The effect of bisulfite modification on the template activity of DNA for DNA polymerase I[1].

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ABSTRACT

Cytosine residues of poly(C) and heat-denatured calf thymus DNA were transformed into 5,6-dihydrouracil-6-sulfonate ($U(SO_3^-)$) residues by treatment with bisulfite. The poly($U(SO_3^-)_2$, C_3) and poly($U(SO_3^-)_9$, C_1) prepared did not form inter-base binding with either poly(A) or poly(I) as judged by the absence of hypochromicity in ultraviolet absorbance. $U(SO_3^-)$ residues in the DNA inactivated it to serve as template for E. coli DNA polymerase I, while the template activity was restored by conversion of the $U(SO_3^-)$ residues into U.

INTRODUCTION

Bisulfite induces mutagenesis in microorganisms[2-5]. On the basis of the reaction at the mononucleotide level, the mutagenesis was deduced to be of the CG-to-TA transition type[2], and this mode of transition was indeed proved in the E. coli and the T4 phage systems[3,4]. Bisulfite adds across the 5,6-double bond of cytosine nucleus and the resulting 5,6-dihydrocytosine-6-sulfonate deaminates to give 5,6-dihydrouracil-6-sulfonate ($U(SO_3^-)$)[6-8]. This reaction proceeds most optimally at pH 5[9,10]. $U(SO_3^-)$ loses HSO_3^- in alkali giving U. A problem in the mechanistic aspect of the bisulfite-induced mutation is whether the base transition occurs through the possible base pairing $U(SO_3^-)$ - A or, rather, after the conversion of $U(SO_3^-)$ into U. An apparent exception to this is the mutation of T4 (ref. 4) whose DNA contains 5-hydroxymethyl C residues in place of C : In view of the fact that 5,6-dihydro-5-methyluracil-6-sulfonate is extremely unstable, readily giving thymine in aqueous solutions[8], the mutagenesis of T4 must be taking place due to the hydroxymethyl-U - A pairing, without significant contribution of the dihydrouracil type residues.

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Recently, Shapiro and coworkers[11,12] studied the effect of bisulfite treatment of poly(U) on the complex formation with poly(A), on the coding ability to direct the synthesis of polyphenylalanine, and on the ability to form a complex with ribosome and phenylalanyl tRNA. They found that the presence of U(SO_3^-) residues abolishes all of these abilities of poly(U). We have now examined the effect of bisulfite modification of DNA on the template activity for DNA polymerase I, a system which should give information of direct relevance to the understanding of chemical mutagenesis[13,14]. Whether poly(U(SO_3^-), C) can form a complex with poly(A) or poly(I) was also studied.

These studies may also contribute to the elucidation of the adverse effects of environmental sulfur dioxide and bisulfite.

MATERIALS AND METHODS

[^{35}S]Na_2SO_3, [^{3}H]TTP and [^{3}H]dGTP were purchased from Radiochemical Centre (England). α-[^{32}P]dATP was obtained from International Chemical and Nuclear Corp. (U.S.A.). Poly(C), poly(A), poly(U) and poly(I) were products of Miles (U.S.A.) and calf thymus DNA (highly polymerized, Type I) that of Sigma (U.S.A.). *E. coli* DNA polymerase I was purified according to the method of Jovin et al.[15], and the enzyme showed a specific activity of 12,000 units per mg protein. A unit of enzyme activity is defined as previously[15] and represents an activity which carries out incorporation of 10 mμmoles of total nucleotides into acid-insoluble product in 30 min incubation at 37°. Radioactivities were determined by a Packard Tri-Carb 3320 liquid scintillation spectrometer. Nearest neighbor frequency analysis was performed using α-[^{32}P]dATP according to the procedure of Josse and Swartz[16].

Preparation of poly(U(SO_3^-), C) Poly(C), 4 mg, was dissolved in 0.4 ml of 1 M $NaHSO_3$-Na_2SO_3 (3 : 1, in mole ratio; pH 6.0) and the solution was incubated at 37°. At 36th hr of the incubation, 0.15 ml of the solution was taken and the rest was incubated for another 36 hrs. The reaction was stopped by loading the solution on a column of Sephadex G-100 (1 x 42 cm) and the product was eluted with 0.05 M sodium acetate buffer (pH 4.5). The modified poly(C) samples were eluted in the void volume fractions, indicating that no extensive chain fission, if any[17], had taken place during the treatment. The poly(U(SO_3^-), C) solutions were stored frozen at -20°. The extent of the modification was determined by hydrolysis of the samples with alkali followed by paper chromatographic

analysis[8] of the mononucleotides produced. The sample modified for 36 hrs contained 39 % U(SO_3^-) residues and the one modified for 72 hrs, 93 % U(SO_3^-). The former sample was designated poly(U(SO_3^-)$_2$, C_3) and the latter poly(U(SO_3^-)$_9$, C_1).

Preparation of poly(U, C) Portions of the poly(U(SO_3^-), C) samples were taken and the U(SO_3^-) residues were transformed into U residues by dialysis of the solution at room temperature against 0.05 M sodium phosphate buffer, pH 9.0. The dialysis for 8 hrs was performed three times to complete the transformation. When the poly(U_2, C_3) and poly(U_9, C_1) samples thus obtained were digested with pancreatic ribonuclease, they gave uridine 3'-phosphate and cytidine 3'-phosphate in 39 : 61 and 93 : 7 mole ratio, as expected.

Mixing of poly(U(SO_3^-), C) and poly(U, C) with homopolymers The solutions of poly(U(SO_3^-), C) and poly(U, C) prepared as described above were dialyzed at 0° against a cacodylate buffer (0.01 M sodium cacodylate - 0.1 M NaCl, pH 6.5). Three 8 hr runs of the dialysis were carried out and then the polymer solutions were made up with the cacodylate buffer to appropriate concentrations. The solution was mixed with a solution of a homopolymer (poly(A) or poly(I) in the cacodylate buffer) and ultraviolet absorbance was measured at 0°.

Preparation of modified DNA templates Calf thymus DNA was denatured by heating a solution (2.58 mg/ml H_2O) at 100° for 10 min followed by a rapid cooling. To 1 ml of the solution were added $NaHSO_3$ (78.1 mg, 0.75 mmole), [^{35}S]Na_2SO_3 (31.7 mg, 0.25 mmole; 1.2 mCi/mmole) and 10 µl of 0.05 M hydroquinone, the resulting pH being 6.0. The hydroquinone added would prevent autoxidation of bisulfite, protecting the DNA from the attack of free radicals[18,19]. The solution was incubated at 37°. Aliquots were removed from time to time,which were dialyzed against 1 liter of a buffer, 5 mM in potassium phosphate and 0.5 mM in hydroquinone, at pH 6.8 and 0° for 4 hrs. Dialysis to 5 mM potassium phosphate (pH 6.8) containing no hydroquinone was then performed three times at 0°, each run taking 4 hrs. Control samples were prepared by incubating for corresponding periods at pH 6 and 37° the heat-denatured DNA samples in a buffer, 0.1 M in potassium phosphate, 0.9 M in sodium chloride and 0.5 mM in hydroquinone, followed by work-up in the manner described for the test samples.

Portions of the [^{35}S] DNA samples were treated with alkali to generate

U residues from the U(SO_3^-) residues. For this, 10 μl of 5 N NaOH was added to 0.50 ml of the DNA solution, and the solution was incubated at 37° for 1 hr. Exhaustive dialysis against 5 mM potassium phosphate buffer (pH 6.8) reduced the amount of the radioactive sulfur in the DNA solution to a value smaller than 2 % of the original. Somewhat stronger alkaline conditions were employed for the extensively modified DNA samples (those which had been treated with bisulfite for 36 hrs and 72 hrs) : 10 μl of 10 N NaOH was added to 0.30 ml of the [^{35}S] DNA solution and the solution was incubated for 1 hr at 37°.

Modification extents in the DNA preparations were calculated on the basis of the radioactivity of the modified DNA solution prior to the alkaline treatment, of the DNA content of the solution (as determined by the Burton procedure[20]), and of the base composition determined for the non-treated DNA. The base composition as determined by the method of Wyatt[21] was, A, 28.9; T, 29.3; G, 20.5; and C, 21.3 in mole %.

Assay for DNA polymerase I-catalyzed DNA synthesis The assay mixture of a total volume 0.15 ml consisted of 70 mM potassium phosphate buffer (pH 7.3), 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 70 μM each of dATP, dGTP, dCTP and TTP (one or more of the nucleotides radioactively labeled), 0.6 unit of DNA polymerase, and template DNA (16 μg). After incubation at 37° for desired time, trichloroacetic acid-insoluble radioactivity was measured by the centrifugation technique of Lehman et al.[22]. No liberation of ^{35}S from the templates was noted by 30 min incubation.

RESULTS AND DISCUSSION

Study on complex formation of poly(U(SO_3^-), C) with poly(A) or poly(I)

A simple approach to examine the ability of U(SO_3^-) to form inter-base binding with A is to modify poly(C) with bisulfite and see if at some degree of modification the polymer starts to complex with poly(A). The complex formation could be observed by the presence of hypochromicity in ultraviolet absorbance. We treated poly(C) with 1 M sodium bisulfite at pH 6.0 and prepared two samples, poly(U(SO_3^-)$_2$, C_3) and poly(U(SO_3^-)$_9$, C_1). The poly(U(SO_3^-), C) samples were kept at pH below 6.5 and low temperatures during the preparative procedures as well as during the complex-formation tests to prevent the conversion of U(SO_3^-) to U. Portions of the samples were incubated at pH 9 in order to prepare corresponding poly(U, C). As Fig. 1 shows, mixing of either the poly(U(SO_3^-)$_2$, C_3) or poly(U(SO_3^-)$_9$, C_1) with poly(A) did not produce detectable hypochromicity. On the other hand,

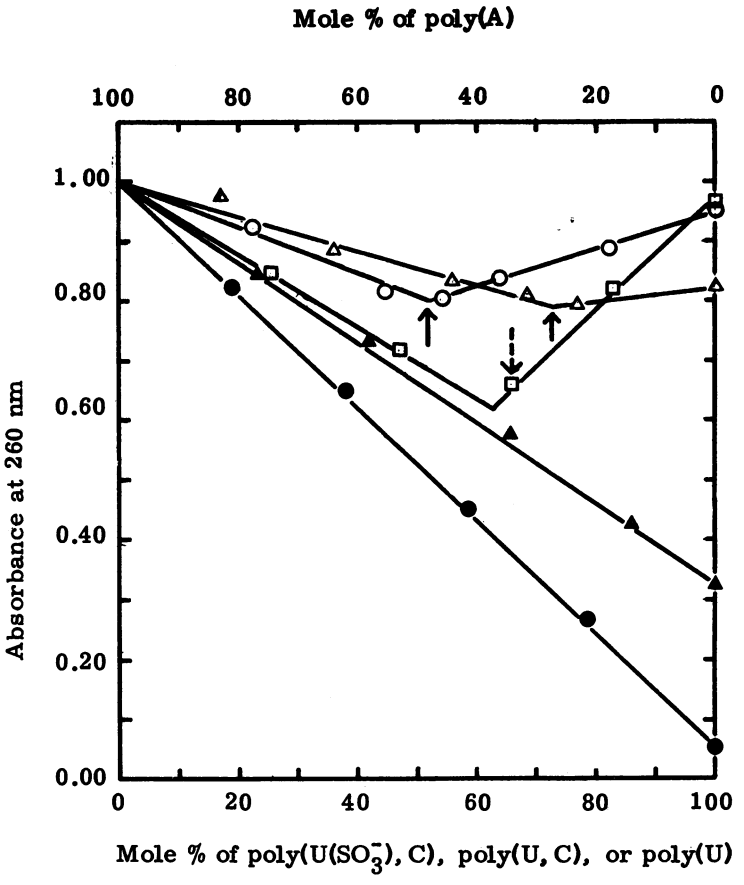

Fig. 1. Mixing curves for poly(A) and bisulfite-treated poly(C). — ● —, Poly($U(SO_3^-)_9, C_1$) ; — ▲ —, poly($U(SO_3^-)_2, C_3$) ; — o —, poly(U_9, C_1) ; — △ —, poly(U_2, C_3) ; — □ —, poly(U). The solid arrow indicates the position where the composition of the polymer mixture is 1 : 1 in U to A ratio, and the arrow with a dashed line the position where it is 2 : 1 in U to A ratio. Experimental conditions are as described in the text.

the mixing curve for poly(A) and the poly(U_2, C_3) or poly(U_9, C_1) showed an inflection point where the mole ratio U/A was unity. The mixing of poly(A) with poly(U) under the same conditions gave the largest hypochromic effect at the mixing ratio U/A, 2, as expected from the literature[23]. As shown in Fig. 2, the samples of poly($U(SO_3^-)$, C) did not produce hypochromicity when mixed with poly(I); that is, 40 % replacement of C residues in poly(C) with $U(SO_3^-)$ resulted in inhibition of the C residues to complex with I. These observations suggest that $U(SO_3^-)$ does not form stable base pairs with A and that the $U(SO_3^-)$ residues rather actively interfere the interaction between the C residues in the copolymer and poly(I). An analogous interferance by $U(SO_3^-)$ was observed in the poly(U, $U(SO_3^-)$) - poly(A) system[11].

Template activity of DNA modified with bisulfite Samples for testing the template activity toward E. coli DNA polymerase I were prepared by treatment of heat-denatured calf thymus DNA with (ca.) 1 M [^{35}S] sodium bisulfite at pH 6 and 37° for varying periods of time up to 72 hrs. Extents of modification of C to $U(SO_3^-)$ were calculated from the ^{35}S radioactivity incorporated and the C content of the original DNA. Thus, the per cent conversion of C to $U(SO_3^-)$ at the period of treatment was 2.2 % at 1 hr, 4.9 % at 3 hr, 11.2 % at 9 hr, 45 % at 36 hr, and 71 % at 72 hr. Control samples were prepared by treating for corresponding period of time the original DNA with phosphate buffer, pH 6, having an equivalent ionic strength in the absence of bisulfite. A portion of each bisulfite-treated DNA was exposed to alkali to bring about the conversion, $U(SO_3^-)$ to U.

Using these samples as templates, the DNA polymerase I-catalyzed polymerization of deoxyribonucleoside triphosphates was investigated. As Fig. 3 shows, the rate of [^{3}H]TMP incorporation in acid-insoluble material in the presence of unlabeled dATP, dCTP and dGTP was greatly reduced by the presence of a small amount of $U(SO_3^-)$ residues in the template. For example, the template bearing $U(SO_3^-)$ residues 2.2 % of the total original C, which corresponded to one $U(SO_3^-)$ per 210 nucleotide residues, exhibited only less than half of the activity of the control sample. As expected, the DNA samples in which the $U(SO_3^-)$ residues were converted into U showed approximately the same activity as the control. Extensively modified DNA templates (45 % and 71 % modified to $U(SO_3^-)$) did not induce any significant incorporation of deoxyribonucleotides (as checked by both [^{3}H]TTP and [^{3}H]dGTP incorporations in the presence of three other unlabeled deoxyribonucleoside triphosphates), while they served as templates

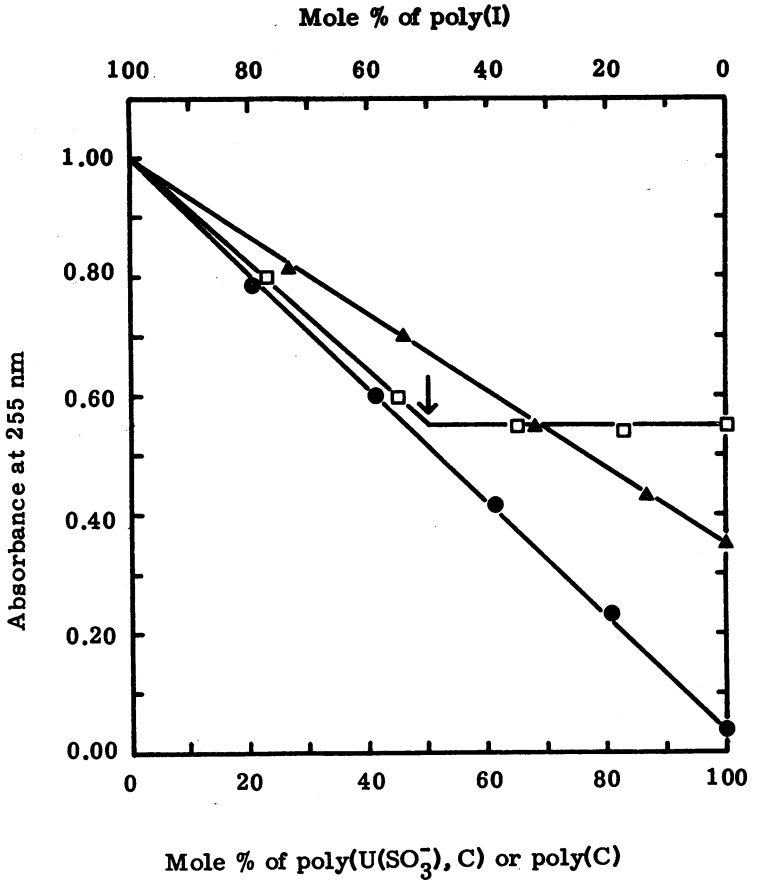

Fig. 2. Mixing curves for poly(I) and poly(U(SO_3^-), C). — ● —, Poly(U(SO_3^-)$_{\underline{9}}$, $C_{\underline{1}}$) ; — ▲ —, poly(U(SO_3^-)$_{\underline{2}}$, $C_{\underline{3}}$) ; — □ —, poly(C).

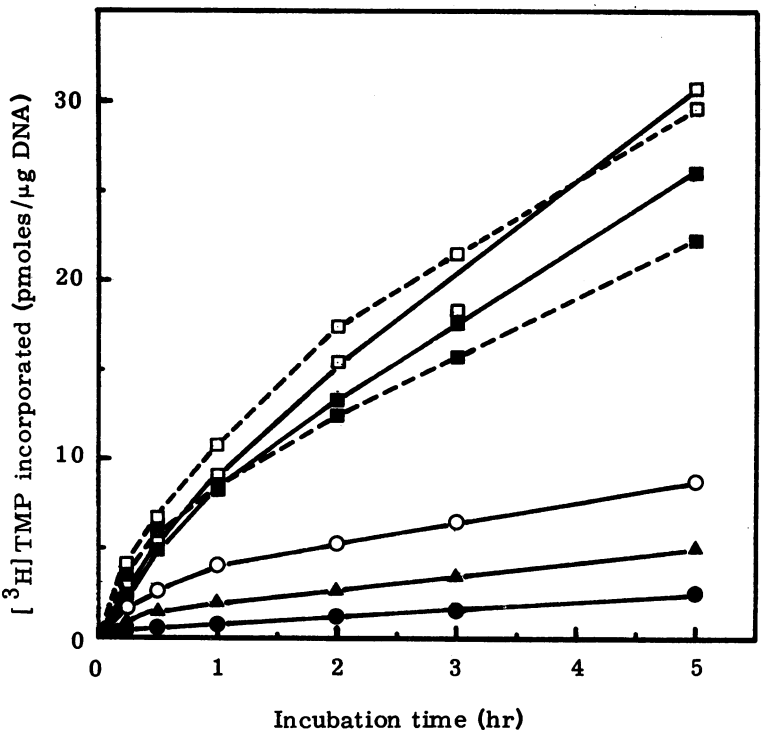

Fig. 3. Template activity of bisulfite-treated DNA samples in the E. coli DNA polymerase I-catalyzed polymerization of deoxyribonucleotides. [^{3}H]TMP incorporated into acid-insoluble products from a mixture of [^{3}H]TTP, dATP, dCTP, and dGTP was measured. The experimental procedure is described in the text. — o —, (U(SO_3^-))DNA, 2.2 % of C modified, as template; — ▲ —, (U(SO_3^-))DNA, 4.9 % modified; — ● —, (U(SO_3^-))DNA, 11.2 % modified ; — □ —, (U)DNA, 2.2 % modified ; — ■ —, (U)DNA, 11.2 % modified ; --- □ ---, control DNA for 2.2 % modified (U(SO_3^-))DNA ; --- ■ ---, control DNA for 11.2 % modified (U(SO_3^-))DNA.

Table I. Template activity of bisulfite-modified DNA in the E. coli DNA polymerase-catalyzed polymerization of deoxyribonucleotides.

Template	Modification extent (%)	Incorporation[a] of radioactive nucleotide from	
		[^{3}H]TTP, dATP, dCTP and dGTP (pmoles/μg DNA)	TTP, dATP, dCTP and [^{3}H]dGTP (pmoles/μg DNA)
(U(SO_3^-))DNA[b]	45	0	0
(U)DNA[b]	45	5.28	3.96
Control DNA	-	4.90	5.14
(U(SO_3^-))DNA[b]	71	0	0
(U)DNA[b]	71	2.43	1.95
Control DNA	-	3.71	3.87

a) Incubation was for 30 min. b) (U(SO_3^-))DNA represents DNA whose C had been transformed into U(SO_3^-), and (U)DNA that in which U(SO_3^-) had been converted to U.

Table II. Simultaneous determination of the incorporation of TMP and dAMP using (U)DNA as templates.

Template	Incorporation from a mixture of [^{3}H]TTP, [^{32}P]dATP, dCTP and dGTP (pmoles/μg DNA)[a]		A/T
	TMP	dAMP	
(U)DNA, 45% modified	3.77	3.57	0.95
Control DNA	3.85	3.08	0.80
(U)DNA, 71% modified	2.00	2.17	1.09
Control DNA	2.90	2.36	0.81

a) Incubation was for 30 min.

Table III. Nearest neighbor frequency of newly synthesized DNA.

Template	Distribution of ^{32}P incorporated from α-[^{32}P]dATP (%)[a]			
	dAp	Tp	dGp	dCp
(U)DNA, 45 % modified	39.2	20.9	18.6	21.3
Control DNA	30.7	19.6	22.0	27.7

a) Incubation for the DNA synthesis was for 30 min.

after treatment with alkali (Table I). The lowered activity of the 71 %-(C to U)-modified DNA as compared with 45 % modified template may be largely due to the long time incubation at pH 6, since the control sample also showed a similarly decreased activity. It should be noted that the incorporation of dGTP is smaller in the (U)DNA systems than in the controls. This is reasonable because the C content in the template was decreased by the modification. In order to further investigate this point, an experiment was carried out in which TMP and dAMP incorporations were simultaneously measured using differentially labeled substrates. As Table II shows, the A to T ratio of the incorporated nucleotide is larger than the control and increases with the modification extent.

Confirmation that we were really observing DNA synthesis in these experiments using (U)DNA as the template was obtained by a nearest neighbor frequency analysis of the product. Thus, results presented in Table III show that the label in α-[^{32}P]dATP was transferred to all four nucleotides. The proportion of ^{32}P transferred to dAp was higher with the modified template than with the control,obviously reflecting the C to U modification of the template.

The strong inhibition of the template activity incurred by the U(SO_3^-) residue suggests that, in addition to the inability of forming base pairs with A, U(SO_3^-) somehow actively interferes the polymerizing reaction; for example, it may abolish the binding of the enzyme to DNA. Such an active role of U(SO_3^-) in the inhibition has been also suggested in the poly(U)-directed polyphenylalanine synthesis[11].

The U(SO_3^-) residues introduced in DNA of organisms by the action of bisulfite would effectively inhibit the organism to replicate. Although there is little doubt that U(SO_3^-) residues cause mainly inactivation, the conclusion that they are not at all responsible for mutagenesis must await further studies: We recently made an unexpected observation that the reactivation of bisulfite-treated phage lambda by mild alkali was not accompanied by an increase of the mutation frequency (mutants/survivors)[1].

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