THE ALKALINE DECOMPOSITION OF SERINE

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INTRODUCTION

A method for the quantitative estimation of arginine is included by Van Slyke (1911–12) in his procedure for the determination of the nitrogen distribution numbers of proteins. The amino acids present in the protein hydrolysate are divided into two groups by means of phosphotungstic acid. The portion precipitated by this reagent is referred to as the basic or diamino fraction and the portion not so precipitated is called the monoamino fraction. Potassium hydroxide, sufficient in amount to give a concentration of approximately 33 per cent, is added to an aliquot part of a solution of the amino acids of the basic fraction and the mixture is boiled for 6 hours under a reflux condenser. The arginine present is thereby decomposed into ornithine, ammonia, and carbon dioxide, and the cystine present is partially decomposed with the evolution of ammonia. At the end of the allotted time, the apparatus is disconnected, water is added, and the ammonia is removed by distillation. The amount of arginine in the original hydrolysate is calculated from the quantity of ammonia evolved, corrections being made for the solubility of arginine phosphotungstate and for the partial decomposition of the cystine.

Plimmer and Rosedale (1925) suggest that for the determination of arginine the division of the amino acids into two groups be omitted. They report that the monoamino fractions from various proteins evolve considerable quantities of ammonia when boiled with alkali. In explanation of this fact, they present the view that the solubility of arginine phosphotungstate is significantly greater than is allowed for by Van Slyke. They conclude: “The arginine value of a protein is the sum of the figures obtained from the di-amino and mono-amino fractions, or the figure obtained
directly, assuming that no other amino-acid behaving like arginine is present in proteins."

In a study of the nitrogen distribution of sericin, a protein of silk which does not yield cystine upon hydrolysis, we determined "arginine" in both the diamino and monoamino fractions. On the assumption that arginine was the only source of the ammonia evolved, the results indicated that considerably less than one-half of this amino acid had been precipitated by phosphotungstic acid. Further experiments showed that the monoamino fraction contained almost no non-amino nitrogen. Since three-fourths of the nitrogen of arginine is in the non-amino form, it was evident that the evolution of ammonia from this fraction could not have been due in its entirety to the decomposition of arginine.

In a search for the compound or compounds which gave rise to the excess ammonia, we turned our attention to serine. The structure of this hydroxy-amino acid suggested that it might be decomposed by alkali with comparative ease. Furthermore, it is well known that sericin contains a large amount of serine. A search of the literature revealed a statement by Baumann (1882) that serine is slowly and incompletely decomposed by boiling its solution with barium hydroxide.

Serine was isolated from sericin and treated as though an arginine determination were being made. Sufficient ammonia to represent a considerable fraction of the nitrogen of the amino acid was obtained. Other decomposition products were isolated and identified.

It seemed to us important to determine the extent to which this instability of serine affects the existing methods for the determination of the nitrogen distribution numbers of proteins. It is clear that an attempt to estimate arginine by the method under discussion will lead to an erroneous result whenever serine is present in the solution to be analyzed. This condition is found in the monoamino fraction, and in the hydrolysate prior to the division of the amino acids, whenever the protein under examination contains serine. The presence or absence of this amino acid in the diamino fraction depends on whether or not it is at all precipitated by phosphotungstic acid. According to our results, it is not so precipitated. The only other point at which the instability of serine to alkali might lead to error is in the determination of
"amide" nitrogen. It was found that the presence of serine causes at most a very small error at this point.

Except in the case of the modification of the arginine determination suggested by Plimmer and Rosedale, therefore, the instability of serine to alkali cannot be used as a basis for criticism of the methods of determination of the nitrogen distribution numbers of proteins.

**EXPERIMENTAL**

**Preparation of dl-Serine**

In the preparation of serine from sericin, we followed, in its general outline, the procedure described by Cramer (1865). We found it advisable, however, to make changes in certain details. For this reason, and because the journal in which Cramer published is somewhat difficult of access, the following description of the method, as modified, is given.

The protein was hydrolyzed with 25 per cent sulfuric acid for 24 hours. Barium hydroxide, sufficient in amount to bring the pH of the hydrolysate to 9, was then added and the mixture was allowed to stand at room temperature for an hour. The solution was next neutralized with sulfuric acid and filtered, and the filtrate concentrated on a steam bath. Crystals of tyrosine, alanine, and serine appeared successively in the concentrate and were removed, as nearly separately as possible, by filtration. The serine was recrystallized by adding to its hot aqueous solution an equal volume of alcohol.

The treatment of the hydrolysate with a base was given in order to racemize the serine. When it was omitted, we were unable to effect the isolation of this amino acid. This need not surprise us, in view of the fact that l-serine has a much greater solubility in water than has dl-serine.

Analysis of the serine showed 13.2 per cent of nitrogen instead of the theoretical 13.3 per cent; a solution of the amino acid showed no optical rotation.

**Effect of Heating Serine in Alkaline Solution**

As previously stated, serine was found to decompose into ammonia and other compounds when its solution was boiled with strong alkali.
In Table I are given the results of some experiments which were undertaken to determine the extent of ammonia formation. After each solution containing potassium hydroxide and serine had been boiled for 6 hours, water was added without disconnecting the apparatus, and the ammonia was removed by distillation. A pressure of approximately 40 mm. of mercury was maintained during the course of the experiment with calcium hydroxide.

Fig. 1 shows additional results from similar experiments. The plotted curve is typical of a number of such curves obtained with

<table>
<thead>
<tr>
<th>Base used</th>
<th>Concentration of base</th>
<th>Amount of serine added</th>
<th>Time</th>
<th>N evolved as ammonia</th>
<th>Fraction of total N of serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium hydroxide</td>
<td>Saturated solution</td>
<td>0.482</td>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>Magnesium hydroxide</td>
<td>Saturated solution</td>
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<td>0.5</td>
<td>0.1</td>
<td>0.2</td>
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<tr>
<td>Potassium hydroxide</td>
<td>Approximately 14 per cent*</td>
<td>0.586</td>
<td>6.0</td>
<td>5.2</td>
<td>6.7</td>
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<td>Potassium hydroxide</td>
<td>Approximately 20 per cent†</td>
<td>0.495</td>
<td>6.0</td>
<td>4.0</td>
<td>6.1</td>
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<td>Potassium hydroxide</td>
<td>Approximately 33 per cent‡</td>
<td>0.101</td>
<td>6.0</td>
<td>1.9</td>
<td>8.9</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>Approximately 33 per cent‡</td>
<td>0.286</td>
<td>6.0</td>
<td>14.1</td>
<td>37.0</td>
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<td>0.181</td>
<td>6.0</td>
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<td>37.7</td>
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<tr>
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<td>0.230</td>
<td>6.0</td>
<td>12.1</td>
<td>39.5</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>Approximately 33 per cent‡</td>
<td>0.289</td>
<td>6.0</td>
<td>14.1</td>
<td>36.6</td>
</tr>
</tbody>
</table>

* The serine was dissolved in 25 cc. of water. 25 cc. of approximately 28 per cent alkali (40 gm. of potassium hydroxide to 100 cc. of water) were added.
† The serine was dissolved in 60 cc. of water. 15 gm. of potassium hydroxide were added.
‡ The serine was dissolved in 50 cc. of water. 25 gm. of potassium hydroxide were added.
varying concentrations of different bases. In this series of experiments, the removal of the ammonia was made continuous by means of the passage of a current of ammonia-free air through the boiling reaction mixture.

The following experiments were carried out with the purpose of identifying additional products of the decomposition of serine in alkaline solution.

60 gm. of crystalline barium hydroxide were dissolved in 40 cc. of hot water and the solution filtered directly into a copper flask.

5 gm. of dl-serine were then added and the solution boiled under a reflux condenser for 72 hours. In order to prevent the absorption of carbon dioxide from the air the condenser was fitted with a tube containing soda-lime. At the completion of the allotted time, the contents of the flask were quickly filtered on a Buchner funnel and the precipitate washed thoroughly with cold water. This precipitate weighed 1.70 gm. after having been dried at 100°.

Examination of Precipitate—It appeared from microscopical examination that, aside from a few flakes of copper, the precipi-
Alkaline Decomposition of Serine

tate was composed entirely of white crystals which were octahedral in form. Addition of dilute acetic acid caused only an exceedingly slight effervescence, not more than might be expected from barium carbonate formed during the filtration. We feel justified, therefore, in stating that no appreciable amount of carbon dioxide was produced from serine during its decomposition.

Dilute hydrochloric acid dissolved the precipitate except for the copper flakes, which were removed by filtration. The barium was then precipitated by the addition of a very slight excess of dilute sulfuric acid, and the filtrate from the barium sulfate concentrated on a steam bath to a volume of about 3 cc. Upon cooling this solution in an ice bath, groups of long needles separated which had the appearance of oxalic acid. They melted at 99°, and when mixed with a known sample of oxalic acid there was no lowering of the melting point. This identified the original precipitate as barium oxalate. A mol for mol transformation of serine into oxalic acid being assumed, the barium oxalate recovered represented 16 per cent of the original serine.

Examination of Filtrate—The filtrate from the barium oxalate was chilled and the resulting crop of barium hydroxide crystals was filtered off on a Buchner funnel and washed with ice water. The combined filtrate and washings were then treated with sulfuric acid. The filtrate from the resulting barium sulfate was concentrated on a steam bath to a small volume. This concentrate was extracted in a separatory funnel four times with ether.

The combined ether extracts were dried over anhydrous sodium sulfate and the ether removed in a vacuum. The ether-soluble material thus obtained, when warmed with o-nitrobenzaldehyde and alkali, gave a negative test for pyruvic acid. The addition of phenylhydrazine produced a beautifully crystalline derivative which was subsequently identified, by means of a mixed melting point with a known sample, as phenylhydrazine lactate, m.p. 103°. While no figures are available, the amount of lactic acid in this fraction must have represented a considerable portion of the original serine.

The solution from which the ether-soluble substances had been removed was evaporated to a small volume on a steam bath and enough absolute alcohol added to make a final concentration of about 70 per cent. After the solution had stood overnight in the
ice box, there were some needles adhering to the sides of the beaker, and in the bottom a liquid layer which crystallized upon stirring. All of the crystals were filtered off together and washed with 70 per cent alcohol. The filtrate and washings contained 2.6 mg. of nitrogen, about half of which was amino nitrogen. No products could be isolated from this solution, however, and no further hint was obtained as to the nature of its contents.

The crystals, insoluble in 70 per cent alcohol, were found, after thorough drying, to weigh 1.01 gm. Analysis showed the presence of 16.9 per cent of nitrogen in this product, all of which was amino nitrogen. This very high nitrogen content was very strongly indicative of glycine. Accordingly, a portion of the material was dissolved in water and treated with picric acid. After concentration to a small volume and cooling, the solution deposited a crop of yellow crystals. Once recrystallized from water, they melted at 202°, both when alone and when admixed with a known sample of diglycine picrate. This identified one of the decomposition products as glycine.

Sulfuric acid was added to the mother liquors from the diglycine picrate and the picric acid removed by extraction with ether. The resulting solution was then neutralized and treated alternately with sodium hydroxide and α-naphthyl isocyanate. At the expiration of the reaction, the solution was filtered and acidified with hydrochloric acid, whereupon a copious precipitate of a uramino acid was obtained. After many recrystallizations this product melted at 191°, and when mixed with α-naphthyl uramino alanine, m.p. 198°, melted at 195°. Due to the small amount of material available, it could not be purified any further. It seems certain, nevertheless, that alanine also was among the decomposition products.

*Pyruvic Acid as an Intermediate Product*—Although all tests for pyruvic acid among the end-products of the decomposition were negative, it still seemed probable that it was an intermediate product. This was shown to be the case by the following experiment.1 70 cc. of 20 per cent potassium hydroxide solution, containing 1.48 gm. of serine and 2.14 gm. of p-hydrazinobenzoic acid, were boiled under a reflux condenser for 25 hours. The solution, after cooling,
was acidified with dilute hydrochloric acid, care being taken to keep the temperature low. Because of the appearance at this point of a voluminous precipitate of silicic acid the solution was made alkaline with ammonia, and the silicic acid was removed by filtration. Upon reacidification of the filtrate, a crystalline precipitate was obtained which was recrystallized several times. A mixed melting point with a known sample of the p-carboxyphenylhydrazone of pyruvic acid showed the two to be identical. Thus, while pyruvic acid did not appear as an end-product in the alkaline decomposition of serine, it was present as an intermediate product.

Treatment of Serine Solutions with Phosphotungstic Acid

$\textit{dl}$-Serine was resolved into its active components by the method of Fischer and Jacobs (1906). The $d$-serine obtained had a specific rotation of $+6.2^\circ$.

1 gm. of $d$-serine and 1 gm. of $dl$-serine were treated separately with phosphotungstic acid under the conditions recommended by Van Slyke (1911–12) for use in the precipitation of the basic amino acids. The mixtures were allowed to stand for 48 hours at $18^\circ$ and subsequently for several days at approximately $5^\circ$. No precipitate formed in either solution.

DISCUSSION

Van Slyke (1911–12) recommends the use of approximately 33 per cent alkali in arginine determinations. To 25 cc. of the solution of amino acids he adds 12.5 gm. of potassium hydroxide. Plimmer (1916) advises the use of a smaller concentration of alkali. He states that in his experiments the concentration of alkali is 20 per cent, but a study of his publications leads us to believe that it is, instead, approximately 14 per cent. As nearly as we can reconstruct his procedure, he dissolves 40 gm. of potassium hydroxide in 100 cc. of water and adds to the solution of amino acids an equal volume of this reagent.

We considered it advisable to obtain data for the production of ammonia from serine under conditions analogous to those employed by Van Slyke and by Plimmer. Accordingly, in the experiments in which potassium hydroxide was used, the concentrations of alkali were approximately 33 per cent, 20 per cent, and 14 per cent, respectively. The amounts of ammonia which were
evolved in these experiments are sufficient to indicate the advisability of separating arginine and serine from one another before the estimation of arginine by the alkaline decomposition method. The percentage error in the determination of arginine in the presence of serine would depend, of course, on the relative amounts of these two amino acids in the solution to be analyzed.

We consider it justifiable to conclude that there is no serine in the basic fractions from protein hydrolysates. Neither dl-serine nor d-serine gave a precipitate with phosphotungstic acid under the conditions recommended by Van Slyke for the precipitation of the bases, despite the fact that the amount of serine used in each experiment was considerably larger than that which usually arises from the hydrolysis of the sample of protein taken for a determination of nitrogen distribution. There is no reason to believe that d-serine and l-serine would yield phosphotungstates of unequal solubilities.

Ammonia is usually removed from protein hydrolysates either by saturating with magnesium hydroxide and distilling under atmospheric pressure or by saturating with calcium hydroxide and distilling at a pressure of 30 to 40 mm. of mercury. As shown by the figures in Table I, neither procedure causes a significant decomposition of serine.

That the breakdown of serine under the influence of hot concentrated alkali is a complex process is apparent from a consideration of the nature of the decomposition products. The presence of glycine and alanine among these products indicates that, regardless of the time of reaction, the serine nitrogen would never be completely transformed into ammonia, as these two substances are not appreciably affected under the conditions used (unpublished experiments). This accounts for the shape of the curve in Fig. 1, which indicates that as the time of the reaction was prolonged, the percentage of the total nitrogen evolved as ammonia was approaching a maximum value in the neighborhood of 55 or 60 per cent.

Of the decomposition products identified, it seems that ammonia and pyruvic acid only could possibly be primary ones, and there is no definite evidence that even these two can be regarded as being in that category. The other products are almost certainly the results of secondary reactions, one of which destroys the pyruvic
acid. That the secondary reactions involve oxidation and reduction is attested to by the formation of oxalic acid, which could only be derived from serine by oxidation, and alanine and lactic acid which are reduction products.

It is not unlikely that amino acids other than serine lose ammonia when heated in alkaline solution. Plans have been made to test this assumption and work on hydroxyglutaminic acid is now in progress.

SUMMARY

1. Serine is decomposed when heated in a strongly alkaline solution; among the products of decomposition are ammonia, glycine, alanine, oxalic acid, and lactic acid. Pyruvic acid is an intermediate decomposition product.

2. It is necessary that serine be absent from solutions in which arginine is to be estimated by the method of alkaline decomposition. Our experiments confirm the usual assumption that this absence is secured by the precipitation of arginine with phosphotungstic acid.

3. Serine is not decomposed in significant amount during the removal of ammonia from protein hydrolysates by the methods commonly employed.

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