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REACTIONS OF BISULFITE, AN ENVIRONMENTAL CHEMICAL, WITH NUCLEIC ACIDS AND OTHER BIOLOGICAL SUBSTANCES

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Abstract - The reactions of bisulfite in aqueous solutions with nucleic acids and their constituents are briefly reviewed. Co-operative actions of bisulfite and certain amines, i.e. semicarbazide, hydrazine, hydroxylamine and methoxyamine, in inducing mutations in bacteriophage lambda are described. Rapid inactivations of lambda during aerobic oxidation of bisulfite was found to be a result of damages in the phage-coat proteins caused by the free radicals generated from bisulfite and oxygen.

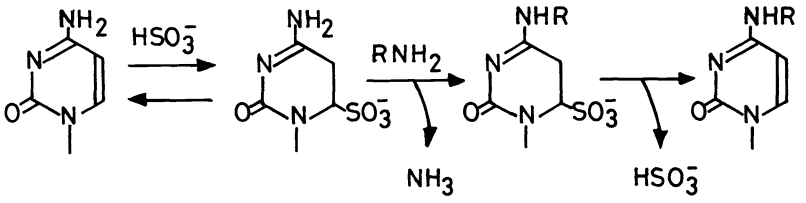
Studies on the interactions of environmental chemicals with biological substances, both in vitro and in vivo, are expected to reveal potentially hazardous nature of such chemicals. We have for several years investigated the reactions, in aqueous solutions, of bisulfite with biological materials, mainly nucleic acids and their constituents. Bisulfite is being used as a food additive and as a drug-preservative. Bisulfite ion is formed from sulfur dioxide in aqueous solution of near of neutrality.

Earlier studies may be summarized as follows. 1. The first of our observations on the reaction between bisulfite and nucleic acid constituents was the facile modification of 4-thiouridine, a minor component of transfer RNA, by bisulfite in the presence of air (1). This modification resulted in the formation of uridine-4-sulfonate. In this process, the reactive species was the sulfite ion radical which was generated during oxidation of bisulfite with the air (2). 2. Treatment of cytosine-nucleotides with bisulfite easily gave nucleotides bearing the 5,6-dihydrouracil-6-sulfonate moiety, which in turn can be converted to uracil-nucleotides under mild conditions (3). 3. This finding has suggested that bisulfite is a mutagen, and the mutagenic activity of bisulfite was actually demonstrated using bacteriophage lambda (4). Shapiro and coworkers also discovered independently the reactivity of bisulfite with cytosine (5) and the mutagenic activity of bisulfite (6). Subsequent work of both our group (7) and Shapiro's (8) has clarified the detailed mechanism of the bisulfite-mediated deamination of cytosine-nucleotide. 4. Free radicals generated by the aerobic oxidation of bisulfite (13) can bring about a variety of reactions with nucleic acids (9-12) as well as with amino acids (13-16). Double-stranded DNA is subject to attack of the radicals, resulting in cleavages of the polynucleotide backbone (9). Transforming activity of DNA is destroyed by the attack of the radicals (11). Amino acids methionine (13,14) and tryptophan (15,16) are easily modified by the radicals. These earlier findings were recently reviewed (17,18). In the following sections, current studies of our laboratory on the subject are described.

Co-operative mutagenic actions of bisulfite and nitrogen nucleophiles (19)

The cytosine-bisulfite adduct can undergo transaminations with various amines (20). The transamination is rapid when strongly nucleophilic amines are used. Thus, semicarbazide (21), hydrazine (22-24), hydroxylamine (19), and methoxyamine (19,25) can substitute the 4-amino group of the cytosine-bisulfite adduct very rapidly (Fig. 1), giving the type 1 compound in a quantitative manner (except for the case of hydrazine, where a dimeric compound is formed depending on the reaction conditions employed (23)). The semicarbazide- and hydrazine-substituted adducts (1a and 1b) can be converted to the pyrimidine derivatives (2a and 2b) by treatment with phosphate buffer, whereas the hydroxylamine- and methoxyamine-substituted adducts (1c and 1d) are stable in the buffer.

It can be asked whether modifications of genome DNA to produce the type 1 and 2 residues will cause mutations or not. If they do, it is expected that bisulfite and these nitrogen nucleophiles will show co-operative functions, i.e., the combinations of the two reagents



1

R =-NHCONH$_2$ (a)
=-NH$_2$ (b)
=-OH (c)
=-OCH$_3$ (d)

2

R =-NHCONH$_2$ (a)
=-NH$_2$ (b)

Fig. 1

will induce mutations much more efficiently than the individual reagents, bisulfite alone or the nucleophile alone.

A typical example of the co-operative action of the reagents for the chemical modification of cytosine-nucleotide is shown in Fig. 2. The rate of the modification by the combined action of 1 M sodium bisulfite and 1 M semicarbazide on cytidine at pH 5 and 37° is estimated at 20 times higher than the rate by 1 M sodium bisulfite alone (7) and more than 200 times higher than that by 1 M semicarbazide alone (26). Similar co-operations were found

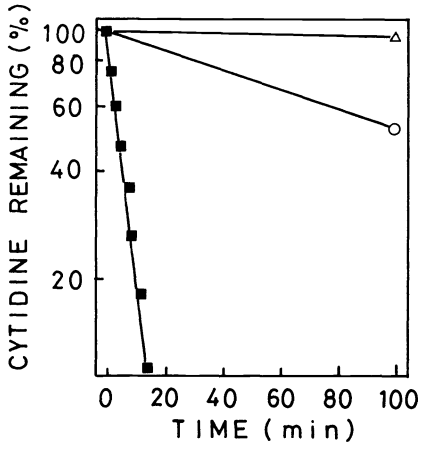


Fig. 2. Co-operative activity of bisulfite and semicarbazide in modification of cytidine. (■) 1 M Semicarbazide plus 1 M sodium bisulfite, (○) 1 M sodium bisulfite, and (△) 1 M semicarbazide. The incubation conditions were pH 5 and 37°.

for combinations of bisulfite with hydrazine, hydroxylamine, and methoxyamine.

As Table 1 shows, the combinations of the two reagents produced strong inactivations and mutation-inductions in bacteriophage lambda, whereas the individual agents produced only much smaller effects both in the inactivation and in the mutation.

Effect of treatment with phosphate buffer on the phages that had been inactivated by the action of the combined reagents was examined. As Table 2 shows, the phages were "re-activated" by the treatment with phosphate when they had been incubated with semicarbazide-bisulfite or with hydrazine-bisulfite. No re-activation, in contrast, was observed for the phages that had been incubated with hydroxylamine-bisulfite or with methoxyamine-bisulfite. Therefore the re-activations occur for those modifications in which type 1 product can be transformed into type 2 derivative but not for those in which 1 cannot be converted to 2. As Table 2 shows, the re-activations did not result in large changes in the mutation frequency.

These observations make it strongly likely that the phenomena observed for phages are real

TABLE 1. Inactivation and mutagenesis of bacteriophage lambda by co-operative actions of bisulfite and nucleophiles

Reagent[a]	pH	Time (min)	Surviving fraction[b]	Number of clear mutant per 10^4 survivors
Semicarbazide + Bisulfite	5	30	0.0034	112
Semicarbazide	5	30	0.57	7
Bisulfite	5	30	1.0	11
Hydrazine + Bisulfite	5	120	0.011	71
Hydrazine	5	120	0.75	5
Bisulfite	5	120	0.60	4
Hydroxylamine + Bisulfite	6	60	0.085	61
Hydroxylamine	6	60	0.63	12
Bisulfite	6	60	1.0	13
Methoxyamine + Bisulfite	5	15	0.0021	248
Methoxyamine	5	15	0.45	11
Untreated			1.0	5

a) Reagent concentrations were 1 M. Reaction temperature was 37°, except for the hydroxylamine-bisulfite reactions where it was 20°. In reactions where individual reagents only were used, 1 M sodium chloride was supplemented in order to normalize the salt concentrations.
b) Determined by plaque forming unit.

reflections of the chemical modifications taking place in the phage DNA. Therefore, it can be concluded that the generation of residues of type 1 and type 2 in DNA can result in mutations.

These results constitute the first clear example of co-operation of two reagents in inducing mutations. A feature in the co-operation is that the two reagents do not interact with each other but they sequentially react with DNA. This observation opens up the possibility that there might be still other examples of co-operations between reagents. Such possibilities should obviously be examined for environmental chemicals. In the case of bisulfite, it is clearly important to extend the study to investigations of possible co-operations with such amines that are abundant in the environment as well as in the human body.

Inactivation of bacteriophage lambda by the oxygen-dependent action of bisulfite (16)
Using again bacteriophage lambda as the substrate, we examined another aspect of bisulfite-reactivity, the free radical reactions. We found that the phage was rapidly inactivated by treatment with dilute, neutral solutions of bisulfite in the presence of air. The co-operative mutagenic action of bisulfite with amines takes place in acidic conditions as described above. Under neutral conditions, strong inactivations were observed for treatments with

TABLE 2. Effect of incubation with phosphate buffer on the bacteriophage lambda that had been pre-treated with the bisulfite-amines

Reagent[a]	Time of treatment (min)	Surviving fraction	Mutation frequency (10^{-4})	After incubation[b]	
				Survival	Mutation frequency (10^{-4})
Semicarbazide + Bisulfite	30	0.0034	112	0.10	100
Hydrazine + Bisulfite	120	0.011	71	0.04	31
Hydroxylamine + Bisulfite	60	0.085	61	0.085	50
Methoxyamine + Bisulfite	10	0.024	96	0.022	103

a) Reagent concentrations, temperature, and pH values for each treatment are given in Table 1.
b) 1 M Sodium phosphate, pH 7 and 37°, for 3 hours

10^{-4}-10^{-2} M bisulfite solutions, while no inactivation of the phage was noted for treatment with 1 M bisulfite (Table 3). The inactivation by 10^{-2} M bisulfite was abolished when the incubation was done under nitrogen atmosphere, indicating that the action of bisulfite is oxygen-dependent and that the phenomenon is associated with the free radical formation during the aerobic oxidation of bisulfite.

TABLE 3. Inactivation of bacteriophage lambda by treatment with sodium bisulfite in the presence of air

Concentration of bisulfite (M)[a]	Atmosphere	Time of treatment (hr)	Surviving fraction
0.0001	Air	3	0.017
0.001	Air	3	0.0006
0.01	Air	3	0.00007
0.1	Air	3	0.25
1.0	Air	3	0.50
0.01	Nitrogen	4	0.62
0	Air	3	0.43

a) The incubation mixture was 0.1 M in sodium phosphate buffer (pH 7), 0.01 M $MgSO_4$, and 0.00025 M $MnCl_2$. The incubation was at 37° without shaking. For more details, see ref. 16.

The DNA was isolated from the inactivated phage particles and its transfection activity (27) was measured. The transfection activity was found to be the same as that of the untreated lambda-DNA. In addition, the size of the DNA from the inactivated phage was the same as that of the intact DNA, as shown by alkaline sucrose-density-gradient centrifugation. Therefore, the DNA was not the target of the radicals. This contrasted sharply to the ease with which chain cleavages of naked DNA (9) take place by the action of radicals generated during aerobic oxidation of bisulfite.

The target of the radicals, therefore, must be the coat proteins of the phage. The activity

of the coat proteins to adsorb to the host bacteria and inject the phage DNA into the host was measured, following the treatment of the phage with bisulfite. As Table 4 shows, this activity decreased in a parallel manner with the decrease of the plaque-forming activity of

TABLE 4. Injection of DNA from bacteriophage lambda to host bacteria

Reagent[a)]	Time of treatment of phage (min)	Surviving fraction	Injection activity[b)]
Bisulfite	0	1.0	0.87
Bisulfite	30	0.25	0.23
Bisulfite	80	0.05	0.07
None	80	1.0	1.0

a) Concentration of bisulfite was 0.01 M and the other conditions of the incubations were as described in Table 3.
b) Injection activities were determined by the method of Scandella and Arber (29).

the phage. As to which part of the coat proteins was damaged by the reagent, no data is yet available. However, the likely target is either the methionine or the tryptophan residues in the proteins. Amino acid methionine is rapidly oxidized in the bisulfite-oxygen system to give methionine sulfoxide (13,14), and tryptophan is converted to several compounds (15, 16) among which formylkynurenine (15) and dioxindolyl-alanine (Kunimoto, M. and Hayatsu, H., unpublished work) have been identified. Although cystine is known to react with sulfite ion to produce cysteine thiosulfate (28), this reaction is apparently not responsible for the observed inactivation, because the high concentration (1 M) of bisulfite was rather ineffective (Table 3).

The rapid inactivation of a functional protein as described above suggests that sulfur dioxide inhaled along with oxygen can damage human organisms by destroying proteins. On the other hand, it is also conceivable that proteins can function as a protective machinery against the incoming sulfur dioxide, by effectively consuming the reactive free radicals and thereby preventing them to attack more vitally important substances such as DNA.

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