CXXXVIII. INVESTIGATIONS ON GELATIN. PART III. THE SEPARATION OF THE PRODUCTS OF HYDROLYSIS OF GELATIN BY THE CARBAMATE METHOD.

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(*Received July 30th, 1924.*)

Until the introduction by Dakin a few years ago of the butyl alcohol method for the isolation of certain of the hydrolysis products of the proteins, the only general process employed for the purpose was that of esterification originally elaborated by Emil Fischer. By Dakin's method a large proportion of the amino-acids could be separated in crystalline form without the necessity of converting them beforehand into their esters. When Dakin applied his method to the separation of the hydrolysis products of gelatin [1920] he met with certain difficulties which were not encountered in the case of the hydrolysis products of caseinogen. It was found, for example, that glycine and hydroxyproline were only very incompletely extracted from the mixture of the hydrolysis products by butyl alcohol, and in the case of the proteins which yield relatively large amounts of these acids on hydrolysis, the method of Dakin does not appear to be entirely satisfactory.

A few years ago Buston and Schryver [1921] showed that amino-acids could be readily separated from carbohydrates and other soluble products by precipitation in the form of their carbamates, a class of compounds originally described by Siegfried, when a solution containing them is treated in the presence of alcohol by barium hydroxide and carbon dioxide. It was found that by this method a very large proportion of the hydrolysis products of caseinogen and gelatin could be readily separated in a crystalline form without submitting them to the esterification treatment.

The objects of the work described in the present communication were, in the first instance, to determine the most suitable conditions under which the carbamates could be precipitated, and in the second instance, to ascertain whether the carbamate method could be adapted to the separation of the individual acids from one another. The investigation has been attended with a certain amount of success and the main outlines are now clear, although a considerable amount of detailed work is still necessary, and is now being carried out in this laboratory to make the process a more complete one.

General method.

The proteins are hydrolysed in the usual manner with dilute sulphuric acid, and the hydrolysis mixture is then treated with barium hydroxide until the solution is just acid with Congo red. The barium sulphate is then filtered off, and more barium hydroxide is added to the filtrate and a second precipitation of barium sulphate is produced. This second precipitate was found to contain appreciable quantities of nitrogen, and on isolating the amino-acid to which it belonged by the method described below, *i*-aspartic acid was obtained.

By adding alcohol (3 vols.) to the filtrate from the second barium sulphate precipitate, a crystalline precipitate was formed, which was found to consist of the barium salts of the dicarboxylic acids (glutamic and aspartic acids only, hydroxyglutamic acid not being present amongst the hydrolysis products of gelatin). The precipitation of the dicarboxylic acids at this stage appears to be complete, no trace of them having been found in any later fraction.

The carbamates were then precipitated in the filtrate by alternate treatment of the ice-cold alcoholic liquid with barium hydroxide and carbon dioxide by the method described in some detail below. The filtrate from the barium carbamates was then concentrated and recarbamated, and the carbamation process repeated until practically no nitrogen was brought down by the treatment of ice-cold alcoholic solutions with barium hydroxide and carbon dioxide. The final filtrate from the carbamates contained generally 6–7 % of the total nitrogen and was almost free from amino-nitrogen. This nitrogen is almost entirely in the form of either proline or prolyl-proline. The exact form has not yet been determined.

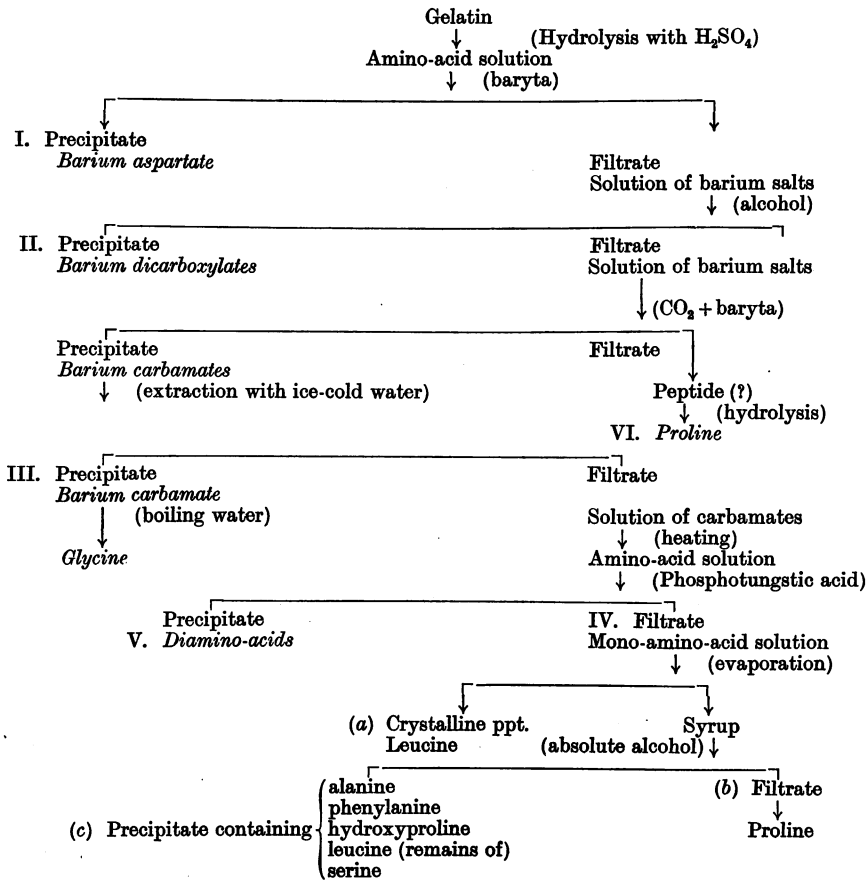
The carbamate fractions were collected together (for details, see below) and when dried, extracted with ice-cold water. This extracts everything except the barium carbamate of glycine. When this is decomposed with boiling water, almost pure glycine separates from the aqueous solution after filtration from the barium carbamate. No glycine has been found in any other fraction.

The soluble carbamates were decomposed by boiling their solution with water. A fraction was thereby obtained which contains also the diamino-acids. These, it has been found by separate experiment, are readily precipitated as carbamates under the conditions generally used. They can be separated as phosphotungstates in the usual way. On evaporating the soluble carbamate fraction after separation of the bases, the greater part of the leucine separated in a nearly pure condition. The filtrate from the leucine, after concentration, was thrown into alcohol, and a crystalline precipitate was produced. The alcoholic filtrate was then concentrated, the residue taken up by absolute alcohol, and filtered from the small amount of precipitated matter, which was added to the precipitate just mentioned. The alcoholic solution was repeatedly evaporated to dryness, and taken up with alcohol until a product was obtained which completely dissolved in absolute alcohol, producing a solution which no

longer formed a crystalline precipitate after prolonged standing in an ice-chest. The only amino-acid obtained from this solution, which contained practically no amino-nitrogen, was proline. It was purified by conversion into the ethyl ester which all distilled within one or two degrees when submitted to fractionation. The amino-acids precipitated by alcohol in obtaining the proline fraction include the remains of the leucine, valine, serine and hydroxyproline. The last named can be estimated by determining the amount of non-amino-nitrogen in the fraction. The phenylalanine can be readily isolated by converting the fraction into the esters and extracting the aqueous suspension by ether in the usual way.

The separation of the amino-acids in this fraction forms the least satisfactory part of the general process, and researches are still being carried out with a view to evolving a more satisfactory process for dealing with them.

Diagram of the Separation of the Hydrolysis Products of Gelatin.



The carbamate method for separating the hydrolysis products of gelatin has been employed in experiments where several hundred grams, and also

where only 10 g. of gelatin have been hydrolysed. Even when only 10 g. of material are used for investigation, fairly accurate estimations of the following amino-acids are possible, viz. glycine, aspartic and glutamic acids, the diamino-acids (in which fraction, of course, the amounts of arginine, histidine, and lysine can be estimated in the usual manner) proline, and hydroxyproline; and the nitrogen of the non-carbamating fraction can also be estimated.

It is possible that a good routine method for the analysis of the proteins when only small amounts of material are available may be based on the carbamate method, but minor details have still to be worked out, and attempts are now being made in the laboratory to improve the process. The chief advantage over other methods seems to consist in the ease with which the glycine, dicarboxylic acids and the proline can be separated in a pure form.

The general scheme of separation is outlined in the table p. 1072.

The following are the quantitative results obtained from a large scale and a small scale experiment. In the large scale experiment, the liquid was not sufficiently diluted when the bases were precipitated by phosphotungstic acid, consequently the diamino-nitrogen found was too high, and the results are not therefore quoted here. Otherwise fair agreement was found between the results of the large scale experiment (with 900 g. gelatin) and the small scale experiment (with 10 g.).

Results in percentages of total N.

Large scale experiment		Small scale experiment		
Glutamic acid	3·2 %	Fraction 1.	Aspartic acid and humin N	4·49 %
Aspartic „	5·6	„ 2.	Dicarboxylic acids*	6·50
Proline ...	14·8	„ 3.	Glycine	17·44
Hydroxyproline	11·25	„ 4 *a*.	Leucine, serine, hydroxyproline, alanine	17·26
Glycine ...	18·2	„ 4 *b*	Proline	12·06
		„ 5.	Diamino-acids	26·67
		„ 6.	Non-carbamating fraction	6·93

* Does not include the aspartic acid of Fraction 1.

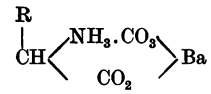
The results in the small scale experiment on addition give 91·3 % of the total nitrogen. To this must be added 2 % to account for the amide nitrogen which is estimated separately. It was found subsequently that the barium carbonate obtained after decomposition of the carbamates always contained a small amount of nitrogen. This, unfortunately, was not estimated in the above experiment. If this had been done, the total N accounted for would have been higher than 93 %.

No particular importance is to be attached to these values as absolute quantities. Subsequent researches on gelatin, which will form the subject of later communications, show that the hydrolysis products of gelatin can vary appreciably, according to the method employed for the preparation (even when different samples are made from the same precursor) and the subsequent treatment.

Some experimental details.

General method of carbamation.

It seemed possible that two types of carbamates could be produced, according to whether the solution of the amino-acids were treated in the first instance with carbon dioxide or with barium hydroxide. In the former case barium salts of the general formula I.



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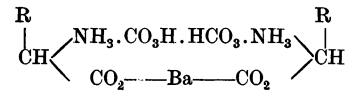

II

might be expected, whereas in the latter case salts of the type II might be expected to be formed, from which, on addition of a further amount of barium hydroxide, another barium salt would be produced, in which the two hydrogen atoms of the CO_3H groups would be replaced by the metal. An extended series of experiments was therefore carried out with the object of determining the conditions under which the amino-acids could be precipitated with the smallest amount of barium carbonate. It was found that large amounts of nitrogen were only precipitated when the ice-cold aqueous-alcoholic solution (two volumes of alcohol to one of water) was never allowed to become acid to phenolphthalein. The process of precipitation can be divided into three stages. (*a*) Recrystallised, moist or finely powdered dry barium hydroxide is added little by little to the aqueous-alcoholic solution kept cool by ice with continuous stirring until no further quantity dissolves. Phenolphthalein is added in sufficient amount to produce a bright pink colour. (*b*) Carbon dioxide is then passed in until the pink colour just fades. (*c*) Recrystallised moist barium hydroxide is then added again, with continuous stirring, until no more dissolves. A thick gelatinous precipitate is thereby produced which contains the carbamates contaminated with relatively small amounts of carbonate. The barium carbamates appear to belong to the second type of salts described above.

Attempts have been made to form carbamates of metals other than barium. The calcium salts are similar in most respects to the barium salts, but the latter are more conveniently prepared owing to the greater solubility of barium, as compared with calcium, hydroxide. The lead salts are difficult to prepare by the direct action of lead hydroxide. The copper salts are soluble. Separate experiments with the mixed diamino-acids show that these are as readily precipitated as the carbamates.

The method may be best illustrated by a description of a complete experiment. 900 g. of gelatin were soaked in five times the weight of water for one day. The mixture was then heated to boiling point on a water-bath and whilst still hot added to a boiling solution of sulphuric acid of such strength that the mixture contained 25 % sulphuric acid (for reasons of employing this method see Knaggs [1923]). Heating was continued for 30 hours. After dilution to

5400 cc. barium hydroxide was added until the mixture was just distinctly acid to Congo red, and the barium sulphate was then filtered off. To the filtrate more barium hydroxide was added in quantity somewhat more than sufficient to convert all the amino-acids into the barium salts, which amount was determined by a formaldehyde titration of a small part of the liquid. The barium sulphate thereby precipitated contains nitrogen; this is nitrogen of *i*-aspartic acid which gives an insoluble barium salt (Fraction 1 of the table, p. 1072). The filtrate from the barium sulphate containing the barium aspartate was concentrated to a volume of 3 litres and mixed with twice the volume of 95 % alcohol, and the mixture was mechanically stirred and then allowed to stand for two days. The barium salts of the dicarboxylic acids separated in a crystalline form.

After filtering off this precipitate, the liquid was cooled with ice, phenolphthalein was added, and then finely powdered dry, or freshly recrystallised moist barium hydroxide, until undissolved crystals remained. The mixture was vigorously stirred the whole time by a mechanical stirrer, the operation being carried out in 10-litre enamelled buckets. Carbon dioxide was then passed in with continual stirring until the phenolphthalein was nearly decolorised; more baryta was then added (until there was an undissolved excess) and the stirring was continued for about two hours and carbon dioxide again passed in. After this alternate treatment with barium hydroxide and carbon dioxide had been repeated twice and excess of baryta added, the gelatinous precipitate was filtered off (precipitate I), first on large ordinary filters, from which it was removed after the greater part of the liquid had separated to a Buchner funnel, when the filtration was completed by suction. The filtrate was again treated alternately twice with baryta and carbon dioxide and a second precipitate was obtained (precipitate II). A third fractional precipitate was obtained in a similar way. These three precipitates were washed by grinding with aqueous alcohol (2 vols. 95 % alcohol:1 vol. water), sucked as dry as possible on a Buchner funnel, then washed with graded strengths of alcohol and finally with ether and air dried. The aqueous alcoholic washings were added to the filtrate from precipitate III, and this liquid was then treated with carbon dioxide to precipitate excess of baryta, and after filtration of the precipitated carbonate, evaporated under diminished pressure to 1 litre. After addition of 2 litres of 95 % alcohol to this liquid and cooling with ice, carbamation was carried out three times in the manner already described. In this way a fourth precipitate was obtained. The following table indicates the course of the precipitation of the amino-acids.

Precipitate	Weight of fraction g.	% of N in fraction	% of N precipitated in fraction
I	1350	4·9	66·15
II	226	3·4	7·46
III	136	4·5	6·12
IV	147	2·2	3·23

In the last column the percentage of the nitrogen in the liquid which was

carbamated (after separation of the dicarboxylates) precipitated in each fraction is indicated. All these precipitates after drying were obtained in the form of fine powders. Further precipitation brings down only very small amounts of nitrogen.

The examination of the various fractions.

The various fractions are designated by the numbers given in the general scheme of separation (on p. 1072).

Fraction I. The greater part of the sulphuric acid used in the hydrolysis is first precipitated by adding baryta, only so much baryta being added as to leave the liquid still markedly acid to phenolphthalein. The first barium sulphate precipitate contains only small quantities of nitrogen. A second fraction of barium sulphate is then produced by adding more than sufficient baryta to combine with all the carboxyl groups (the amount necessary being determined by a formalin titration). The second precipitate contains appreciable amounts of nitrogen. In one experiment when 900 g. of gelatin were hydrolysed this amounted to 1·2 g. It was boiled with dilute sulphuric acid, and from the extract thus obtained the sulphuric acid was precipitated quantitatively by barium hydroxide. The filtrate from the barium sulphate was concentrated to a syrup, which was then thrown into absolute alcohol. A granular precipitate weighing 12·14 g. was obtained. It was purified by reprecipitation as barium salt by the addition of barium hydroxide to its aqueous solution. The product obtained by regeneration of the acid from the barium salt contained 10·44 % N (calculated for $C_4H_7O_4N$ 10·5 %). The substance agreed in its properties with *i*-aspartic acid, which is known to give a barium salt which is almost insoluble in water.

Fraction II. The crystalline alcoholic precipitate produced by the addition of alcohol was filtered off, and the free acids generated therefrom. They consisted of aspartic and glutamic acids, but no trace of hydroxyglutamic acid was found. The two former acids could be readily separated from one another, the glutamic acid being separated in the form of its hydrochloride.

Fraction III. The various fractions of the carbamate precipitates described above, which contain considerable amounts of barium carbonate, weighing altogether when dried 1840 g., were mixed with 5 litres of ice-cold water, with which they were mechanically stirred for two hours in an enamelled bucket surrounded with ice. After filtration the precipitate remaining undissolved was again extracted with ice-cold water (2·5 litres), and this extraction was repeated after filtration twice more with quantities of 1·5 and 1 litres of water. The aqueous solution contained the soluble carbamates. The insoluble fraction was decomposed by blowing steam through a suspension in hot water for about two hours. The carbamate was thereby decomposed into barium carbonate and free amino-acid. The solution of the latter was filtered off and concentrated. Crystals separated out. Various fractions were obtained and the mother-liquor from the last crop was thrown into absolute alcohol. A granular pre-

cipitate was thereby produced. The nitrogen was estimated in all the crops and found to be about 17·9 %. Nothing but glycine could be obtained from this fraction, nor could any glycine be isolated from other fractions; all attempts to isolate an insoluble barium carbamate from them led to a negative result. No precipitate was produced by phosphotungstic acid (although such a precipitate has been obtained in the corresponding fraction from other kinds of gelatin, S.B.S.). The glycine was further characterised by its conversion into the hydrochloride of the ethyl ester.

Fraction IV. The soluble barium carbamates obtained by the method described above were decomposed by boiling this solution. The barium carbonate thus precipitated was filtered off. The filtrates and washings were diluted so that the solution contained about 2 % amino-acids, and the diamino-acids were precipitated from this by phosphotungstic and sulphuric acids in the usual manner, this yielding *Fraction V.* (This was not further examined.) After excess of phosphotungstic acid and sulphuric acid, etc., had been separated in the usual manner, the solution freed from diamino-acids was concentrated *in vacuo.* Crystals separated. The first fraction weighed 13·01 g. and contained 10·06 % N. This consisted of almost pure leucine. The filtrate from this was concentrated to a small bulk and then thrown into absolute alcohol. A granular precipitate was obtained, weighing, after washing with alcohol and drying, 158 g.; it contained 11·9 % N of which 9·4 % was amino N, as determined by the method of Van Slyke. This sub-fraction consists of the remainder of the leucine which had not separated out, phenylalanine, alanine and serine. The amount of hydroxyproline could be determined indirectly by estimating the amount of non-amino N in the fraction. Investigations are still taking place on the separation of the amino-acids in this fraction. It was esterified in the ordinary way, and the free esters were obtained from the hydrochlorides. The conversion of the hydrochlorides into the free esters by Foreman's method [1919] was not found to be satisfactory, as the decomposition of the hydrochlorides in chloroform solution by barium oxide was very slow and incomplete. This may be due to the presence of hydroxyproline, as a similar difficulty with the corresponding fraction from caseinogen, which contains only a little hydroxyproline, was not experienced. In the case of gelatin, the decomposition was effected by sodium ethoxide in alcoholic solution. From the mixture of esters thus obtained, that of phenylalanine was readily obtained by extracting the aqueous suspension with ether.

The alcoholic filtrate from the precipitation of the amino-acids after separation of the leucine contains the proline. The alcoholic solution was evaporated down, and the residue was taken up with absolute alcohol and this solution filtered, the filtrate was evaporated and taken up with absolute alcohol, and these processes were repeated until a product was obtained which was completely soluble in absolute alcohol, giving a solution which yielded no deposit after prolonged standing in an ice-chest. This solution was almost free from amino-nitrogen. The ester hydrochloride of the alcohol-soluble portion

was prepared in the usual manner. The conversion into free ester was readily effected by Foreman's method. The ester had a nearly constant B.P. of 80° at 15 mm.: and was found to be the ester of pure proline, which was identified by the estimation of copper and nitrogen in the copper salt, and by conversion into the hydantoin derivative.

Fraction VI. This fraction, which formed the filtrate from the carbamates, was obtained in the form of a thick syrup by concentration *in vacuo*. This was dissolved in water and hydrolysed for 12 hours with 25 % sulphuric acid. After the quantitative separation of the acid by barium hydroxide and concentration a syrup was obtained which upon throwing into alcohol gave only a slight precipitate. The alcoholic solution was again concentrated, and the resultant syrup again hydrolysed with 25 % acid, and the processes just described repeated. A syrup was finally obtained which was completely soluble in alcohol. After concentration of the alcoholic solution a product was obtained which formed on standing a semi-crystalline mass. By means of cupric hydroxide a crystalline salt soluble in alcohol was obtained, with all the properties of the copper salt of proline. The copper and nitrogen were determined in this, and the results confirmed the assumption that the substance was the salt of proline.

The examination of this fraction has not been completed. The results do not indicate definitely whether it contained the prolyl-proline of Dakin [1920] or simply proline which had escaped precipitation by the carbamate method.

This work was carried out during the tenure of a Studentship of the Department of Scientific and Industrial Research by one of the authors.

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