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THE DETERMINATION OF BASE RATIOS OF VERY SMALL SAMPLES OF RIBONUCLEIC ACID USING THIN LAYER CHROMATOGRAPHY

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SUMMARY

A technique is described for the quantitative estimation of guanine, adenine, uridylic acid, cytidylic acid and thymine by separation on thin layers of cellulose, elution and spectrophotometric analysis. The method is highly reproducible both for authentic standards and for the estimation of base ratios in plant nucleic acid hydrolysates.

INTRODUCTION

The determination of the base composition of ribonucleic acid (RNA) is usually carried out by the separation of the products of RNA hydrolysis by paper chromatography[1,2], paper electrophoresis[3], a combination of electrophoresis and thin layer chromatography[4] or on strong anion exchange resins[5]. However, these methods in their standard forms are not suited to the accurate analysis of small samples (less than 100 μg RNA).

There have been several reports of rapid one-dimensional separation of bases of nucleic acids on thin layers of cellulose[6–10] and Silica Gel G[6,7], but despite its potential superiority over paper chromatography, both in sensitivity and in the cleanness of separation of the spots[7,11], there is no available method for the quantitative thin layer chromatographic base analysis of RNA. This would require a particularly clear separation of the spots, as was obtained by HOLGATE AND GOODWIN[10] using an isopropanol–HCl–water (65 : 17.2 : 17.8, v/v/v) solvent system on cellulose plates.

This paper describes the development of a similar solvent system for the quantitative analysis of the base composition of small samples of RNA (15–75 μg). Careful attention to standardization at all stages of the procedure is essential but, taking these precautions, the method is very reliable and gives 95–100 % reproducibility both using authentic standards of purine and pyrimidine derivatives and with acid hydrolysates of plant nucleic acids.

METHODS

Thin layer chromatography was carried out on glass plates (20 cm square) with

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a 0.2 mm cellulose layer (Whatman CC41 Chromedia) and isopropanol–HCl–water (68:16.4:15.6, v/v/v) as the solvent[1]. The solvent was made up by weighing the solutions in the proportions 53.5:19.3:15.6, w/w/w).

The preparation of the plates

20 g CC41 and 100 ml Analar grade 2 *N* HCl were stirred in an M.S.E. Top Drive macerator at top speed for one minute. The slurry was filtered using Whatman No. 1 paper in a Buchner funnel and the cellulose pad washed with 100–200 ml glass distilled water. The wet pad was weighed (composition *ca.* 1 g water/1 g cellulose) and resuspended in glass-distilled water by mixing in the M.S.E. macerator at top speed for 15 min, using a ratio 40 g pad material/*ca.* 80 ml water* (3 min at top speed is adequate if a Servall Omnimixer is used).

The plates were wiped with alcohol just before spreading to free them completely from grease and dust. The plates were spread using a Shandon Spreader (Shandon Scientific Co., Ltd.) with a 0.2 mm gap and left to dry for 10 min at room temperature (18–20°) followed by 3 h at 40°. Both the time and temperature for drying are critical for subsequent good separation of the spots. The plates were kept over $CaCl_2$ or silica gel in a desiccator at room temperature for not more than two days before use.

Plates were spotted with 10–50 μl samples (50–250 mμ moles) of purine and pyrimidine derivatives using a Hamilton microsyringe. Adenine and guanine from B.D.H.** and uridylic acid (disodium salt) and cytidylic acid (mixed isomers of the nucleoside 2′ and 3′ monophosphoric acids***) were used as standards. The spots were dried in a stream of cool air. The plate was first given a preliminary run in 0.1 *N* HCl§ (Analar grade) at room temperature until the solvent had run to a distance of *ca.* 4 cm from the origin (*ca.* 10 min). This facilitates the separation of guanine and adenine. The plate was air-dried and then run in the same direction, in isopropanol–HCl–water (68:16.4:15.6, v/v/v) for 3–4 h at 40° in a previously equilibrated tank (Shandon Universal TLC Chromatotank). After 4 h the solvent front was 3–5 cm from the top of the plate.

Removal of the spots from the plates

The plates were dried in a stream of cool air and the spots removed immediately. (If the spots were left on the plates overnight and eluted the following day quantitative reproducibility of duplicates was not obtained.) The spots were viewed under a U.V. lamp (254 mμ emission band) and a rectangle marked round each spot with a dissecting needle (Fig. 1).

In preliminary experiments an equal amount of cellulose was removed with each sample and used as a blank. The whole operation was carried out in a draught-free room. Single-edge flexible razor blades were used to scrape off the samples which were collected onto squares of black smooth surfaced paper and transferred to 5 ml conical centrifuge tubes. 3–4 ml of the acid of appropriate normality (see Table I) for

* Whatman Chromedia CC41 is not consistent in texture and different batches may require small changes in maceration time and in the weight of Chromedia CC41 needed/100 ml glass-distilled water.

** British Drug Houses, Poole, Dorset.

*** Sigma Chemical Co. Ltd., 12 Lettice Street, London.

§ Standardized grade HCl should not be used as it contains impurities which interfere with the chromatography.

each spot was added and each suspension mixed on a Whirlimixer (Scientific Industries, International Inc. (U.K.) Ltd.). The covered tubes were left to stand at + 2° overnight, the precipitate removed by centrifugation at 2650 × *g* for 20 min and the supernatant carefully removed with a fine Pasteur pipette for optical density determinations. Measurements were made using cells of 1 cm path in a Hilger Uvispek spectrophotometer. The wavelengths and factors used for calculating μmoles/ml of each component are given in Table I.

Hydrolysis of the RNA samples

Hydrolysis was carried out in closed Pyrex tubes in 1.0 *N* HCl (Analar Grade) for 1 h at 100°. 1–10 mg RNA were hydrolysed in 0.25 ml *N* HCl and 10–40 μl of the hydrolysates applied directly to the TLC plates.

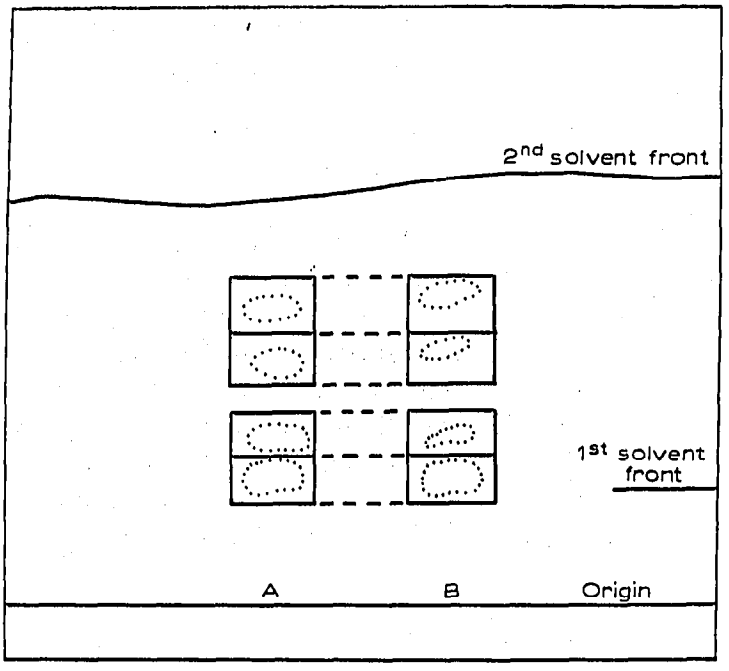

Fig. 1. A tracing of the U.V. absorbing spots on a TLC plate after separation of purine and pyrimidine derivatives from acid hydrolysis of RNA. (A) 10 μl of a mixture of guanine, adenine, uridylic acid and cytidylic acid standards. (B) A hydrolysate of a fraction of ribosomal RNA from leaves of *Vicia faba* L. var. Royal Dwarf Fan. 1st solvent: 0.1 *N* HCl; 2nd solvent: Isopropanol–HCl–water (68:16.4:15.6, v/v/v).

TABLE I

ULTRAVIOLET ABSORPTION DATA ON PURINE AND PYRIMIDINE DERIVATIVES

Substance	*Normality of HCl*	*Absorption max (mμ)*	*Reference wavelength (mμ)*	$\Delta E^{1}_{1\,cm}$ *μ mole/ml between* λ_{max} *and* λ_{ref}
Guanine	1.6	249	290	8.15[12]
Adenine	0.1	262.5	290	12.64[12]
Cytidylic acid	0.01	278	300	10.7[12]
Uridylic acid	0.1	260.5	280	6.94[14]
Thymine	0.1	265	290	6.93[13]

RESULTS

The separation of the two purines, adenine and guanine, and the pyrimidine nucleotides, cytidylic and uridylic acids, was always very clean and a tracing of one chromatogram is shown in Fig. 1. The R_F values are given in Table II. The spots were distinct and never overlapping. The solvent system can also be used for the separation of DNA derivatives. Thymine has an R_F value of 0.75 in a mixture and 0.745 when alone, *i.e.* it runs very close to the position of uracil in the RNA samples but well-separated from the other spots. Optimum separation was obtained when the material was applied as a 10 μl spot. Streaking occurred if the spot contained more than 250 mμmoles of material.

TABLE II

R_F VALUES OF PURINE AND PYRIMIDINE DERIVATIVES OF HCl HYDROLYSIS OF NUCLEIC ACIDS

Solvent: isopropanol–HCl–water (68:16.4:15.6, v/v/v)

Substance	R_F
Guanine	0.33
Adenine	0.42
Cytidylic acid	0.60
Uridylic acid	0.74
Thymine	0.75

TABLE III

QUANTITATIVE ESTIMATIONS OF PURINE AND PYRIMIDINE DERIVATIVES

Triplicate samples of each standard compound applied as a 10 μl spot. The method is described in the text.

Substance	*mμmole/sample*		*Recovery (%)*
	*Applied to plate**	*Recovered*	
Guanine	180, 180, 184 → 181	179, 183, 188 → 183	101
Adenine	192, 196, 196 → 195	188, 192, 188 → 189	97
Uridylic acid	136, 136, 136 → 136	136, 134, 140 → 137	100
Cytidylic acid	200, 200, 192 → 197	197, 199, 199 → 198	100

* The amount of material applied to the plate was calculated by dissolving triplicate samples in 4 ml of elution acid of appropriate concentration and calculating the concentration of substance in the sample using the values given in Table I.

Quantitative recoveries

Triplicate samples (0.1–0.2 μmole) of each standard were applied to the plates and taken through the complete procedure to test its reproducibility. The controls were samples of the standards in 4 ml of the appropriate elution acid (Table I). From the difference in absorption at the maximum and at a reference wavelength the amount of material applied to each plate could be calculated. A comparison of the amount of material applied to the plate and the amount recovered from it is shown in Table III. For guanine, uridylic acid and cytidylic acid the recoveries were always of the order of 100 % but the adenine values were usually in the region 96–98 %. By using samples in triplicate the occasional loss of material during one of the transfer stages could be readily detected. Thus repetition of the assay, which would be necessary with duplicates could be avoided. In several preliminary experiments, readings of the eluted spots were taken against a "cellulose" blank from the plate and against an acid blank. The readings against the acid were more consistent on every occasion so this procedure was adopted.

A comparison was also made to see if the same degree of reproducibility could be obtained when four spots, each containing a simple standard compound, were applied on top of each other and run together. The comparison with each component run singly is shown in Table IV. The % composition giving the base ratios for the artificial mixture and for the single spots were very similar. Thus the presence of more than one derivative does not appear to interfere with the accurate assay of any component. The analyses of the same mixture on different occasions were highly reproducible.

TABLE IV

COMPARISON OF ESTIMATION OF NUCLEIC ACID DERIVATIVES WHEN APPLIED (A) AS SINGLE* SPOTS AND (B) AS A MIXTURE

Substance	*mμmole/sample*		*Moles % of each base*	
	A	*B*	*A*	*B*
Guanine	46	51	18	18.3
Adenine	73	78	28.5	28
Uridylic acid	64	72	25	25.4
Cytidylic acid	73	78	28.5	28

* Each compound was applied as six 10 μl spots which were scraped off and combined in 4 ml of elution acid for spectrophotometric assay.

The analysis of authentic RNA hydrolysates

The method was next used to analyse HCl hydrolysates of RNAs from leaves of *Vicia faba* L. The separation of the components of a hydrolysate of leaf ribosomal RNA is shown in Fig. 1B. (The RNA sample was prepared by phenol extraction according to DYER AND LEECH[15].) The separation of guanine, adenine, uridylic acid and cytidylic acid is clean and comparable with the separation of standards shown in Fig. 1A. Spots were eluted and assayed as for the standards and the results of one analysis are shown in Table V. Four 10 μl samples of the hydrolysates were run on

TABLE V

ANALYSIS OF AN HCl HYDROLYSATE OF LEAF RIBOSOMAL RNA

In each experiment three samples of the hydrolysate were run and similar spots eluted and combined in 4 ml of the appropriate acid for spectrophotometric assay.

Exp.	*mμ moles/sample*				*Base ratios*			
	Guanine	*Adenine*	*Cytidylic acid*	*Uridylic acid*	*G* :	*A* :	*C* :	*U*
26/27	164	152	142	122	28.3	26.5	24.5	21.2
28	168	149	138	128	28.8	25.6	23.7	22.0
29	164	144	135	121	29.1	25.5	23.9	21.9
Mean:	165	148	138	124	28.7	25.9	24.0	21.7

each occasion and combined to give one reading in Table V. Each successive assay was completed a week after the previous one and, as can be seen from Table V, successive assays of the same material gave consistent results.

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