COMPARISON OF THE MERITS OF THE NEW METHOD OF HYDROLYSIS OF NUCLEIC ACID BY P_2O_5 WITH THOSE OF THE STANDARD PERCHLORIC ACID METHOD

M. V. NARURKAR AND M. B. SAHASRABUDHE

Biology Division, Department of Atomic Energy, Government of India, Indian Cancer Research Centre, Parel, Bombay (India)

In a previous communication[1] a novel approach to the hydrolysis of nucleic acids was presented. It was shown that by dehydrating the ribose bridges of the internucleotide linkages to the carbon stage with P_2O_5, it was possible to obtain the purine and pyrimidine bases in a free form. It was also shown that the recoveries were quantitative and that the bases were not altered in any way by the P_2O_5 treatment. This was confirmed by comparing the R_F values and the absorption spectra of the P_2O_5 liberated bases with samples of the pure bases. In the present communication an attempt has been made to assess the comparative merits of the method in terms of standard procedures. Hydrolysis with $HClO_4$ has been taken as a representative of standard methods of hydrolysis of nucleic acids[2].

EXPERIMENTAL

Purified commercial samples of RNA and DNA, and DNA isolated from spleen and calf thymus have been used in these studies. The isolation of DNA was carried out by CHARGAFF's modification[3] of MIRSKY AND POLLISTER's method[4].

The actual hydrolysis procedure was as follows: 40 mg of dry nucleic acid powder were placed in a dry vial and sufficient P_2O_5 was added to cover the nucleic acid powder. The vial was then stoppered and the contents thoroughly mixed by gentle shaking. It was then heated in an electric oven at 150° C for 2 hours. To the cooled charred mass, 5 ml of 0.1 *N* HCl were then added to dissolve the bases; the solution was filtered to remove the carbon particles. For comparison, another portion of 40 mg, of nucleic acid powder was treated with $HClO_4$ (72 % w/v) at 100° C for 2 hours. After adequate dilutions, the liberated bases by both methods were separated by paper chromatography as detailed below.

In the present investigation, larger amounts (40 mg) of nucleic acids have been used for hydrolysis studies, although smaller amounts give equally good results. This was done in order to obtain sufficient quantity of the hydrolysates for the various chromatographic and other studies carried out here. For example, the hydrolysates were chromatographed with six different solvent systems enumerated in Table I along with samples of pure bases. This was done for two purposes. (1) To select a good solvent system for the separation of the bases and (2) to compare the R_F values of the liberated bases with the pure samples, and thus indirectly indicate that the bases that are liberated in P_2O_5 hydrolysis are identical with those of the authentic samples. Additional proof that the hydrolysates contained only free bases and no nucleosides and nucleotides was obtained by staining auxiliary chromatograms for sugars[5] and phosphates[6].

For quantitative estimation, 0.1 ml portions of the clear solution obtained by both methods of hydrolysis, containing hydrolysates from 800 to 1000 μg of nucleic acids, were spotted on a Whatman No. 1 filter paper. Since this quantity of material is comparatively large for the purposes of paper chromatography, the spotting was done in the form of a long streak along the starting line. Descending chromatograms were run for 20–24 hours using butanol for DNA hydrolysates and *iso*propanol–5 *N* HCl[7] in the proportion of 68 to 32 for RNA hydrolysates.

References p. 371.

TABLE I

COMPARISON OF THE R_F VALUES OF THE BASES LIBERATED IN P_2O_5 HYDROLYSATES WITH

No.	Solvent	*Adenine*		*Guanine*		*Cytosine*	
		Standard	*Hydrolysate*	*Standard*	*Hydrolysate*	*Standard*	*Hydrolysate*
1	Butanol alone	0.35	0.35	0.13	0.13	0.23	0.24
2	Butanol–H_2O[8], 87:13	0.38	0.38	0.18	0.18	0.22	0.22
3	Butanol–formic acid[9]–H_2O, 77:10:13	0.33	0.33	0.13	0.13	0.16	0.17
4	Butanol–10% urea[10]	0.41	0.41	0.09	0.09	0.27	0.26
5	Saturated $(NH_4)_2SO_4$[11]–*iso*propanol–H_2O, 79: 2:19	0.39	0.40	0.28	0.28	0.77	0.78
6	*Iso*propanol: conc. HCl[7]–H_2O, 68:16:16	0.62	0.62	0.50	0.51	0.69	0.69

Solution of bases in 0.1 *N* HCl. Chromatographs run at room temperature.

These two solvents were selected because they give the best separation of the bases (see Table I). After thoroughly drying the chromatogram at room temperature, the positions of the separated bases were located by taking a contact print of the chromatograms with ultraviolet light[12] on Kodak rapid reflex document paper (Doustat 23). The corresponding areas on the chromatogram were cut out into small pieces and eluted by over-night extraction with 10 ml of 0.1 *N* HCl. Quantitative estimations were carried out with a Beckman spectrophotometer (Model D.U.) at 265 mμ. Additional recovery experiments were carried out with both the methods by adding 5 mg of each of the bases to 40 mg of RNA and DNA samples and submitting these to hydrolysis by P_2O_5 or $HClO_4$, as the case may be, under identical conditions. The results of these investigations are given in Tables II to VII.

RESULTS AND DISCUSSION

It will be seen from Table I that R_F values of the bases liberated after P_2O_5 hydrolysis are identical with those of the pure bases for the six solvent systems that have been investigated. This clearly indicates that the bases themselves are not affected by the P_2O_5 treatment. Tables II, III and IV gives a comparative evaluation of the results with P_2O_5 and $HClO_4$ hydrolysis for DNA samples obtained from different sources. In each case, the sum of the calculated nitrogen values for the various bases corresponded with the one determined by independent Kjeldahl nitrogen determinations in the respective samples; this gives added evidence that all the bases in the nucleic acids are recovered on the chromatogram and that no loss of bases occurs in the hydrolysis of nucleic acids with P_2O_5 or with $HClO_4$. Table V gives the recovery figures for the bases added to the DNA sample. The recovery figures in the case of adenine and guanine are high for both the methods and may be due to some experimental error in the preparation of the mixture of bases. The fact that no such high recovery figures were obtained in Table VII proves the point.

The results with RNA samples are given in Table VI. Here again there is a close correspondence between the values obtained for the bases by both methods. Recovery figures for the bases added to RNA before hydrolysis are given in Table VII; they are uniformly good and range from 95.2% in the case of adenine to 101.2% in the case of guanine with the $HClO_4$ method, as compared with 92.8% for cytosine and 103.6 for guanine by the P_2O_5 method.

References p. 371.

HOSE OF PURE BASES

Uracil		*Thymine*	
Standard	*Hydrolysate*	*Standard*	*Hydrolysate*
0.36	0.35	0.45	0.44
0.37	0.37	0.52	0.52
0.33	0.33	0.53	0.53
0.39	0.39	0.62	0.63
0.49	0.50	0.39	0.39
0.78	0.79	—	—

It would be clear from the foregoing results that the new method obeys the general criteria of a good method of hydrolysis, and that it compares very favourably with the existing standard procedures. Hydrolysis by $HClO_4$ however has two disadvantages. (1) $HClO_4$, being a powerful oxidizing agent, explodes in presence of organic impurities if the temperature is inadvertently raised to more than 105°C and (2) occasionally filter papers get charred while spotting the diluted $HClO_4$ hydrolysates for chromatography. No such difficulties arise with P_2O_5 hydrolysis. Further, on theoretical considerations it appears likely that the new method might be applicable for the hydrolysis of nucleic acids even in the presence of proteins. This aspect is under investigation and will be reported elsewhere.

TABLE II

COMPARATIVE STUDIES OF THE HYDROLYSIS OF NUCLEIC ACID BY THE P_2O_5 AND $HClO_4$ METHODS

(Purified commercial DNA sample)

No.	*Base*	*P_2O_5 method*		*$HClO_4$ method*	
		Base µg/mg DNA	*Calculated N_2 value µg/mg DNA*	*Base µg/mg DNA*	*Calculated N_2 value µg/mg DNA*
1	Adenine	74	38.44	74	38.44
2	Guanine	98	45.06	99	45.88
3	Cytosine	39	14.96	40	15.22
4	Thymine	77	18.97	76	18.87
	% N_2		11.74 %		11.84 %

Nitrogen determination by Kjeldahl method: 12.31 %.

Bases separated on paper chromatogram using butanol as the solvent, and the purines and pyrimidines estimated at 265 mµ using Beckman spectrophotometer.

TABLE III

COMPARATIVE STUDIES OF THE HYDROLYSIS OF NUCLEIC ACID BY THE P_2O_5 AND $HClO_4$ METHODS

(Thymus DNA sample)

No.	*Base*	*P_2O_5 method*		*$HClO_4$ method*	
		Base µg/mg DNA	*Calculated N_2 value µg/mg DNA*	*Base µg/mg DNA*	*Calculated N_2 value µg/mg DNA*
1	Adenine	110	58.42	110	58.42
2	Guanine	63	29.39	63	29.39
3	Cytosine	48	18.39	49	18.65
4	Thymine	91	22.41	92	22.71
	% N_2		12.86 %		12.91 %

Nitrogen determination by Kjeldahl method: 13.61 %.

Bases separated on paper chromatogram using butanol as the solvent, and the purines and pyrimidines estimated at 265 mµ using Beckman spectrophotometer.

References p. 371.

TABLE IV

COMPARATIVE STUDIES OF THE HYDROLYSIS OF NUCLEIC ACID BY THE P_2O_5 AND $HClO_4$ METHODS

(Spleen DNA sample)

No.	Base	P_2O_5 method		$HClO_4$ method	
		Base μg/mg DNA	Calculated N_2 value μg/mg DNA	Base μg/mg DNA	Calculated N_2 value μg/mg DNA
1	Adenine	103	53.35	100	51.95
2	Guanine	68	31.76	71	32.94
3	Cytosine	51	19.40	51	19.40
4	Thymine	96	23.70	98	24.18
	% N_2		12.82 %		12.84 %

Nitrogen determination by Kjeldahl method: 13.97 %.

Bases separated on paper chromatogram using butanol as the solvent, and the purines and pyrimidines estimated at 265 mμ using Beckman spectrophotometer.

TABLE V

COMPARATIVE STUDY OF THE RECOVERY OF BASES ADDED TO DNA SAMPLE BY THE P_2O_5 AND $HClO_4$ METHODS

5 mg of each of the bases are added to 40 mg of commercial DNA sample prior to hydrolysis and the hydrolysis carried out with P_2O_5 and $HClO_4$ as detailed in the text.

No.	Base	Recovery by P_2O_5 method %	Recovery by $HClO_4$ method %
1	Adenine	116	116
2	Guanine	118	116
3	Cytosine	104	104
4	Thymine	96	100

Bases separated on paper chromatogram using butanol as the solvent, and the purines and pyrimidines estimated at 265 mμ using Beckman spectrophotometer.

TABLE VI

COMPARATIVE STUDIES OF THE HYDROLYSIS OF NUCLEIC ACID BY THE P_2O_5 AND $HClO_4$ METHODS

No.	Base	P_2O_5 method		$HClO_4$ method	
		Base μg/mg RNA	Calculated N_2 value μg/mg RNA	Base μg/mg RNA	Calculated N_2 value μg/mg RNA
1	Adenine	100.4	51.9	100	51.9
2	Guanine	155	72.02	152	70.55
3	Cytosine	37	14.61	36	13.60
4	Uracil	98	24.60	102	25.15
	Per cent N_2		16.31 %		16.15 %

Nitrogen determination by Kjeldahl method: 15.91 %.

Bases separated on paper chromatogram using *iso*propanol–5 *N* HCl as the solvent, and the purines and pyrimidines estimated at 265 mμ using Beckman spectrophotometer.

References p. 371.

TABLE VII

COMPARATIVE STUDY OF RECOVERY OF BASES ADDED TO RNA SAMPLES

5 mg of each of the bases are added to 40 mg of RNA sample and P_2O_5 and $HClO_4$ hydrolysis carried out as detailed in text.

No.	*Base*	*Recovery by P_2O_5 method* %	*Recovery by $HClO_4$ method* %
1	Adenine	96.4	95.2
2	Guanine	103.6	101.2
3	Cytosine	92.8	96.4
4	Uracil	98.8	97.6

Bases separated on paper chromatogram using *iso*propanol: 5 *N* HCl and the purines and pyrimidines estimated at 265 mμ with Beckman spectrophotometer.

ACKNOWLEDGEMENTS

Thanks are due to Prof. J. BRACHET, Director, Laboratory of Animal Morphology, University of Brussels, who is currently visiting Professor at this Institute, and to Dr. V. R. KHANOLKAR and Dr. A. R. GOPAL-AYENGAR for helpful criticism and advice.

SUMMARY

1. A new method of hydrolysis of nucleic acids with P_2O_5 to purine and pyrimidine bases has been presented and its performance compared with the standard method of hydrolysis by $HClO_4$.

2. By comparing the nitrogen values obtained by independent Kjeldahl determinations and those accounted for by the isolated bases, it was shown that the recoveries of the bases were complete and that no loss took place in the hydrolysis.

3. Recoveries of the bases added before the hydrolysis have also been studied and shown to be good.

4. The bases were not in any way affected by the P_2O_5 treatment.

5. The method is equally suitable for both the RNA as well as the DNA.

REFERENCES

[1] M. V. NARURKAR AND M. B. SAHASRABUDHE, *Nature*, 176 (1955) 883.
[2] A. MARSHAK AND H. J. VOGEL, *J. Biol. Chem.*, 189 (1951) 597.
[3] E. CHARGAFF, B. MAGASANIK, E. VISCHER, C. GREEN, R. DONIGER AND D. ELSON, *J. Biol. Chem.*, 186 (1950) 51.
[4] A. E. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 101.
[5] S. M. PARTRIDGE, *Nature*, 164 (1949) 443.
[6] C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
[7] G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
[8] R. D. HOTCHKISS, *J. Biol. Chem.*, 175 (1948) 315.
[9] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.
[10] J. G. BUCHAMAN, *Nature*, 168 (1951) 1091.
[11] R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 49 (1951) 401.
[12] R. MARKHAM AND J. D. SMITH, *Nature*, 163 (1949) 250.

Received June 12th, 1956