DETERMINATION OF BASE RATIOS ON SUB-MICROGRAM QUANTITIES OF RNA

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1. Introduction

The determination of base compositions of ribonucleic acids is an important step in sequence analysis.

As RNAs are often not available in milligram quantities for the determination of base compositions using spectrophotometric procedures [1–6], more sensitive radioactive labelling methods have been developed. The procedures of Sanger et al. [7] can be used directly for base composition analysis of RNA uniformly labelled with ^{32}P to a high specific activity. But not all RNAs can be so labelled in vivo, and ^{32}P also has an inconveniently short half-life. The Randeraths [8,9] developed a method using sodium boro-[^{3}H]hydride to reduce periodate-oxidised nucleosides to stable tritiated nucleoside tri-alcohols. Tritium has a low energy of radiation so it is difficult to detect by autoradiography, especially when picomole quantities of tritiated nucleoside tri-alcohols are analysed.

We previously described a simple, sensitive procedure for chemically labelling RNA and its derivatives which uses *p*-hydrazinobenzene-[^{35}S]sulphonic acid (^{35}S-*p*-HBSA) as the radioactive labelling reagent [10]. We now wish to describe routine labelling procedure for determining base compositions of RNA and ribo-oligonucleotides. The procedure is simple and very sensitive requiring only 0.5 μg of the polymer for at least a triplicate determination.

2. Materials and methods

2.1. *Sample preparations*

^{35}S-*p*-HBSA was prepared as described earlier [10] from [^{35}S]sulphanilic acid (The Radiochemical Centre, Amersham, England). Turnip Yellow Mosaic Virus RNA and Cowpea Chlorotic Mottle Virus (CCMV) RNA were isolated from purified virus preparations by a modification [11] of the two phase phenol method of Fraenkel-Conrat et al. [12]. Alfalfa Mosaic Virus RNA was a gift from Roger Hull of this Institute.

Ribo-oligonucleotides were obtained from unlabelled CMV RNA digested with RNAase T_1 and fractionated by fingerprinting on DEAE cellulose sheets using the water cooled flat plate method of Trim and Dickerson [13,14]. Figure 1 shows an ultraviolet photograph of the fingerprint of the digestion products. All clearly resolved ultraviolet absorbing components were cut out from the DEAE cellulose sheet and the oligonucleotides eluted with M NH_4HCO_3 solution adjusted to pH 8.6 with ammonia. Each eluate was collected in a small tube and freeze-dried for 72 h in a desiccator containing P_2O_5, concd. H_2SO_4 and NaOH pellets. The residue was dissolved in water and oligonucleotide concentration was determined by spectrophotometry.

2.2. *Digestion of viral RNA ribo-oligonucleotides to nucleosides*

2.2.1. Enzymic digestion

Viral RNA or ribo-oligonucleotide (approximately 0.5 μg) in unbuffered aqueous solution was incubated, in a small covered tube, for 16 h at 37°C with 0.1 μg pancreatic RNAase A (Boehringer Mannheim), 5 μg *E. coli* Alkaline phosphatase (Sigma Chemical Company), 2 units of snake venom phosphodiesterase (Calbiochem), 10 μl 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6), and 10 μl 0.01 M $MgCl_2$ solution.

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2.2.2. Alkaline digestion

Viral RNA or ribo-oligonucleotide solution (approx. 0.5 μg) was dried in a stream of nitrogen. The residue was dissolved in 50 μl 0.3 M KOH, and incubated at 37°C for 16 h. This hydrolyzate was brought to about pH 8 with perchloric acid. The supernatant was pipetted off from the insoluble potassium perchlorate and after transferring to a small clean tube, the mononucleotides were dephosphorylated by incubation at 37°C for 16 h with 5 μg *E. coli* alkaline phosphatase.

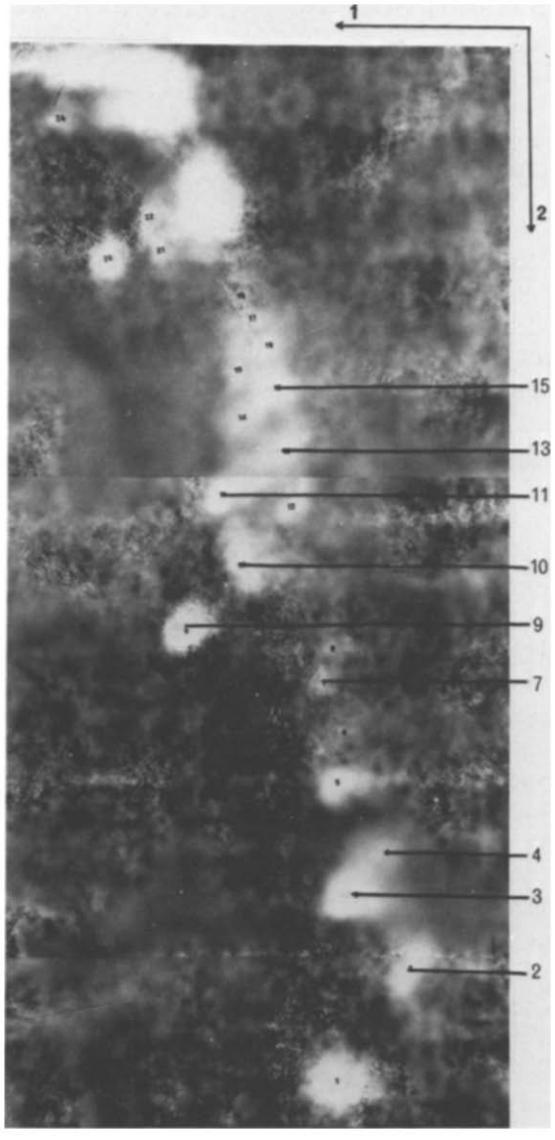

Fig.1. An ultraviolet photograph of the nucleotide map of unlabelled CCMV RNA digested completely with RNAase T_1. The RNA (1.5 mg) was digested with 75 units of the enzyme and fingerprinted as described by Trim and Dickerson [13, 14]. The base compositions of the following numbered oligonucleotides have been determined using the ^{35}S-*p*-HBSA labeling process: (2) CpGp, (3) ApGp, (4) (ApCp)Gp, (7) $(Ap)_3Gp$, (9) UpGp, (10) (CpUp)Gp, (11) (UpAp)Gp, (13) (ApCpCpUp)Gp, (15) (CpCpApApUp)Gp.

2.3. *Labelling of the nucleosides with ^{35}S-p-HBSA*

The enzymic hydrolyzate was oxidised with a five times molar excess of sodium periodate for 90 min in the dark. Excess periodate was converted to iodate by adding 10 μl of 50% ethylene glycol and leaving the mixture in the dark for 60 min. The solution was then dried in a stream of nitrogen to remove formaldehyde. A blank containing no nucleosides was similarly processed.

The residue was dissolved in 20 μl of 0.5 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6), and labelled by adding about a two-times molar excess of ^{35}S-*p*-HBSA. In some experiments, depending on the fractionation method to be used, excess unreacted ^{35}S-*p*-HBSA was removed by adding 2 μl alkaline phthaldehydic acid solution [10].

2.4. *Fractionation and measurement of labelled nucleosides*

Five microlitres of the labelled nucleoside solution was used for analysis by thin-layer electrophoresis or chromatography followed by autoradiography and scintillation counting of individual components removed from the thin-layer sheets [10].

3. Results and discussion

Table 1 shows the base composition of the three viral RNAs determined by the ^{35}S-*p*-HBSA labelling procedure compared with previously published data. There is good agreement between the two sets of figures.

Table 2 shows the base compositions of some of the ribooligonucleotides; numbers correspond to those given in fig.1. The base compositions of the dinucleotides, trinucleotides and many of the tetranucleotides could be derived simply by visual inspection of the autoradiographs.

Table 1
Base Compositions of some viral RNAs

Virus	Base compositions in moles per cent								Ref.
	Measured[a]				Literature				
	U	G	C	A	U	G	C	A	
Turnip Yellow Mosaic	21.3	17.0	39.7	22.0	22.2	17.2	38.0	22.6	[19]
Cowpea Chlorotic Mottle	27.8	26.0	20.0	26.2	28.2	26.4	20.3	25.8	[20]
Alfalfa Mosaic	27.1	25.3	20.9	26.7	28.2	24.3	21.1	26.4	[21]

[a]The viral RNAs were digested to nucleosides and subjected to the ^{35}S-*p*-HBSA labeling process as described in the text. The radioactivity of each labeled nucleoside of an RNA is expressed as percentage of total radioactivity of the four labeled nucleosides of the RNA. Each analysis was done using approx. 0.1 μg RNA

The ^{35}S-*p*-HBSA labelling procedure has also been used to determine the base composition of ribo-oligonucleotides derived from pancreatic RNAase A digestion of unlabelled CCMV RNA [15]. The base composition results obtained agreed with those determined by Trim et al. [13] who used a combination of in vitro ^{32}P-labelling and spectrophotometric procedures. Trim's method [13,14], which increases the sensitivity of detection by spectrophotometry compared with the earlier methods of Holley et al. [16], is also the first step in a new procedure being developed for the nucleotide sequencing of unlabelled RNA. The determinations of base compositions of the finger-printed unlabelled oligonucleotides have previously been done mainly by spectrophotometry which has required all the material from a fingerprint. Our new ^{35}S-*p*-HBSA labelling procedure, uses only 5% of each oligonucleotide, thus leaving enough material for other sequencing analyses. In fact the ^{35}S-*p*-HBSA labelling procedure for determining base compositions requires only 0.5 μg of RNA for at least a triplicate determination. Except for the NaB^3H_4 labelling procedure, which has been greatly improved by Randerath and his associates [17,18], no other chemical labelling method has been demonstrated

Table 2
Base compositions of RNAase T_1-oligonucleotides (CCMV RNA) as determined by the ^{35}S-*p*-HBSA labelling procedure

Spot Nos. cf. fig.1.	Radioactivities of labeled nucleosides				Base compositions[a]				Empirical formulae
	G	A	C	U	G	A	C	U	
2	86	–	86	–	1	–	1	–	CG
4	75	66	71	–	1	0.88	0.95	–	(AC)G
7	65	183	–	–	1	2.82	–	–	A_3G
9	127	–	–	125	1	–	–	0.98	UG
10	160	–	150	150	1	–	0.94	0.94	(CU)G
11	82	74	–	71	1	0.90	–	0.87	(UA)G
13	53	50	107	60	1	0.94	2.01	1.13	$(AC_2U)G$
15	70	123	136	72	1	1.76	1.94	1.03	$(C_2A_2U)G$

[a]Each ribo-oligonucleotide was digested with enzymes to nucleosides and subjected to the ^{35}S-*p*-HBSA labeling process as described in the text. Base compositions are expressed in terms of the yield of a labeled nucleoside relative to the yield of labeled guanosine in each ribo-oligonucleotide

to be useful for an accurate determination of base compositions of RNA and ribo-oligonucleotides. Sulphur-35 is easier to detect on chromatograms and electrophoretograms by autoradiography than tritium, so the ^{35}S-*p*-HBSA labelling procedure has an advantage over the NaB^3H_4 labelling procedure. One limitation of the ^{35}S-*p*-HBSA procedure is that electron pair acceptors (acids and strong oxidising agents) disturb the labelling reaction [15]. This is relatively unimportant because adequate methods which exclude acids and oxidising agents have been developed for labelling ribonucleosides.

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