CCCXVIII. THE PREPARATION OF ORNITHINE, ORNITHURIC ACID AND α-BENZOYLORNITHINE.

BY WILLIAM ROBERT BOON AND WILLIAM ROBSON.

From the Department of Physiology, University of London King's College.

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AN investigation contemplated in this laboratory involved the preparation of ornithuric acid and its derivative α-benzoylornithine in large amounts. The poor yields obtained by the existing methods and their tedious character made it desirable to improve these methods. Success has attended our efforts in this direction and it is thought that our results may be of value to other workers requiring these compounds and their derivatives.

The method to be described is essentially the same as those already published and consists of the following steps: arginine (or carbamido-arginine) → ornithine → ornithuric acid → benzoylornithine. Ornithine can be obtained either synthetically or by the hydrolysis of arginine, and of these alternatives the latter appeared to us the more promising, especially as arginine monohydrochloride can be fairly readily isolated from a gelatin hydrolysate [Cox, 1928; Felix and Dirr, 1928]. In later experiments the lengthy preparation of arginine monohydrochloride was avoided by substituting for it the carbamido-arginine described by us [1935] in a previous paper.

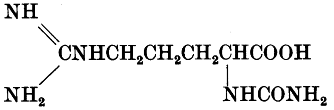
The first workers to prepare ornithine in any quantity from arginine were Schulze and Winterstein [1898]. Their method consisted essentially of the hydrolysis of an arginine salt with baryta and the isolation of the ornithine so produced in the form of its dibenzoyl derivative ornithuric acid. The method has two disadvantages. The yield of ornithuric acid is only 27 % of that demanded by theory; moreover, its subsequent hydrolysis and the isolation of the ornithine are by no means easy, so that the yield of the latter compound suffers accordingly.

Bergmann and Zervas [1926] overcame the second of these difficulties by preliminary isolation of the ornithine as a salicylidene derivative of the barium salt, a compound which is practically insoluble in cold water and is readily decomposed by dilute acids. By this method they were able to obtain a 23 % yield of ornithine dihydrochloride.

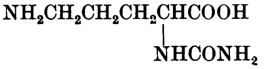
Bergmann and Zervas suggested that the greatest loss occurred during hydrolysis of the arginine salt with baryta. At first sight this seems justified, especially since ornithine can be converted quantitatively into ornithuric acid. It is very unlikely that any appreciable decomposition of the ornithine occurs since Van Slyke [1911] and Plimmer [1916] showed that the amount of ammonia evolved on boiling an arginine salt with caustic alkali is always less than that required by theory if all the urea produced in the reaction were completely hydrolysed. Plimmer, in fact, showed that the amount of ammonia evolved only approached the theoretical figure if all the urea were completely hydrolysed, and that this took place only when the concentration of sodium hydroxide was greater than 20 % and the time of boiling several hours.

Another possible source of loss is suggested by the observation of Baumann and Hoppe-Seyler [1874] that when glycine is boiled with urea and baryta, ammonia is evolved and carbamidoacetic acid (hydantoic acid) is produced. Lippich [1906] showed that this reaction was not confined to glycine by preparing α-carbamido-*iso*hexanoic acid from leucine. Later, this author [1908] showed that the reaction was applicable to most of the monoamino-acids obtainable by the hydrolysis of proteins and also to such aromatic amino-acids as anthranilic acid and sulphanilic acid. It appeared to us therefore that the urea produced in the initial stages of the reaction might react with the constituents of the mixture to give one or more of the following products:

(1)

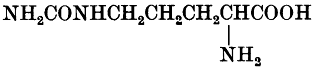


(2)



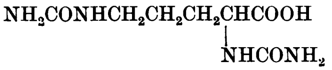
$NH_2CH_2CH_2CH_2\underset{\substack{|\\ NHCONH_2}}{C}HCOOH$

(3)



$NH_2CONHCH_2CH_2CH_2\underset{\substack{|\\ NH_2}}{C}HCOOH$

(4)



$NH_2CONHCH_2CH_2CH_2\underset{\substack{|\\ NHCONH_2}}{C}HCOOH$

Compound (1) is already known to us as carbamido-arginine; compound (3) is citrulline [Koga and Odake, 1914; Wada, 1930] and is characterised by an extremely insoluble copper salt. Compounds represented by the formulae (2) and (4) are as yet unknown, but their existence is possible, especially in view of the fact that Hoppe-Seyler [1933] has isolated δ-carbamido-*n*-butylhydantoin from the urine of a cystinuric, it being formed presumably from the lysine and urea contained therein.

Examination of the reaction mixture, however, after hydrolysis of the arginine under the conditions specified either by Schulze and Winterstein or by Bergmann and Zervas, failed to reveal the presence of either carbamido-arginine or of citrulline. Nevertheless, we are not prepared to state that either of these substances or their derivatives are not present in the mixture.

The problem of the decomposition of arginine by alkali was then examined from another angle. Any change in the conditions which would lead to increased hydrolysis of the urea produced in the reaction should lead to an increased yield of ornithine. Examination of the quantity of baryta used by Schulze and Winterstein and by Bergmann and Zervas shows that the actual concentration of baryta available for hydrolysis is extremely small after allowance has been made for the amount used in liberating the arginine from its salt and the formation of the barium salt of arginine.

Comparing the amounts of free alkali present with those employed by Werner [1923] in his studies of the decomposition of urea by alkali, we arrived at the conclusion that this is where the difficulty lay. In point of fact there can be no appreciable hydrolysis of the urea produced when Bergmann and Zervas's quantities are employed since there is practically no barium carbonate precipitated during the reaction.

In order to see if the yield of ornithuric acid were increased with increasing hydrolysis of urea, we undertook a series of semi-quantitative experiments in which the amounts of ammonia evolved and of ornithine produced, isolated as ornithuric acid, were studied in relation to the time of boiling and the concentration of baryta employed. It was found that up to a certain concentration of baryta, the amounts of ammonia evolved and of ornithuric acid isolated were increased. From an examination of the curves of Fig. 1 which represent the amount of ammonia evolved plotted against time, it will be seen that the slope of curves I and II remains approximately constant, that of III changes slightly, whilst in the case of IV the change is so marked that the total amount of ammonia evolved in the course of the reaction is less than that corresponding to III. This decrease is paralleled by the yield of ornithuric acid.

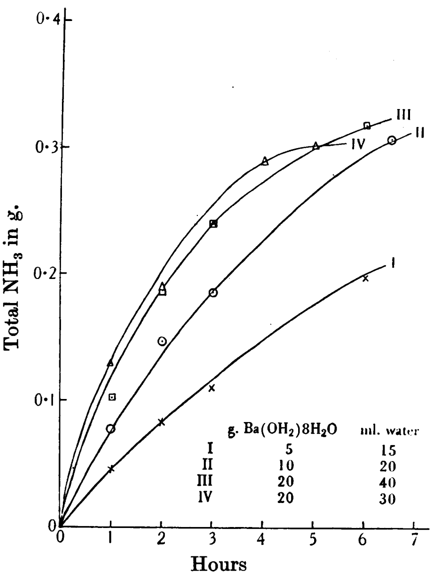


Fig. 1.

The observation that increase in the concentration of baryta employed beyond a certain optimum value produced a decrease in the yields of both ammonia and ornithuric acid, suggested that the large amount of barium carbonate produced under these conditions was causing an appreciable loss by absorption of the reactants. This idea seemed to be borne out by the fact that prolonging the time of boiling to 24 hours had no appreciable effect on the yield in case IV, whilst in the other cases the increase though small was definite. To overcome this difficulty caused by the formation of a precipitate during the course of the reaction, sodium hydroxide was substituted for barium hydroxide as the hydrolysing agent. We had a precedent for so doing in the observations of Van Slyke [1911] and Plimmer [1916], already referred to above. It was found that on heating an arginine salt or the carbamido-compound with ten times its weight of 20 % NaOH for 6 hours a 70 % yield was obtained, whilst if the time of boiling was extended to 15 hours the yield of ornithuric acid obtained was theoretical. The modification can also be used for the isolation of ornithine salts if the hydrolysate is first acidified in order to remove carbonate, then treated with an excess of baryta and finally shaken with salicylaldehyde, according to the procedure of Bergmann and Zervas.

For the preparation of benzoylornithine from ornithuric acid two methods, essentially the same, are available in the literature. The first due to Sørensen *et al.* [1912] involves the repeated hydrolysis of ornithuric acid with successive quantities of dilute baryta on the water-bath and the recovery of the unchanged ornithuric acid. The yield is good (60 %) but the very large number of operations makes the process laborious and time-consuming. The second method, due to Wada [1930], is essentially the same but involves only one operation, boiling under reflux with baryta. His yield however drops to only 41 %. There appears to be an anomaly in this paper in so far as the quantity of baryta employed is less than that required to dissolve all the ornithuric acid. By employing a slight excess of baryta over that required to form the barium salt of ornithuric acid and of the benzoic acid produced during the course of hydrolysis, and by increasing the duration of refluxing the mixture, we have succeeded in one simple operation in obtaining a 76 % yield of α-benzoylornithine of excellent purity.

EXPERIMENTAL.

Semi-quantitative examination of the decomposition of arginine by various concentrations of baryta solution.

The apparatus consisted of a 150 ml. round-bottomed flask, fitted with a reflux condenser and so arranged that a stream of washed air could be drawn through into a bubbling tube containing standard acid.

2·75 g. of arginine hydrochloride were taken for each experiment, the baryta and water added and the whole boiled over a small flame with a continuous stream of ammonia-free air passing through the apparatus. At intervals the tube containing the standard acid was removed and another substituted; the excess acid being titrated against standard NaOH. After 6 hours NaOH was added to the reaction mixture in moderate excess of that required to liberate the barium from its salts and the solution while still hot was saturated with carbon dioxide and filtered. The precipitated barium carbonate was repeatedly boiled out with water and the washings saturated with carbon dioxide and filtered. The combined washings and filtrate were then made more strongly alkaline with NaOH and treated with benzoyl chloride as described by Schulze and Winterstein. The mixture was acidified to Congo red with HCl and filtered. The precipitate was washed on the filter with water, twice with cold alcohol and finally with ether until the washings were free from benzoic acid. The product had M.P. 187°, unchanged after recrystallisation from 50 % alcohol. The benzoic acid was removed as described, in preference to boiling it out with water, as this procedure sometimes gave a sticky product.

The following table shows the yield of ornithuric acid obtained, corresponding to the concentration of baryta used:

Time of boiling in hours	$Ba(OH)_2$, $8H_2O$/water	Yield %
6	5/15	33·2
6	10/20	51
6	20/40	57
6	20/30	54·5
17	20/40	68·5

Preparation of carbamido-arginine from gelatin.

Gelatin (1 kg.) was boiled with 20 % HCl (2 l.) for 14 hours. The hydrolysate was evaporated *in vacuo* to a thick syrup, which was then dissolved in water and the volume made up to 4 l. 40 % NaOH was added until the solution was just acid to Congo red. It was then decolorised with charcoal in the usual way. To the hot filtrate was added a hot solution of flavianic acid (200 g.) in water (1 l.) and the whole allowed to cool overnight. The precipitate was collected at the pump and repeatedly washed with cold water until the washings were free from chloride. The solid was dried, heated on the water-bath with conc. HCl (200 ml. per 100 g. of flavianate) for 2 hours, cooled overnight in the ice-chest and filtered. The solid remaining was repeatedly washed with conc. HCl until the washings were practically colourless. The filtrate and washings were combined and evaporated *in vacuo* to a thick syrup. This was dissolved in water (1 l.), the solution neutralised to litmus and the neutral solution decolorised with charcoal. The practically colourless filtrate was then treated with a solution of potassium cyanate (70 g.) in water and the whole heated on a steam-bath for an hour. On cooling the carbamido-arginine separated. It weighed 82 g., and was pure enough for the next experiment.

Ornithuric acid from carbamido-arginine.

Carbamido-arginine (40 g.) and 20 % NaOH (400 ml.) were heated under reflux for 15 hours. After partial neutralisation of the alkali the solution was treated with benzoyl chloride in the usual way and the product worked up as described above. The yield was 57·5 g., approximately theoretical.

Ornithine hydrochloride from carbamido-arginine.

Carbamido-arginine was hydrolysed as in the previous experiment, the solution being finally acidified to remove carbonate and then treated with a large excess of baryta. It was then shaken with salicylaldehyde, as described by Bergmann and Zervas. The barium salt of the salicylidene compound which separated was collected, washed with cold water and decomposed by dilute HCl. The mixture was cooled, extracted with ether, the aqueous solution evaporated to dryness *in vacuo*, the residue extracted with hot methyl alcohol and the alcoholic extract concentrated to a syrup. The residue was dissolved in water, neutralised to litmus with ammonia and concentrated. To the resulting syrup rectified spirit was added, according to the method of Jaffé [1877], until it became cloudy. On standing for several days the ornithine monohydrochloride separated as a colourless crystalline mass. This was collected at the pump and air-dried. The yield was approximately theoretical.

Benzoylornithine from ornithuric acid.

Sørensen *et al.* [1912] found it necessary, in carrying out this operation, to use a silver-lined flask in order to obtain an ash-free product. We found a pyrex flask suitable for the purpose. Ornithuric acid (48 g.), baryta (45 g.) and water (750 ml.) were boiled under reflux with exclusion of carbon dioxide for 15 hours. While the solution was still hot, the barium was quantitatively removed by the addition of sulphuric acid. The precipitated barium sulphate was removed from the solution and repeatedly boiled out with water. The combined filtrates were evaporated under reduced pressure to about 200 ml. and extracted with ether to remove the benzoic acid. The solution was then concentrated to a stiff syrup and treated with absolute alcohol. The white crystalline precipitate which separated was filtered off and air-dried. The product was ash-free, and when heated in the manner described by Sørensen *et al.* melted at 264–267°. The yield was 24 g. or 76 % of theory.

REFERENCES.

Baumann and Hoppe-Seyler (1874). *Ber. deutsch. chem. Ges.* **7**, 37.
Bergmann and Zervas (1926). *Z. physiol. Chem.* **152**, 298.
Boon and Robson (1935). *Biochem. J.* **29**, 2573.
Cox (1928). *J. Biol. Chem.* **78**, 475.
Felix and Dirr (1928). *Z. physiol. Chem.* **176**, 29.
Hoppe-Seyler (1933). *Z. physiol. Chem.* **214**, 267.
Jaffé (1877). *Ber. deutsch. chem. Ges.* **10**, 1926.
Koga and Odake (1914). *J. Tokyo Chem. Soc.* **35**, 519.
Lippich (1906). *Ber. deutsch. chem. Ges.* **39**, 2953.
—— (1908). *Ber. deutsch. chem. Ges.* **41**, 2953.
Plimmer (1916). *Biochem. J.* **10**, 115.
Schulze and Winterstein (1898). *Z. physiol. Chem.* **26**, 1.
Sørensen, Andersen and Höyrup (1912). *Z. physiol. Chem.* **76**, 44.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
Wada (1930). *Biochem. Z.* **224**, 427.
Werner (1923). The chemistry of urea. (Longmans, Green and Co., London.)