XIII. THE ANALYSIS OF PROTEINS. II. THE ACTION OF NITROUS ACID UPON THE HEXONE BASES.

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THE estimation of the hexone bases, in the fraction precipitated by phosphotungstic acid, according to Van Slyke's method [1911, 2] depends upon the action of nitrous acid. Lysine loses the whole of its nitrogen, histidine one-third, and arginine one-quarter in the reaction. As the amount of arginine is determined by decomposition with alkali, the amount of histidine can be calculated from the non-amino nitrogen of the solution. The difference from the total nitrogen then expresses the amount of lysine. The values for histidine and lysine are thus obtained indirectly, those for lysine being entirely dependent upon the accuracy of the other two estimations.

The estimation of arginine was investigated by Plimmer [1916] and found to be accurate. Concordant results for the amount of arginine in proteins have since been obtained, but the results for histidine and lysine have never been as concordant as was desired. It thus became necessary to study the action of nitrous acid upon the three hexone bases. Van Slyke [1911, 1] studied the reaction with his original apparatus. He found that the α -amino group of amino acids reacted completely in five minutes. The ϵ -amino group of lysine reacted in half an hour, and in the case of other amino compounds still longer times of reaction were needed for complete evolution of the nitrogen. In the analysis of the hexone bases, Van Slyke stated that a reaction time of half an hour at 20°, or longer at lower temperatures, should be given. No exact time was mentioned for the reaction at the lower temperatures. It was possible, if a longer time was given, that the guanidine complex of arginine might react and cause discrepancies in the estimation.

It has now been found that a time of one hour should be given for the reaction of lysine at temperatures below 20° and that arginine in this time gives amounts of nitrogen in excess of that expected from the α -amino group. This excess is greater in still longer reaction times, which indicates that the guanidine complex reacts slowly. The excess in one hour is very small and not sufficient to cause any appreciable error in the analysis. As far as these three bases are concerned the method is therefore satisfactory.

EXPERIMENTAL.

The large form of apparatus described by Van Slyke [1912] was used. After the air had been expelled from the apparatus, a known volume of solution was introduced into the burette with a pipette and allowed to enter the reaction vessel. The sides of the burette were then washed twice with 1 cc. of water. A control for the determination of air in the reagents was made in a similar way with the same volume of water. In making the determinations it is advisable always to have the same amount of reagent in the reaction vessel; this can be attained by making the reagents stand at a certain mark scratched on the vessel before the solution of amino compound is introduced. The apparatus was shaken continuously for the shorter times of reaction up to one hour; in the case of longer times the reagent and solution were mixed by shaking, allowed to stand and shaken for the last half hour. The evolved gas was treated with permanganate and measured in the usual way. Its volume was then converted to mg. of nitrogen by the table. The control figure was deducted and the amount calculated for the volume of solution made at the start. A total nitrogen determination of the solution was made by Kjeldahl's method, except in the case of lysine picrate, which was weighed out and its amino nitrogen calculated from the formula.

(1) *Action of Nitrous Acid upon Lysine.*

For these experiments a specimen of lysine picrate was kindly supplied by Prof. Drummond. Both the picrate and a solution of lysine prepared from it were used.

(a) *Lysine picrate.* 0.4902 g. was dissolved in water containing a small amount of sodium hydroxide and made up to 50 cc. Five or 10 cc. were taken for the estimations. The room temperatures at the time of the experiments varied from 11 to 15°.

Reaction time	N evolved calc. as g. per 10 cc.	Percentage of N	Percentage of N calc. for	
			1 atom N	2 atoms N
15 min.	0.0056	5.71	3.01	7.67
30 "	0.0055	5.61	—	—
3.5 hours	0.0067	6.84	—	—
5.5 "	0.0067	6.84	—	—
15.5 "	0.0076	7.75	—	—
16.5 "	0.0075	7.65	—	—

(b) *Lysine solution.* A solution of lysine was prepared from 0.5 g. of the picrate by solution in water, acidification with hydrochloric acid, removal of picric acid by filtration and extraction with ether, separation of ether by evaporation *in vacuo*. The solution was then made up to about 100 cc. Five or 10 cc. were taken for the estimations of amino nitrogen and 10 cc. for that of total nitrogen. The temperature at the time of the experiments was from 15 to 16°.

Reaction time	N evolved in g. per 100 cc.	Total N in g. per 100 cc.
15 min.	0.0278	0.035
30 "	0.0283	—
30 "	0.0304	—
30 "	0.0358	—
1 hour	0.0343	—
1 "	0.0394	—
2.5 hours	0.0364	—
6.5 "	0.0344	—

Thus, in the case of lysine picrate, at temperatures of 11 to 15° complete evolution of nitrogen did not occur until after five and a half hours.

The solution of lysine reacted more rapidly; in one experiment the whole of the nitrogen was given off in 30 minutes. A time of one hour is required for the reaction at 15 to 16° for complete decomposition of the two amino groups.

(2) *Action of Nitrous Acid upon Ornithine.*

In order to confirm the slow reaction of the end amino group of the diamino acids an experiment was made with a solution of ornithine. This was prepared by decomposing arginine carbonate by boiling with 20 % sodium hydroxide for six hours [Plimmer, 1916]. The alkaline solution so obtained was acidified with glacial acetic acid and diluted to 100 cc. Five or 10 cc. portions were used for the amino determinations, which were made at from 14 to 16°.

(1) 0.1540 g. arginine carbonate = 0.077 g. N per 100 cc. ornithine			(2) 0.07 g. arginine carbonate = 0.035 g. N per 100 cc. ornithine		
Reaction time	N evolved calc. in g. per 100 cc.		Reaction time	N evolved calc. in g. per 100 cc.	
30 min.	0.0519		1 hour	0.0133	
45 "	0.0576		2 hours	0.0151	
60 "	0.0679		3.5 "	0.0218	
			17 "	0.031	

The whole of the nitrogen was thus not evolved from ornithine in one hour; complete evolution occurred in 17 hours. This long time of reaction is probably connected with the excess of sodium acetate present in the solution derived from the neutralisation of the sodium hydroxide; unfortunately a specimen of ornithine was not available.

(3) *Action of Nitrous Acid upon Histidine.*

A solution of about 0.3 g. of histidine hydrochloride in 105 cc. water was used. Ten cc. portions were taken for the determinations. The temperature varied from 17 to 19°. Total nitrogen was determined in another portion of 10 cc.

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g. per 100 cc.
5 min.	0.0245	30 min.	0.0267
10 "	0.0251	1 hour	0.0271
20 "	0.0257	1 "	0.0264
30 "	0.0260	17 hours	0.0265
30 "	0.0243		

Total N per 100 cc. = 0.0257 g.

Complete reaction was not attained in 5 minutes at 17°, but in 30 minutes the nitrogen of the amino group was completely given off. There was no reason to expect that reaction would occur with the nitrogen atoms of the glyoxaline ring; this was shown to be the case by the long reaction for 17 hours. The stability of the glyoxaline ring was also confirmed by an experiment with glyoxaline, a specimen of which was kindly supplied by Prof. Barger. No nitrogen was evolved on treatment with nitrous acid under the same conditions.

(4) *Action of Nitrous Acid upon Arginine.*

The same specimen of arginine carbonate as previously used for the study of its decomposition with alkali was employed. Three series of experiments were made:

(a) with 0.6 g. dissolved in about 105 cc. water. Temperature from 14 to 17°:

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g. per 100 cc.
15 min.	0.0375	4.5 hours	0.0501
30 "	0.0407	16 "	0.0591
30 "	0.0390	16.5 "	0.0667
1 hour	0.0400	23 "	0.0611
1 "	0.0406	40 "	0.0890
3 hours	0.0497		

Total N per 100 cc. = 0.1540 g. $1/4 = 0.0385$ g.

(b) with 0.3 g. in about 105 cc. water. Temperature 14 to 17°:

Reaction time	N evolved calc. as g. per 100 cc.	Reaction time	N evolved calc. as g. per 100 cc.
.5 hour	0.0267	2 hours	0.0280
1 "	0.0294	4 "	0.0304
1 "	0.0280	21.5 "	0.0504

Total N per 100 cc. = 0.1078 g. $1/4 = 0.0267$ g.

(c) with 0.2 g. in 100 cc. water. Temperature 14 to 16°:

Reaction time	N evolved calc. as g. per 100 cc.
30 min.	0.0173
60 "	0.0170

Total N per 100 cc. = 0.07 g. $1/4 = 0.0175$ g.

The amino group of arginine thus reacts completely at 14 to 17° in half an hour. In one hour there was a greater evolution of nitrogen and in longer times of reaction the evolution increased progressively. The guanidine nucleus of arginine is evidently slowly attacked by nitrous acid. The excess of nitrogen produced in one hour was small and amounted in one experiment to about 5 %. In carrying out estimations of arginine by this method the time of reaction should not exceed one hour. In this time the other amino acids react completely and any error introduced by excess of nitrogen from arginine is inappreciable.

SUMMARY.

1. Lysine requires a time of one hour for complete reaction with nitrous acid at temperatures from 14 to 17°.

2. The amino nitrogen of histidine and arginine is given off in 15 to 20 minutes at this temperature.

3. A longer time of reaction than one hour has no effect upon histidine, but leads to a greater evolution of nitrogen from arginine.

4. In estimating a mixture of the three hexone bases, it is necessary and sufficient to give a reaction time of one hour at temperatures of 14 to 17° with nitrous acid. The excess of nitrogen given off by arginine will not cause an appreciable error.

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