CLXVII. ON THE HYDROLYSIS OF GUANINE.

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SOME two years ago when examining certain autoclaved material by the diazo-test for thymine recently described [Hunter, 1936], a greenish blue colour was observed as soon as the test solution was mixed with the alkaline diazo-reagent. The colour faded rapidly to yellow in the presence of the weak alkali, but if a few drops of sodium hydroxide were added quickly after the test solution, the green colour changed to a beautiful blue which was stable for many hours. In the writer's experience such a test was unique and seemed to call for further investigation. A small amount of commercial yeast nucleic acid was heated with 25 % sulphuric acid at 150–160° for 5 hours (the usual conditions for the hydrolysis of nucleic acid to yield pyrimidines) and a little of the neutralized hydrolysate tested with the diazo-reagents; a strong positive test was obtained. Adenine and guanine were then separately hydrolysed in a similar manner with the result that the guanine hydrolysate yielded a very strong test, whilst the adenine hydrolysate was quite negative. It was thus established that guanine was the source of the substance giving the blue colour with the diazo-reagents.

Experiments were made with quantities of 10–50 mg. guanine hydrochloride to establish the optimum conditions of strength of acid, time of heating and temperature, for the production of maximum yields of the substance as measured by the colour given by the diazo-reagents. Great accuracy could not easily be attained and was not aimed at, the best conditions being finally somewhat arbitrarily decided upon. In the earlier work sulphuric acid was employed, but to simplify the isolation hydrochloric acid was later substituted.

The unknown substance was apparently very stable in acid solution; it was not precipitated by silver in the purine fraction, but was precipitated quantitatively with silver and barium hydroxide and was recoverable by the usual H_2S treatment; it was also precipitable by mercuric chloride near neutrality and by phosphotungstic acid; although its effective recovery from the phosphotungstate was rendered doubtful by the blackish blue colour given in excess of $\text{Ba}(\text{OH})_2$. Means, in short, seemed to be available for the separation of the substance from both purines and pyrimidines, but since pure guanine was employed pyrimidines were not likely to complicate the isolation. As the sequel will show, no metallic precipitant is actually necessary in the isolation of the pure substance.

The first crystalline material was obtained as sulphate from the silver-baryta precipitate. When excess of absolute alcohol was added to the concentrated silver-free fraction acidified with H_2SO_4 , an oil separated. After cooling and scratching with a glass rod discrete well-formed crystals appeared. The material thus obtained, however, was not ash-free, and after repeated crystallization it still retained traces of what appeared to be silica. The experience of Fischer [1910], who obtained a 50–60 % yield of xanthine by boiling guanine for 30 hours with 20 % HCl was similar to this, in that his xanthine was always contaminated with silica, which was avoided only by recourse to a platinum vessel for hydrolysis.

The main bulk of the base has, however, been prepared as hydrochloride. The dihydrochloride crystallizes readily by the method described below, and there is no difficulty in obtaining ash-free preparations. From various batches totalling 40 g. guanine hydrochloride, 7.2 g. of the dihydrochloride of the new base $C_4H_7N_5 \cdot 2HCl$ were obtained, a yield of 15.6%. A special batch of 6.21 g. guanine hydrochloride, the quantitative degradation of which is reported below yielded 1.16 g. $C_4H_7N_5 \cdot 2HCl$, or just over 16% on a weight basis.

*Analyses.*¹

Sulphate. Found: C, 21.93, 21.60; H, 4.35, 4.26; N, 30.75, 31.19, 30.87; S, 14.28, 14.29; ash, 0.36%. $C_4H_7N_5 \cdot H_2SO_4$ requires C, 21.52; H, 4.03; N, 31.40; S, 14.35%.

Dihydrochloride. Found: C, 24.41, 24.34; H, 4.48, 4.58; N, 35.10, 35.33, 35.17; Cl, 36.06, 36.05%. $C_4H_7N_5 \cdot 2HCl$ requires C, 24.24; H, 4.58; N, 35.38; Cl, 35.81%.

Evidence pointing to the constitution of the new base as 4- (or 5-) guanidino-glyoxaline is presented in the following paper.

A small amount of the new base remaining in the mother-liquors from the above preparation was precipitated, after removal of chloride, by silver and baryta at pH 8.0. Large excess of baryta was then added to the filtrate and a further appreciable precipitate was obtained, which was decomposed, the solution being freed from Ag, Ba and SO_4 , evaporated *in vacuo* to small volume and made acid with HCl. By treatment with alcohol about 60 mg. of small well-formed rhombohedra, m.p. 200°, were obtained, which proved to be glycoeyamine hydrochloride. Addition of picric acid to the filtrate from this yielded a mixture of the picrates of guanidine and glycoeyamine.

The filtrate from the silver-baryta precipitate was treated with H_2S in the usual manner; excess of HCl was added and the material taken to dryness to get rid of HNO_3 . By treatment with alcohol 0.63 g. of white spicular crystals was obtained melting fairly definitely at 180°. The filtrate brought to dryness *in vacuo* and taken up in hot absolute alcohol gave another crop of crystals, m.p. 147°.

On micro-analysis (Dr Stantial) the two substances gave respectively: C, 21.80; H, 5.51; N, 12.99; Cl, 31.73% and C, 17.54; H, 4.34; N, 20.86%. They were thus glycine hydrochloride (requires C, 21.52; H, 5.38; N, 12.57; Cl, 31.80%) and glycine nitrate (requires C, 17.39; H, 4.35; N, 20.29%).

A small amount of substance having 11.42% N and m.p. 225° had been isolated from the same fraction, which proved to be glycine sulphate (m.p. 225°, 11.29% N). The latter had arisen from a slight excess of H_2SO_4 left when removing Ba.

The disintegration of guanine having thus been fairly exhaustively studied (all products with the exception of glycoeyamine having at this stage been identified) it was thought worth while to fractionate a hydrolysate as quantitatively as the removal of test portions at various stages permitted. The following summarized account is described in detail in the experimental section.

It is clear from this account that any degradation product of guanine that may have escaped detection must be present in very small amount. Considering the relative complexity of the analysis and the frequent removal of small test portions of which no account was kept the recovery is for practical purposes complete.

¹ Micro-analyses performed through the courtesy of Prof. W. Lash Miller, Department of Chemistry, University of Toronto, by Dr Helen Stantial.

Material used:	g. N	% Total N
6.21 g. guanine hydrochloride = 4.574 g. guanine	2.120	100
<i>Recovery, in order of separation:</i>		
1. 2.402 g. Xanthine (gravimetrically)	0.885	41.8
2. Ammonia-N (by titration)	0.600	28.3
3. 1.26 g. C ₄ H ₇ N ₅ .2HCl (colorimetric estimation)	0.446	21.0
1.16 g. C ₄ H ₇ N ₅ .2HCl isolated as such	—	—
0.07 g. C ₄ H ₇ N ₅ .2HCl isolated as dipicrate	—	—
i.e. 1.23 g. C ₄ H ₇ N ₅ .2HCl isolated = 97.6% of estimated	—	—
4. 0.326 g. Glycine (by ninhydrin and amino-N determination)	0.061	2.8
0.180 g. Glycine isolated as glycine sulphate = 55% of estimated	—	—
5. 0.037 g. } Guanidine. Colorimetric estimation	0.026	1.2
6. } Glycocycamine.	—	—
0.026 g. isolated as mixed picrate = 70% of estimated	—	—
Total accounted for	2.018	95.1

DISCUSSION.

When guanine is heated with strong acids under the conditions described the changes taking place are more complex than have been previously recognized. It is clear that the main reaction is:

As shown 4.574 g. guanine yielded 2.402 g. xanthine or a yield of 52%. This is comparable with the yields obtained by Fischer¹ [1910].

Proceeding alongside this reaction is the apparent removal of the CO group in position 6 with the production of the new base assumed (see following paper) to be 4- (or 5-) guanidinoglyoxaline and CO₂ according to:

As shown 4.574 g. guanine yielded 1.26 g. guanidinoglyoxaline dihydrochloride = 0.796 g. of the free base. For the production of 0.796 g. guanidinoglyoxaline 0.962 g. guanine is required; so that the reaction outlined above proceeds to the extent of 21.5% on the basis of guanine used.

If the guanine utilized for the formation of xanthine and of guanidinoglyoxaline (2.386 + 0.962 = 3.348 g.) be deducted from the total guanine used, (4.574 g.) there is left 1.226 g. guanine, available for the production of glycine, guanidine, glycocycamine and ammonia. From 1.226 g. guanine there could theoretically be produced 0.609 g. glycine. The yield of glycine actually obtained, namely 0.326 g., thus represents 53% of that obtainable from the guanine available for its formation, or just over 14% yield from the total guanine employed. The production of glycine is thus not insignificant from a quantitative

¹ It should be noted that by Fischer's method of hydrolysing for 30 hours with 20% HCl at 100°, an appreciable amount of the base giving the blue diazo-test is formed.

aspect. It appears probable that 4-(or 5)-guanidinoglyoxaline is an intermediate product in its formation (*vide* p. 1190).

The guanidine theoretically derivable from 1.226 g. guanine is 0.479 g. compared with a yield of about 0.030 g., about 6% on the basis of guanine available or less than 2% of the total guanine. The glycoyammine obtained is even less and is unlikely to represent more than 1% of the available guanine. It is not unlikely that both products are derived through 4-(or 5)-guanidinoglyoxaline.

The total ammonia-N produced is recorded above as 0.60 g. Of this 0.22 g. is produced when 2.402 g. xanthine are formed. There thus remains to be accounted for 0.38 g. N. There is this amount of N in 0.82 g. guanine. If 0.82 g. be subtracted from 1.226 g. there is left 0.406 g. guanine. From this there is theoretically obtainable only 0.203 g. glycine which is less than that (0.326 g.) actually obtained; from which it can be concluded that one N of the glyoxaline ring takes part in the formation of glycine while the other forms ammonia.

EXPERIMENTAL.

In all experiments a well crystallized commercial product sold as guanine hydrochloride was used. This showed a loss of 8.93% moisture on heating at 105°, and by micro-Kjeldahl 34.70% N (calculated for $C_5H_5N_5O \cdot HCl$, H_2O , 8.76% moisture and 34.10% N).

6.21 g. guanine hydrochloride were put in a short-necked 300-ml. Kjeldahl flask with 250 ml. 3.4 N HCl. A small beaker was inverted over the opening and the flask placed in a gas-heated autoclave; 23 min. were required to raise the temperature to 158°, at which point it was held for 90 min.; the flame was then extinguished and after 65 min. the temperature had fallen just below 100° when the autoclave was opened and the flask was removed.

The contents were transferred with washings to a 500 ml. balloon flask and distilled *in vacuo* to dryness. About 50 ml. water were added and again distilled off. This was repeated. Most of the HCl was thus removed. Finally about 75 ml. water were added, and the contents were left in a refrigerator overnight.

The precipitate, which is practically pure xanthine, was carefully collected on a small Hirsch funnel, well washed with water and dried; wt. 2.402 g. The material gave a picrate and a nitrate identical with the corresponding salts prepared from authentic xanthine. Its behaviour with the murexide test was also typical of that of xanthine.

The filtrate and washings from the xanthine were brought to 100 ml. 0.5 ml. was removed and diluted to 5 ml. for ammonia-N determination by aeration, and by micro-Kjeldahl distillation. The aeration method yielded 0.586 mg. N/ml.; the distillation method 0.61 mg. N/ml., or an average of 0.6 mg. N/ml. of the 1 in 10 dilution.

1 ml. of the same solution contained 2.94 mg. Cl; the whole solution thus containing nearly 3.0 g. Cl required rather more than 7.0 g. $Ba(OH)_2$ for neutralization.

The solution was again transferred with washings to a 500 ml. balloon flask and 7.8 g. finely powdered $Ba(OH)_2$ were added. Distillation *in vacuo* was continued until a drop of the contents of the flask no longer gave a Nessler reaction. When the solution had thus been freed from NH_3 and the volume brought to about 100 ml. by addition of water, the calculated amount of 5N H_2SO_4 was added to precipitate the Ba. The solution was freed from both Ba and SO_4 ions by final adjustment with dilute H_2SO_4 and $Ba(OH)_2$. The mixture was centrifuged and the $BaSO_4$ well washed with water. The fluid and washings were transferred to a 500 ml. balloon flask and evaporated *in vacuo* to about 15 ml. The slightly brown solution was brought to the boil, treated with a very small amount

of powdered charcoal and filtered through a small funnel into a boiling-tube, the paper being well washed with hot water. It was then evaporated *in vacuo* to about 7 ml. when solid began to appear on the sides of the tube. Evaporation was continued until crystals appeared in the body of the fluid. The tube was then removed from the pump, heated over a flame to dissolve the crystals, and absolute ethyl alcohol was added from a pipette until crystals appeared in the still warm solution (about 10 ml. alcohol). The tube was then cooled under the tap, 5–7 ml. alcohol were added, and the tube was left in the refrigerator overnight. The crystals were collected, washed with absolute alcohol and dried at 105°; wt. 1.003 g. The filtrate was concentrated, again treated with charcoal and yielded a second crop of white crystals, 0.114 g. The material even of the second crop was practically pure 4-(or 5-)guanidinoglyoxaline dihydrochloride, and represents at this stage more than 90 % of that estimated by colour test.

From the diluted aliquot used above for NH_3 and Cl determinations, a portion was removed and again diluted 10 times. A standard solution containing 0.1 mg./ml. pure 4-(or 5-)guanidinoglyoxaline dihydrochloride was prepared. Into each of two tubes were put 5 ml. 1.1 % sodium carbonate and 2 ml. Koessler and Hanke diazo-reagent. To one tube was added 0.5 ml. of the standard, to the other 0.5 ml. of the unknown, followed after mixing in each case by 1 ml. 2.5 *N* NaOH. Compared in a Duboscq colorimeter the test read 15.0 mm. against the standard at 18.9 mm., so that the amount of dihydrochloride present was 1.26 g.

The filtrate and washings from the second crop of the dihydrochloride were distilled *in vacuo* to remove alcohol, and made to a vol. of about 30 ml. with water. A solution of 10 % AgNO_3 was then added until a drop of the mixture showed slight blackening with a solution of $\text{Ba}(\text{OH})_2$ on a tile. The precipitate was centrifuged, washed with a little water and discarded. To the fluid a hot solution of $\text{Ba}(\text{OH})_2$ was very carefully added to pH 8.0 (bromothymol blue). The precipitate was separated, washed with a little water, transferred to a small flask, made acid with H_2SO_4 , treated with H_2S and stoppered (*Fraction A*). The filtrate was made strongly alkaline with a hot saturated solution of $\text{Ba}(\text{OH})_2$, the precipitate was separated, washed with a solution of $\text{Ba}(\text{OH})_2$, transferred to a small flask, made acid with H_2SO_4 , treated with H_2S and stoppered (*Fraction B*). The silver-baryta filtrate was transferred to a flask, made acid with H_2SO_4 and treated as above (*Fraction C*).

Fraction A was freed from Ag_2S , and from H_2S by distillation of the acid solution *in vacuo*. Ba and H_2SO_4 were removed as usual. To a volume of about 10 ml. about 5 ml. of hot saturated aqueous picric acid were added. The copious picrate which separated was dissolved by heat and the tube allowed to cool. It was separated and dried; wt. 0.205 g., M.P. 200°. After recrystallization from water the M.P. was 216°, or the same as that of the dipicrate prepared from pure 4-(or 5-)guanidinoglyoxaline (*vide* p. 1193).

Fraction B was treated as fraction A, the Ba- and H_2SO_4 -free solution being strongly alkaline to litmus. To a volume of about 25 ml. were added 15 ml. of saturated aqueous picric acid. The precipitate was dissolved by heat and left overnight to crystallize. Under the microscope there appeared two crystalline species distinguished by both form and colour. The mixed picrate was filtered and weighed 170 mg., M.P. 220–225°. The filtrate was concentrated and a crop of about 8 mg. sulphur-yellow needles, M.P. 200°, was obtained. The first crop was recrystallized from water and quickly yielded crystals typical of guanidine picrate, M.P. 325°. The crystals were of a deep golden colour with zigzag or swastika forms. The sulphur-yellow needles on recrystallization had M.P. 207°, the melt being typically blood-red in colour.

It has been mentioned that in the treatment of this fraction obtained from 40 g. guanine hydrochloride, a small amount of the hydrochloride of a base, M.P. 200°, had been obtained. This substance was now found to form a picrate similar in all respects to that here described. Despite some difference in M.P. of the hydrochloride and the picrate recorded for glycoyamine, this substance was suspected. It was accordingly synthesized by the method of Ramsay [1908] from guanidine and chloroacetic acid. Its hydrochloride was found to melt at 200°, its picrate at 207° and its picolonate at 245°. In crystalline form and colour all the derivatives of our base were identical with the corresponding ones from glycoyamine. By heating a few mg. of the hydrochloride of glycoyamine, and a few mg. of the hydrochloride in question to 170° and dissolving in a little water, a strong Jaffé test was obtained from each solution. The Weyl test, characterized by a burgundy colour on acidification [Barger, 1914], was equally good evidence for the conversion into glycoyamidine.

An attempt was made to estimate the guanidine in fraction B, before the treatment with picric acid just described. The volume of the solution had been brought to 25 ml. A standard solution of guanidine carbonate containing 1 mg./ml. was made. The Marston modification of the Tiegs test was used.¹ It was found that 0.1 ml. of the test solution gave approximately the same colour as 0.3 ml. of the standard guanidine carbonate. The 25 ml. of fraction B thus contained the equivalent of 75 mg. guanidine carbonate or 37 mg. guanidine. The estimate is a maximum as the mol. wt. of glycoyamine is double that of guanidine.

Fraction C was treated as fractions A and B, taken to a small volume and made to 25 ml. Preliminary tests with ninhydrin indicated that the solution contained about 12 mg. glycine per ml. Two determinations in the Van Slyke micro-amino-N apparatus showed the presence of 2.40 mg. $\text{NH}_2\text{-N/ml.}$ or 0.060 g. in fraction C. This amount corresponds to 0.326 g. glycine.

Very slightly more than the theoretical amount of H_2SO_4 was now added to about 23 ml. remaining from the solution mentioned. It was evaporated *in vacuo* in a small tube to a vol. of 1–2 ml. and absolute alcohol was added. The crystals were filtered off; yield 0.183 g., M.P. 226°, identical with the sulphate made from glycine. A further crop of 0.107 g. was obtained giving a total of 290 mg. glycine sulphate against a theoretical yield of 496 mg. glycine sulphate.

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¹ The reagent used here was Weber's modification of Marston's reagent as described by Robertson [1929], and is made by mixing in order 5 ml. 10% sodium nitroprusside, 5 ml. 10% potassium ferricyanide, 5 ml. 10% sodium hydroxide, with the final addition of 45 ml. water. The test is carried out by adding to about 1 ml. unknown solution, made alkaline with 1 drop 2.5 N NaOH, 0.5 ml. of the reagent after it has been kept for 30 min.

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