Double Platinum Salt.—This crystallizes in needles which melt at 165–166°.

Calc. for $(C_7H_{10}N_2S)_2.H_2PtCl_6$: Pt, 27.17. Found: Pt, 27.14, 27.26.

A description of the products obtained by hydrolysis of this pyrimidine will be given in a future paper.

NEW HAVEN, CONN.

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

STUDIES ON NITRATED PROTEINS. II. THE SYNTHESIS OF 3,5-DINITROTYROSINE.[1]

BY TREAT B. JOHNSON AND EDWARD F. KOHMANN.

Received July 13, 1915.

In his paper entitled "Über das Tyrosin," Städeler[2] states that if special precautions are not taken in the preparation of *mono*-nitrotyrosine[3] from tyrosine, and the proper proportions of nitric acid, water and tyrosine are not used, the nitrate of *mono*-nitrotyrosine will not separate from the acid solution. If such a condition develops and the resulting solution is allowed to stand and is then evaporated at a low temperature a new amino acid is obtained which crystallizes from boiling water in golden yellow plates. He concluded that this acid is *dinitrotyrosine*. The same amino acid is also obtained, according to him, by treatment of *mono*-nitrotyrosine with a mixture of equal parts of water and nitric acid (sp. gr. 1.3) at ordinary temperature, and then evaporating the acid solution at a low temperature. This transformation, however, was not quantitative because of the secondary formation of a large amount of oxalic acid and also a secondary nitrogenous substance which Städeler did not examine. According to this investigator the same dinitro compound is also formed by saturating tyrosine, suspended in water, with gaseous nitrous acid. Städeler described this amino acid as a yellow compound which crystallizes in plates that are difficultly soluble in cold and hot water, but easily soluble in alcohol and moderately soluble in ether. It possessed a sour taste and was characterized by the property of staining the skin, linen and other objects intensely yellow. No melting point was assigned to the compound, but Städeler states that at 100–115° it loses no weight, and when heated at a higher temperature decomposes with effervescence. He found by analysis 39.30% of carbon and 3.40% of hydrogen while the calculated values for these two elements are 39.85% and 3.32%, respectively. The percentage of nitrogen was not determined. Städeler

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[1] Part of a dissertation presented by Mr. Edward F. Kohmann to the Faculty of the Graduate School of Yale University, 1915, in candidacy for the Degree of Doctor of Philosophy.

[2] *Ann.*, 116, 82 (1860).

[3] Johnson and Kohmann, THIS JOURNAL, 37, 1863 (1915).

described the potassium, barium, ammonium and lead salts of his acid which were all dark red in color.

The only other paper dealing with the chemistry of this amino acid, which has appeared since Städeler's work, is that published by Thudicum and Wanklyn[1] in 1869 under the title: "Researches on the Constitution and Reactions of Tyrosine." These investigators commented on the fact that Städeler did not determine nitrogen in his dinitrotyrosine and that his analytical results therefore did not exclude the possibility of there being O_3 in the place of an NO_2 group in the formula of his supposed dinitrotyrosine and called attention to the fact that his analytical results agreed quite as well with $C_9H_{10}(NO_2)NO_6$ as with $C_9H_9(NO_2)_2NO_3$. On repeating Städeler's work they failed in their attempts to make dinitrotyrosine and concluded from the peculiar results obtained that Städeler's dinitrotyrosine was probably nothing but an oxidized tyrosine. This, on prolonged oxidation, gave an abundant yield of oxalic acid. Thudicum and Wanklyn also made the interesting statement that there is no production of picric acid by oxidation of tyrosine with nitric acid. Furthermore, they were unable to verify Städeler's observation that tyrosine interacts with nitrous acid to form *dinitrotyrosine*. On the other hand, they obtained a highly colored combination whose structure was not established.

In connection with our investigations on nitrated proteins, it was necessary to determine under what conditions *dinitrotyrosine* is formed by the action of nitric acid on tyrosine, and also to establish definitely what positions in the benzene nucleus are occupied by the two nitro groups. Our first attempts to prepare the acid were unsuccessful, notwithstanding the fact that Städeler's directions were followed as closely as possible. In no case did we obtain evidence of its formation. If the nitric acid solution was allowed to evaporate spontaneously at ordinary temperature, or at higher temperatures, a mixture of oxalic acid and mononitrotyrosine was always obtained. Too energetic treatment led exclusively to the production of oxalic acid. In other words, nitrotyrosine was completely oxidized by vigorous treatment, and it consequently seems very doubtful whether dinitrotyrosine was actually obtained by Städeler.

We now find, that while the employment of nitric acid alone does not lead to the smooth formation of dinitrotyrosine, on the other hand, its synthesis can easily be accomplished by nitration of tyrosine with a mixture of concentrated nitric and sulfuric acids. Under proper conditions, which are discussed in detail in the experimental part of this paper, the acid can easily be obtained in excellent yields. Our amino acid differed, in its chemical behavior, in many ways from that described by Städeler. He states that his acid would not form a hydrochloride. Our acid, on

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[1] *J. Chem. Soc.*, **22**, 283 (1869).

the other hand, forms smoothly such a salt. According to him dinitrotyrosine is soluble in alcohol and also ether, whereas our acid was found to be only very slightly soluble in alcohol and practically insoluble in ether. Our acid possessed no definite melting or decomposition point.

Städeler published analytical results agreeing with the calculated values for an anhydrous acid, and states that his product lost no weight when heated at 115°. We now find that dinitrotyrosine crystallizes with one molecule of water which is expelled from the acid by heating at about 140°. In the hydrated condition the acid is bright yellow in color, but when dehydrated assumes a bright red color. This is one of the most characteristic properties of this acid and serves for its identification. From the above data we are forced to conclude, therefore, that Städeler was dealing with some other substance, or, what seems most probable, impure mononitrotyrosine.

The constitution of our dinitrotyrosine was established in the following manner: The free amino acid was first condensed with ammonium thiocyanate in acetic anhydride solution according to the method of Johnson and Nicolet[1] and converted into the corresponding thiohydantoin represented by Formula IV. The latter compound, on digesting with an excess of 25% chloroacetic acid, was easily desulfurized and converted into the corresponding hydantoin (V). The yield was excellent. This dinitrohydantoin (V) was then reduced and converted into the hydrochloride of the diaminohydantoin represented by Formula VI. The latter, after diazotization, was then digested with cuprous chloride (Sandmeyer's reaction) and transformed into the corresponding dichlorohydantoin corresponding to Formula VII. This dichlorohydantoin proved to be identical in every respect with the hydantoin of 3,5-dichlorotyrosine, which has previously been described in a paper from this laboratory by Wheeler, Johnson and Hoffmann.[2] *Therefore, tyrosine, when it interacts with nitric acid in the presence of sulfuric acid, undergoes nitration with substitution in the two positions ortho to the hydroxyl group giving 3,5-dinitrotyrosine represented structurally by Formula III.*

These various changes are represented by the structural formulas below.

It is a well-known fact that *diortho*-substituted tyrosines like 3,5-diiodotyrosine (iodogorgoic acid) (VIII) and the corresponding dibromo- and dichloro-acids (IX and X) fail to give Millon's reaction. It was, therefore, of especial interest to determine whether dinitrotyrosine (III) would conform to this behavior. We now find that it does not give the test. This observation is of interest and points to the conclusion that the production of the red color does not involve a nitration of tyrosine

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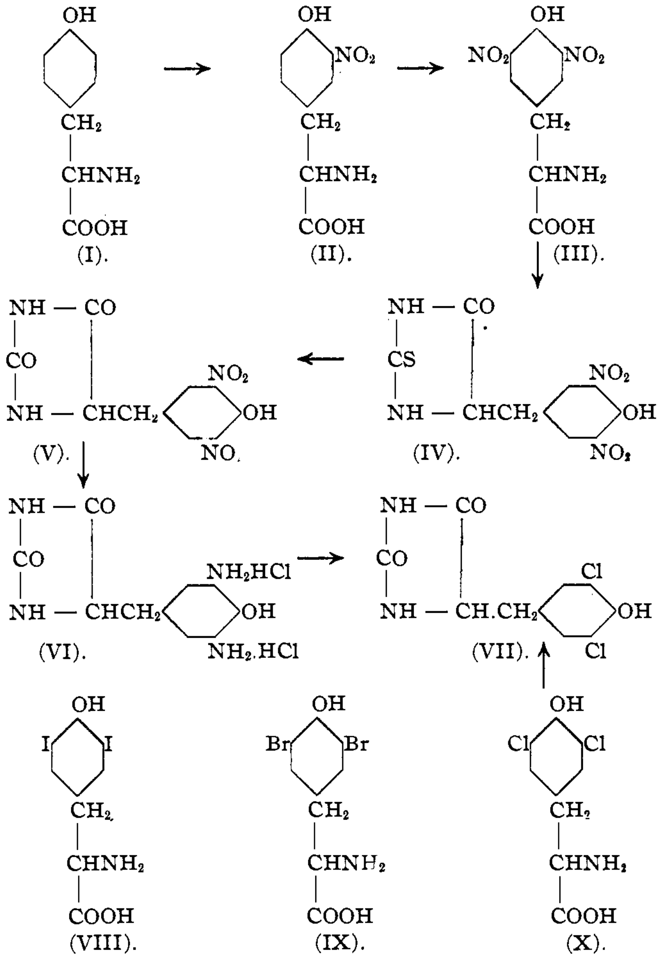
[1] THIS JOURNAL, 33, 1973 (1911).

[2] *J. Biol. Chem.*, 10, 147 (1911).



by the Millon reagent. The chemistry of this reaction will receive further attention in a later publication. In our first paper on nitrated proteins[1] attention was called to the fact that Loew examined the behavior of nitric acid and mixtures of nitric acid and sulfuric acids towards proteins. He made the observation that the proteins used failed to respond to Millon's test after such a treatment.[2] Moreover, it was noted that in one case mononitrotyrosine was obtained by hydrolysis of a nitrated protein (nitro-fibroin).[3] Does this mean that when proteins are subjected to vigorous nitration that the tyrosine in converted largely into dinitrotyrosine? Furthermore, if it is formed, is it the precursor of picric

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

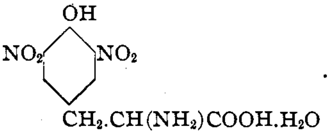
[2] Loew, *J. prakt. Chem.*, [2] 3, 180.

[3] Inouye, *Zeit. physiol. Chem.*, 72, 486 (1911).

acid, which Welter[1] first obtained by vigorous treatment of the silk proteins with strong nitric acid? Our researches on nitrated proteins will be continued, and will include a study of polypeptide combinations of nitro- and dinitrotyrosine (II and III).

Experimental Part.

3,5-Dinitrotyrosine,



.—After several preliminary experiments the following procedure was finally adopted for the preparation of this interesting amino acid: First, an acid mixture is prepared by combining 22 g. of pure concentrated sulfuric acid and 3 g. of concentrated nitric acid. This mixture is then cooled to 10°, and 2 g. of finely pulverized tyrosine added slowly in small portions while the solution was constantly stirred and the temperature kept below 10°. It is not advisable to nitrate more than 2 g. of tyrosine at a time and these should be added to the acid as rapidly as possible. A complete solution of the tyrosine is not effected by the operation and after final addition of the acid the mixture is then poured immediately into water. This operation was repeated six times and the combined solutions worked for dinitrotyrosine. In order to isolate the free acid, the sulfuric acid is exactly precipitated as barium sulfate and the barium free filtrate then made distinctly alkaline with ammonia and the resulting solution finally evaporated to dryness, by heating at 60° under diminished pressure. On treating the resulting residue with about 25 cc. of cold water the dinitrotyrosine is obtained in the form of a bright red ammonium salt moderately soluble in water. In order to obtain the free acid this salt is decomposed with a little dilute hydrochloric acid and the dinitrotyrosine dissolved in boiling water. It is well to digest with bone-coal here to clarify the solution. On cooling, the dinitrotyrosine crystallizes in beautiful, golden-yellow plates containing water of crystallization. From 12 g. of tyrosine we obtained 6 g. of pure dinitrotyrosine. Especially characteristic is the behavior of this acid when heated. When heated at 140–150° it loses its water of crystallization and assumes a brick-red color. At 220–230° it decomposes with effervescence. The acid does not lose its water of crystallization when heated at 110° as was shown by the following nitrogen determination:

Calc. for $C_9H_9O_7N_3.H_2O$: N, 14.51. Found: N, 14.47.

When dried to constant weight at 150° the following results were obtained:

I. 0.4022 and 0.3714 g.; lost 0.0260 and 0.0237 g. H_2O.

Calc. for $C_9H_9O_7N_3.H_2O$: H_2O, 6.23. Found: H_2O, 6.47, 6.38.

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[1] *Ann. chim.*, **29**, 301 (1796).

Nitrogen determinations in the anhydrous acid:

Calc. for $C_9H_9O_7N_3$: N, 15.50. Found: N, 15.45, 15.36.

The Behavior of Dinitrotyrosine towards Millon's Reagent.—This amino acid does not give a red color with Millon's reagent, but when boiled with the reagent is converted into a characteristic insoluble mercury salt. It can be prepared as follows: To a boiling Millon's solution is added an equal volume of a hot, saturated, aqueous solution of the amino acid when there immediately separates the yellow mercury salt. This is separated by filtration, washed with nitric acid and dried for analysis in the air. The salt is insoluble in the common solvents. It begins to decompose at 170° and effervesces at 185°. This salt was not examined further but will receive attention at a later period. It is not improbable that we may be able to develop a practical method for the quantitative separation of dinitrotyrosine from nitrotyrosine and tyrosine by application of this interesting reaction.

Calc. for $C_9H_8O_7N_3.HgNO_3$: N, 10.5. Found: N, 10.17, 10.22, 10.13.

The Hydrochloride of Dinitrotyrosine, $C_7H_9O_9N_3.HCl$.—This salt is prepared by dissolving dinitrotyrosine in hydrochloric acid and then adding to the solution strong hydrochloric acid. It separates in the form of bright yellow plates which begin to darken at 220°, when heated in a capillary tube, and decompose at 230° with effervescence. The salt dissolves in water and is precipitated by dilution with concentrated hydrochloric acid. The hydrochloride of *o*-nitrotyrosine is characterized by similar properties. For analysis, the salt was dried at 110°.

Calc. for $C_9H_9O_7N_3.HCl$: N, 13.60. Found: N, 13.51.

Ammonium Salt of Dinitrotyrosine.—This is prepared by dissolving the acid in a small volume of warm ammonia solution and then diluting this with strong aqueous ammonia. On allowing the solution to cool the salt finally deposits as beautiful, red prisms. The salt decomposed when heated above 230°. It was dried for analysis at 110°.

Calc. for $C_9H_8O_7N_3.NH_4$: N, 19.45. Found: N, 19.31, 19.25.

The Thiohydantoin of 3,5-Dinitrotyrosine (IV).—One and two-tenths grams of dinitrotyrosine, 0.6 g. of anhydrous ammonium thiocyanate and 5.7 cc. of acetic anhydride were warmed on a steam bath for one hour. A clear, orange-colored solution was obtained which was poured into an excess of cold water, when a dark-colored oil deposited, which soon solidified. This was separated by filtration, suspended in strong hydrochloric acid and the mixture finally evaporated to dryness to hydrolyze any 3-acetyl hydantoin present. The 2-thiohydantoin was left behind as a yellow powder. This was purified by crystallization from 50% acetic acid and separated in the form of burrs of short prisms. It melted at 225–230° with decomposition. From 3.7 g. of dinitrotyrosine we obtained 4 g. of crude hydantoin.

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

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[1] *Loc. cit.*