CCLXXVI. ON THE QUANTITATIVE DETERMINATION OF ADENINE IN THE PRESENCE OF GUANINE.

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THE quantitative analysis of the amount of total purine bases present in organs or in specimens of nucleic acids can be carried out with great accuracy. The same cannot be said however of the estimation of the individual purine bases. After acid hydrolysis, the total bases may be quantitatively precipitated, either by means of an ammoniacal silver solution or by the copper bisulphite reagent. The quantitative separation of the two main purine bases guanine and adenine is a much more difficult task. Generally their different solubilities in dilute ammonia or hot water have been used for this purpose. Adenine itself however is not soluble enough in neutral or weakly alkaline solution to allow of a quantitative separation of the two purine bases in this way.

Several attempts have therefore been made to estimate the amount of guanine in another way. The adenine content has been calculated indirectly from the content of purine bases containing an amino-group. Most recently Schmidt [1933] has made extensive use of a new principle. The guanine is deaminised with a purified solution of liver guanase. The ammonia formed is distilled over *in vacuo* and titrated in the usual way. The method gives reliable results even if applied on a micro-scale. There is however difficulty in obtaining strongly active purified extracts and some uncertainty as to the quantitative course of the reaction in the presence of possibly inhibiting substances.

For guanine analysis, Robertson [1929] oxidised guanine with permanganate and determined the guanidine formed colorimetrically with Marston's nitroprusside reagent. In using this method the present author obtained good results only with pure guanine solutions; applied to the purine precipitate from nucleic acid hydrolysates, the method proved unreliable, very low figures being generally obtained. Robertson considered the most important source of error to be adsorption of guanidine on the precipitate of manganese dioxide. Owing to the extreme sensitivity of the colour production by the nitroprusside reagent with guanidine, it seems more plausible to believe that impurities thrown down with the ammonia silver precipitate prevent the colour formation.

For the discussion of the structure of the ribopolynucleotide of pancreas discovered independently by Hammarsten [1920] and Feulgen [1919-20] it was desirable to know the exact guanine:adenine ratio. A more reliable method than those outlined above was therefore necessary.

In oxidising guanine with permanganate according to Robertson, some observations were made which led to the elaboration of the technique of analysis given below. If the oxidation of guanine is performed in boiling *N* HCl in a porcelain dish in the presence of adenine, the end-point is distinctly marked by

a change in the colour of the solution. As long as there is guanine left the permanganate disappears very quickly from the greenish solution. As soon as the guanine is destroyed, the colour fades and on the addition of further permanganate turns yellow. The appearance of this yellow tint is very sharp and allows the fixation of the end-point of the oxidation as sharply as in ordinary volumetric titrations. Owing to the well-known resistance of adenine to oxidising agents this purine base is still untouched and can be quantitatively determined. Using this finding, an attempt was made to estimate the amount of guanine and adenine in a mixture by analysis of the purine-N before and after destruction of guanine. By this technique, the desired accuracy of purine analysis was reached.

The utilisation of this lability of guanine as well as of xanthine and of uric acid towards oxidising agents has been suggested for quantitative analysis by Grynberg [1932]. On titrating ribose according to the Willstätter-Schudel method, he found that the consumption of iodine by guanine corresponds to 2 mols. per mol. of purine base, whereas adenine and hypoxanthine do not react with iodine. The consumption of iodine as well as the decoloration of permanganate can however be influenced by other substances. Far greater accuracy is obtained if the total amount of purine bases is determined both before and after the destruction of the guanine.

The accuracy of this method depends on the complete oxidation of the guanine without any destruction of adenine. For this reason the oxidation is to be performed under certain definite conditions. If the purine bases are dissolved in N H_2SO_4 , a brownish colour of manganese dioxide is obtained as soon as the guanine is destroyed. The end-point however is difficult to distinguish, hence the oxidation of the guanine may be incomplete. If the reaction is performed in boiling 0.5–1 N HCl it goes further, since the adenine is oxidised by the chlorine developed from the manganese dioxide and the hydrochloric acid. Fortunately, there develops at the same time a very strong yellow colour which can be distinguished after the addition of one or two drops of $N/10$ $KMnO_4$. When permanganate is added to a boiling hydrochloric acid solution of adenine, the solution turns brownish yellow after the first drop of the oxidising agent. Hence this colour change was taken as an indicator of the completeness of the oxidation of guanine in the presence of adenine. In the titration of pure guanine solutions about 20 mg. of adenine sulphate were added as an indicator.

When pure solutions of the purine bases guanine and xanthine were titrated in the presence of adenine the same accuracy was obtained as in ordinary volumetric titrations, as may be seen from Table I. On oxidation of nucleic acid solutions directly after acid hydrolysis however the end-point could not be distinguished owing to the presence of permanganate-consuming impurities. The purine bases were therefore first precipitated with ammoniacal silver solution, and the precipitate was washed three times with dilute silver solution. The silver was then removed by boiling with N HCl and the volume made up to 50 or 100 ml. In aliquot portions of the solution, the purine bases were precipitated with the copper bisulphite reagent both before and after oxidation with permanganate. After washing, nitrogen analysis was performed on the purine precipitates.

The consumption of permanganate by guanine and xanthine.

Two samples of guanine hydrochloride prepared from thymus nucleic acid and yeast nucleic acid respectively and one sample of xanthine sulphate were submitted to oxidation. The two specimens of guanine hydrochloride were recrystallised twice from a dilute hydrochloric acid solution. Specimen I con-

tained 31.25 % N and 16.45 % moisture (115°) and specimen II 31.45 % N and 16.3 % moisture. Calculated for $C_5H_5ON_5 \cdot HCl$, $2H_2O$ 31.32 % N and 16.1 % moisture. Hence the air-dried hydrochloride contains two molecules of water of crystallisation, and not one, as stated by Feulgen in his monograph.

The xanthine sulphate was recrystallised once. After drying it contained 22.05 % N. Calculated for $C_5H_4O_2N_4 \cdot H_2SO_4$ 22.4 % N.

Solutions were prepared by dissolving 194.2 mg. of specimen I of the guanine hydrochloride and 200.6 mg. of specimen II in 50 ml. of *N* HCl each. The oxidation was performed with 0.126 *N* $KMnO_4$ solution in a porcelain dish after about 20 mg. adenine sulphate had been added. The results are given in Table I.

Table I. *Oxidation of guanine hydrochloride with permanganate.*

1	2	3	4	5	6
Specimen	ml. of guanine solution	ml. of 0.126 <i>N</i> permanganate solution	ml. of <i>M</i> guanine solution	ml. of <i>N</i> permanganate solution	Ratio 5 : 4
I	10	7.85	0.174	0.995	5.71
		7.85			
	5	3.90	0.087	0.495	5.68
II		3.90			
	5	4.05	0.0895	0.513	5.73
	2.5	2.05	0.045	0.260	5.77

On another occasion the figure 5.76 was found for the ratio ml. *N* $KMnO_4$: ml. *M* guanine solution.

When a 0.0046 *M* xanthine solution was titrated in the presence of adenine the following figures were found for the ratio given above: 5.01, 4.90, 4.95, 5.03, 4.85, 5.08, 5.06 and 5.13. Mean 5.00.

Hypoxanthine behaved exactly like adenine and could also be used to indicate the end-point of the oxidation of guanine and xanthine in *N* HCl.

It is understood therefore that this method will not allow any differentiation between the aminopurines and their corresponding oxypurines.

Analysis of yeast nucleic acid.

2 (4) g. of yeast nucleic acid (Merck) were hydrolysed for 2.5 hours in 90 ml. of 5 % H_2SO_4 in a water-bath. After cooling and diluting to 100 (200) ml. nitrogen analysis was made (Kjeldahl). Thereafter a portion of 75–90 ml. was neutralised with NaOH and made alkaline with ammonia. The purine bases were precipitated with ammoniacal silver solution, and the precipitate was washed three times with diluted ammoniacal silver solution in a centrifuge-tube. Then the precipitate was treated with hot *N* HCl, and the silver chloride removed by filtration. The volume was made up to 100 ml. with *N* HCl.

In 7.5 or 10 ml. of the solution the amount of total purine bases was determined by precipitation with the copper bisulphite reagent. The same procedure was repeated on portions of 15 or 20 ml. of the solution after previous oxidation of the guanine with permanganate. On the copper purine precipitates ordinary nitrogen analysis was made. If the precipitation with copper bisulphite was performed on the acid hydrolysate directly without previous separation of the purine bases by the ammoniacal silver solution, the figures obtained for the purine-N were constantly too high.

The permanganate oxidation was performed in a porcelain dish heated on a free flame. Generally the strength of the permanganate solution chosen was

such that 30 to 50 drops of it were required for complete oxidation of the guanine. The end-point was generally clearly distinguished to one or two drops.

In the purine analysis the technique of Schmidt [1933] was applied. The solution was made alkaline with NaOH and then acidified with 0.5 ml. glacial acetic acid. After the addition of sodium acetate and 5 ml. of 30 % bisulphite solution the specimen was heated. To the boiling solution 5 ml. of 10 % CuSO₄ were added. Boiling was continued for 2 mins. The precipitate was then collected in a centrifuge-tube and washed three times with 1 % acetic acid solution. The Swedish angle-centrifuge¹, which runs 27 tubes simultaneously without previous weighing, proved very useful here. The precipitate, which contained about 10 mg. N, was suspended in sulphuric acid for analysis.

Table II. *Guanine : adenine ratio found in yeast nucleic acid (Merck).*

Exp.	N in 100 ml hydrolysate mg.	Volume taken for the precipitation with silver ml.	Volume taken for the copper precipitation after diluting to 100 ml. ml.	Total purine-N mg.			
I	240.5	88	7.5	10.35	10.45	10.50	
				Mean	10.43		
II	244.0	90	7.5	10.64	10.54	10.52	
				Mean	10.57		
III	241.5	75	10.0	12.46	12.31	12.46	
				Mean	12.41		
IV	182.8	75	10.0	8.80	8.70	8.74	8.75
				Mean	8.75		
Total purine-N in 100 ml.							
Volume taken for the oxidation with permanganate ml.	Adenine-N mg.			As found after precipitation with silver mg.			
				As calculated from the nitrogen content of the hydrolysate mg.			
15	10.90 10.90			139.1			
	Mean 10.90			141.1			
15	10.55 9.45* 7.87†			140.9			
20	12.06	12.22 12.20 12.27 12.46		124.1			
		Mean 12.24		120.7			
20	8.85	8.81 8.75 8.07*		87.5			
		Mean 8.81		91.2			

* Five drops of the permanganate solution in excess.

† About ten drops of the permanganate solution in excess.

The results of the purine analysis are given in Table II. As the table shows, the purine-N of the adenine fraction corresponds closely (within a range of 1-3 (5) %) to 50 % of the amount of total purine-N.

It must be admitted however that such a good agreement in the guanine : adenine ratio as is indicated by Table II was not always found. In the analyses, particularly on specimens of the pancreas nucleic acid, the determination of the end-point sometimes proved difficult, and consequently the figures for the adenine content found were throughout the series somewhat too low or

¹ Furnished by A. B. Vinkelcentrifug, Kungsgatan 33, Stockholm.

too high. (See next paper.) If the oxidation were performed in $N H_2SO_4$ the oxidation tended to be incomplete and the adenine content to be too high. On the other hand the presence of salts in the HCl solution gives rise to higher temperatures and may cause a destruction of adenine. The reliability of the technique could easily be checked by analysing a sample of yeast nucleic acid with guanine : adenine ratio 1 : 1 simultaneously.

When this technique was applied to micro-analysis with 1–1.5 mg. N (2–3 mg. adenine) for a single analysis, the figures found for the adenine content were from 5 to 10 % too low. The oxidation with permanganate in 5 or 10 ml. of HCl proceeded too far, since the amount of adenine present was insufficient to produce the colour change used as indicator. Somewhat better results were obtained when the oxidation was performed in $N H_2SO_4$, the error being about 4 %. Possibly here an oxidation by iodine in weakly alkaline solution would give better results.

SUMMARY.

A method is described for the quantitative determination of guanine and adenine when they occur as they do in the nucleic acids in the absence of their corresponding oxypurines. Owing to its instability towards oxidising agents, guanine can be quantitatively destroyed by the addition of permanganate to a boiling acid solution without any impairment of the adenine present. The two purine bases can consequently be determined by analysing for total purine-N before and after oxidation with permanganate. The purine bases are precipitated by the copper-bisulphite reagent.

The amount of purine base used for a single analysis was about 15–20 mg. On hydrolysates of yeast nucleic acid the method gave theoretical results. Its applicability to nucleic acid chemistry will be shown in the next paper.

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