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On the Mechanism of Inactivation of Papain by Bisulfite¹⁾

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The mechanism of inactivation of papain (EC 3.4.4.10) by sodium bisulfite was investigated. The inactivation was pH-dependent, and the rate was rapid around pH 6 to 8. Exchange of oxygen for nitrogen gas or addition of radical scavengers largely prevented the inactivation. The results indicate that the inactivating effect of sodium bisulfite is oxygen-dependent and is caused by free radicals formed during the autooxidation of (bi)sulfite. The inactivation was accompanied by a decrease of the essential sulfhydryl group of papain. Treatment of inactivated papain with 2-mercaptoethanol led to partial reactivation with concomitant restoration of the sulfhydryl group. The inactivated papain was judged not to be dimeric on the basis of molecular weight determination.

Treatment of papain with ³⁵S-labeled sodium bisulfite resulted in incorporation of a significant amount of radioactivity into the protein. However, the incorporation into iodoacetate-treated papain was slight. Treatment of the labeled protein with 2-mercaptoethanol led to some reduction of the specific radioactivity incorporated with concomitant restoration of the enzyme activity.

Based on these results, it is likely that (bi)sulfite inactivates papain through modification or oxidation of the essential sulfhydryl group of the enzyme.

In addition, yeast alcohol dehydrogenase (EC 1.1.1.1) was also readily inactivated by (bi)sulfite while lysozyme (EC 3.2.1.17) was resistant to inactivation.

Keywords—papain; inactivation; bisulfite; autooxidation; radical scavenger; alcohol dehydrogenase; lysozyme

Papain (EC 3.4.4.10) is a well known thiol protease which depends for its enzyme activity on the sulfhydryl (SH) group of a cysteine residue. This enzyme possesses a single essential SH group.²⁾ It has been reported that papain was inactivated by sodium bisulfite,³⁾ and that this inactivation was due to reaction with an aldehyde group in the papain molecule.⁴⁾ However, the mechanism of the inactivation has not been clarified, and the presence of the aldehyde group in papain has not yet been confirmed directly.

On the other hand, extensive studies on the autooxidation of (bi)sulfite to (bi)sulfate have shown that the reaction mechanism involves free radicals.⁵⁾ Free radicals formed by autooxidation are involved in a number of biologically important reactions, including the following: oxidation of reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide admine dinucleotide phosphate (NADPH)⁶⁾ and methionine or its thioether analogs,⁷⁾ destruction of indole-3-acetic acid,⁸⁾ tryptophan,⁹⁾ β -carotene¹⁰⁾ and vitamin B₁,¹¹⁾ the addition of $\cdot\text{SO}_3^-$ across the double bonds of alkenes¹²⁾ and of various nucleotides and nucleic acid,¹³⁾ the peroxidation of corn oil¹⁴⁾ and rat liver homogenate,¹⁵⁾ and the cleavage of deoxyribonucleic acid (DNA).¹⁶⁾

These results led us to consider that the mechanism of the inactivation of papain by sodium bisulfite should be reinvestigated. The present paper shows that the inactivation of papain by (bi)sulfite probably involves modification or oxidation of the essential SH group of papain by reactive species produced during the aerobic oxidation of (bi)sulfite.

Materials and Methods

N- α -Benzoyl-DL-arginine-*p*-nitroaniline (BAPA) was obtained from Aldrich Chemical Company Inc., U.S.A. ^{35}S -Labeled sodium bisulfite was obtained from Amersham International Ltd., England. The initial specific activity was 18.5 mCi/mmol. Sodium bisulfite, cysteine hydrochloride, dimethyl sulfoxide (DMSO), Tiron, iodoacetic acid and *p*-chloromercuribenzoic acid (PCMB) were purchased from Nakarai Chemicals Ltd., Japan. All other chemicals were of the highest purity available and were used without further purification.

Crystalline papain was prepared by the method of Kimmel and Smith.¹⁷⁾ It is well known that the enzyme preparation obtained by this method contains an enzyme fraction in unactivated form. In the present study, therefore, papain fully activated by treatment with cysteine was used unless otherwise stated. The cysteine-activated papain was prepared as follows.^{4a)} Crystalline papain was dissolved in 25 mM citrate buffer (pH 6.0) containing 50 mM cysteine and 2 mM ethylenediaminetetraacetic acid. The mixture was incubated at 37°C for 30 min, and then the activator was removed by gel filtration on Sephadex G-25. Borohydride- and cyanide-activated papains were prepared according to the methods of Glazer and Smith¹⁸⁾ and Morihara,^{4a)} respectively. Protein concentration was determined spectrophotometrically using a value of $E_{1\%}^{1\text{cm}} = 24.0$ at 280 nm¹⁹⁾ and a molecular weight of 23400.¹⁹⁾ Papain activity was determined by measuring the rate of liberation of *p*-nitroaniline from BAPA at 37°C. The assay mixture contained, in a final volume of 2.5 ml, 250 μmol of citrate buffer (pH 6.0), 1.4 μmol of BAPA and enzyme fraction. The reaction was stopped by addition of 0.5 ml of 0.3% iodoacetate and then the absorbance was measured at 410 nm. Superoxide dismutase (SOD, from human blood) and lysozyme (from egg white) were obtained from Sigma Chemical Co., U.S.A. Lysozyme was assayed by following the hydrolysis of *Micrococcus lysodeikticus* cells.²⁰⁾ Alcohol dehydrogenase (YADH, from yeast) was obtained from Oriental Yeast Co., Ltd., Japan, and was assayed by the method of Racker.²¹⁾

Alkylation of papain with iodoacetate was done at pH 8.2 in Tris-HCl buffer, and the product was purified by passage through a column of Sephadex G-25.²²⁾

Molecular weight determinations were made by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 4 M urea,²³⁾ and by molecular sieve chromatography on Sephadex G-100.²⁴⁾

For determination of radioactivity, aliquots of solution were poured into scintillation vials. To each vial was added 10 ml of ACS-II (Amersham Co., U.S.A.). The vials were counted in a Searle Analytic Mark III (Analytic 81) liquid scintillation counter.

Samples for amino acid analysis were hydrolyzed with 6 N HCl at 110°C for 24 h in evacuated sealed tubes. Amino acid analysis was performed with a Hitachi 034 liquid chromatograph by the procedure of Spackman *et al.*²⁵⁾ Tryptophan and SH contents were determined by the method of Spies and Chamber²⁶⁾ with *p*-dimethylaminobenzaldehyde and by that of Boyer²⁷⁾ with PCMB, respectively.

Electron spin resonance (ESR) measurement was performed using a JES-FE-3X spectrometer.

Results

Inactivation of Papain by Sodium Bisulfite

When the activity of papain was determined in the presence of sodium bisulfite in the assay mixture, the activity was found to be reduced, as shown in Table I. In addition, similar results were obtained for the borohydride- and cyanide-activated enzymes. This

TABLE I. Effect of NaHSO_3 on the Activity of Papain

NaHSO_3 added (M)	Relative activity (%)
0	100
4.0×10^{-6}	99
4.0×10^{-5}	73
1.2×10^{-4}	53
2.0×10^{-4}	36
3.2×10^{-4}	25
4.0×10^{-4}	16
4.0×10^{-3}	0

Reaction mixtures contained 80 μg of papain, 1.4 μmol of BAPA, the specified amount of NaHSO_3 , and 250 μmol of citrate buffer, pH 6.0, in a final volume of 2.5 ml. Incubation was carried out at 37°C for 15 min.

inactivation was time-dependent as described in a later section (Fig. 3).

Factors Modifying the Inactivation of Papain by Sodium Bisulfite

a) Effect of pH—Papain was incubated with sodium bisulfite in buffer solutions having various pH values at 37°C. As shown in Fig. 1, the inactivation was dependent on pH. The rate of the inactivation was rapid at around pH 6 to 8.

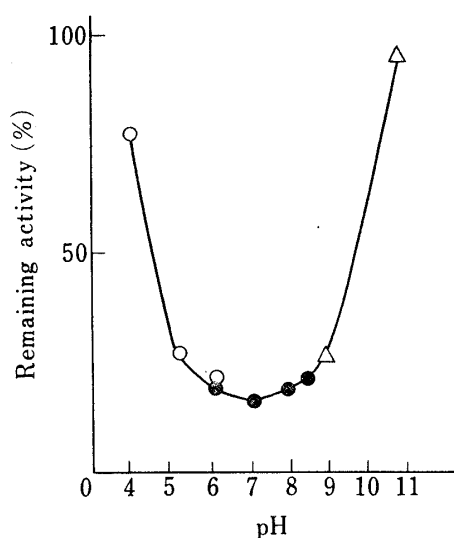
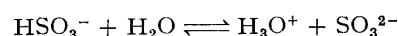


Fig. 1. Effect of pH on the Inactivation of Papain by Sodium Bisulfite

Papain (800 µg) was incubated at 37°C in 2.0 ml of 0.05 M citrate buffer (○), or of 0.05 M phosphate buffer (●), or of 0.05 M carbonate buffer (△), containing NaHSO₃ at a final concentration of 5 mM. After 10 min, the reaction mixtures were passed through a Sephadex G-25 column equilibrated with 0.1 M citrate buffer (pH 6.0). Aliquots of the protein fractions were taken for determination of the enzyme activity.

Bisulfite dissociates according to the following reaction.



The equilibrium between bisulfite and sulfite depends upon the pH and also the acidic dissociation constant, which is affected by the temperature and ionic strength of the solution. At 25°C, pK_a values ranging from 6.25 in concentrated salt to 7.20 at low ionic strength have been cited for the dissociation of bisulfite to sulfite.²⁸⁾ Therefore, from the pH dependence of the inactivation observed here, it was thought that the reactive species were both bisulfite and sulfite. The term bisulfite is used hereafter to designate the sum SO_3^{2-} and HSO_3^- .

b) Effect of Oxygen—The role of molecular oxygen was studied by performing the reaction under a nitrogen atmosphere. As shown in Table II, the inactivation of papain by bisulfite was found to be reduced under a nitrogen atmosphere. The result suggests the participation of oxygen in the inactivation of papain by bisulfite. The small extent of the inactivation observed even under a nitrogen atmosphere was probably due to residual traces of oxygen, since

TABLE II. Inhibitory Effect of NaHSO₃ on Papain Activity under Anaerobic Conditions

NaHSO ₃ added (M)	Relative activity (%)	
	in air	in N ₂ gas
0	100	100
3.2×10^{-4}	25	77
2.0×10^{-4}	36	83
1.2×10^{-4}	53	90

Reaction mixtures contained 160 µg of papain, 2.8 µmol of BAPA, the specified amount of NaHSO₃, and 500 µmol of citrate buffer, pH 6.0, in a final volume of 5.0 ml. Incubation was carried out at 37°C for 15 min in air or in N₂ gas.

in this experiment no attempt was made to remove dissolved oxygen completely from the solutions of the reactants.

c) Effects of Various Substances—The actions of various substances on the inactivating effect of bisulfite are summarized in Table III. Radical scavengers, such as cysteine, KBr, KI, ethanol, formate, mannitol, thiocyanate, Tiron and SOD, largely prevented the inactivation of papain by bisulfite. On the other hand, manganese(II), which is an effective initiator for aerobic oxidation of bisulfite, significantly accelerated the inactivation. The other metal

TABLE III. Effects of Various Substances on the Inactivation of Papain by NaHSO_3

Substance added	Concentration (M)	Relative activity (%)
None	—	100
NaHSO_3	4×10^{-4}	16
+cysteine	4×10^{-3}	92
cysteine	4×10^{-4}	78
cysteine	4×10^{-5}	33
+KI	4×10^{-3}	94
KI	4×10^{-4}	87
KI	4×10^{-5}	41
+KBr	4×10^{-4}	72
KBr	4×10^{-5}	59
+ethanol	4×10^{-3}	42
+DMSO	4×10^{-3}	71
+HCOONa	4×10^{-3}	74
+D-mannitol	4×10^{-2}	66
D-mannitol	4×10^{-3}	52
+NaSCN	4×10^{-3}	66
NaSCN	4×10^{-4}	23
+SOD	10 μg	62
+boiled SOD ^{a)}	10 μg	18
+tiron	4×10^{-3}	72
tiron	4×10^{-4}	68
NaHSO_3	4×10^{-5}	73
+ $\text{Mn}(\text{CH}_3\text{COO})_2$	8×10^{-7}	20
+ FeSO_4	8×10^{-7}	77
+ FeCl_3	8×10^{-7}	79
+ $\text{Cu}(\text{CH}_3\text{COO})_2$	8×10^{-7}	76
+ CoCl_2	8×10^{-7}	74
+ $\text{Zn}(\text{CH}_3\text{COO})_2$	8×10^{-7}	74
+ $\text{Ni}(\text{NO}_3)_2$	8×10^{-7}	77

Reaction mixtures contained 80 μg of papain, 1.4 μmol of BAPA, the specified amount of various substances, and 250 μmol of citrate buffer (pH 6.0), in a final volume of 2.5 ml. Incubation was carried out at 37°C for 15 min.

a) Boiled SOD: SOD heat-treated at 90°C for 15 min.

TABLE IV. Amino Acid Composition and SH Group Content of NaHSO_3 -treated Papain

Amino acid ^{a)} and SH	Amino acid residues and SH/mol of protein	
	Native	NaHSO_3 -treated
Trp	5.0	4.9
Tyr	19.0	19.1
His	1.9	1.8
Phe	4.2	4.1
Met	0	0
-SH	1.0	0.1
Remaining activity (%)	100	4

Papain (3 mg) was incubated with NaHSO_3 (5 mM) in 6 ml of 0.1 M citrate buffer (pH 6.0) at 37°C for 60 min. After removal of the reagents by passage of the reaction mixture through a Sephadex G-25 column, protein fractions were analyzed for amino acid composition, free sulphydryl group (-SH) and remaining enzyme activity as described in "Materials and Methods."

a) The values in the table denote the numbers of residues per protein molecule, assuming the number of leucine residues to be 11.0 and the number of arginine residues to be 12.0. No correction was made for decomposition during acid hydrolysis.

ions tested had little effect. These results indicate that free radicals formed during aerobic oxidation of bisulfite are responsible for the inactivation of papain.

Amino Acid Analysis of Bisulfite-treated Papain

To obtain some information about the mechanism of inactivation of papain by bisulfite, amino acid analyses of bisulfite-treated enzyme were performed. The amino acid compositions of native papain and bisulfite-treated papain with an activity of only 4% of that of intact enzyme are summarized in Table IV. The most significant change was seen in SH group content. No appreciable change was found in the content of the other amino acids, which are not listed in the table. This analysis also indicated that no cysteic acid formation had occurred during the treatment with bisulfite.

In addition, the amount of SH group and the enzyme activity were determined after papain had been treated with various amounts of bisulfite. The results are shown in Table V. The extent of the inactivation and the decrease of SH group were in parallel.

TABLE V. Inactivation and SH Content of Papain

NaHSO ₃ added (M)	Relative activity (%)	SH/papain
0	100	1.00
2.5×10^{-3}	74	0.85
4.0×10^{-3}	54	0.68
5.0×10^{-3}	33	0.48
1.0×10^{-2}	18	0.24

Papain (1.6 mg) was incubated at 37°C for 10 min in 5.0 ml of 0.1 M citrate buffer (pH 6.0) containing NaHSO₃ at the indicated final concentration. Then the reaction mixtures were passed through a Sephadex G-25 column equilibrated with 0.1 M acetate buffer (pH 4.6). Aliquots of the protein fractions were taken for determinations of the enzyme activity and the contents of SH group.

Reactivation of Inactivated Papain by 2-Mercaptoethanol

The above results suggest that the inactivation is a consequence of modification or oxidation of the essential SH group of papain. If so, the inactivated papain might be reactivated by reduction of the protein under mild conditions. Papain (3 mg) was incubated with 30 μmol of sodium bisulfite in 6 ml of 0.1 M citrate buffer (pH 6.0) at 37°C for 30 min, and then the mixture was passed through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.5). Aliquots of the protein fractions were taken for determinations of the enzyme activity and the content of SH group. A 4 ml fraction of bisulfite-treated papain, in which the activity was reduced to 21% of the initial activity with concomitant loss of 0.76 SH group per mol of the enzyme, was incubated with 400 μmol of 2-mercaptoethanol at 37°C for 60 min. The enzyme activity and SH group content of the protein fractions were determined after separation of the protein from the reagents by gel filtration. As shown in Table VI, treatment

TABLE VI. Restoration of Enzyme Activity and SH Content of NaHSO₃-treated Papain by 2-Mercaptoethanol

Sample	SH/papain	Specific activity (%)
Papain	1.00	100
Papain + 2-mercaptoethanol	1.00	100
NaHSO ₃ -treated papain	0.24	21
NaHSO ₃ -treated papain + 2-mercaptoethanol	0.53	60

The experimental conditions are described in the text.

of bisulfite-inactivated papain with 2-mercaptoethanol resulted in partial restoration of both the activity and SH group content. Although the reactivation by 2-mercaptoethanol is partial, these results strongly support the conclusion that the inactivation is a consequence of modification or oxidation of the essential SH group of papain.

Molecular Weight Determination of Bisulfite-treated Papain

If the decrease of SH group was due to the formation of a disulfide bond, one would expect an increase in molecular weight, because the enzyme contains only a single SH group per molecule. Molecular weight determinations for bisulfite-inactivated papain were made by both the SDS-polyacrylamide gel electrophoresis method and the molecular sieve method. The gel filtration and electrophoretic patterns of intact and bisulfite-treated papains showed that there was no significant difference in terms of molecular weight between the two proteins (not shown). This indicates that the inactivation does not involve disulfide bond formation.

Incorporation of ^{35}S -Labeled Bisulfite into Papain

The $\cdot\text{SO}_3\text{H}$ ($\cdot\text{SO}_3^-$) is considered to be one of the species produced by autooxidation of bisulfite.⁵⁾ Thus, we attempted to detect free radicals formed in an aqueous solution of bisulfite by using the ESR technique. Figure 2 shows the ESR signal obtained at 77°K when sodium bisulfite was dissolved in 0.1 M phosphate buffer (pH 7.0). A radical signal in the region of $g=2.008$ was observed. This signal may be due to a sulfur radical rather than an oxygen radical.²⁹⁾ No signal was observed when ESR measurement was carried out at room temperature.

The sulfur radical is anticipated to react readily with the SH group of papain. This possibility was examined with ^{35}S -labeled sodium bisulfite. A reaction mixture (4.0 ml) containing 1.6 mg papain, 40 μmol $\text{NaH}^{35}\text{SO}_3$ (specific radioactivity, 1.8×10^6 cpm/ μmol NaHSO_3) and 400 μmol of citrate buffer (pH 6.0) was incubated at 37°C in an open vessel. After 60 min, the mixture was passed through a Sephadex G-25 column equilibrated with 0.1 M citrate buffer (pH 6.0). Radioactivity and remaining enzyme activity of the protein fractions were determined. The enzyme activity was found to have been completely lost. The specific radioactivity of the protein was found to be 5.8×10^4 cpm/mg which represented an incorporation of 0.75 mol bisulfite group per mole of papain. No significant loss of the incorporated radioactivity was found on further dialysis of the labeled protein. In a control experiment, iodoacetate-treated papain, in which the SH group was completely blocked, was treated with $\text{NaH}^{35}\text{SO}_3$ under the same reaction conditions as described for native papain. The incorporated specific radioactivity of the protein in this case was found to be 8.7×10^3 cpm/mg, which represents an incorporation of 0.11 mol bisulfite group per mol of papain. These results indicate strongly that a significant amount of sulfur derived from bisulfite is incorporated into papain, and that the incorporation occurs mainly at the SH group of the protein.

In addition, the role of molecular oxygen in the incorporation of radioactivity was examined by performing the reaction under anaerobic conditions with nitrogen gas bubbling through the reaction mixture. The extent of incorporation of radioactivity into papain was greatly reduced (to less than 20%), indicating that oxygen was essential for the incorporation. Furthermore, the effect of DMSO on the incorporation was examined. In the presence of DMSO, the specific radioactivity of bisulfite-treated papain, in which the remaining enzyme activity was 65%, was found to be 1.2×10^4 cpm/mg which represented an incorporation of 0.16 mol bisulfite group per mol of papain. That is to say, the extent of the incorporation was found to be reduced to about 21% by the addition of DMSO to the reaction mixture. This result suggests the participation of certain radical species in the incorporation of bisulfite group into papain.

The ^{35}S -labeled papain prepared as described above was treated with 0.1 M 2-mercaptoethanol in 0.1 M phosphate buffer (pH 7.5) at 37°C for 90 min. After removal of the reagents by gel filtration on Sephadex G-25, the protein fractions were assayed for protein-bound radioac-

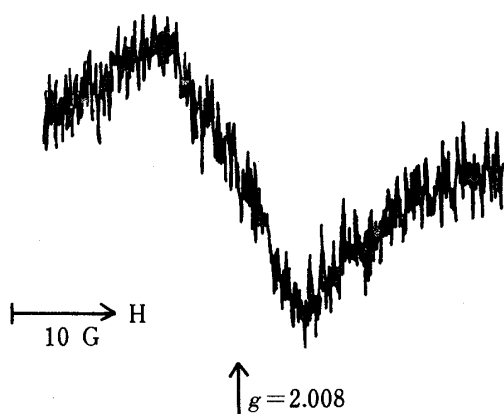


Fig. 2. ESR Spectrum of an Aqueous Solution of Sodium Bisulfite

About 10 mg of sodium bisulfite was dissolved in about 100 μ l of 0.1 M phosphate buffer (pH 7.0), and the solution was frozen immediately. The ESR spectrum was measured under the following conditions: field, 3300 G \pm 100 G; modulation frequency, 100 kHz; amplitude, 3.2×10^3 ; response, 0.01 s; sweep rate, 5 G/min; and temperature, 77 $^{\circ}$ K.

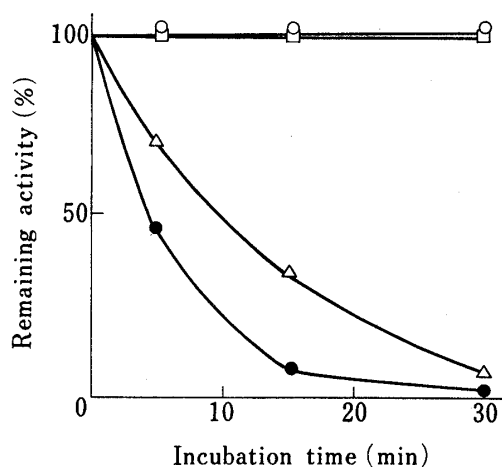


Fig. 3. Reactions of Sodium Bisulfite with Papain, Alcohol Dehydrogenase and Lysozyme

Each enzyme (2.4 mg) was incubated at 37 $^{\circ}$ C in 6.0 ml of 0.1 M phosphate buffer (pH 7.0) containing NaHSO₃ at a final concentration of 5 mM. At various intervals, aliquots of the reaction mixtures were taken and passed through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.0).

Aliquots of the protein fractions were taken for determination of the enzyme activities as described in "Materials and Methods."

●, papain+NaHSO₃; △, YADH+NaHSO₃; □, lysozyme+NaHSO₃; ○, control experiments (papain only, YADH only and lysozyme only).

tivity and enzyme activity. The thiol treatment resulted in a reduction of incorporated bisulfite group to 0.31 mol from 0.75 mol per mol of papain with a concomitant recovery of the enzyme activity to 45%.

Reaction of YADH and Lysozyme with Sodium Bisulfite

The results obtained for papain indicate that the inactivation by bisulfite is due to modification or oxidation of an essential SH group of the enzyme. For comparison, the data for YADH and lysozyme are shown in Fig. 3. YADH contains SH groups essential for enzyme activity, but lysozyme does not. As would be expected, the enzyme activity of YADH was readily destroyed by bisulfite in the same manner as that of papain, while lysozyme activity was not affected.

Discussion

The present results strongly suggest that the inactivation of papain that occurs upon treatment with bisulfite results mainly from modification or oxidation of the essential SH group of papain by free radicals formed during the aerobic oxidation of bisulfite.

A possible mechanism for the modification of the SH group of papain by bisulfite is the formation of protein S-sulfonate by sulfur radicals formed during aerobic oxidation of bisulfite. This speculation is based on the following findings and facts. It has been documented that $\cdot\text{SO}_3\text{H}$ ($\cdot\text{SO}_3^-$) besides $\cdot\text{OH}$ and $\cdot\text{O}_2^-$ is formed during autooxidation of bisulfite. We also confirmed the formation of a sulfur radical (possibly $\cdot\text{SO}_3\text{H}$ ($\cdot\text{SO}_3^-$)) in bisulfite aqueous solution by using the ESR technique. The treatment of papain with ³⁵S-labeled sodium bisulfite resulted in incorporation of a significant amount of radioactivity into the protein. However, the

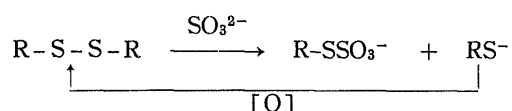
incorporation into S-carboxymethylated papain was found to be less than 15% of that into native papain. When the reaction was performed under anaerobic conditions, the extent of incorporation of radioactivity was greatly reduced. In all the above cases, the degrees of inactivation of papain and of incorporation of radioactivity into the protein were in parallel. The incorporation was found to be reversible by 2-mercaptoethanol, though the recovery was not complete. S-Sulfonated proteins are known to be reconstituted by treatment with sulfhydryl compounds such as 2-mercaptoethanol.³⁰⁾

The findings (Table III) that $\cdot\text{OH}$ and $\cdot\text{O}_2^-$ scavengers prevented the inactivation of papain by bisulfite might be explicable as follows: both $\cdot\text{OH}$ and $\cdot\text{O}_2^-$ propagate the bisulfite-oxygen chain reaction.⁵⁾ Therefore, the addition of these radical scavengers to the reaction mixture results in a reduced formation of sulfur radicals.

On the other hand, there is a possibility that the SH group of papain was oxidized directly by $\cdot\text{OH}$ or $\cdot\text{O}_2^-$ to $-\text{SOH}$ or $-\text{SOOH}$, though the formation of cysteic acid or of an intermolecular disulfide bond was not detected. Indeed, the oxidation of the SH group of papain by $\cdot\text{O}_2^-$ was postulated to occur during radiolysis under aerobic conditions.³¹⁾ We also observed that papain was readily inactivated by the xanthine-xanthine oxidase system, which generates $\cdot\text{O}_2^-$ (our unpublished data).

In addition, the incomplete reversibilities of the inactivation (Table VI) and of the incorporation of radioactivity in the case of experiments using ^{35}S -labeled sodium bisulfite suggests that some irreversible inactivation of papain takes place as a consequence of reaction of various groups in addition to the SH group in the protein, though no significant change was found in the content of any amino acid (other than the SH group; Table IV) under the analytical conditions employed.

It is well known that the scission of disulfides by sulfite ions to form S-sulfonates occurs as follows:^{30,32)}



Sulfitolysis has frequently been used for the cleavage of disulfide bonds in proteins, usually in the presence of a protein-denaturant or oxidizing agent or both these agents. In the case of the present study on papain, it appears that sulfitolysis of disulfide bonds in the protein by bisulfite is not the main reaction responsible for the incorporation of a sulfur group derived from bisulfite into papain with consequent inactivation of the enzyme. This view is based on the following findings and facts. Treatment of bisulfite-inactivated papain with 2-mercaptoethanol resulted in significant restoration of the enzyme activity (Table VI). The inactivation of the enzyme by bisulfite was largely prevented in the presence of radical scavengers such as cysteine and potassium iodide (Table III). Disulfide bonds in papain are resistant to reducing agents such as 2-mercaptoethanol, and are susceptible to reduction only in the presence of concentrated protein-denaturant such as urea or guanidine hydrochloride.²²⁾ In the experiments using ^{35}S -labeled sodium bisulfite, incorporation of radioactivity into S-carboxymethylated papain was found to be less than 15% of that into the native enzyme. In the presence of DMSO as a radical scavenger, the extent of incorporation of radioactivity into papain was greatly reduced.

In conclusion, it appears that bisulfite shows a relatively high degree of specificity for the SH group in papain. This conclusion is also supported by the findings that YADH (containing essential SH groups) is almost as susceptible to inactivation as papain, while lysozyme (containing no essential SH group) is highly resistant to bisulfite (Fig. 3).

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