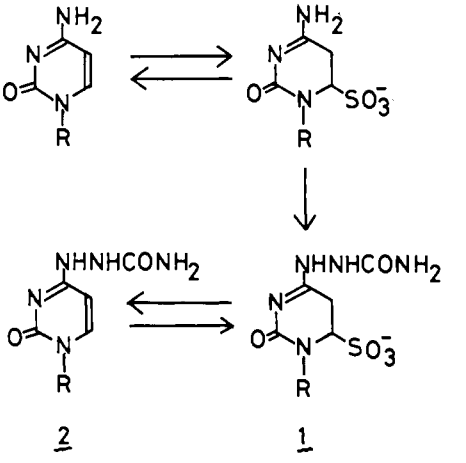
Reaction of Cytidine with Semicarbazide in the Presence of Bisulfite. A Rapid Modification Specific for Single-Stranded Polynucleotide†

Hikoya Hayatsu

ABSTRACT: Semicarbazide reacted rapidly with 5,6-dihydrocytidine-6-sulfonate, which was formed from cytidine by addition of bisulfite across the 5,6-double bond. The transaminated product, 5,6-dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate ribofuranoside, was identified by comparison with that formed by treatment of 4-semicarbazido-2-ketopyrimidine ribofuranoside with bisulfite. The progress of the transamination was monitored spectrophotometrically by use of a strong absorbance of the product in alkali. The reaction between cytidine and the semicarbazide–bisulfite mixture was optimal at pH 4.5. Complete transformation of cytidine into the product required only 5 min with the use of 3 M semicarbazide–1 M sodium bisulfite, pH 5.0, at the reaction temperature 37 °C. The product was stable in unbuffered solution, but in phosphate buffers it underwent elimination of bisulfite to give 4-semicarbazido-2-ketopyrimidine ribofuranoside. The rate of the elimination at pH 7.0 and 37 °C increased proportionally with the increase of the phosphate concentration. Complete elimination was obtained by treatment with 1 M sodium phosphate for 2 h. When heat-denatured calf-thymus DNA was treated with 3 M semicarbazide–1 M bisulfite at 37 °C and pH 5.0, the transamination of reactive cytosine residues was completed by 10 min of incubation. At 20 °C, it required 85 min of incubation. Cytosine residues in native DNA did not react at all even by prolonged incubations. The modified DNA samples were further treated with a phosphate buffer at pH 7, producing 4-semicarbazido-2-ketopyrimidine residues in the DNA. Analysis of the base compositions of these samples by perchloric acid hydrolysis showed that the modification was selective to cytosine, which had been expected from studies with monomers. It also showed that the reactive cytosine residues in the denatured DNA constitute about 80% of the total cytosine, which was consistent with the view that heat-denatured DNA still contains a considerable amount of secondary structure. The semicarbazide–bisulfite modification is expected to be a sensitive method to locate cytosine residues in single-stranded regions of polynucleotides.

Addition of bisulfite across the 5,6-double bond of cytidine leads either to the deaminated compound (Shapiro et al., 1970; Hayatsu et al., 1970a,b), or to the transaminated derivatives in the presence of certain amines (Shapiro and Weisgras, 1970; Boni and Budowsky, 1973). For the amine, methoxyamine has been shown to undergo a rapid reaction to give a very stable product, 5,6-dihydro-N^4-methoxycytidine-6-sulfonate (Sverdlov et al., 1974). Semicarbazide transaminates cytidine by itself (Hayatsu and Ukita, 1964; Hayatsu et al., 1966), like methoxyamine (Kochetkov et al., 1963), and is therefore expected to be efficient in the bisulfite-catalyzed transamination system. In this paper, the rapid modification of cytidine with the semicarbazide–bisulfite system and its single-strand specific nature are described (Scheme I).

Scheme I



Experimental Section

Semicarbazide–bisulfite mixtures were always freshly prepared before use. For this, a mixture of semicarbazide hydrochloride, NaOH, and $NaHSO_3$ was dissolved in water to make up a solution of desired concentrations and pH. Bisulfite content of the mixture did not change during 1-day standing at room temperature, as checked by the absorbance, at 276 nm, of H_2SO_3 in 0.06 N HCl. Denatured DNA used in this work was prepared immediately before use by heating 1% DNA solution (in 5 mM sodium phosphate, pH 7.5) at 100 °C for 10 min and cooling rapidly in an ice bath. Uv spectra were recorded on Beckman Acta CIII spectrophotometer. Solvents employed in ascending paper chromatography were (1) ethanol–1 M ammonium acetate, pH 7.5 (7:3), (2) 1-butanol–acetic acid–water (2:1:1), and (3) 1-butanol–water (86:14). R_f values of compounds are summarized in Table I.

Reaction of Cytosine with Semicarbazide–Bisulfite. Cytosine (888 mg) was suspended in a mixture of 2 M semicarbazide hydrochloride–NaOH (25 ml), pH 5.0, and $NaHSO_3$ (5.2 g). On warming at 37 °C for 10 min with shaking, the cytosine went into solution, and soon a product started to

† From the Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan. *Received December 15, 1975.* This work was partly supported by a grant from the Ministry of Welfare of Japan.

TABLE I: R_f Values of Compounds.

Compound	R_f in Solvent 1	2	3
5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate (**1a**)	0.34		0.03
4-Semicarbazido-2-ketopyrimidine (**2a**)	0.44		0.09
Cytosine	0.62		0.19
Uracil	0.69		0.40
5,6-Dihydrouracil-6-sulfonate	0.52		0.00
5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate ribofuranoside (**1b**)	0.38	0.23	0.01
4-Semicarbazido-2-ketopyrimidine ribofuranoside (**2b**)	0.47	0.31	0.04
Cytidine	0.63	0.40	0.13
Uridine	0.68	0.45	0.24
5,6-Dihydrouridine-6-sulfonate	0.53	0.15	0.11

precipitate. After a total period of 30 min, the mixture was chilled in ice and the precipitate was collected by filtration. The crystals were washed with cold water (20 ml) and then with acetone and ether, successively. The dried material weighed 1.48 g (68%). For analysis, the product was recrystallized from 0.2 M $NaHSO_3$-Na_2SO_3, pH 7. Anal. Calcd for $C_5H_8O_5N_5SNa \cdot H_2O$: C, 20.62; H, 3.46; N, 24.05; S, 11.01. Found: C, 20.63; H, 3.68; N, 23.96; S, 10.48. Uv, in water, λ_{max} 243 nm (ϵ 17.0 × 10^3), λ_{min} 214 nm (ϵ 7.0 × 10^3); in 0.1 N NaOH, λ_{max} 265 nm (ϵ 22.2 × 10^3), λ_{min} 227 nm (ϵ 3.6 × 10^3).

Time Course of the Reaction of Cytidine with Semicarbazide-bisulfite. The reaction was started by adding an aqueous solution of cytidine into a large excess of semicarbazide-bisulfite solution. The final concentration of cytidine was 5 mM. Aliquots were removed at desired periods and diluted with 100-fold volume of 0.1 or 0.2 N NaOH, and A_{275} nm values of the resulting solutions were determined. The value, $(A_\infty - A_t)/(A_\infty - A_0)$, where A_∞ is the A_{275} value at completion of the reaction, A_t that at time t, and A_0 that at time zero, represents the fraction of cytidine remaining.

Uv Spectral Data for 5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate Ribofuranoside (**1b**). In water, and in 0.5 M Tris-HCl, pH 7.0, λ_{max} 245 nm (ϵ 17.7 × 10^3), λ_{min} 215 nm (ϵ 7.5 × 10^3); in 0.1–1 N NaOH, λ_{max} 268 nm (ϵ 23.8 × 10^3), λ_{min} 228 nm (ϵ 4.5 × 10^3). For determination of the extinction coefficients, the following experiments were carried out. Ten micromoles of 4-semicarbazido-2-ketopyrimidine ribofuranoside (**2b**) was treated with 50 μl of 1 M $NaHSO_3$, pH 5.5, for 1 h at room temperature. The total mixture was subjected to paper chromatography run in ethanol-water (7:3, v/v). The zone (R_f ~0.3) corresponding to **1b** was eluted with water to give an aqueous solution of **1b** which was free of **2b** and most of the salts. The yield was 160 A_{246} nm units and the concentration was 272 A_{246} units/ml. A portion of the solution was diluted 300-fold with either water, 0.5 M Tris-HCl, pH 7.0, or aqueous NaOH (0.1, 0.2, or 1 N), and the spectra were recorded. Another portion of the solution was diluted 330-fold with 0.5 M sodium phosphate buffer, pH 7.0, the solution was incubated at 37 °C for 15 h to quantitatively revert **1b** to **2b**, and the spectrum was determined. Employing the extinction coefficient 1.00 × 10^4 at 275 nm for **2b** (determined with a pure sample of **2b**), the ϵ values of **1b** were calculated.

Rates of the Elimination of Bisulfite from 5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate Ribofuranoside (**1b**). The adduct **1b** was treated with a desired buffer solution. Aliquots were taken and diluted with tenfold volumes of 0.2 N NaOH, and the A_{315} nm values of the resulting solutions were recorded within 10 min after the dilution. The value, $(A_\infty - A_t)/(A_\infty - A_0)$, where A_∞ is the A at completion of the conversion, A_t that at time t, and A_0 that at time zero, represents the fraction of **1b** remaining. Good pseudo-first-order plots were generally obtained and the k_{obsd} values were calculated by the equation, 0.693/half-time.

Reaction of DNA with Semicarbazide-Bisulfite. One volume of a 1% solution of calf-thymus DNA (Sigma, Type I) in 5 mM sodium phosphate buffer, pH 7.5, was added to 10 volumes of 3.3 M semicarbazide-1.1 M $NaHSO_3$, pH 5.0. Aliquots were withdrawn at appropriate intervals and added to 50-fold volumes of 0.2 N NaOH, and A_{275} nm values of the resulting solutions were determined.

For base composition analysis, heat-denatured DNA was treated with 3 M semicarbazide-1 M bisulfite in the same manner as above, and the reaction was terminated at desired period by addition of 0.25 volume of 5 N NaOH, which brought the pH of the solution to 7. The solution was dialyzed at room temperature against 0.1 M sodium phosphate buffer, pH 8.8, for 1 day, by which the bisulfite adducts of the thymine and cytosine residues were reverted to the parent pyrimidines. The solution was further dialyzed against water overnight and then adjusted to pH 7.0 and to 0.66 M in sodium phosphate concentration. The solution was allowed to stand at room temperature for 20 h. In this process, the residues of 5,6-dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate were converted to those of 4-semicarbazido-2-ketopyrimidine. The mixture was warmed at 37 °C for 2 h to ensure the complete conversion and dialyzed extensively toward water. Base compositions were determined by heating the DNA (15 A_{260nm} units) with 60% $HClO_4$ (10 μl) at 100 °C for 1 h and fractionating the resulting bases by paper chromatography. In the chromatography (run descendingly with isopropyl alcohol-concentrated HCl-water, 170:41:39, by volume), 4-semicarbazido-2-ketopyrimidine (**2a**) traveled together with guanine. The contents of **2a** and guanine in this mixture were determined spectrophotometrically, utilizing the molar extinction coefficients, ϵ_{282nm} 14.1 × 10^3 (**2a**), 6.7 × 10^3 (guanine), and ϵ_{260nm} 5.45 × 10^3 (**2a**), 8.0 × 10^3 (guanine) in 0.1 N HCl. Corrections were made for the contents of **2a** in DNA, by doubling the amounts found in hydrolysates.

Results

Reaction of Cytosine with Semicarbazide-Bisulfite. When cytosine was treated with a mixture of semicarbazide and sodium bisulfite at pH 5 and 37 °C, it rapidly gave 5,6-dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate (**1a**). The dihydro compound **1a** was isolated as a crystalline material, which was analyzed as its monohydrate. The uv spectra of **1a**, both in water and in 0.1 N NaOH, were very similar to those of 5,6-dihydro-4-semicarbazido-2-ketopyrimidine (Janion and Shugar, 1968). NMR spectrum of the 5-deuterio derivative of **1a**, which was formed by a reaction of cytosine with semicarbazide-bisulfite in D_2O, gave a singlet at 2.96 ppm (in D_2O, 3-(trimethylsilyl)propanesulfonate as a standard) corresponding to the 5-H, and a singlet at 4.42 ppm corresponding to the 6-H. The chemical shifts and the small coupling constant for 5-H and 6-H were analogous to those observed for 5,6-dihydrouracil-6-sulfonate (Shapiro et al., 1970; Hayatsu et al., 1970b) and suggested a diequatorial configuration for the 5-H and the 6-H. When heated in 0.07 M sodium phosphate

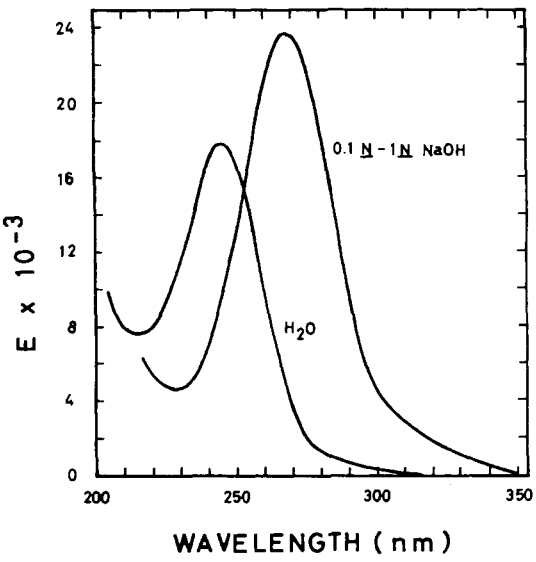


FIGURE 1: Ultraviolet absorption spectra of 5,6-dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate ribofuranoside (**1b**).

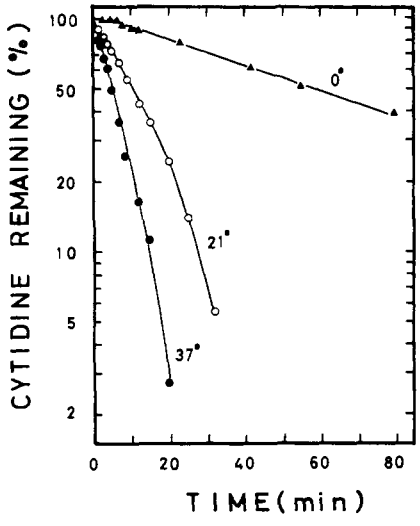


FIGURE 2: Time courses of the reaction of cytidine with 1 M semicarbazide-1 M sodium bisulfite, pH 5.0, at various temperatures.

buffer, pH 7, at 100 °C for 15 min, the adduct **1a** generated 4-semicarbazido-2-ketopyrimidine (**2a**), which was identified by its uv spectra at pH 1, 7, and 13 (Janion and Shugar, 1968). In 0.1–0.5 N HCl, **1a** rapidly underwent hydrolysis to give 5,6-dihydrouracil-6-sulfonate. The conversion was complete, either with 0.1 or 0.5 N HCl, in 2 h at room temperature. 5,6-Dihydrouracil-6-sulfonate thus formed was identified both by paper chromatography in solvent (**1**) and by its reversal to uracil upon treatment with alkali (Shapiro et al., 1970; Hayatsu et al., 1970a).

Reaction of Cytidine with Semicarbazide-Bisulfite. The reaction between cytidine and the semicarbazide-bisulfite agents was analyzed first by paper chromatography. When cytidine was incubated in 1 M semicarbazide-1 M sodium bisulfite at pH 5.0 and 37 °C for 1 h, 5,6-dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate ribofuranoside (**1b**) was formed as a single product. This compound gave R_f values in several solvent systems (Table I) identical with those of a sample prepared by treatment of 4-semicarbazido-2-ketopyrimidine ribofuranoside (**2b**) (Hayatsu et al., 1966) with sodium bisulfite (in 1 M bisulfite, pH 5.5, incubation for 10 min at room temperature completed this reaction). Uv spectra at pH 7 and 13 of the product **1b** obtained from cytidine were also identical with those of the sample obtained from **2b** (Figure 1).

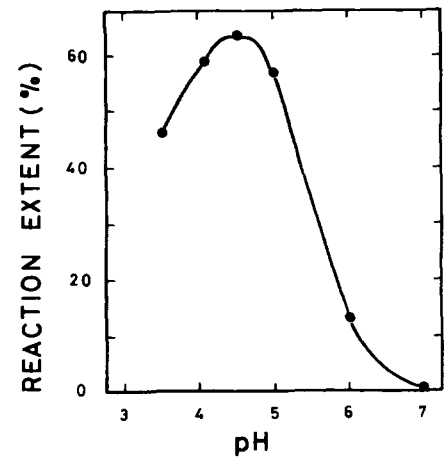


FIGURE 3: Effect of pH on the velocity of the reaction of cytidine with semicarbazide-bisulfite. Cytidine (5 mM) was incubated in 1 M semicarbazide-1 M sodium bisulfite at 20 °C for 15 min.

TABLE II: Effect of Concentrations of Semicarbazide and Bisulfite on the Velocity of the Conversion, Cytidine to 5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate Ribofuranoside (**1b**).[a]

Concn of Reagents (M)		Time Required for 50% Conversion (min)
Semicarbazide	Sodium Bisulfite[b]	
1.0	0.2[c]	21
1.0	1.0	12.5
0.2	1.0[c]	65

[a] The reactions were run at 21 °C and pH 5.1. [b] The concentrations represent total buffer concentrations, i.e., HSO_3^- + SO_3^{2-}. [c] NaCl (0.8 M) was added to this mixture in order to normalize the salt concentration.

By use of the strong uv absorbance in alkali, the progress of the conversion, cytidine to **1b**, was followed. The wavelength selected for this measurement was 275 nm, where the reagent blank gave negligible absorbance. 5,6-Dihydrocytidine-6-sulfonate, that was formed very rapidly in the reaction mixture (Hayatsu et al., 1970b), was immediately reverted to cytidine in this alkaline solution. Time courses of the reaction at 37, 21, and 0 °C are presented in Figure 2. These reactions were run by mixing cytidine with a large excess of 1 M semicarbazide-1 M sodium bisulfite at pH 5.0. Initial lag period was observed for the reaction at 0 °C, and the conversions at 21 and 37 °C became faster with the progress of the reaction. It is seen that the conversion is very rapid at 37 °C, the half-time being about 5 min. For completion of the reaction, it took 30 min at 37 °C, or 60 min at 21 °C. Cytidine 5′-phosphate reacted at a similar rate with 1 M semicarbazide-1 M bisulfite; at 21 °C, the time required for 50% reaction was 11 min.

The effect of pH on the velocity of the reaction was examined with 1 M semicarbazide-1 M bisulfite at 20 °C. As Figure 3 shows the pH optimum was about 4.5. The velocity decreased sharply as the pH increased from 5 to 6. At pH 7, no transamination was observed by the 15-min incubation.

Effect of changing the concentrations of either semicarbazide or bisulfite was studied. It is seen from Table II that the rate was proportional to the concentration of semicarbazide,

TABLE III: The Pseudo-First-Order Rate Constants of the Conversion, 5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate Ribofuranoside (**1b**) to 4-Semicarbazido-2-ketopyrimidine Ribofuranoside (**2b**) at 37 °C.

pH	Buffer and Its concn (M)	k_{obsd} (min^{-1})
	Sodium Phosphate	
5.0	0.5 M	0.017
6.0	0.5 M	0.017
7.0	1.0 M	0.033
7.0	0.5 M	0.017
7.0	0.2 M	0.008
7.0	0.2 M + 0.8 M NaCl	0.008
8.0	0.5 M	0.007
	Tris-HCl	
7.0, 8.0	0.5 M	0.002
9.0	0.5 M	0.003

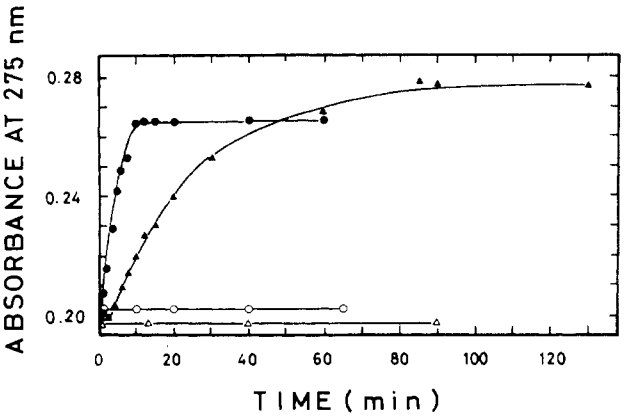


FIGURE 4: Time course of the transamination of cytosine residues in DNA. Calf thymus DNA, heat-denatured or native, was treated with 3 M semicarbazide–1 M sodium bisulfite, pH 5.0, and the A_{275nm} value in 0.2 N NaOH was determined. See Experimental Section for detail. At 37 °C: (●) denatured DNA; (○) native DNA. At 20 °C: (▲) denatured DNA; (△) native DNA.

whereas the rate was not so much affected by the change in the bisulfite concentration. Therefore, it is advantageous to increase the concentration of semicarbazide rather than that of bisulfite for the purpose of obtaining faster reactions. At nearly saturated reagent concentrations of 3 M semicarbazide–1 M $NaHSO_3$, the transformation of cytidine into **1b** at pH 5.0 and 37 °C was completed by 5 min of treatment.

Specificity of the Reaction. Adenosine 5′-phosphate or guanosine 5′-phosphate was unchanged when treated with 1.8 M semicarbazide–1.8 M $NaHSO_3$ at 37 °C and pH 5.1 for 2 h (checked by paper chromatography in solvent (**1**) and by uv spectra). Under the identical conditions, thymidine and uridine gave their bisulfite adducts, i.e., 5,6-dihydrothymidine-6-sulfonate and 5,6-dihydrouridine-6-sulfonate, but no other products were formed. These bisulfite adducts can be reverted to their parent pyrimidines by treatment with weak alkali (Shapiro et al., 1970; Hayatsu et al., 1970b).

Conversion of 5,6-Dihydro-4-semicarbazido-2-ketopyrimidine-6-sulfonate Ribofuranoside (**1b**) *to 4-Semicarbazido-2-ketopyrimidine Ribofuranoside* (**2b**). It was found that a facile elimination of bisulfite from the adduct **1b** to give **2b** takes place in phosphate buffers of near neutrality. The product **2b** was identified by paper chromatography in three solvent systems (Table I) and by ultraviolet spectra at pH 7 and 13.4 (Hayatsu et al., 1966). Using paper-chromatographically purified sample of **1b** (70% ethanol was used as the chromatographic solvent), the pseudo-first-order rate constants for the elimination at 37 °C were determined (Table III). The rate increased proportionally with the increase of the phosphate buffer concentration. In the absence of phosphate, i.e., in water, the compound **1b** was completely stable at least for 2 days at room temperare. The rate in phosphate buffer did not vary in the pH range 5 to 7, but it was considerably lower at pH 8. The effect of the phosphate buffer concentration was not a mere salt effect since addition of sodium chloride did not affect the rate. In Tris-HCl buffer at pH 7 to 9, the conversion was very slow. For complete conversion of **1b** to **2b**, either of the following incubations may be employed: in 0.1 M sodium phosphate buffer, pH 7.0, at 20 °C for 30 h; in 1 M sodium phosphate, pH 7.0, at 37 °C for 2 h.

Over a wide range of pH value, the elimination of bisulfite from the adduct **1b** to give **2b** appears to take place. In contrast to the base **1a**, the nucleoside **1b** did not deaminate in acid, but instead slowly gave **2b**. Thus, by 23-h standing at room temperature in 0.1 N HCl, about 30% conversion of **1b** into **2b** was detected by spectroscopic measurement. In 0.1 N NaOH, about 15% conversion of **1b** to **2b** occurred by 2-h standing at room temperature. This was detected by the rise in A_{315}-nm value of the solution. The A_{275}-nm value, which was used for the measurement of the rate of the transamination (see above), was unchanged, since **2b** possesses a molar absorbance not very much different from that of **1b** at this wavelength (Hayatsu et al., 1966).

2′-Deoxycytidine 5′-phosphate also underwent transamination with semicarbazide in the presence of bisulfite, and the adduct **1c** formed was convertible to **2c** by treatment with phosphate buffers at pH 7.

Modification of DNA with Semicarbazide–Bisulfite. It is known that the addition of bisulfite to the 5,6-double bond of cytosine residues in polynucleotide occurs only for those in single-stranded region (Shapiro et al., 1973). The single-strand specific nature of the semicarbazide–bisulfite reagents was demonstrated by use of native and denatured DNA. The method used for determining the progress of the modification was to measure the A_{275nm} value of DNA in alkali. Heat-denatured calf-thymus DNA reacted very rapidly with 3 M semicarbazide–1 M bisulfite at pH 5.0 and 37 °C (Figure 4). The reaction came to a stop after 10-min incubation and no further increase in the A_{275nm} value was observable up to 60-min incubation. At 20 °C, the reaction reached the plateau by 85-min incubation. The increase of the A_{275nm} value was approximately equal to that observed for the reaction at 37 °C. Native DNA, in contrast, did not react with the agents even by a prolonged treatment at 37 °C.

Denatured DNA was treated with 3 M semicarbazide–1 M bisulfite at pH 5.0 and 20 °C, for 20 and 90 min. After removal of the agents by dialysis, the residues of **1c** in the DNA were converted to those of **2c** by treatment with 0.66 M phosphate buffer. This conversion was monitored by measuring the A_{310nm} value of the DNA in 0.1 N NaOH (Hayatsu et al., 1966; Hayatsu and Ukita, 1966). Treatment for 20 h at room temperature completed the conversion since no further increase in the A_{310nm} value was observed upon additional incubation for 2 h at 37 °C. The modified DNA samples were hydrolyzed with perchloric acid. The bases produced were fractionated by paper chromatography, and the amounts of the bases were determined spectrophotometrically. When 4-semicarbazido-2-ketopyrimidine 2′-deoxyribofuranoside 5′-phosphate (**2c**) was subjected to the identical treatment, 50% recovery of the base **2a** was obtained. Therefore, corrections were made for the content of the modified base in the DNA, by doubling the

amount of **2a** found in the DNA hydrolyzate. The results in Table IV show that selective modification of cytosine residues did take place, as expected. In the 90-min-treated DNA, 82% of the total cytosine residues had been modified. The extent of the modification in the 20-min-treated sample (47%) was consistent with the results in Figure 4. Thus, if the increase of the A_{275nm} value by the 90-min incubation was taken as representing 82% modification, the A_{275nm} value at 20 min corresponded to 44% modification.

TABLE IV: Base Compositions of DNA Modified with 3 M Semicarbazide–1 M Bisulfite at pH 5.0 and 20 °C.

	Mol %				
Treatment[a]	Cytosine	4-Semicarbazido-2-keto-pyrimidine	Thymine	Guanine	Adenine
None	20.7		25.8	26.1	27.4
20 min	10.3	9.1	25.1	26.5	28.3
90 min	3.6	16.5	24.2	26.1	29.7

[a] Subsequent to the treatment with semicarbazide-bisulfite, the DNA was incubated in a phosphate buffer at pH 7 to transform the residues of **1c** to those of **2c**. See text for detail.

Discussion

The modification by semicarbazide–bisulfite is very rapid under appropriate conditions. Since the rate of the transamination with semicarbazide–bisulfite is nearly two orders of magnitude greater than that of the deamination (compare the results reported by Sono et al., 1973; Shapiro et al., 1974), the product is exclusively the transaminated compound **1**. With other amines except for methoxyamine (Sverdlov et al., 1974), concomitant deamination is difficult to avoid. The half-life of cytidine 5′-phosphate in 1 M methoxyamine–1 M sodium bisulfite at pH 6 and 20 °C was reported to be 0.5 h (pH–rate profile was not described; Sverdlov et al., 1973). At comparable reagent concentrations and temperature, the transamination with semicarbazide–bisulfite at pH 5 is about three times more rapid. The rate of uncatalyzed transamination of cytidine with semicarbazide (Hayatsu and Ukita, 1964; Hayatsu et al., 1966) is two orders of magnitude smaller than that of the bisulfite-catalyzed reaction.

Owing to the two-step nature of the reaction (Scheme I), the time course (Figure 2) shows that the velocity of the accumulation of the transaminated product **1b** is slow in the initial stage and is later accelerated. Since the semicarbazide concentration affects the rate more strongly than that of bisulfite (Table II), the transamination step must be rate determining. This indicates the possibility that there could still be some other nucleophiles which, in combination with bisulfite, can modify cytidine even faster than the semicarbazide–bisulfite.

The low pK_a of semicarbazide (3.5) makes the nonprotonated form of the amine predominant over the protonated (unreactive) form at pH 4.5 and above, which will attack the protonated form of 5,6-dihydrocytidine-6-sulfonate ($pK_a \sim 5.1$; Shapiro et al., 1974). In addition, it is known that the equilibrium between cytidine and its bisulfite adduct becomes more favorable to the adduct side as the pH value drops down (Shapiro et al., 1970; Hayatsu et al., 1970b). The pH–rate profile (Figure 3) may in this way be accounted for.

The facile acid hydrolysis of the 4-semicarbazido group of adduct **1a** to give 5,6-dihydrouracil-6-sulfonate is analogous to the ease with which 5,6-dihydro-4-semicarbazido-2-ketopyrimidine undergoes acid hydrolysis (Janion and Shugar, 1968). It is surprising that the corresponding riboside **1b** does not undergo acid hydrolysis. This stability, however, is similar to that of the methoxyamino group of 5,6-dihydro-4-methoxycytidine-6-sulfonate (Sverdlov et al., 1973). The 5,6-dihydro-6-sulfonate structure of this methoxyamino derivative has been reported to be very stable both in acid and alkali (Sverdlov et al., 1973). In contrast, the semicarbazido derivative **1b** undergoes elimination of bisulfite over a wide range of pH value, although the rates are much lower than those for 5,6-dihydrocytidine-6-sulfonate (Hayatsu et al., 1970b).

The mechanism of the phosphate-catalyzed elimination of bisulfite from **1b** (Table III) is not known. It may be that $H_2PO_4^-$ is acting as a general acid catalyst. Alternatively, the protonated form of **1b** may be reacting with HPO_4^{2-} as a general base catalyst.

The reaction of heat-denatured DNA with 3 M semicarbazide–1 M bisulfite at pH 5.0 proceeded with striking rapidity until about 80% of total cytosine residues was modified (Figure 4 and Table IV). The unreactive fraction of the cytosine residues must have been in the structured portion of the denatured DNA. It is known that a considerable portion of heat-denatured DNA possesses secondary structure (Lepecq and Paoletti, 1967). The strict single-strand specificity of this reaction, in combination with the rapidity, suggests its usefulness as a conformational probe for DNA and RNA. For such purposes, it would be an advantage that pancreatic ribonuclease and snake venom phosphodiesterase can cleave the phosphodiester bonds of the 4-semicarbazido-2-ketopyrimidine ribonucleotide residues in RNA (Hayatsu et al., 1966; Hayatsu and Ukita, 1966). The methylamine–bisulfite system (Shapiro et al., 1972) has already been utilized in conformational analysis of transfer RNA (Schulman et al., 1974). [^{14}C]Semicarbazide hydrochloride of specific activity 5 mCi per mmol is commercially available. If one uses this material for the modification of cytosine residues after, for example, 100-fold dilution with cold semicarbazide, one would obtain $\sim 10^3$ dpm per 0.01 μmol of cytosine.

It may be expected that hydrazides, like semicarbazide, will substitute the amino group of cytosine with the catalysis of bisulfite. For example, uncatalyzed transamination of cytidine takes place with Girard-P reagent (Kikugawa et al., 1967) and with isoniazid (Kikugawa et al., 1969). Usefulness of the reaction will be strengthened if the hydrazide is attached to a fluorescent group or to a dye. Hydrazides carrying functional groups of other types, for instance, a photoactivatable group, may also be quite useful if they can be introduced into cytosine residues.

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Adenosine Triphosphatase of Rat Liver Mitochondria: Detergent Solubilization of an Oligomycin- and Dicyclohexylcarbodiimide-Sensitive Form of the Enzyme†

John W. Soper‡ and Peter L. Pedersen*

ABSTRACT: The hydrolytic activity of the ATPase bound to purified inner membrane vesicles of rat liver mitochondria can be increased threefold by washing extensively with a high ionic strength phosphate buffer. The specific ATPase activities of such phosphate-washed membranes are the highest reported to date for a mitochondrial membrane preparation (21–24 μmol of ATP hydrolyzed min^{-1} mg^{-1} in bicarbonate buffer at 37 °C). Deoxycholate (0.1 mg/mg of protein) extracts from these membranes a soluble, cold-stable ATPase complex which exhibits a specific activity under optimal assay conditions of 12 μmol of ATP hydrolyzed min^{-1} mg^{-1}. This complex is not sedimented by centrifugation at 201 000*g* for 90 min, and readily passes through a 250-Å Millipore filter. The ATPase activity of the soluble complex is inhibited 95% by 2.4 μM oligomycin. In addition, inhibitions of 60% or better are obtained in the presence of 1–8 μM dicyclohexylcarbodiimide, *p*-chloromercuribenzoate, venturicidin, and aurovertin. While a similar complex may be extracted with Triton X-100, this preparation is always lower in both specific activity and in inhibitor sensitivities than the complex extracted with deoxycholate. Detergents of the Tween and Brij series and other detergents of the Triton series are also much less effective than deoxycholate in solubilizing the oligomycin-sensitive ATPase complex of rat liver. It is concluded that deoxycholate is superior to other detergents as an extractant of the oligomycin-sensitive ATPase complex of rat liver mitochondria, and that the complex extracted with deoxycholate possesses a closer similarity to the membrane-associated ATPase than does the complex extracted with Triton X-100. These studies document the first report of a detergent-solubilized, oligomycin-sensitive ATPase preparation from rat liver mitochondria.

Lardy et al. (1958) first discovered that the antibiotic oligomycin can simultaneously block the terminal step in oxidative phosphorylation (phosphoryl transfer to ADP) and inhibit ATPase activity in submitochondrial particles. Since this time a number of workers have described conditions for extraction of a particulate form of the oligomycin-sensitive ATPase (OS-ATPase)[1] from a variety of sources. The bile salts, deoxycholate (Tzagoloff et al., 1968; Yamamoto, 1970) and cholate (Kopaczyk et al., 1968; Kagawa and Racker, 1966) have been used to carry out the majority of these extractions. Additional methods used to obtain insoluble preparations have included acetone extraction of mitochondria (Vallejos et al., 1968) and treatment of submitochondrial particles with lysolecithin (Sadler et al., 1974).

Tzagoloff and Meagher (1971) have solubilized from yeast mitochondria a dispersed OS-ATPase of high specific activity. The nonionic detergent Triton X-100 (TX-100) was used for this extraction, while a similar preparation has been described also by Ryrie (1975). TX-100 extractions of beef heart mitochondria have been carried out by Linnett et al. (1975) and by Swanljung et al. (1973).

Although the oligomycin-insensitive ATPase (F_1) has been solubilized and purified from rat liver (Catterall and Pedersen, 1971; Lambeth and Lardy, 1971), no reports have yet appeared on the properties of an oligomycin-sensitive preparation from

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received January 14, 1976.* This work was supported by a grant from the National Institutes of Health (CA 10951).

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[1] Abbreviations used are: OS-ATPase, oligomycin-sensitive adenosine triphosphatase; F_1, oligomycin-insensitive ATPase; DCCD, dicyclohexylcarbodiimide; ClHgBzO, *p*-chloromercuribenzoate; TX-100, Triton X-100; cmc, critical micelle concentration; Cl_3CCOOH, trichloroacetic acid; mitoplast, inner membrane + matrix; H medium, PA buffer and P buffer, see Methods and Results; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.