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The 5,6-Double Bond of Pyrimidine Nucleosides, a Fragile Site in Nucleic Acids

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Our studies have revealed reagents that can attack the 5,6-double bond of pyrimidine nucleosides; potassium permanganate and bisulfite. This review is a personal account of these studies, with a discussion on the vulnerable nature of this particular double bond to external nucleophiles and oxidizing agents. The finding that N(4)aminocytidine, produced on treatment of cytidine with bisulfite and hydrazine, is a strong mutagen is also described.

Key words: N(4)aminocytidine, bisulfite, permanganate, pyrimidine nucleotides.

A current issue in the chemistry of nucleic acids is elucidation of base modifications in relation to mutation. Since mutations are rare events, most of the base modifications being dealt with are ones occurring only slowly or rarely. Mutations can arise through deamination of DNA bases, and depurination and depyrimidination of DNA (*1*). 8-Oxoguanine, which may be formed in DNA through the actions of active oxygen species, is known to induce transversion mutations (*2, 3*). These reactions, which probably contribute to spontaneously occurring mutations, take place only slowly. For example, cytidine is deaminated on treatment at 97°C for 21 h in a neutral aqueous solution only to the extent of 2% (*4*). Deoxyguanosine in a denatured DNA is oxidized to 0.16% 8-oxodeoxyguanosine on treatment with 14 mM ascorbate for 3 h at 37°C and pH 6.8 (*5*). These slow changes of base molecules are important because of their biological consequences. However, from an ordinary sense in chemistry, the bases in DNA and RNA may be regarded as being quite stable. This stability is a necessity for living organisms to preserve genetic information in the form of nucleotide alignment within DNA and RNA.

There are reagents that can attack nucleic acid bases rapidly, either at the polynucleotide- or base-level. Examples are methylating agents, *e.g.*, dimethylsulfate and methyl methanesulfonate. Chemical methylation by these reagents takes place at a high velocity at the nitrogen and oxygen atoms in the purine and pyrimidine rings (*1*). These electrophilic methylating agents attack these electron-rich sites. It is now known that the 5,6-double bonds of pyrimidine bases in nucleic acids are susceptible to nucleophilic agents, the attack of the agents occurring at position 6 to give 5,6-dihydropyrimidine type products (*1, 6, 7*). This type of reaction takes place not only with chemical reagents but also with certain enzyme proteins, *e.g.*, thymidylate synthase (*8*). Since the attack of a nucleophile should in theory occur perpendicularly from above or below the 5,6-double bond and the nucleophile has some size, steric constraints may govern the speed of the addition. Indeed, these nucleophilic agents react preferentially with loosely structured portions of polynucleotides, *e.g.*, pyrimidines in single-stranded polynucleotides can react with bisulfite but those in double-stranded polymers cannot (see below) (*9, 10*).

Permanganate oxidizes carbon-carbon double bonds by forming either parallel or perpendicular electron overlaps with the double bonds (*11*). The 5,6-double bond of pyrimidine nucleobases, particularly that of thymine, is susceptible to oxidation by permanganate (see below) (*12*).

Thus, the 5,6-double bond of pyrimidine bases may be viewed as one of the fragile sites in nucleic acids.

During the past two and a half decades, we have discovered several typical examples of such a reaction at the 5,6-double bond of pyrimidine nucleosides, and have explored the scope of them (*9, 13*). In this review, I would like to describe these reactions and our exploration of their use.

Permanganate

A commonly used oxidizing agent, potassium permanganate ($KMnO_4$), is very soluble in water and deeply violet-colored. In the early 1960s, studies on the chemistry of extensive degradation of nucleosides with permanganate were carried out by British scientists (*14*). Searching for a base-selective chemical reaction, I observed in 1967 that a dilute $KMnO_4$ solution is decolorized by pyrimidine nucleotides but not by purine nucleotides. When individual nucleotides were treated with excess $KMnO_4$, thymine nucleotide was degraded most rapidly among the pyrimidines, the rate of degradation being in the order, dpT > pU ≫ dpC ≫ dpG, dpA. The reaction took place rapidly with heat-denatured DNA but not with native DNA (Fig. 1) (*12*). These results indicated that permanganate may be used as a probe for thymines in the single-stranded portions of polynucleotides. Investigation of the chemistry of the permanganate reaction with thymine and thymidine revealed that the major products were *cis*-thymine glycol and its nucleoside (1 in Fig. 2), and that the other products were 5-hydroxy-5-methylbarbituric acid and its nucleoside (2) (*15, 16*).

Since we reported these fundamental features of the modification of nucleic acids by permanganate, it has been applied to biochemical studies in many laboratories. One of the standard protocols of Maxam-Gilbert nucleotide sequencing involves permanganate oxidation to cause specific chain cleavage at thymine nucleotide residues (*17*). A

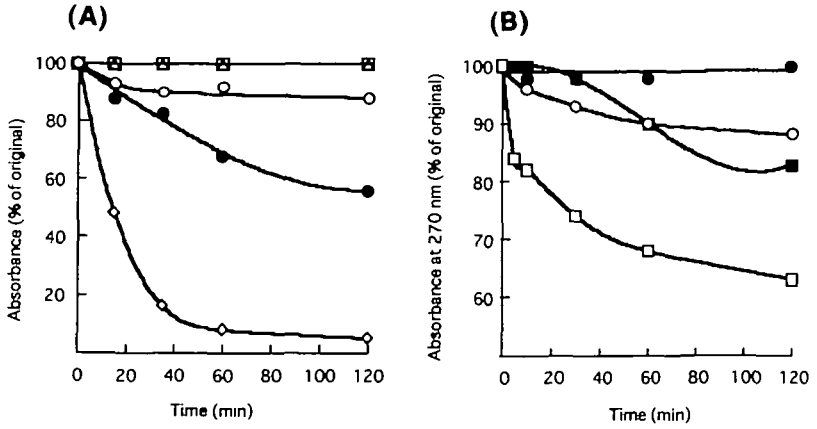

Fig. 1. **Thymine- and single strand-selective modification of DNA with permanganate.** (A) The oxidation of mononucleotides with 0.4 mM $KMnO_4$, and (B) the permanganate oxidation of DNA. (A) The 50-ml reaction mixture contained 2.5 mmol of nucleotide, 0.2 M phosphate buffer, pH 6.7, and 20 mmol of $KMnO_4$. The reaction was performed at 0°C. ◇, thymidine 5′-phosphate (267 nm); ●, uridine 5′phosphate (270 nm); ○, deoxycytidine 5′-phosphate (270 nm); △, deoxyguanosine 5′-phosphate (260 nm); □, deoxyadenosine 5′-phosphate (260 nm). (B) ●, native DNA in 0.8 mM $KMnO_4$; ○, heat-denatured DNA in 0.8 mM $KMnO_4$; ■, native DNA in 26.4 mM $KMnO_4$; □, heat-denatured DNA in 26.4 mM $KMnO_4$.

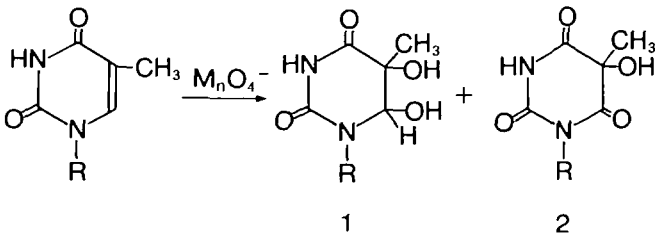

Fig. 2. **Permanganate oxidation of thymine (R=H) and thymidine (R=deoxyribose).**

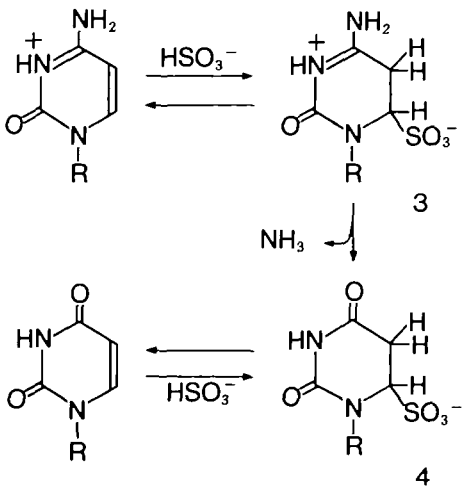

Fig. 3. **Bisulfite-mediated deamination of cytidine.**

variation of this protocol is simultaneous degradation of 5-methylcytosine and thymine by permanganate, a procedure which allows positive band assignment for 5-methylcytosine residues in DNA (*18*).

Thymine-specific permanganate degradation is widely used for probing protein nucleic-acid interactions (*19-22*). A protein contact with a particular nucleotide sequence will result in "protection" of the thymines in that sequence. For example, this principle was used to elucidate the interaction of a transcription start site of DNA with an RNA polymerase (*22*). Treatment of naked DNA samples with permanganate allowed the detection of loose areas in the B-Z and Z-Z junctions of DNA (*23*). Also, a triple-helix DNA conformation was investigated by permanganate probing (*24*).

A class of spontaneously occurring DNA damage in the human body comprises oxidative reactions due to active oxygen radicals, and the products include thymine glycol (*25*). In these studies, the permanganate oxidation of thymine has been useful in providing a standard sample of *cis*-thymine glycol.

It is noteworthy that, although 8-oxoguanine can be formed under various oxidative stresses (*26*), permanganate is inert to guanine, therefore 8-oxoguanine is not produced on the treatment of DNA or guanine nucleosides with $KMnO_4$ (*27*).

As discussed above, permanganate can react with the pyrimidine ring of common nucleotides. Easy oxidation cannot occur with the extra-ring residues, *i.e.*, the amino- and methyl-residues of nucleobases. However, with some "minor" nucleosides, the situation is different. We reported extensive studies on the permanganate oxidation of 4-thiouridine; uridine 4-sulfonate is formed very rapidly, without oxidation of the pyrimidine ring (*28*). The 4-sulfonate derivative is a useful compound, because the sulfonate residue can be replaced readily with various nucleophilic reagents, *e.g.*, amino compounds and imidazole. One such application is the production of a fluorescent derivative, which may be expected to allow sensitive detection of 4-thiouracil nucleosides (*29*).

Bisulfite

The sulfur atom of the bisulfite ion (HSO_3^-) is strongly nucleophilic, and therefore may be expected to add to the 5,6-double bond of uracil, thymine, and cytosine. We luckily discovered that this addition can indeed take place with remarkable ease (*9, 30, 31*). We knew, as mentioned above, that the permanganate oxidation of 4-thiouridine yields uridine-4-sulfonate. We then wanted to determine the kinetics of this reaction. To achieve this, it was necessary to stop the reaction as desired, by degrading the permanganate. When sodium bisulfite was added to the reaction mixture for this purpose, bisulfite itself reacted with 4-thiouridine. This bisulfite-thiouridine interaction required a supply of oxygen for its progress. To our surprise, the reaction product turned out to be uridine-4-sulfonate, the same as that of the permanganate oxidation (*32*). Having become aware that bisulfite is an agent of

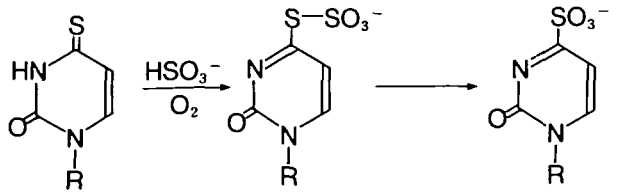

Fig. 4. **Mechanism of 4-thiouridine-bisulfite interactions.**

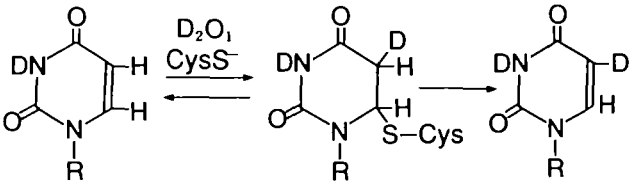

Fig. 6. **Cysteine-catalyzed hydrogen isotope exchange in uridine.**

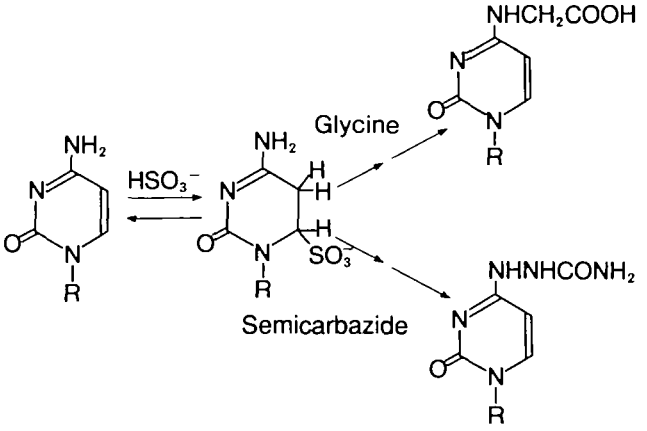

Fig. 5. **Bisulfite-mediated transamination of cytidine.**

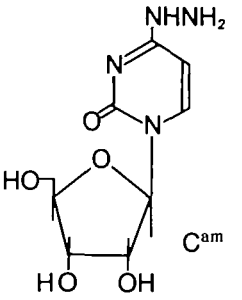

Fig. 7. **N(4)Aminocytidine.**

extreme interest, we began to wonder whether or not it might react with common nucleosides. To make a long story short, the reactions of bisulfite with pyrimidine nucleosides are summarized in Fig. 3 (*9, 30, 31, 33*). In Fig. 4, a scheme for the reaction of bisulfite with 4-thiouridine (*34*) is shown.

Both cytidine and uridine undergo reversible addition of HSO_3^- at the 5,6-double bond, yielding 5,6-dihydro-6-sulfonate derivatives **3** and **4**. Uridine-bisulfite adduct **4** is stable in a neutral aqueous solution, but reverts to uridine under slightly alkaline conditions (pH >9). Cytidine-bisulfite adduct **3** has a remarkable property; in this adduct the amino group at position 4 is susceptible to attack by nucleophiles including OH^- and certain amino compounds (*35*). The result is deamination to **4** or, with amino compounds, transaminated derivatives such as those in Fig. 5 (*36, 37*). The deamination by water molecules, as shown in Fig. 3, may be viewed as a means of converting cytosine to uracil. It is noteworthy that bisulfite does not interact with adenine or guanine. Thymine, like uracil, gives a bisulfite addition compound in a reversible manner, but the adduct is unstable at neutral pH, thymine being readily regenerated (*38, 39*). The presence of the 5-methyl moiety obviously decreases the ease of bisulfite addition to position 6, presumably due to an electron donating effect of the methyl group. Consistent with this concept, 5-methylcytosine is deaminated by bisulfite much more slowly than cytosine (*38*).

Thus, bisulfite is an agent with which cytosine-specific deamination in DNA and RNA can be achieved. The deamination occurs at pH 5-6, and with subsequent alkaline treatment the conversion of C to U can be completed.

Again, the conformation of nucleic acids influences the reactivity of a reagent. The HSO_3^- ion must position itself above or below the plane of the pyrimidine ring in order to make the addition to the 5,6-double bond possible. Thus, the reaction occurs readily with heat-denatured DNA but only very slowly with native double-stranded DNA (*9, 40*).

We discovered the reactions illustrated in Fig. 3 in 1969, and our report was published at the beginning of 1970 (*30*). Shapiro and his associates at the New York University published almost simultaneously a paper reporting similar observations (*41*). Their discovery was made in a systematic search for catalysts that hydrolyze cytosine.

It is noteworthy that the addition of bisulfite at position 6 of pyrimidine rings also results in labilization of the hydrogen at position 5: an accelerated hydrogen isotope exchange has been observed for cytosine and uracil through the action of bisulfite (*33, 42, 43*). This observation lead to the finding of a similar action of cysteinyl SH, which catalyzes the hydrogen isotope exchange at position 5 (see below).

Other than 4-thiouracil, some minor bases in nucleic acids can react with bisulfite: 5-hydroxymethylpyrimidines (*44*) and N(6)isopentenyladenine (*45*).

By virtue of the single-strand specific nature and high selectivity, bisulfite-mediated cytosine deamination has been used as a standard method for site-specific mutagenesis (*46*). An interesting application is seen in the use of different deamination rates between cytosine and 5-methylcytosine (*38*) for prescribing a protocol for sequencing 5-methylcytosine residues in genomic DNA (*47*).

Attack by sulfhydryl at position 6 of pyrimidines

Analogous to the sulfur atom in bisulfite, sulfhydryl sulfur is expected to add to the 5,6-double bond of pyrimidine nucleosides. Indeed, we have observed cysteine-catalyzed isotope exchange at position 5 of uridine 5′-phosphate (*48*) (Fig. 6). Also, cysteine and glutathione can cause debromination of 5-bromo-2′-deoxyuridine (*49*). These reactions presumably involve an initial attack by the SH at position 6 of the pyrimidine ring.

Mutagenic nature of modified pyrimidines

At the time when we were exploring the kind of nitrogen nucleophiles that can replace the 4-amino group of bisulfite adduct **3** (Fig. 3), we were engaged in mutation studies involving the *Salmonella typhimurium* TA strains that were developed earlier by B.N. Ames, University of California, Berkeley, as sensitive tester bacteria for mutagens (*50*). We then found that N(4)aminocytidine (C^{am}) (Fig. 7), a product formed through transamination of cytidine with hydrazine plus bisulfite, was an extremely strong mutagen in *S. typhimurium* TA1535, a tester strain for detecting base pair substitutions (*51*). For this mutagenic activity, metabolic transformation of the compound by externally added mammalian microsomal enzymes was unnecessary; N(4)aminocytidine as such caused mutation in the bacteria. Subsequent work has shown that N(4)-aminocytidine is mutagenic in mammalian cells (*52, 53*) and *Drosophila* as well (*54*), and that the types of mutation induced are base pair transitions, AT-to-GC and GC-to-AT (*55*). We have studied the mechanism of this mutagenesis to a certain depth. The results of these studies may be summarized as follows. (i) N(4)Aminocytidine is metabolized in cells to $dC^{am}TP$, which is then incorporated into DNA during proliferation of the organism (*56*), (ii) in this process $dC^{am}TP$ can be used by the organism as a substitute of either dCTP or dTTP (*57*), and (iii) once incorporated into DNA, the C^{am} base in the DNA serves as a template for the incoming dGTP and dATP (*57, 58*), thus leading to the transitions, AT-to-GC and GC-to-AT (*59*).

What, then, will be the behavior of a bisulfite adduct of type **3**, if such an adduct is formed in DNA? Using a stable adduct formed from N(4)aminocytosine and bisulfite, we have explored this problem, and it was shown in *in vitro* experiments with *Escherichia coli* DNA polymerase that this adduct in a DNA template is a strong block for elongation of the DNA chain being synthesized by the polymerase (*60*).

Prospects

As described above, we have identified two agents, permanganate and bisulfite, that can attack the 5,6-double bond of pyrimidine bases of nucleic acids when that part of the polynucleotide is sterically exposed. Osmium tetroxide, like permanganate, oxidizes the 5,6-double bond (*61*). Hydrazine, hydroxylamine, and methoxyamine have long been known to attack the position 6 to give, like bisulfite, 5,6-dihydropyrimidine type adducts (*7*).

We may pose the question; Has the existence of this **fragile** site, 5,6-double bond of pyrimidines, in nucleic acids any biological significance? At least two enzymes use this property for their actions. Thymidylate synthase catalyzes methylation at position 5 of 2-deoxyuridine 5′-phosphate through the formation of a covalent bond intermediate in which the SH of a cysteinyl residue of the enzyme is linked to position 6 of the uracil ring (*8*). DNA cytosine-5-methyltransferases act on DNA to carry out methylation at position 5 of certain cytosine residues in the polynucleotide. Again, activation of position 5 as to electrophilic attack by a carbo-cation is mediated by transient covalent bond formation between a cysteinyl SH in the enzyme molecule and the carbon at position 6 of the cytosine ring (*62*). Thus, organisms can utilize the susceptibility of the 5,6-double bond of pyrimidines to create carbon-carbon linkages at position 5.

My supposition is that there could be more examples of this class of biological reactions yet to be discovered in nature.

I would like to express my thanks to the late Professor E. Ochiai of the University of Tokyo for encouraging me, when I started as a researcher, to shift my studies to bioorganic chemistry from pure organic chemistry. A fascinating world of nucleic acid chemistry was introduced to me by the late Professor T. Ukita of the University of Tokyo, to whom I am very grateful. My way of thinking about science was molded by Professor H.G. Khorana of the University of Wisconsin (now at the Massachussetts Institute of Technology), to whom I express my deep gratitude.

The work described in this review was the culmination of collaboration with many young enthusiastic scientists. Some of these scientists' names are among those cited in the reference section. I owe these collaborators very much for the accomplishment of the work.

REFERENCES

1. Singer, B. and Grunberger, D. (1983) *Molecular Biology of Mutagens & Carcinogens*, Plenum Press, New York
2. Shibutani, S., Takeshita, M., and Grollman, A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* **349**, 431-434
3. Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., and Loeb, L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G→T and A→C substitutions. *J. Biol. Chem.* **267**, 166-172
4. Shapiro, R. and Klein, R.S. (1966) The deamination of cytidine and cytosine by acidic buffer solutions. Mutagenic implications. *Biochemistry* **5**, 2358-2362
5. Kasai, H. and Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res.* **12**, 2137-2145
6. Kochetkov, N.K., and Budovskii, E.I. (1972) Substitutions and additions in the heterocyclic rings of nucleic acid bases and their derivatives in *Organic Chemistry of Nucleic Acids, Part B*, pp. 269-348, Plenum Press, New York
7. Brown, D.M. (1974) Chemical reactions of polynucleotides and nucleic acids in *Basic Principles in Nucleic Acids Chemistry* (Ts'o, P.O.P., ed.) Vol. II, pp. 2-90, Academic Press, London
8. Carreras, C.W. and Santi, D.V. (1995) The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **64**, 721-762
9. Hayatsu, H. (1976) Bisulfite modification of nucleic acids and their constituents in *Progress in Nucleic Acid Research and Molecular Biology* (Cohn, W.E., ed.) Vol. 16, pp. 75-124, Academic Press, New York
10. Shapiro, R., Braverman, B., Louis, J.B., and Servis, R.E. (1973) Nucleic acid reactivity and conformation. *J. Biol. Chem.* **248**, 4060-4064
11. Freeman, F., Fuselier, C.O., Armstead, C.R., Dalton, E., Davidson, P.A., Karchesfski, E.M., Krochaman, D.E., Johnson, M.N., and Jones, N.K. (1981) Permanganate ion oxidations. 13. Soluble manganese (IV) species in the oxidation of 2,4(1*H*,3*H*)-pyrimidinediones (uracils). *J. Am. Chem. Soc.* **103**, 1154-1159
12. Hayatsu, H. and Ukita, T. (1967) The selective degradation of pyrimidines in nucleic acids by permanganate oxidation. *Biochem. Biophys. Res. Commun.* **29**, 556-561
13. Hayatsu, H. (1993) Chemical modification of nucleic acids and the mutagenicity of modified bases, with special reference to permanganate- and bisulfite-mediated modifications (in Japanese). *Yakugaku Zasshi* **113**, 19-31
14. Jones, A.S. and Walker, R.T. (1963) The permanganate oxidation of nucleosides. *J. Chem. Soc.* 3554-3557
15. Iida, S. and Hayatsu, H. (1970) The permanganate oxidation of thymine. *Biochim. Biophys. Acta* **213**, 1-13
16. Iida, S. and Hayatsu, H. (1971) The permanganate oxidation of thymidine and thymidylic acid. *Biochim. Biophys. Acta* **228**, 1-8
17. Rubin, C.M. and Schmid, C.W. (1980) Pyrimidine-specific chemical reactions useful for DNA sequencing. *Nucleic Acids Res.* **8**, 4613-4619
18. Fritzsche, E., Hayatsu, H., Igloi, G.L., Iida, S., and Kössel, H. (1987) The use of permanganate as a sequencing reagent for

identification of 5-methylcytosine residues in DNA. *Nucleic Acids Res.* **15**, 5517-5528
19. Kassavetis, G.A., Blanco, J.A., Johnson, T.E., and Geiduschek, E.P. (1992) Formation of open and elongating transcription complexes by RNA polymerase III. *J. Mol. Biol.* **226**, 47-58
20. Panigrahi, G.B., Beatty, L.G., and Sadowski, P.D. (1992) The FLP protein contacts both major and minor grooves of its recognition target sequence. *Nucleic Acids Res.* **20**, 5927-5935
21. Lee, D.N. and Landick, R. (1992) Structure of RNA and DNA chains in paused transcription complexes containing *Escherichia coli* RNA polymerase. *J. Mol. Biol.* **228**, 759-777
22. Suh, W.-C., Ross, W., and Record, M.T., Jr. (1993) Two open complexes and a requirement for Mg^{2+} to open the λP_R transcription start site. *Science* **259**, 358-361
23. Jiang, H., Zacharias, W., and Amirhaeri, S. (1991) Potassium permanganate as an in situ probe for B-Z and Z-Z junctions. *Nucleic Acids Res.* **19**, 6943-6948
24. Häner, R. and Dervan, P.B. (1990) Single-strand DNA triple-helix formation. *Biochemistry* **29**, 9761-9765
25. Adelman, R., Saul, R.L., and Ames, B.N. (1988) Oxidative damage to DNA: Relation to species metabolic rate and life span. *Proc. Natl. Acad. Sci. USA* **85**, 2706-2708
26. Kasai, H. and Nishimura, S. (1993) Formation of 8-hydroxyguanine by oxidative DNA damage, its repair and its mutagenic effects in *Advances in Mutagenesis Research* (Obe, G., ed.) Vol. 4, pp. 31-45, Springer-Verlag, Berlin
27. Nawamura, T., Negishi, K., and Hayatsu, H. (1994) 8-Hydroxyguanine is not produced by permanganate oxidation of DNA. *Arch. Biochem. Biophys.* **311**, 523-524
28. Yano, M. and Hayatsu, H. (1970) Permanganate oxidation of 4-thiouracil derivatives. Isolation and properties of 1-substituted 2-pyrimidone-4-sulfonates. *Biochim. Biophys. Acta* **199**, 303-315
29. Hayatsu, H., Nawamura, T., Kubo, T., Negishi, K., Gohda, R., Hamase, K., and Imai, K. (1994) A fluorescent derivative of 4-thiouridine. *Nucleic Acids Res. Symp. Ser.* **31**, 33-34
30. Hayatsu, H., Wataya, Y., and Kai, K. (1970) The addition of sodium bisulfite to uracil and to cytosine. *J. Am. Chem. Soc.* **92**, 724-726
31. Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. (1970) Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. *Biochemistry* **9**, 2858-2865
32. Hayatsu, H. (1969) The oxygen-catalyzed reaction between 4-thiouridine and sodium sulfite. *J. Am. Chem. Soc.* **91**, 5693-5694
33. Sono, M., Wataya, Y., and Hayatsu, H. (1973) Role of bisulfite in the deamination and the hydrogen isotope exchange of cytidylic acid. *J. Am. Chem. Soc.* **95**, 4745-4749
34. Hayatsu, H. and Inoue, M. (1971) The oxygen-mediated reaction between 4-thiouracil derivatives and bisulfite. Isolation and characterization of 1-methyluracil 4-thiosulfonate as an intermediate in the formation of 1-methyluracil-4-sulfonate. *J. Am. Chem. Soc.* **93**, 2301-2306
35. Shapiro, R. and Weisgras, J.M. (1970) Bisulfite-catalyzed transamination of cytosine and cytidine. *Biochem. Biophys. Res. Commun.* **40**, 839-843
36. Boni, I.V. and Budowsky, E.I. (1973) Transformation of noncovalent interactions in nucleoproteins into covalent bonds induced by nucleophilic reagents. *J. Biochem.* **73**, 821-830
37. Hayatsu, H. (1976) Reaction of cytidine with semicarbazide in the presence of bisulfite. A rapid modification specific for single-stranded polynucleotide. *Biochemistry* **15**, 2677-2682
38. Shiragami, M., Kudo, I., Iida, S., and Hayatsu, H. (1975) Formation of diastereomers of 5,6-dihydrothymine-6-sulfonate by deamination of 5-methylcytosine with bisulfite. *Chem. Pharm. Bull.* **23**, 3027-3029
39. Itadani, A., Shiragami, M., Kawada, R., Wataya, Y., and Hayatsu, H. (1981) Sulphite-ion-catalysed reversion of 5,6-dihydrothymine-6-sulphonate to thymine. A general-base-catalysed reaction. *J. Chem. Soc.* 1287-1292
40. Shapiro, R. (1977) Genetic effects of bisulfite (sulfur dioxide). *Mutation Res.* **39**, 149-176
41. Shapiro, R., Servis, R.E., and Welcher, M. (1970) Reactions of uracil and cytosine derivatives with sodium bisulfite. A specific deamination method. *J. Am. Chem. Soc.* **92**, 422-424
42. Kai, K., Wataya, Y., and Hayatsu, H. (1971) Bisulfite-catalyzed isotope labeling of cytidine 5′-phosphate at its 5 position. *J. Am. Chem. Soc.* **93**, 2089-2090
43. Iida, S., Wataya, Y., Kudo, I., Kai, K., and Hayatsu, H. (1974) Bisulfite-catalyzed tritium labeling of DNA and RNA. *FEBS Lett.* **39**, 263-266
44. Hayatsu, H. and Shiragami, M. (1979) Reaction of bisulfite with the 5-hydroxymethyl group in pyrimidines and in phage DNAs. *Biochemistry* **18**, 632
45. Furuichi, Y., Wataya, Y., Hayatsu, H., and Ukita, T. (1970) Chemical modification of $tRNA^{Tyr}$ yeast with bisulfite. A new method to modify isopentenyladenosine residue. *Biochem. Biophys. Res. Commun.* **41**, 1185-1191
46. Watson, J.D., Tooze, J., and Kurtz, D.T. (1983) In vitro mutagenesis in *Recombinant DNA* pp. 106-116, W.H. Freeman and Company, New York
47. Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827-1831
48. Wataya, Y., Hayatsu, H., and Kawazoe, Y. (1972) Cysteine-catalyzed hydrogen isotope exchange at the 5 position of uridylic acid. *J. Am. Chem. Soc.* **94**, 8927-8928
49. Wataya, Y., Negishi, K., and Hayatsu, H. (1973) Debromination of 5-bromo-2′-deoxyuridine by cysteine. Formation of deoxyuridine and *S*-[5-(2′-deoxyuridyl)]cysteine. *Biochemistry* **12**, 3992-3998
50. McCann, J., Choi, E., Yamasaki, E., and Ames, B.N. (1975) Detection of carcinogens as mutagens in the *Salmonella*/microsome test: Assay of 300 chemicals. *Proc. Natl. Acad. Sci. USA* **72**, 5135-5139
51. Negishi, K., Harada, C., Ohara, Y., Oohara, K., Nitta, N., and Hayatsu, H. (1983) N^4-Aminocytidine, a nucleoside analog that has an exceptionally high mutagenic activity. *Nucleic Acids Res.* **11**, 5223-5233
52. Nomura, A., Negishi, K., Hayatsu, H., and Kuroda, Y. (1987) Mutagenicity of N^4-aminocytidine and its derivatives in Chinese hamster lung V79 cells. *Mutation Res.* **177**, 283-287
53. Takahashi, M., Nishizawa, M., Negishi, K., Hanaoka, F., Yamada, M., and Hayatsu, H. (1988) Induction of mutation in mouse FM3A cells by N^4-aminocytidine-mediated replicational errors. *Mol. Cell. Biol.* **8**, 347-352
54. Negishi, T., Negishi, K., Ryo, H., Kondo, S., and Hayatsu, H. (1988) The genotoxicity of N^4-aminocytidine in the *Drosophila* wing spot test. *Mutagenesis* **3**, 11-13
55. Bessho, T., Matsumoto, K., Nomura, A., Hayatsu, H., and Negishi, K. (1989) Spectrum of N^4-aminocytidine mutagenesis. *J. Mol. Biol.* **205**, 659-664
56. Negishi, K., Tamanoi, K., Ishii, M., Kawakami, M., Yamashita, Y., and Hayatsu, H. (1988) Mutagenic nucleoside analog, N^4-aminocytidine: Metabolism, incorporation into DNA, and mutagenesis in *Escherichia coli*. *J. Bacteriol.* **170**, 5257-5262
57. Negishi, K., Takahashi, M., Yamashita, Y., Nishizawa, M., and Hayatsu, H. (1985) Mutagenesis by N^4-aminocytidine: Induction of AT to GC transition and its molecular mechanism. *Biochemistry* **24**, 7273-7278
58. Takahashi, M., Negishi, K., and Hayatsu, H. (1987) Proofreading of a mutagenic nucleotide, N^4-aminodeoxycytidylic acid, by *Escherichia coli* DNA polymerase I. *Biochem. Biophys. Res. Commun.* **27**, 104-109
59. Negishi, K., Bessho, T., and Hayatsu, H. (1994) Nucleoside and nucleobase analog mutagens. *Mutation Res.* **318**, 227-238
60. Bessho, T., Nitta, N., Negishi, K., and Hayatsu, H. (1992) Blockage of polymerase-catalyzed DNA chain elongation by chemically modified cytosine residues in templates and the release of blockage for readthrough. *Nucleic Acids Res.* **20**, 4213-4220
61. Nielsen, P.E. (1990) Chemical and photochemical probing of DNA complexes. *J. Mol. Recogn.* **3**, 1-25
62. Verdine, G.L. (1994) The flip side of DNA methylation. *Cell* **76**, 197-200