BISULFITE-CATALYZED TRITIUM LABELING OF DNA AND RNA

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1. Introduction

In vitro chemical labeling of DNA and RNA has received considerable attention [1] because the so-labeled polynucleotides may be used for hybridization studies, sequence analysis, and other investigations of biochemical interest. The principle so far employed is the hydrogen–tritium ($H \rightarrow {}^3H$) exchange at the 8-positions of purines which occurs on heating a solution of nucleic acids in $[^3H]H_2O$. We recently reported that bisulfite catalyzes the $H \rightarrow {}^3H$ (^2H) exchange at position 5 of pyrimidine nucleotides CMP [2, 3] and UMP [4]. The catalysis is believed to occur through reversible formation of a type of adduct between the pyrimidine nucleotides and bisulfite, 5,6-dihydropyrimidine nucleotide 6-sulfonate. For cytosine nucleotide, bisulfite also brings about easy deamination of the 4-NH_2 group at pH 4–6 [3, 5–7], but at pH 7.5 the H-exchange reaction exclusively takes place [2]. For the labeling of UMP, not only bisulfite ion but also an amine is required, which apparently promotes the cleavage of the C(5) – H bond by donating electrons to the hydrogen [4]. The method was successfully applied to the labeling of uridylyl (3′-5′)cytidine with tritium, without concomitant degradation of the dinucleoside phosphate. In contrast to the easy back exchange ($^3H \rightarrow H$) of 8-[^{3}H] purines in H_2O, the 5-[^{3}H]pyrimidines do not lose the isotope under usual reaction conditions. We now report the results of the ammonium bisulfite-catalyzed tritium labeling of DNA and RNA.

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2. Materials and methods

[^{32}P] Denatured lambda DNA was obtained from [^{32}P] phage lambda [8] as described by Davis et al. [9]. [^{3}H] Lambda DNA was prepared from [^{3}H]-lambda [10] by extraction with water-saturated phenol. Both phages had been purified by CsCl-density gradient centrifugation. Deoxyuridine 5′-phosphate, which was used as a marker in the chromatography of enzyme digest of DNA, was obtained by deamination of deoxycytidine 5′-phosphate with sodium bisulfite [7].

2.1. *Labeling of DNA. Determination of base composition and ^3H incorporated into each nucleotide*

A stock solution of calf thymus DNA (Sigma, Type V, 3 mg/ml) in H_2O was used either as such (designated 'native') or after heating at 100°C for 15 min followed by rapid cooling ('denatured'). The DNA solution was mixed with $[^3H]H_2O$ and $(NH_4)_2SO_3$ (a 3 M sulfite solution of pH 7.5 containing $(NH_4)_2SO_3$ and $NaHSO_3$ in 20:1 mole ratio): the final concentrations of DNA, tritiated water, and $(NH_4)_2SO_3$ were 1.5 mg/ml, 0.15 Ci/ml, and 0.91 M, respectively. The total volume of a reaction mixture was 0.66 ml. Control experiments were run in which the bisulfite salt was replaced by 0.3 M sodium phosphate buffer (pH 7.5). The reaction mixtures were incubated in sealed tubes at 37°C for 24 hr and then dialyzed extensively against 4 mM Tris–HCl buffer (pH 7.2) at room temperature; the dialysis solution was renewed five times, the total volume was 40 litres and the time of dialysis was 156 hr in total. The first dialysis solution contained 5 mM EDTA in order to maintain a loose structure of the DNA. After the

Table 1
Incorporation of ^3H into each base of DNA and RNA.

		Buffer	Radioactivity [^3H], cpm/0.1 μmole (Base composition, mole %)			
			C	*G*	*T*	*A*
DNA	Denatured	Bisulfite	1120 (20.6)	1730 (21.5)	0 (29.7)	1130 (28.2)
	Native	Bisulfite	195 (20.3)	1470 (21.7)	0 (27.8)	770 (27.4)
	Denatured	Phosphate	10 (21.2)	1560 (23.1)	0 (28.1)	720 (27.6)
	Native	Phosphate	10 (20.3)	1090 (20.1)	0 (29.0)	450 (30.6)
	Untreated		(21.2)	(22.4)	(29.1)	(27.2)
			C	*G*	*U*	*A*
RNA		Bisulfite	5680 (20.1)	6900 (38.8)	16 550 (18.3)	6930 (22.8)

dialysis, the labeled DNA was digested first with pancreatic DNase and then with snake venom phosphodiesterase [11]. The resulting mononucleotides were separated by two-dimensional paper chromatography: solvent run to the first dimension was (i) isopropanol–conc. NH_4OH–H_2O (7:1:2, by vol), which separated salts from the nucleotide mixture; solvent for the second dimension (ii) isobutyric acid–0.5 N NH_4OH (10:6, v/v); and then the solvent (iii) isopropanol–conc. HCl–H_2O (75:17:8, by vol) was run to the direction of the first dimension. By the chromatography with the strongly acidic solvent iii, the purine deoxynucleotides were decomposed to give the purines. Each compound was eluted with 0.01 N HCl and counted for radioactivity in Kinard Scintillator [12] by use of Packard Tri-Carb 3320 liquid scintillation spectrometer, the counting efficiency being 20%. Quantities of the bases and nucleotides were determined by UV measurement on a Hitachi 124 recording spectrometer. It was confirmed by a separate experiment that deoxyuridine 5′-phosphate, if it was present in the digestion mixture, should appear as a discrete spot on the chromatogram.

2.2. *Labeling of RNA and analysis of the [^3H] RNA*

Yeast RNA (Kohjin, once dialyzed against H_2O and lyophylized, 38 OD units at 260 nm) was incubated with $(NH_4)_2SO_3$ (1 M, pH 7.5) in [^3H]H_2O (0.5 Ci/ml) containing EDTA (5 mM). The total volume was 0.1 ml. The incubation was at 37°C for 24 hr and the RNA was precipitated by addition of 2ml of 5% trichloroacetic acid under cooling. The RNA was collected by centrifugation and reprecipitated from a 1 M Tris-HCl (pH 9.0, 0.1 ml) solution by addition of 4 ml of cold ethanol. The RNA was dissolved again in 1 M Tris–HCl (pH 9.0, 0.1 ml) and the solution incubated at 37°C for 2 hr in order to regenerate uracil residues from the bisulfite adduct [7]. By repeated ethanol-precipitations and drying of aqueous solutions over P_2O_5, all the easily exchageable tritium was removed. Yield, 18 OD units.

The [^3H]RNA was digested with RNase T_2 (Sankyo) and the nucleoside 3′-phosphates that resulted were separated and their quantities determined by two dimensional cellulose thin layer chromatography using the same solvent systems as those described for DNA analysis.

2.3. *Sucrose density gradient centrifugation analysis of bisulfite-treated DNA*

Denatured [^{32}P] lambda DNA was treated with 1 M ammonium bisulfite in H_2O at pH 7.5 and 37°C for 24 hr, and the solution was diluted with 2.8 vol of 0.33 N NaOH. A portion of the diluted solution was mixed with 0.8 vol of an EDTA–NaOH–NaCl (0.25 M–1.25 M– 0.6 M) solution and a standard [^3H] lambda DNA, and the resulting mixture was centrifuged through a gradient of 5–20% sucrose containing 0.2 M NaOH, 1 M NaCl, and 10^{-3} M EDTA. The centrifugation was on a Hitachi 55-P ultracentrifuge for 3 hr at 47 000 rpm and 10°C, using an RPS-50 rotor. Fractions, two drops each, were collected on filter paper discs by Isco density gradient fractionator model 182 and counted for radioactivity.

3. Results and discussion

Calf thymus DNA and yeast RNA were treated with ammonium bisulfite in [^3H]H_2O at 37°C and pH 7.5, conditions suitable to bring about H → ^3H exchange at position 5 of both cytosine and uracil without concomitant deamination of cytosine [2–4]. It was expected that purines will also be labeled with ^3H at position 8 under these conditions [1]. The la-

beled nucleic acids, after easily exchangeable ^3H was replaced by H, were analyzed for radioactivity in each base and the results obtained are summarized in table 1.

Cytosine in denatured DNA and RNA was labeled to an extent similar to that of purines by treatment with bisulfite, while no significant incorporation was observed for cytosine in DNA when phosphate buffer was used instead of bisulfite, indicating that the incorporation was indeed effected by catalysis of bisulfite. Little incorporation was obtained for cytosine residues in native DNA by the bisulfite treatment. As expected, uracil in RNA was labeled effectively whereas thymine in DNA, possessing a CH_3 instead of H at position 5, was not. The labeling of purines was apparently not due to specific action of the catalyst used, since they were labeled in all cases studied. Purines in denatured DNA were somewhat more highly labeled than those in native DNA, but the difference was not so great as in the case of cytosine.

Chromatography of the enzymic digest of the bisulfite-treated DNA gave no deoxyuridine 5′-phosphate, which confirmed that deamination of cytosine residues did not occur during the bisulfite treatment. No drastic changes in base compositions of the treated- and untreated- DNAs were observed. In a separate experiment, stability of the DNA backbone during the bisulfite treatment was examined. For this, ^{32}P-labeled denatured lambda DNA was treated with 1M ammonium bisulfite at 37°C for 24 hr and the treated DNA was analyzed by alkaline sucrose density gradient centrifugation. As fig. 1 shows, there was little cleavage of the phosphodiester bond by the bisulfite treatment. These results indicate that nucleic acids can be labeled by the bisulfite method with little damage during the treatment.

The extent of the bisulfite-catalyzed $H \rightarrow {}^3H$ exchange at position 5 of cytosine in denatured DNA was about 2% of complete exchange. Under comparable conditions, cytidine 5′-phosphate underwent 10% exchange [2]. In case a higher level of labeling is required, a longer time of incubation with a higher bisulfite concentration and $[^3H]H_2O$ having a higher specific activity may be employed.

This method may become a practical and general means to prepare labeled polynucleotides containing cytosine and uracil residues. The method may also be useful to study conformation of nucleic acids since the labeling of cytosine is dependent on DNA conformation.

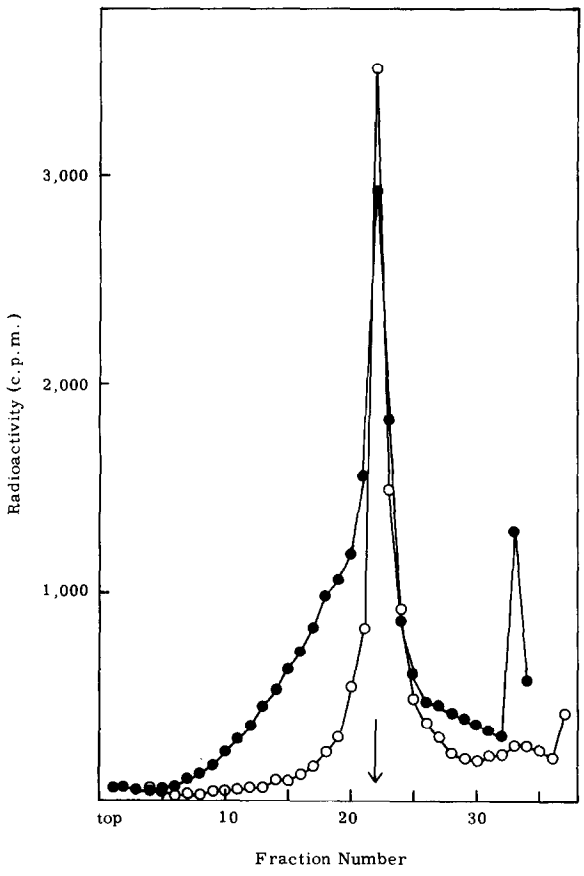

Fig. 1. Alkaline sucrose density gradient centrifugation of denatured lambda DNA treated with 1 M ammonium bisulfite at pH 7.5 and 37°C for 24 hr. (●–●–●) Treated DNA; (○–○–○) untreated DNA. The arrow indicates the position where the [^3H] lambda DNA-peak was located.

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