THE CLEAVAGE OF DNA BY THE OXYGEN-DEPENDENT REACTION OF BISULFITE

Hikoya Hayatsu and Robert C. Miller, Jr.

Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan, and Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, U. S. A.

Received October 18, 1971

Summary. Sodium bisulfite (10^{-2}M) reacts with DNA leading to the cleavage of internucleotide bonds as shown by alkaline sucrose density gradient centrifugation. This is an oxygen-dependent reaction occurring rapidly at pH 7 and room temperature in the presence of Mn^{2+} ions. The reaction can be inhibited by hydroquinone, a free radical scavenger. Bisulfite therefore possesses two modes of action toward nucleic acids: one is the ionic reactions with pyrimidine occurring at high concentrations, and another is the free radical reactions at low concentrations.

Bisulfite adds, at high concentrations (>1 M), across the 5,6-double bond of pyrimidine nucleosides to give the 5,6-dihydro-6-sulfonate derivatives (1-3). Such an addition compound formed from cytidine is readily hydrolyzed to the corresponding uridine derivative under mild conditions (1, 3, 4). This reaction has been used for the chemical mutagenesis of phage λ (5), E. coli (6), and phage T4 (7). The bisulfite-modification of pyrimidine bases has also been used for the studies of the structure-function relationship of transfer RNA (8, 9). On the other hand, bisulfite reacts, at low concentrations (10^{-2}M), with 4-thiouridine (10, 11), with isopentenyladenosine (8, 12) and with methionine (13, 14). Since these reactions are oxygen-dependent and can be blocked by a free radical scavenger, hydroquinone, they are assumed to involve free radical mechanisms. The free radical chain reaction that takes place in the autoxidation of bisulfite, forming the sulfite- and the hydrogen peroxide-radicals, is well documented (15). The sulfite radical is the reacting species in the reactions of 4-thiouridine and isopentenyladenosine,

whereas the hydrogen peroxide radical is probably acting in the formation of methionine sulfoxide from methionine. We now wish to report that a DNA chain can be readily cleaved by the oxygen-dependent reaction of bisulfite.

Materials and methods. [^{3}H]DNA of phage T7 was prepared by infecting E. coli B96 (Ade$^-$) with T7 phage in TCG medium (16) containing [^{3}H]adenine at a specific activity of 2 mc/mg. [^{32}P]DNA of phage T7 was prepared by infecting E. coli B23 with T7 phage in TCG containing i^{32}P at 2 mc/mg. Both phage preparations were purified by differential centrifugation and banded in CsCl density gradients. The labeled DNA was extracted by gentle rocking with an equal volume of phenol. The phenol layer was discarded and the aqueous phase was dialyzed exhaustively against 0.01 M Tris·HCl, pH 7.4–0.15 M NaCl–10^{-3}M EDTA at 4°. 10^{-1}M sodium bisulfite solution, pH 7, was freshly prepared before use by dissolving a 0.075 mole : 0.025 mole mixture of Na_2SO_3 and $NaHSO_3$ into de-ionized distilled water to the final volume of 100 ml. The reactions were run in open small tubes at 24°. The reaction was started by addition of 10 µl of the 10^{-1}M bisulfite solution into 90 µl of a [^{32}P]DNA solution (about 10,000 cpm) in sodium phosphate buffer, pH 7, containing $MnCl_2$. The final concentration of the phosphate buffer in the reaction mixture was 10^{-1}M; Mn^{2+}, 10^{-3}M; and EDTA, 10^{-4}M. The reaction was stopped by addition of 40 µl of 1.25 M NaOH–1.25 M NaCl–0.25 M EDTA solution. (In a separate experiment it has been observed that no change in the size of the DNA occurs by 30 min-treatment with the alkaline sulfite). [^{3}H]DNA reference was added and the solution was centrifuged in an alkaline sucrose density gradient (5-20 % sucrose supplemented with 0.2 M NaOH, 1 M NaCl, and 10^{-3}M EDTA). The gradients were centrifuged in a Beckman-Spinco SW 50.1 rotor at 35,000 rpm for 5 hrs. Droplets from a pinhole at the bottom of the tube were fractionated and counted for ^{3}H and ^{32}P on a scintillation spectrometer. In the treatment of DNA with 1 M bisulfite [^{32}P]DNA was allowed to stand for 10 min in a solution containing 10^{-1} M phosphate buffer, 1 M

Na_2SO_3-$NaHSO_3$(3:1), and 10^{-3}M Mn^{2+}. The reaction mixture then was diluted with an alkaline EDTA solution, and submitted to the centrifugation as before.

Results and discussion. It is known that in the presence of transition metal ions, such as Mn^{2+}, bisulfite undergoes a rapid autoxidation forming free radicals, presumably $\cdot SO_3^-$ and $\cdot HO_2$, during the oxidation (13). Double stranded [^{32}P] DNA of phage T7 (M. W. 2.6×10^7) was treated at room temperature with 10^{-2}M bisulfite at pH 7, in the presence of Mn^{2+} ions. As judged by a separate comparable experiment, no pH change must have occurred during this treatment. The resulting DNA was then analyzed by centrifugation in an alkaline sucrose density gradient. The centrifuge patterns given in Fig. 1-a show the time dependent decrease in the size of the DNA molecule. In the 30 min bisulfite-treated sample, the DNA molecules had been degraded into smaller molecules having average chain lengths $\leq 1/50$ of the original DNA. An intermediate stage can be seen for the 5 min sample. The 10 min sample gave a pattern similar to that of the 30 min sample. No

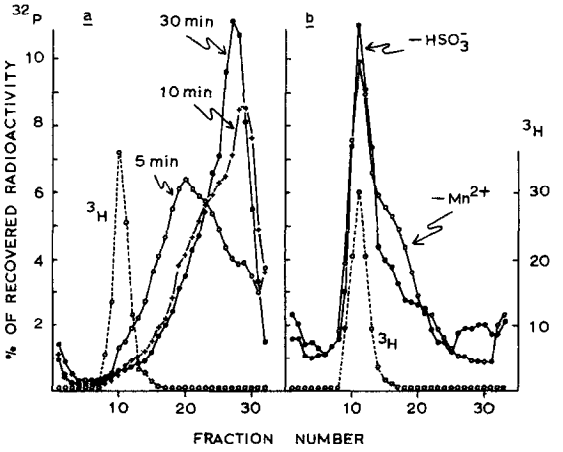

Fig. 1. Alkaline sucrose density gradient centrifugation of phage T7 DNA treated with bisulfite. (a) Each three reaction mixtures contained [^{32}P] DNA, 10^{-2} M bisulfite, 10^{-1} M phosphate buffer (pH 7), and 10^{-3} M Mn^{2+}. Incubation was at 24° for the period indicated. (b) Control experiments omitting bisulfite or Mn^{2+} from the reaction mixture. Incubation was for 30 min.

significant breakdown of the DNA was detected in the absence of bisulfite or in the absence of Mn^{2+} ions (Fig. 1-b). These results strongly suggest that the agents responsible for the chain cleavage of the DNA involve free radicals generated by the autoxidation of bisulfite. This view was supported by the fact that the cleavage was much less extensive either under the nitrogen atmosphere or in the presence of 5×10^{-3} M hydroquinone.

When the DNA was treated with 1 M bisulfite, instead of 10^{-2}M bisulfite, little degradation of the DNA was obtained, whereas with 10^{-3}M bisulfite a degradation similar to that with 10^{-2}M bisulfite was again observed. Thus, at high concentrations, bisulfite undergoes ionic reactions with pyrimidine bases, while at low concentrations, it brings about free radical reactions leading to the cleavage of the phosphodiester bonds of DNA. This feature of bisulfite is analogous to that of hydroxylamine (17,18), another cytosine-specific chemical mutagen. The inhibition of the reaction in concentrated bisulfite solutions has an analogy also in the oxygen-dependent reaction between 4-thiouridine and bisulfite, where the optimal concentration of bisulfite is about 10^{-2}M. This phenomenon has been explained, in analogy to the case of hydroxylamine, in terms of a competition between the substrate and the bisulfite molecules to react with the active free radicals (11). The same argument may be applied to the oxygen-dependent reaction between bisulfite and DNA. It is to be investigated whether the free radicals generated from bisulfite and oxygen cleave the internucleotide bonds directly, or indirectly by removing the bases. It is possible that the alkali-treatment in the centrifugation step could have led to the cleavage after such indirect action of radicals. It is known that the Mn^{2+}-oxygen-sulfite system is far more efficient than H_2O_2 for the oxidation of methionine (13). It is therefore possible that the nature of the internucleotide bond cleavage caused by Mn^{2+}-oxygen-sulfite is different from that by H_2O_2(19), and hence that by oxygen-hydroxylamine (20) as well. The cleavage of DNA by the oxygen-dependent reaction of bisulfite may be correlated to the inactivation of a transforming activity of B. subtilis DNA

at low concentrations of bisulfite (M. Inoue, H. Hayatsu, and H. Tanooka, unpublished results). Finally, the possibility should be pointed out that the environmental bisulfite, at its low concentrations, may cause breakage of DNA in the living organisms.

Acknowledgement. We thank Dr. H. G. Khorana of Massachusetts Institute of Technology and Dr. T. Ukita of the University of Tokyo for their giving us the opportunity of carrying out this research.

References:

1. Shapiro, R., Servis, R. E. and Welcher, M., J. Amer. Chem. Soc., 92, 422 (1970).
2. Hayatsu, H., Wataya, Y. and Kai, K., J. Amer. Chem. Soc., 92, 724 (1970).
3. Hayatsu, H., Wataya, Y., Kai, K. and Iida, S., Biochemistry, 9, 2858 (1970).
4. Hayatsu, H. and Sono, M., Chem. Commun., in press.
5. Hayatsu, H. and Miura, A., Biochem. Biophys. Res. Commun., 39, 156 (1970).
6. Mukai, F., Hawryluk, I. and Shapiro, R., Biochem. Biophys. Res. Comm 39, 983 (1970).
7. Summers, G. A. and Drake, J. W., News letter of the Environmental Mutagen Society, No. 4, 31 (1971).
8. Furuichi, Y., Wataya, Y., Hayatsu, H. and Ukita, T., Biochem. Biophys. Res. Commun., 41, 1185 (1970).
9. Kućan, Z., Freude, K. A., Kućan, I. and Chambers, R. W., Nature New Biology, 232, 177 (1971).
10. Hayatsu, H., J. Amer. Chem. Soc., 91, 5693 (1969).
11. Hayatsu, H. and Inoue, M., J. Amer. Chem. Soc., 93, 2101 (1971).
12. Hayatsu, H., Wataya, Y., Furuichi, Y. and Kawazoe, Y., Abstract of XXIIIrd International Congress of Pure and Applied Chemistry, Boston, O-C-3 (1971).
13. Yang, S. F., Biochemistry, 9, 5008 (1970).
14. Inoue, M. and Hayatsu, H., Chem. Pharm. Bull. (Tokyo), 19, 1286 (1971).
15. Abel. E., Monatsh. Chem., 82, 815 (1951).
16. Kozinski, A. W. and Szybalski, W., Virology, 9, 260 (1959).
17. Freese, E. and Freese, E. B., Biochemistry, 4, 2419 (1965).
18. Freese, E., Freese, E. B. and Graham, S., Biochim. Biophys. Acta, 123, 17 (1966).
19. Schweitz, H. and Luzzati, D., J. Chim. Phys., 60, 1173 (1963).
20. Bendich, A., Borenfreund, E., Korngold, G. C., Krim, M. and Balis, M. E. (1964), as quoted in ref. 18.