ANALYSIS OF NUCLEIC ACID DERIVATIVES AT THE SUBNANOMOLAR LEVEL. (IV) ANALYSIS OF POLYRIBONUCLEOTIDES BY CONVERSION TO TRITIATED NUCLEOSIDE DERIVATIVES

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1. Introduction

A previous communication of this series [1] reported on a procedure for quantitative ultramicroanalysis of ribonucleosides involving periodate oxidation, reduction of the resulting nucleoside dialdehydes with tritiated sodium borohydride, chromatographic separation, and liquid scintillation counting. As described in the present communication, this procedure can be utilized to analyze the base composition of ribooligo- and ribopolynucleotides after appropriate enzymatic digestion. The entire procedure is represented in fig. 1 *.

2. Materials and methods

Tritiated sodium borohydride (specific activity 200 mC/mmole) was from New England Nuclear Corp. All operations involving this compound were carried out under a hood. The RNA copolymer poly (AGUC) was Miles lot no. 329/1-4382. Yeast RNA, type XI, purified according to Crestfield et al. [2], was Sigma lot no. 35B-8530. Di- and trinucleotides were from Gallard Schlesinger Co. and Miles Laboratories. The oligonucleotides were shown by chromatography on PEI-cellulose [3] to contain less than 3–5% impurities. For complete digestion of oligonucleotides, the incubation mixture contained per microliter: 0.5–1 μg oligonucleotide, 0.05 μg snake venom phosphodiesterase (Worthington

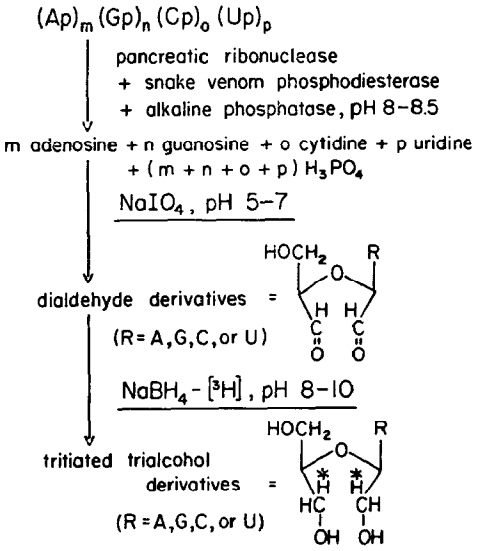

Fig. 1. Quantitative analysis of ribopolynucleotides by enzymatic digestion and tritium labelling.

Code VPH), 0.04 μg *E. coli* alkaline phosphatase (Worthington Code BAPC, dialyzed exhaustively against distilled water), 20 nanomoles $NaHCO_3$, and 10 nanomoles $MgCl_2$. Incubation was at 37° for 4 hours. For complete digestion of RNA, the mixture contained per microliter: 1–5 μg RNA, 0.2 μg snake venom phosphodiesterase, 0.2 μg alkaline phosphatase, 0.2 μg pancreatic ribonuclease A (Sigma, Type 1–A), 100 nanomoles $NaHCO_3$, and 10 nanomoles $MgCl_2$. Incubation was at 37° for 6–8 hours. The enzymatic digest was appropriately diluted and used for

* For abbreviations, see "Abbreviated Instructions to Authors" as published in the issues of J. Biol. Chem.

the oxidation step without prior removal of enzymes and electrolytes. Oxidation was carried out in aqueous solution in the dark for 30 min at room temperature. The mixture contained per microliter: 0.15–0.25 nanomole nucleoside and an approximately two-fold molar excess of $NaIO_4$. A 25-fold molar excess of $NaBH_4$-[^{3}H] (0.5 M in 0.1 N NaOH) was then added, and the reaction was allowed to proceed for 30 min at room temperature, also in the dark. Excess acetic acid was added to destroy residual borohydride (caution: tritium gas is liberated). After 15 min the solution was evaporated to dryness in a stream of air at room temperature, the residue was dissolved in water to make a solution containing 2–50 picomoles per microliter of individual nucleoside trialcohols, and the solution was again evaporated to complete dryness. Finally the residue was taken up in water to make a solution containing 10–100 picomoles per microliter of each derivative. An aliquot of this solution was mixed with an equal volume of an aqueous solution containing about 2 nanomoles per microliter of carrier nucleoside trialcohols [4].

For separation of the ribonucleoside derivatives, 1 µl aliquots of this solution were applied to a cellulose thin layer (Eastman No. 6064) at 2.5 cm from the lower edge. The chromatogram was developed with ethyl acetate/n-butanol/isopropanol/7.5 N aqueous ammonia (3 : 1 : 2 : 2, by vol.) until the solvent front was 12-14 cm above the origin. R_F values for the nucleoside trialcohols were: 0.56 (A), 0.45 (C), 0.34 (U), 0.23 (G).

After the layer had been dried thoroughly and evenly in a stream of hot air for at least five min, the compounds were visualized with a short wave UV lamp. Rectangles of equal size (about 1 cm^2) containing the compounds were cut out and placed, layer side down, in counting vials. Elution was carried out with 500 µl water at room temperature for 60 min. One hundred µl 4 N ammonia and 11 ml scintillation fluid [5] were then added, and the vials were vigorously shaken. Radioactivity was measured in a liquid scintillation counter.

For calculating the base composition, use was made of the fact that the resulting radioactivity of each derivative was directly proportional to the concentration of the corresponding nucleoside in the original solution [1]. Count rates obtained by subjecting a blank (without nucleotide) to the entire procedure were subtracted from each nucleoside trialcohol count rate. In all cases the count rate of the blank was only slightly above background.

Table 1
Base ratios of oligonucleotides by tritium labelling.

Oligonucleotide	Base ratio	(mean * ± s **)	s_{rel} ***
ApG	A:G	1.01 ± 0.014	1.4
ApU	A:U	1.02 ± 0.030	2.9
ApC	A:C	0.96 ± 0.019	2.0
UpA	U:A	0.97 ± 0.020	2.1
UpC	U:C	0.97 ± 0.032	3.3
ApApU	A:U	2.02 ± 0.062	3.1
UpUpG	U:G	2.00 ± 0.035	1.8

* Mean of ten determinations.
** Standard deviation.
*** Relative standard deviation, $s_{rel} = \frac{s \cdot 100}{\text{mean}}$ %.

3. Results

The results of a number of experiments in which the base compositions of oligonucleotides were analyzed by tritium labelling are listed in table 1. It can be seen that the observed base ratios conform to within 4% or less with the predicted ratios. The small differences between predicted and observed ratios are probably due to an overlapping of traces of labelled impurities with nucleoside trialcohols on the chromatograms. The data for poly (AGUC) and yeast RNA listed in table 2 demonstrate good agreement between tritium labelling and ultraviolet spectrophotometry.

4. Discussion

The conversion of ribonucleosides to nucleoside trialcohols with periodate and unlabelled sodium borohydride has been studied by Viscontini et al. [6] and Khym and Cohn [4]. As recently shown by us [1,7], these reactions can be utilized for the quantitative ultramicroanalysis of ribose derivatives if the reduction is carried out with tritiated sodium borohydride. RajBhandary [8] recently reported a method for end group labelling of ribopolynucleotides based on the same sequence of reactions.

Table 2
Base analysis of polynucleotides by tritium labelling.

Polynucleotide	Method	A (%)	G (%)	U (%)	C (%)
Poly (AGUC)	Labelling	26.4	21.5	23.6	28.5
	Spectrophotometry *	25.8	21.4	23.6	29.2
Yeast RNA	Labelling	25.5	28.8	23.5	22.2
	Spectrophotometry *	25.1	29.6	22.7	22.6

* Procedure to be published.

The most sensitive and accurate methods for quantitative and sequential analysis of nucleic acids and oligonucleotides described in the literature require homogeneous biological labelling with ^{32}P [9,10]. The method reported in the present communication allows one to study the base composition in cases where only minute quantities of material are available and homogeneous *in vivo* labelling is not possible (e.g., nucleic acids of human origin). Our method is sensitive, with great accuracy and precision, to about 1 picomole (10^{-12} mole) of nucleoside trialcohol derivatives [1,7]. The lower limit for standard spectrophotometric analysis is about 1 nanomole (10^{-9} mole) per compound. Enzymatic digestion is considerably milder than conventional acid or alkaline hydrolysis and incurs no danger of deamination of cytosine derivatives [11] or conversions of minor bases from transfer RNA [12]. However, reactions of some minor bases can be expected to occur at the labelling step [13]. This aspect has to be studied further before the procedure can be applied to digests of transfer RNA.

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