but we worked with much larger quantities than were used in our original investigation. In applying the reaction on a large scale, it is interesting to note that we again confirmed our original observation, namely, that the nitration of fibroin under the conditions described was productive of a remarkably constant amount of nitro-fibroin. From 479 g. of fibroin, dried at 100°, we obtained 345 g. of the nitrated protein or about 72% of the weight of the original silk fibroin. This result is in close agreement with those recorded in our previous paper. In other words, this yield calculated upon the basis of 20 g. of fibroin gives the figure 14.40, while in our original work we actually obtained in two experiments 14.26 and 14.20 g. of the nitro-fibroin from 20 g. of fibroin. This experiment was repeated with the same result. The nitric acid filtrates left after filtration of nitro-fibroin were saved (see below).

Hydrolysis with Hydrochloric Acid.—Our method of hydrolyzing nitro-fibroin was as follows: Three hundred grams of the nitrated protein, corresponding to 416.5 g. of silk fibroin, were dissolved in 1200 cc. of hydrochloric acid (sp. gr. 1.19). Solution was brought about very quickly and was attended with some foaming, but there was no evolution of carbon dioxide. This solution was then digested in an oil bath for 15 hours at a temperature of 120–130°. It was then allowed to stand at ordinary temperature for 3 days when a dense, brownish precipitate was observed to have deposited in the acid solution. The yield of this material was increased by concentration of the acid solution and then saturating the same at 0° with hydrochloric acid gas. After repeating this operation several times the precipitates were combined and decolorized by digestion in strong hydrochloric acid with animal charcoal. The material was then purified by recrystallization from the same solvent. We obtained in this manner 20 g. of pure material. This substance crystallized from hydrochloric acid in the form of characteristic yellow needles which melted with effervescence at 237°. This compound contained chlorine and proved to be identical with the hydrochloride of 3-nitrotyrosine (II) previously synthesized by Johnson and Kohmann[2] and also isolated by Johnson[2] from the products resulting from the hydrolysis of Inouye's nitrated protein with sulfuric acid.

Calc. for $C_9H_{11}O_5N_2Cl$: N, 10.70; Cl, 13.51. Found: N, 10.85, 10.95; Cl, 13.61.

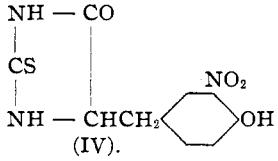
The identity of this salt was also established by the fact that it underwent decomposition with alkali giving 3-nitrotyrosine II melting at 237°

[1] Johnson, Hill and O'Hara, *Loc. cit.*

[2] *Loc. cit.*

with effervescence. A mixture of our hydrolytic product and the synthetical 3-nitrotyrosine melted at 237°.

Our nitrotyrosine also interacted smoothly with ammonium thiocyanate in acetic anhydride solution giving 2-thio-4(3-nitro-4-hydroxybenzyl)-hydantoin (IV)

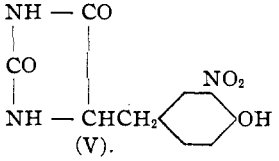


(IV).

which has already been described by Johnson and Kohmann.[1] It melted at 238–240° with decomposition.

Calc. for $C_{10}H_9O_4N_3S$: N, 15.74. Found: N, 15.65.

When this 2-thiohydantoin (IV) is desulfurized with chloroacetic acid it is transformed into 4-(3-nitro-4-hydroxybenzyl)-hydantoin (V).[2] We applied the same reaction with our 2-thiohydantoin and obtained the same sulfur free hydantoin. It melted at 224° to a clear oil. We also obtained the same hydantoin (V) by the action of potassium cyanate on the hydrochloride of our 3-nitrotyrosine from nitro-fibroin. Two



(V).

grams of the hydrochloride of 3-nitrotyrosine were dissolved in water with 2 g. of potassium cyanate and the solution evaporated to dryness. The residue was then triturated with 15 cc. of concentrated hydrochloric acid and again evaporated to dryness. In this manner we obtained smoothly the hydantoin (V) which crystallized from hot water or glacial acetic acid in the form of rosets of needles. They melted at 224° to a clear oil. A mixture of this product and the synthetical hydantoin melted at exactly the same temperature.

Calc. for $C_{10}H_9O_5N_3$: N, 16.75. Found: N, 16.62.

It is interesting to note here that during all these operations we obtained no evidence of changes indicating the presence of any 2-nitrotyrosine or 3,5-dinitrotyrosine. The dinitrotyrosine exhibits a behavior similar to that of 3-nitrotyrosine, when dissolved in strong hydrochloric acid, giving a very insoluble, characteristic hydrochloride.

Examination of the Hydrochloric Acid Solution.—The mother liquor containing the other amino acids of nitro-fibroin (300 g.) was concentra-

[1] *Loc. cit.*

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

ted, after complete separation of 3-nitrotyrosine, to a thick syrup by heating in a vacuum at 100°. Absolute alcohol was then added in excess and the α-amino acids esterified according to the directions given by Fischer.[1] Glycocoll was obtained in large amount in the form of its ester hydrochloride. The total yield of this salt was 141.5 g. and it melted at 144°. A mixture of this material with the pure hydrochloride of ethylaminoacetate melted at the same temperature.

Calc. for $C_4H_{10}O_2NCl$: N, 10.05. Found: N, 9.85, 9.97.

After complete separation of the hydrochloride of ethylaminoacetate the alcoholic filtrate was concentrated by heating under diminished pressure at 100° and the excess of alcohol removed. A black, syrupy residue was obtained which was diluted with one-half its volume of water and the hydrochloric acid neutralized at a low temperature by addition of 33% sodium hydroxide solution. The free amino acid esters were then extracted in the usual manner with ether after saturating the solution with potassium carbonate.[2] After drying carefully and removal of the ether, we obtained 50.7 g. of oil which was fractionally distilled under diminished pressure with the following result:

Fraction 1, 53–63° at 13 mm. = 21.50 g.
Fraction 2, 63–85° at 13 mm. = 24.00
Fraction 3, 85–95° at 15 mm. = 3.00

Total = 48.5

These fractions were saponified and examined for amino acids. Alanine was the only one identified. The usual tests for phenylalanine and leucine were applied to Fraction 3, but this gave, on saponification, nearly pure alanine. From the 3 fractions were obtained 30 grams of this α-amino acid.

Calc. for $C_3H_7O_2N$: N, 15.75. Found: N, 15.90, 15.80.

The results obtained by hydrolysis are recorded in Table I.

TABLE I.

Percentages and Amounts of Amino Acids Produced by Hydrolysis of 300 g. of Nitro-fibroin.

Glycine ester hydrochloride.	Actual glycine.	% of glycine.	Nitrotyrosine hydrochloride.[3]	% of nitrotyrosine.	Alanine.	% of alanine.	Oxalic acid.	Percentage of nitrofibroin accounted for as glycine alanine and nitrotyrosine.
Grams.	Grams.	%.	Grams.	%.	Grams.	%.	Grams	%.
141.5	76.0	25.36	20.0	5.74	30.0	10.0	2.78	41.10

[1] *Z. physiol. Chem.*, 33, 177 (1901).

[2] Fischer, *Loc. cit.*

[3] It is important to note here that this represents pure nitrotyrosine hydrochloride. The actual quantity present was larger but it could not be completely separated on *account of its solubility.*

Examination of the Nitric Acid Filtrate.—After the separation of nitro-fibroin from the nitric acid, it was thoroughly washed with water and the washings added to the original strong nitric acid solution. This was not dicarded by us, but submitted to a careful examination in order to determine its contents. It was of interest to determine whether it contained any characteristic products of oxidation, formed by the prolonged action of nitric acid, and also any nitrotyrosine and other amino acid constituents of the original fibroin. In order to avoid any further oxidation this solution was concentrated by pouring into shallow glazed pans and allowed to evaporate spontaneously at a temperature of 35°. A specially constructed oven was used for this purpose and the operation hastened by passing a swift current of air over the surface of the liquid. We obtained in this manner a yellow, viscous oil containing in suspension hard, dense crystals. In our analysis of this residue we applied the methods utilized by Mörner in his examination of the decomposition products obtained by intense treatment of protein material with strong nitric acid.[1] He demonstrated the formation of oxalic, picric, benzoic and *p*-nitrobenzoic acids and another acid of unknown constitution (*x*-saüre) as products of the reaction. Following his directions in our work we were able to establish conclusively the presence of oxalic acid only. In fact, we found that practically the entire residue apart from the oxalic acid consisted of ammonium nitrate.

In a second experiment we modified our procedure and subjected the residue, after evaporation of nitric acid, to an esterification in order to determine whether any amino acids were dissolved in the liquor. This operation was not productive of any evidence that such combinations were present. In fact, after extraction with ether in the same manner and evaporating the solvent only a very small amount of resinous material was obtained. In other words, the nitric acid solution contained only end products of oxidation.

Specific Rotation of Nitro-Fibroin.—Fibroin is optically active and its rotatory power has been carefully measured by Vignon.[2] This investigator used hydrochloric acid as a solvent for his protein and determined the specific rotation of fibroins from nine different sources. His results were remarkably constant. This protein in every case was laevorotatory and the specific rotation ranged from —39.40° to —48.20°. We now find that our nitro-fibroin is likewise laevorotatory and that the specific rotation is about the same as that of pure fibroin. We obtained the values $[\alpha]_{17}^{D} = -43.39°$ and —44.10°. Solution of the nitro-fibroin was effected by dissolving a weighed amount of the protein in 50

[1] *Z. physiol. Chem.*, **95**, 263 (1915).

[2] *Compt. rend.*, 113, 802 (1891); 114, 129 (1892); *Bull. soc. chim.*, [3] 7, 139, 773, 799 (1892).

cc. of concentrated hydrochloric acid at 0° and then diluting to 100 cc. with distilled water. The solution was then filtered to clarify it. Several rotations were taken at 17° and remarkably constant results were obtained. In fact, the measurements ranged between the values recorded above.

While nitro-fibroin was found to be optically active, on the other hand, the alanine and 3-nitrotyrosine obtained by hydrolysis were both optically inactive. In this connection it is interesting to note that 3-nitrotyrosine, prepared from natural tyrosine (from silk) by action of nitric acid,[1] is likewise optically inactive.

Summary.

Nitro-fibroin[2] has been hydrolyzed by digestion with hydrochloric acid and 41.10 per cent of its molecule accounted for in the form of glycocoll, alanine, and 3-nitrotyrosine. The protein is optically active, but on hydrolysis leads to the formation of optically inactive alanine and 3-nitrotyrosine. The relative proportion of tyrosine to alanine and glycocoll in silk fibroin is 1 : 2.1 : 3.6, while in our nitro-fibroin the ratio of nitrotyrosine to alanine and glycocoll is 1 : 1.7 : 4.4.

Tyrosine linked in fibroin is not attacked by nitric acid of specific gravity 1.12 at ordinary temperature with substitution of two nitro groups *ortho* to the phenolic hydroxyl group of this amino acid.

NEW HAVEN, CONN.

[CONTRIBUTION FROM THE BUREAU OF PLANT INDUSTRY.]

VOLATILE OIL OF EUTHAMIA CAROLINIANA (L). GREENE.

BY G. A. RUSSELL.

Received May 2, 1916.

Euthamia Caroliniana (L). Greene (Family Compositae), is found growing in moist, sandy old fields throughout the United States from Massachusetts to Florida and west as far as Texas. The plant is indigenous to these states and occurs in greatest abundance near the coast, especially in Florida. It is rarely ever found on new land or in woods, but is very frequently found in abundance in old, moist lake bottoms or abandoned fields. It flowers from September to November and each plant produces an abundance of small lemon-yellow blossoms.

Distillation of the Volatile Oil.—On September 8, 1914, Mr. S. C. Hood, scientific assistant in charge of the Florida station of the Office of Drug and Poisonous Plant Investigations of the Bureau of Plant Industry, distilled 83 kilograms of the fresh herb gathered just previous to the flowering stage. The material was gathered in old fields adjacent to the city of Orlando in Orange County, Fla. While yet in the fresh unwilted

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

[2] Johnson, Hill and O'Hara, *Ibid.*