Calc. for $C_{11}H_8O_6N_4S$: N, 17.93. Found: N, 17.91.

4-(3,5-Dinitro-4-hydroxybenzyl)-hydantoin (V).—This was prepared as follows: Four grams of the above thiohydantoin were desulfurized by digesting, in an oil bath at 140°, with 15 g. of chloroacetic acid and 45 cc. of water for 6 hours. A clear solution was obtained, from which on cooling, 2.9 g. of the hydantoin separated. This was purified for analysis by crystallization from glacial acetic acid and separated in large golden-yellow blocks, which decomposed at 235°. The hydantoin is insoluble in water.

Calc. for $C_{10}H_8O_7N_4$: N, 18.90. Found: N, 18.65, 18.85.

Formation of 4-(3,5-Dichloro-4-hydroxybenzyl)-hydantoin (VII) from 4-(3,5-Dinitro-4-hydroxybenzyl)-hydantoin (V).—Two grams of the hydantoin of 3,5-dinitrotyrosine were reduced by warming with 50 cc. of dilute hydrochloric acid and an excess of tin. After the reduction was complete the excess of acid was removed by evaporation, 400 cc. of water added to dissolve the residue and the tin finally precipitated with hydrogen sulfide. After separating from tin sulfide by filtration the filtrate was then concentrated to a volume of 10–15 cc. under diminished pressure and finally diluted with 10 cc. of concentrated hydrochloric acid. Two grams of sodium nitrate dissolved in 5 cc. of cold water, were then added slowly while keeping the solution cold. The corresponding diazonium salt separated as a yellow solid. After complete diazotization the mixture was then poured into 10 g. of strong cuprous chloride solution. On heating, nitrogen was evolved. After complete decomposition of the diazonium compound and cooling the solution, a crystalline substance separated. This contained chlorine and was identified as the hydantoin of 3,5-dichlorotyrosine. It was very soluble in alcohol and crystallized in rhombohedral prisms which melted at 202–203°. A mixture of this compound and a sample of the hydantoin of 3,5-dichlorotyrosine prepared by Wheeler, Johnson and Hoffmann[1] melted at the same temperature.

Calc. for $C_{10}H_8O_3N_2Cl_2$: N, 10.1. Found: N, 9.9.

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

STUDIES ON NITRATED PROTEINS. III. THE CONVERSION OF FIBROIN INTO NITRO-FIBROIN (FIBROIN-XANTHOPROTEIC ACID).

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In our study of the chemistry of the xanthoproteic reaction for proteins, it was apparent to us, after considering what had already been done by previous investigators and also from what we learned by making

[1] *Loc. cit.*

several preliminary tests on our part, that the success of any research dealing with the action of nitric acid on proteins depended to a large extent on the selection of the proper protein material for investigation. It was necessary that the latter be easily obtainable in a high state of purity and be composed of a small number of the characteristic protein α-amino acids. After considering several proteins of both animal and plant origin, we decided that no representative of this class of natural substances, which was available in quantity, fulfilled so well the essential conditions as the characteristic protein—*fibroin*. This can be obtained extremely pure without apparent alteration of its molecular structure, and practically constant in composition. According to the best analyses about 70% of the molecule of fibroin from Italian silk is composed of the three α-amino acids, namely, glycocoll, alanine and tyrosine, which are present in the proportions of 36, 21 and 10%, respectively. This protein is practically free from histidine, and the presence of arginine has been detected by only two investigators.[1] They obtained by hydrolysis of a purified fibroin about 1.0% of its weight in this amino acid. Fibroin contains a small percentage of aspartic acid and in only one case has it been shown that glutaminic acid is present in greater proportion than 3.5% of the molecule.[2] Canton and Italian silk fibroin contain only 1.6 and 1.25%, respectively, of phenylalanine. Fibroin does not contain tryptophane.

It is well known that silk fibroin is colored strongly yellow by the action of strong nitric acid, and by vigorous treatment with the concentrated acid is completely destroyed with the production of picric acid.[3] By some investigators the production of the yellow color is assumed to be due to the formation of xanthoproteic acid,[4] while others believe that picric acid in the reagent involved, which then dyes the silk yellow. The action is undoubtedly a very peculiar one and regarding the real nature of the first phases of the reaction we have very little knowledge. It remains to be established whether the reaction between a protein and nitric acid can be so regulated as to lead to a nitro protein possessing chemical individuality.

The behavior of nitric acid towards silk was investigated as early as 1859 by Vogel.[5] This investigator used apparently crude ungummed material and obtained by action of nitric acid and by a subsequent treatment with ammonia a yellow substance to which he assigned the formula

[1] Fischer and Skita, *Z. physiol. Chem.*, **35**, 221 (1902).

[2] Fischer, *Z. physiol. Chem.*, **53**, 126 (1907).

[3] See our first paper by Johnson and Kohmann, THIS JOURNAL, **37**, 1863 (1915).

[4] Mulder, *Ann.*, **28**, 73 (1838).

[5] *Buchner's neues Repert*, **8**, 1; *Handwörterbuch der reinen u. angew. Chem.*, **7**, 743 (1859).

$C_{48}H_{38}O_9N_6$. He states that different substances are obtained according to the length of time of action of the acid on the protein.

Vignon and Sisley[1] investigated in 1891 the behavior of silk fibroin towards both nitric and nitrous acids. Nitric acid of density 1.33 was used and the observation made that the protein was colored intensely yellow. This was fast to light and was intensified by treatment of the fibroin with alkaline solutions. They made the observation that nitrous acid probably functionates in this change and observed that nitric acid free from nitrous acid will not produce the yellow color. When they reversed their process and treated silk first with nitrous acid a yellow color was also produced, but it was not fast to light. However, if the protein was afterwards treated with nitric acid the yellow color was rendered permanent. Their method of treatment led to an increase of 2% in the weight of the silk and they found 21.6% of nitrogen in their nitrated protein while the original fibroin contained 18.8%. The differences between fibroin and Vignon and Sisley's nitrated protein are recorded in Table I.

TABLE I.

Reagent.	Fibroin.	Nitrated protein.
Conc. H_2SO_4	Soluble giving a colorless solution	Swells up and gives a transparent, viscous mass like albumen
Strong caustic potash	No reaction in cold. On warming dissolves with evolution of ammonia	Turns red and dissolves on warming with evolution of ammonia
Conc. HCl	Soluble	Soluble
$ZnCl_2$ solution	Soluble	Soluble
Dry distillation	Carbonizes with evolution of ammonia	Burns without deflagration. Burns more rapidly than fibroin

This work of Vignon and Sisley[2] was followed by that of Inouye[3] in 1912, who prepared a nitro-fibroin by nitration of fibroin, and investigated the character of the nitroamino acids formed by its hydrolysis with sulfuric acid.

In our work, the results of which are described in this paper, we have confined ourselves to a study of the behavior of fibroin towards nitric acid of specific gravity 1.12. The temperature has been kept uniform (18–25°) and the time period has been carefully regulated. The change in the protein has been studied quantitatively as well as qualitatively. After carrying out, under specific conditions, a series of nitration experi-

[1] *Bull. soc. chim.*, [3] **6**, 898 (1891); *Ber.*, **25**, R. 14 (1892); *Compt. rend.*, **113**, 701 (1891).

[2] *Loc. cit.*

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

ments on pure fibroin, we have made the interesting observation that the action of the acid on the protein is a gradual process until about 30% of the fibroin is carried into solution. This condition obtains after exposure to the action of the acid for about 192 hours. The remaining 70% of the original fibroin is left behind, suspended in the acid, as a pulverulent, orange-colored powder which is very resistant to further action of the acid. This modified protein, the properties of which are described in the experimental part of this paper, remained practically unchanged after submersion in nitric acid of sp. gr. 1.12 for thirteen days. It is questionable whether our nitrated product is identical with the so-called nitrofibroin described by Inouye.[1] Before this can be established it will be necessary to develop methods of determining quantitatively the relative proportions of the characteristic, hydrolytic products containing nitro groups. (This work is now in progress.)

Inouye's product was obtained after 48 hours' treatment with nitric acid and he states that his yield was equivalent to 85–90% of the weight of silk taken. We now know that the action of nitric acid (sp. gr. 1.12) on fibroin is not complete at the end of this period.

So far as the action of nitric acid is concerned the behavior of our final product conforms to that of an organic combination of definite constitution. Whether a nitric acid weaker than that used will lead to the formation of the same combination by longer treatment remains to be established. There are undoubtedly side reactions involved which can be regulated by choosing an acid of the proper strength.

It is an interesting fact that the nitration reaction can be followed qualitatively by Millon's test for only a few hours. After 24 hours' treatment the nitrated protein failed to respond to the test. This would indicate that the reaction was complete, but it was on observation that the reaction continued beyond this stage. Whether this indicates that the tyrosine nucleus of the protein (fibroin) is completely converted after 24 hours into a dinitro combination will be determined by a careful study of the products of hydrolysis. Our new hydantoin derivatives, which we have described in previous papers, should prove of great value[2] for the isolation and identification of the nitro combinations formed. This work is now in progress.

Experimental Part.

Preparation of the Silk for Nitration.—The protein (fibroin) that was used in our work was a very fine grade of silk-noils, which was furnished to us gratuitously by the manufacturer.[3] The noils were of excellent quality and exceptionally free from foreign material (dirt). Al-

[1] *Loc. cit.*

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

[3] The Cheney Brothers, silk manufacturers, South Manchester, Connecticut.

though the material used was a thoroughly degummed product, care was taken, however, to carefully cleanse it of any adhering fatty material previous to nitration. The cleansing procedure was as follows: One pound of silk-noils was boiled with 15 liters of a neutral 1.0% red-oil soap solution for a period of six hours. The silk was then thoroughly washed with warm and cold water to remove the soap solution and finally desiccated at a constant temperature of 100°. This material was then preserved in a desiccator over sulfuric acid and used as needed.

Physical Condition of the Silk.—It has been our experience in conducting nitration experiments on silk fibroin that the results obtained are dependent, to a great extent, upon the physical nature of the fibre. In order to obtain consistent results under a given set of conditions, it is necessary that the natural condition of the fibre, as obtained after the degumming process, be preserved as much as possible. If pure spun silk in skein form or a silk yarn of close texture is used for experimental purposes, entirely different results will be obtained, under prescribed conditions, than when silk-noils are used. In the case of skein silk the individual fibres are closely packed and when the silk is moistened with acid there is a tendency for the fibres to swell, thus producing a very close texture. With such material the action of the acid is superficial and it takes a much longer treatment to obtain a uniform nitrated product. On the other hand, when silk-noils are used the action of the acid is quicker and also more uniform. The silk fibres in this material are very short and more porous, due to the mechanical treatment to which the silk has been subjected. Consequently, a greater surface is offered for the action of the acid. The action is not superficial and a stirring process can be successfully applied during the nitration which enables one to effect a thorough incorporation of the acid into the fibre.

Nitration of the Fibroin.—The nitration experiments were carried out in tall, slender, cylindrical beakers of 1200 cc. capacity. Twenty grams of the dried noils were carefully weighed into the latter for each experiment and then covered with 1000 cc. of an aqueous solution of nitric acid (sp. gr. 1.12). The purest, concentrated nitric acid was used. The beakers were covered with watch glasses and the nitration allowed to proceed at the temperature of the laboratory (18–25°), with frequent stirring of the silk suspension in order to have a uniform action. At the end of the given nitration period the nitrated product was filtered by suction and then washed repeatedly by maceration with cold water until the wash liquor gave no acid reaction. The nitrated protein was then dried at 100° and weighed to determine the yield. The nitrogen content of the nitrated product was estimated by the Kjeldahl method, modified to include the nitrogen of nitrates. Salicylic acid was used in every digestion.

TABLE II.—YIELDS OF NITROFIBROIN AND CORRESPONDING NITROGEN PERCENTAGES.

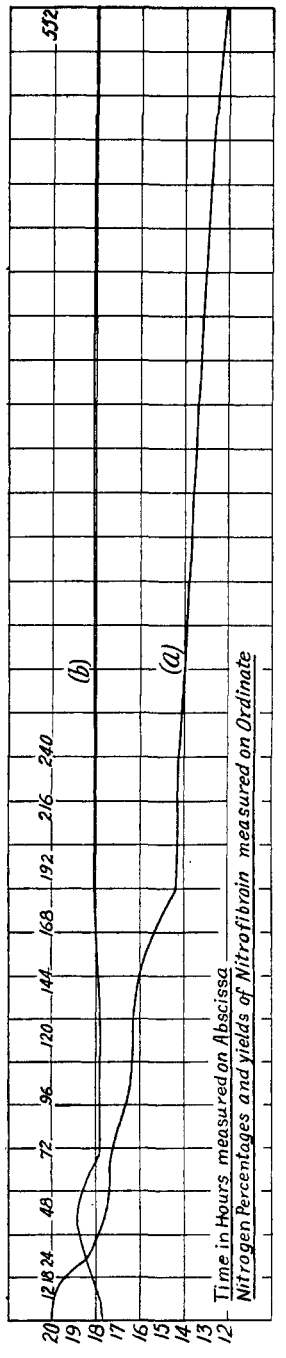
	Amount of silk used. Grams.	Specific grav. of HNO_3 used.	Time period of nitration. Hours.	Dehydration temperature.	Amount of acid used. Cc.	Yield of nitrated silk.	Nitrogen content of nitrated silk. (a).	(b).
1.....	20	1.12	12	100°	1000	19.9608	17.92	17.90
2.....	20	1.12	18	100°	1000	19.4584	18.12	18.17
3.....	20	1.12	24	100°	1000	18.7224	18.34	18.29
4.....	20	1.12	48	100°	1000	17.5459	18.77	18.76
5.....	20	1.12	72	100°	1000	17.2848	17.78	17.78
6.....	20	1.12	96	100°	1000	16.4710	17.82	17.70
7.....	20	1.12	120	100°	1000	16.3221	17.84	17.65
8.....	20	1.12	144	100°	1000	16.1102	17.76	17.75
9.....	20	1.12	168	100°	1000	15.3647	18.03	18.12
10.....	20	1.12	192	100°	1000	14.3452	18.08	18.08
11.....	20	1.12	216	100°	1000	14.2902	18.02	18.01
12.....	20	1.12	240	100°	1000	14.2402	18.12	18.05
13.....	467.5	1.12	240	100°	23,730	33.350	18.00	18.01
14[1].....	20	1.12	240	100°	1000	14.2670	18.00	18.01
15[2].....	20	1.12	552	100°	1000	12.0000	18.10	18.05

Following in every detail such a procedure as described above, a series of nineteen experiments were carried through differing from each other only as regards the length of time of nitration, which ranged from 12 to 552 hours. The various yields of nitrofibroin obtained from these different experiments, together with the corresponding nitrogen contents in percentages, are recorded in Table II. Both the percentages of nitrogen found and the respective yields of the several experiments have been expressed graphically in Table III by the Curves *a* (yields of nitrofibroin) and *b* (percentages of nitrogen found). Divisions of time, expressed in hours, have been plotted on the abscissa and the percentages of nitrogen and yields of nitrofibroin on the ordinate. Curve *a* commences on the ordinate at 20, the amount of fibroin taken for each experiment. Its nitrogen content (of the fibroin) is the intersection of *b* with the ordinate at 17.74. Curve *a* falls steadily below 20 finally intercepting curve *b* at 28 hours, and remaining below it from that point on.

No decisive conclusions can be drawn from inspection of the two curves below the 72-hour period. After this period *b* shows a constant valuation, while *a* constantly falls until the 192-hour period, when it then runs nearly parallel to curve *b* for the remaining 240 hours. This parallelism points to the interesting fact that we are dealing here with a definite product of nitration. The chemical deportment of our product was in accord with such an assumption. When our nitrofibroin, which we obtained by treatment of fibroin with nitric acid for 240 hours, was allowed to stand in freshly prepared nitric acid (sp. gr. 1.12) for a second period of

[1] 14 is 131 calculated on the basis of 20 g. silk, instead of 467.5 g.

[2] At the end of 240 hrs. treatment, the suspension was filtered and then immersed in a fresh quantity of 1000 cc. of HNO_3 (1.12).



13 days at ordinary temperature only a comparatively small loss of weight was incurred. It was no more than what would be expected from a slight solubility of the product in the acid. Furthermore the content of nitrogen remained constant at 18%. In order to substantiate further the conclusion that our treatment of fibroin with nitric acid of specific gravity 1.12, at ordinary temperature, is productive of a definite nitrated product which is very resistant to further action of the acid, a nitration experiment was carried through on a much larger unit. 467.5 g. fibroin were subjected to the action of the nitric acid (using the usual proportion as given in Table I) for 240 hours. After washing free from nitric acid and drying at 100° we obtained 333.5 g. of the nitrated protein, which gave on analysis (Kjeldahl) exactly 18% of nitrogen. In other words, the yield of nitrofibroin was exactly proportional to that obtained when only 20 g. of fibroin were nitrated.

$$467.5 : 333.5 :: 20 : 14.267.$$

On inspection of Table II, it will be seen that we actually obtained in the 216 and 240 hours' experiments with 20 g. of fibroin the values—14.2902 and 14.2670 g., respectively.

Description of the Nitration Experiments.—The behavior of the noils, after submersion in the nitric acid, was followed very closely. At first there was no apparent action. After 15 minutes the first evidence of nitration was generally noticed. The action, however, was local and at first the silk became yellow in spots which rapidly diffused through the individual fibres as the action was continued. At the end of 12 hours the entire mass generally assumed a light, golden-yellow color of even distribution. After 12 hours' treatment the silk fibres were coherent in appearance, and, in fact, did not lose their identity until after several hours' longer treatment. After 96 hours, disintegration of the fibres was

seen to commence. The material had then assumed a deep, golden-yellow color and the protein, after drying, could easily be pulverized by rubbing in the hand. Further nitration produced nothing but a powder of nitrated protein. The final products obtained after nitration for 240 and 552 hours, respectively, consisted of deep, golden-yellow, pulverulent material which resembled very much mosaic gold in its physical characteristics. It did not possess a crystalline structure.

The progress of the nitration reaction was followed by application of the Millon's test for tyrosine. Samples of nitrated silk obtained from 0 to 44 hours' treatment with the nitric acid (sp. gr. 1.12) responded positively to the test. The intensity of the color, however, continually decreased by prolonged treatment and at the end of 24 hours the protein gave only a very slight coloration. None of the nitrated products obtained after treatment of the silk for 24–552 hours responded to the Millon reaction. The acid filtrates from these were also examined but in no case was a positive tyrosine test obtained.

TABLE IV.—ACTION OF MILLON'S REAGENT ON NITRATED FIBROIN.

Time in hours.	Nitrated protein.		Acid filtrates.	
	Warm Millon's reagent.	Boiling sol.	Warm Millon's reagent.	Boiling sol.
0	Deep red	Deep red	....	....
12	Faint red	Faint red	....	....
18	Slight red color	Slight red color	....	....
24	Slight red color	Slight red color	....	....
25 to 552	No Millon's test given in any case			

Properties of the Nitrofibroin.—Table V exhibits the principal differences between fibroin and our nitrofibroin.

TABLE V.

Reagent.	Fibroin.	Nitrofibroin.
Millon's sol.	Red color	No red color
Aqueous ammonia	Insoluble	Insoluble
Alkaline carbonates (in water)	Insoluble	Insoluble
1% caustic soda solution	Insoluble	Insoluble
Strong caustic soda solution	Soluble (hot) and is reprecipitated by dilution with water	Insoluble in 10% solution. Color changes from yellow to red which becomes more intense on heating. Alkaline solution takes on a light orange color and is decolorized on making acid. No precipitate is produced Insoluble in 50% sodium hydroxide solution. Solution, however, is colored orange. This color disappears at neutral point or when solution is made slightly acid

TABLE V(*continued*).

Reagent.	Fibroin.	Nitrofibroin.
Glacial acetic acid	Soluble in hot solution	Insoluble
Conc. HCl	Soluble	Soluble
Conc. H_2SO_4	Soluble	Soluble
Conc. HNO_3	Soluble	Soluble
Application of heat (Melting point)	Disintegrates at about 170°. No definite decomposition point	Begins to turn brown at about 240° and at 300° is almost black. No definite decomposition point
Copper oxide in ammoniacal solution (Schweitzer's reagent)	Soluble in hot solution	Insoluble
Nickel oxide in ammoniacal solution	Soluble in hot solution	Insoluble
Biuret reaction	Positive	Color does not develop on account of orange color of the alkaline solution
Strong KOH solution (50%)	Soluble with evolution of ammonia	Soluble in hot solution with slight evolution of ammonia

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF WESLEYAN UNIVERSITY.]

THE SEPARATION OF MONO-β-, 2,6- AND 2,7-SULFONIC ACIDS OF ANTHRAQUINONE.

BY M. L. CROSSLEY.

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Mono-β-, 2,6- and 2,7-sulfonic acids of anthraquinone, the products of direct sulfonation, are said to be easily separated as calcium salts which ultimately can be converted into the acids.[1] In preparing these acids, I have observed that when the lime treatment is used a considerable portion of the calcium salts of mono-β- and 2,6-acid is thrown out of solution and discarded with the calcium sulfate. As a result a poor yield of these acids is obtained. Especially is this true when the sulfonation is intended to give the 2,6- and 2,7-acids as the chief products.

After considerable experimentation with different methods, I find that the best yields are obtained on separating the acids as their sodium salts by the direct neutralization of the sulfonated mixture with sodium hydroxide. By careful concentration of the solution of sodium sulfonates the mono-β- and 2,6-salts can be separated together, leaving the bulk of the sodium sulfate in the filtrate with the 2,7-salt, from which it can be precipitated by ethyl alcohol. The first two salts are separated from each other by their different solubilities in water. In addition to the three salts already mentioned, I have obtained a new substance which has not previously been described.

[1] Beilstein's *Handbuch der Organische Chemie*, **2**, 406.