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A Rapid and Specific Gas Chromatographic Analysis for Cysteine-S-Sulfonate to Determine the Distribution of Sulfite in Mammalian Plasma

Joseph Don deBethizy

A RAPID AND SPECIFIC GAS CHROMATOGRAPHIC ANALYSIS FOR CYSTEINE-S-SULFONATE TO DETERMINE THE DISTRIBUTION OF SULFITE IN MAMMALIAN PLASMA

by

Joseph Don deBethizy

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NOTE OF EXPLANATION

This thesis is written in a form acceptible for publication in Analytical Toxicology and will be submitted to the journal October, 1979. A general review of literature on cysteine-S-sulfonate and sulfite exposure has been included in the appendix.

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ABSTRACT

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by

Joseph Don deBethizy, Master of Science

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Major Professor: Dr. Joseph C. Street
Department: Animal, Dairy, Veterinary Sciences (Toxicology)

It has been shown in previous studies that when sulfite is absorbed by rabbits via either inhalation of SO_2 or oral exposure to sulfite, the hydrated form, bisulfite, interacts with plasma disulfides where it is suspected to be in the form, cysteine-S-sulfonate. A rapid and specific gas chromatographic analysis procedure for cysteine-S-sulfonate has been developed to better study the distribution of sulfite in biological systems. Sulfonated proteins are enzymatically hydrolyzed to ensure stability of the acid labile S-sulfonate disulfide. The hydrolysate is then applied to a 6 cm cation-exchange column and eluted with 0.1 N HCl which elutes the acidic cysteine-S-sulfonate with the void volume of the column leaving behind any remaining cysteine. The silylated derivatives of the column effluent are prepared using Tri-Sil/BSA. These derivatives are injected into a gas chromatograph equipped with a flame-photometric detector operating in the sulfur mode, 2% OV-101 on Chromosorb W/HP 1/4 inch glass column, oven temperature 140°C, and carrier flow rate of 86 ml/min. The presence of cysteine-S-sulfonate in sulfite treated rabbits has been directly determined by the described method.

(45 pages)

INTRODUCTION

Man is exposed to sulfite principally through the inhalation of the gaseous pollutant, SO_2, and by ingestion of sulfite used in the processing of food and beverages (1). Regardless of the route of exposure, SO_2/sulfite is rapidly absorbed into the circulatory system of mammals as bisulfite (2). However, the distribution of bisulfite among blood constituents and the molecular forms in which it is bound in mammals have not been completely elucidated. Yokoyama, et al (3) followed the distribution of radio-labeled sulfur after treating nine dogs to 22 and 50 ppm $^{35}SO_2$. These investigators ascertained the rate at which ^{35}S entered the circulation from the mucosa of the upper airways, the extent of deposition in peripheral tissues and the rate at which it was excreted from the dogs, but were unable to determine the molecular forms of the bound ^{35}S.

Gunnison and Benton (4) suggested that cystine and other physiological disulfides may act as the primary sinks for bisulfite in mammalian plasma by the following reaction:

$$RSSR + HSO_3^- \underset{\text{pH 7.4}}{\rightleftharpoons} RSSO_3^- + RSH$$

This reaction, known as sulfitolysis, has been used as a gentle method for breaking disulfides in structural investigations of proteins (5). Confirmation of the formation of cysteinyl-S-sulfonate residues in sulfonated proteins has only been indirectly demonstrated because of the relative lability of the S-sulfonate moiety to conventional acid hydrolysis of proteins. Gunnison and Benton circumvented this problem by developing a colorimetric method for following the uptake of inor-

ganic sulfite by disulfides and the subsequent displacement of the bound sulfite with alkaline cyanide. Adapting this indirect method to physiological samples, Gunnison was able to implicate the involvement of cysteine-S-sulfonate as the major sink for bisulfite in mammalian plasma (6,7,8). However, the Gunnison method lacks specificity for cysteine-S-sulfonate and is subject to interfering plasma constituents which limit the sensitivity of the colorimetric assay (4).

The purpose of the present investigation was to develop a rapid and specific gas chromatographic determination for cysteine-S-sulfonate in proteins using enzymatic hydrolysis and the specificity of the flame-photometric detector operated in the sulfur mode. Reports of the use of GLC for separation and detection of S-containing amino acids, however, are limited. Caldwell and Tappel (9) separated the silylated derivatives of a variety of sulfo-and seleno-amino acids and their oxidation products by GLC. In a more thorough study of the gas chromatography of sulfur amino acids, Shahrokhi and Gehrke (10) quantitatively prepared the TMS derivatives of twelve sulfur-containing amino acids. Separation and detection was achieved using a 0.5% w/w OV-1 column and a flame ionization detector. The TMS derivatives were confirmed by elemental analysis. Neither group attempted the derivatization of cysteine-S-sulfonate.

Earlier methods for direct determination of free S-sulfonates did not lend themselves to routine evaluation of physiological samples. DeMarco, et al (11) demonstrated the chromatographic behavior of cysteine-S-sulfonate on the long cation-exchange column of a Model 120 Beckman-Spinco Amino Acid Analyzer. They were able to show elution of

the acidic S-sulfonate and cysteic acid within the void volume of the column. Ruffin and Biserte (12) achieved separation of cysteine-S-sulfonate and cysteic acid by using an 0.8 cm x 35 cm anion exchange column fitted to a Multichrom Amino Acid Analyzer. Sensitivity of these methods was limited by the ninhydrin detection system.

To chromatographically determine cysteine-S-sulfonate residues in sulfonated proteins, the proteins must be quantitatively hydrolyzed. Enzymatic hydrolysis was the method of choice to prevent hydrolysis of the acid-labile S-sulfonate disulfide. Complete enzymatic hydrolysis of proteins was demonstrated by Hill and Schmidt (13). They used a battery of three enzymes; papain, leucine amino peptidase, and prolidase to achieve "close to theoretical amounts" of amino acids ordinarily quite labile during acid hydrolysis of proteins. These investigators pointed out that cysteine was the most resistant amino acid to enzymatic hydrolysis. The use of pronase for enzymatic protein digestion has been promoted as a relatively non-specific endopeptidase producing a mixture of short peptides and free amino acids (14, 15). It was hoped that pronase would be more active than papain on the more resistant cysteine residues.

With these considerations, we have employed a method utilizing enzymatic hydrolysis, ion-exchange column chromatography, and GLC to directly determine cysteine-S-sulfonate in sulfonated proteins.

MATERIALS AND METHODS

Materials

Crystalized and lyophilized bovine serum albumin; L-cysteic acid; prolidase from pig kidney (highly purified suspension in 2.7M $(NH_4)SO_4$ solution, pH 8); leucine amino peptidase from hog kidney, Type III-CP; and protease from Streptomyces griseus (Pronase), repurified Type VI, were obtained from Sigma Chemical Co. Anhydrous sodium sulfite, sodium metabisulfite, and dichloromethane (nanograde solvent) were obtained from Mallinckrodt Chemical Works. N,O-bis(Trimethylsilyl)-acetamide in silylation grade dimethyl formamide (Tri-Sil BSA) was obtained in 1.0 ml ampules from Pierce Chemical Co. Sodium tetrathionate was synthesized by the method of Gilman, et al, (16). Sodium cysteine-S-sulfonate monohydrate was synthesized by the method of Segel and Johnson (17). Sulfonated bovine serum albumin (S-BSA) was prepared by the method of Bailey and Cole (5).

Enzymatic Hydrolysis

All proteolytic enzymes were used as commercially supplied except for the removal of exogenous amino acids by dialysis against three changes of 200 volumes of buffer (5mM Tris, pH 8.0, 5mM $MgCl_2$) for four hours ($MgCl_2$ excluded for Pronase). The method for enzymatic hydrolysis was an adaptation of the method by Hill and Schmidt (13). Sulfonated-bovine serum albumin (S-BSA), 20 mg, was dissolved in 4.0 ml of glass distilled water and adjusted to pH 5.0 with 1N NaOH.

Sufficient stock Tris, pH 8.0, was added to produce a final concentration of 5mM Tris. The buffered protein solution was equilibrated to 39°C in a shaking water bath. The first stage of hydrolysis was started by adding 1.0 ml Pronase solution (2 mg Pronase/1.0 ml 5mM Tris, pH 8.0) to the protein solution. After 12 hours of digestion, the pronase was inactivated by bringing the digest to pH 2.0 with 1N HCl. After 20 minutes, the digest was returned to pH 8.0 with 1N NaOH and made 5mM in $MgCl_2$.

The second stage of hydrolysis was started with the addition of 0.5 ml LAP (2mg/ml) and 0.2 ml of prolidase (10 mg/ml) to the digest. After 12 hours of incubation the digest was adjusted to pH 2.0 with 1.0 M HCl for 20 minutes. Any protein that precipitated at this point was sedimented and the supernatent lyophilized. The lyophilized samples were stored dessicated and frozen until needed. The progress of the enzymatic hydrolysis was monitored spectrophotometrically with ninhydrin at 570 nm, (18).

Short Cation-Exchange Column Chromatography

(Column Preparation) Dowex 50-X4, 100-200 mesh (Bio Rad) was used in preparing a 5.8 cm x 1.8 cm column. The resin was equilibrated with several washes of the eluant, 0.1 N HCl.

(Elution) Samples, dissolved in 1.0 ml of eluant, were placed on the column and rinsed into the column with 1.0 ml of the eluant. The cysteine-S-sulfonate was eluted with a continuous flow of 0.1 N HCl. During method development, successive 1.0 ml fractions were collected to insure quantitative recovery of the sulfonated amino acid. An

appropriate aliquot of each fraction was transferred to the silylation reaction vial for derivatization. For routine analyses, the cysteine-S-sulfonate was recovered by batch elution.

Silylation

Prior to gas chromatography, samples were silylated with commercially prepared Tri-Sil/BSA. Aqueous samples were transferred to silylation reaction vials and taken to dryness under a N_2 stream. The samples (1.0umole amino acid/100 ul Tri-Sil) were silylated for 15 minutes at 100±2°C in a constant temperature oil bath. It was important to submerge the reaction vials in the oil to the level of the sample to allow adequate refluxing. After 15 minutes the reaction vial was rapidly cooled and 5 ul injected onto the GLC column. Screw-cap vials* with teflon septa were used so that aliquots could be removed by syringe. Cysteine required dilution with dichloromethane (1:10 v/v) prior to injection on the column.

GLC Apparatus

Samples were chromatographed in a Tracor (Tracor, Inc.) MT 220 gas chromatograph equipped with a Melpar, Inc. flame photometric detector operable to 165°C. A narrow band optical filter with maximum transmission at 394 nm and a half-width of 5 nm was used with the detector. Nitrogen served as the carrier gas while the detector was serviced by hydrogen, oxygen, and air. All gas cylinders were equipped

*Reactivials, 2 ml, Pierce Chemical Company

with Tracor filter-driers to remove trace hydrocarbons and water. A two pen, 1 mv Micro-tek recorder monitored both FID and FPD responses. A 6.4×10^{-9} amp signal produced a full-scale deflection at maximum input and output settings on the two-channel electrometer. A 1.8 m x 4 mm i.d. boro-silicate glass column containing 2.0% (w/w) OV-101 on 80/100 mesh Chromosorb W-HP was used for separation and quantitation of the S-amino acids. The column was prepared and conditioned by the method of Leibrand, et al (17).

Method Application, in vivo

One male, New Zealand White rabbit, 4 kg, was treated with 0.7 mmole sulfite/kg body weight by intravenous injection in the marginal ear vein. The sulfite solution was prepared by dissolving sodium metabisulfite in normal saline and adjusting to pH 7.4 with NaOH. Total injection volume was 1.34 ml and was administered at a rate of approximately 1 ml/min. Whole blood was collected (20 ml) by cardiac puncture 90 min. after IV injection and immediately transferred to vacutainer tubes containing citrate at 4°C. Plasma was prepared and stored frozen until analysis. Gunnison and Palmes showed that plasma S-sulfonates were stable when stored at -15°C for 46 days (7). Plasma aliquots of 1.0 ml were analysed for cysteine-S-sulfonate by the described method as shown in Fig. 1 and for cyanolytic sulfite by the Gunnison method. Plasma aliquots were also analysed after dialysis against 3-1 liter changes of 0.9% NaCl-6 mM Tris buffer, pH 7.4 for 24 hours to determine the level of low molecular weight S-sulfonates. The described method allowed us to analyse the dialysate for the presence of cysteine-S-sulfonate.

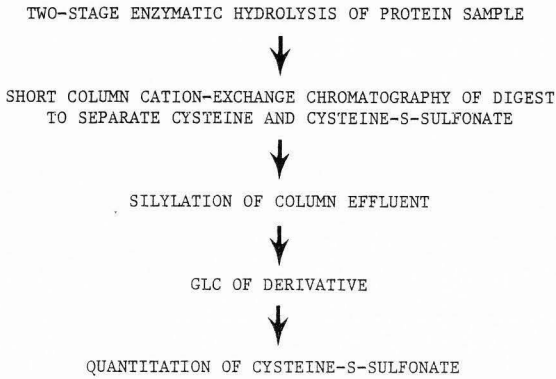


Figure 1. Flow chart of the proposed method for quantitation of cysteine-S-sulfonate in physiologically important proteins.

RESULTS

Method Development: Research

The effectiveness of the proteolytic enzyme regimen employed for the complete hydrolysis of sulfonated proteins was examined using S-BSA. Bovine serum albumin was chosen as a model for sulfonation because it is a well characterized protein with 17 disulfide bridges per molecule and offers a good substrate for sulfitolysis. In addition, albumin represents nearly two-thirds, by weight, of the plasma proteins in humans and thus is the major protein that would be encountered in an analysis of human plasma. Employment of the adaptation of the Hill and Schmidt method for complete enzymatic hydrolysis of proteins resulted in 95% hydrolysis of S-BSA (based on 154 umole amino acids/20 mg S-BSA) as illustrated in Fig. 2. Liberation of total amino acids was followed with ninhydrin and confirmed using amino acid analysis. A burst of proteolytic activity was observed following the addition of both pronase and the LAP-prolidase combination to the hydrolysate. The 24 hour hydrolysis period was convenient and achieved sufficient hydrolysis of S-BSA to quantitatively liberate cysteinyl-S-sulfonate residues. As presented in Table 1, 93% of the original cysteine in bovine serum albumin was recovered as cysteine-S-sulfonate from S-BSA. The 0.57mole% cysteine found in the S-BSA hydrosylate was most likely exogenous amino acid contributed by the proteolytic enzyme preparations as well as a small amount of cysteine that was not sulfonated. Enzymatic hydrolysis of unreacted bovine serum albumin is also presented in Table 1 as both a comparison and as a check on the

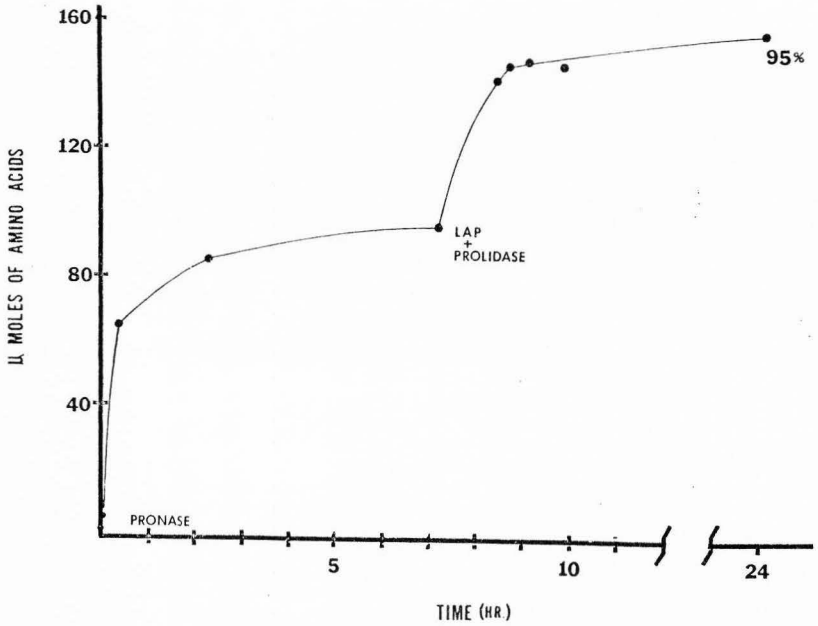


Figure 2. Enzymatic hydrolysis of S-BSA(20mg). Pronase(2mg), leucine amino peptidase(LAP,1mg), and prolidase(2mg) were employed at pH 8-8,5. Progress of the reaction was monitored spectrophotometrically with ninhydrin at 570nm (16).

Table 1. Sulfur-containing Amino Acid Composition of S-BSA and BSA Determined by GLC Analysis of Enzymatic Hydrolysate

	S-BSA		BSA	
Amino Acid	Found mole %	Theory mole %	Found mole %	Theory mole %
Cysteine	0.57	0	6.00	6.18
Cysteine-S-sulfonate	5.75	6.18	0	0
Cysteic acid	0	0	0	0
Methionine	0.70	0.71	0.70	0.71

specificity of the described analysis for cysteine-S-sulfonate. As would be expected, no sulfonated cysteine was found in the bovine serum albumin. The cysteine and methionine values presented in Table 1 were determined by amino acid analysis of the total enzymatic hydrolysate on a Beckman Automatic Analyzer using a Durrum single column system. Methionine was also determined by gas chromatography of the total hydrolysate.

Using synthetic standards of cysteine-S-sulfonate and cysteine, derivatives were sought which would be amenable to gas chromatography. The TFA-butyl ester derivatization was attempted but only a stable derivative for cysteine was detected by GLC. Silylation in dimethylformamide produced stable trimethylsilyl (TMS) derivatives of both cysteine and cysteine-S-sulfonate. Typical GLC chromatograms of the derivatives are presented in Fig. 3. As evidenced in these chromatograms, the retention times for both derivatives were similar.

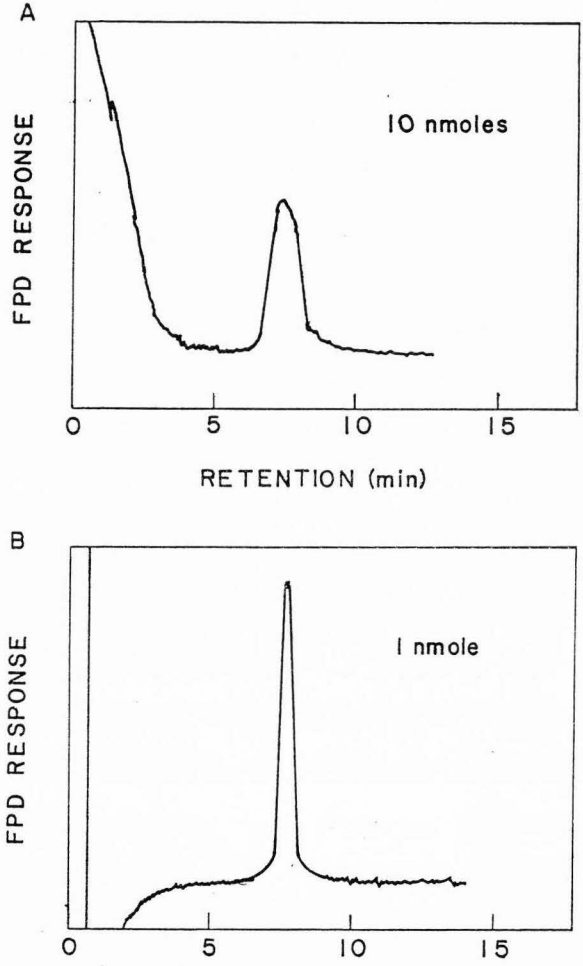


Figure 3. GLC analysis of the TMS derivatives of synthetic standards of cysteine-S-sulfonate(A) and cysteine(B). Sample, column, and conditions are the same as Figure 4.

Calibration curves, shown in Fig. 4, illustrate a linear response with a slope of 2 on a log-log plot for both the cysteine-S-sulfonate and cysteine TMS derivatives which is characteristic of the sulfur-mode flame photometric response (20). There was a 10-fold greater sensitivity for the cysteine derivative over the cysteine-S-sulfonate derivative. The minimal detectable amount for cysteine-S-sulfonate was 6 nmoles with a peak height of 10mm. In addition, the cysteine-S-sulfonate TMS derivative was more susceptible to degradation in air than the cysteine TMS derivative. This problem was overcome by the use of teflon-lined septa which permitted the direct removal of the derivative without opening the reaction vial.

Conditions for effective separation of cysteine-S-sulfonate and cysteine TMS derivatives by gas chromatography could not be found, so it proved necessary to separate these prior to gas chromatography. Rapid separation of the two analogs was possible before silylation using short column cation-exchange chromatography with an acidic eluant. This step of the procedure takes advantage of the low pKa of the S-sulfonate moiety. The elution profile of a run of synthetic standard of cysteine-S-sulfonate on the short cation-exchange column is shown in Fig. 5. Cysteine was not eluted under these conditions, and it did not interfer with the recovery of cysteine-S-sulfonate. Although cysteic acid did elute at this pH, its TMS derivative was separable from the cysteine-S-sulfonate derivative on the GLC. Running the S-BSA hydrolysate on this column resulted in the elution of only cysteine-S-sulfonate as determined by gas chromatography; this finding was confirmed by the absence of cysteic acid in the complete

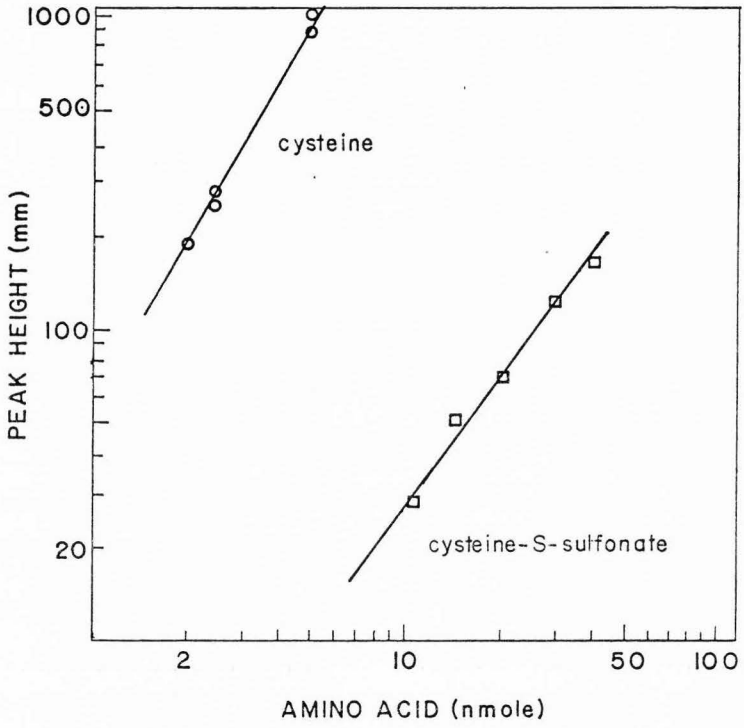


Figure 4. Calibration curves for the trimethylsilyl derivatives of cysteine and cysteine-S-sulfonate. Column: 2.0% OV-101 on Chromosorb W-HP, 1.8m X 4mm I.D. glass. Sample: silylation volume, 50ul; silylation concentration, 10nmole amino acid/ul Tri-Sil in DMF. Flow rate: N_2, 86ml/min; H_2, 140ml/min; air, 35ml/min; O_2, 28ml/min. Temperature: inlet, 240°C; column, 140°C; flame photometric detector, 165°C. Sensitivity: 64 X 10^{-8} a.f.s.

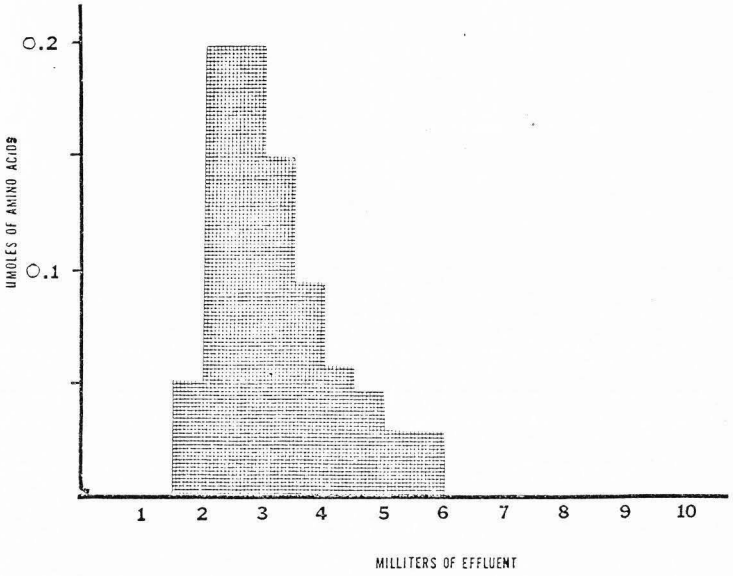


Figure 5. Elution profile of a synthetic standard of cysteine-S-sulfonate(1.0umole) dissolved in 0.5ml 0.1N HCl(eluant) and chromatographed on a short column(1.8cm X 5.8cm) of Dowex 50-X4 resin. The sample was washed in with 0.5ml of the eluant followed by continuous flow of the eluant. Fractions(0.5ml) were collected and assayed for the presence of cysteine-S-sulfonate using ninhydrin (16) and gas chromatography of the TMS derivative.

amino acid analysis [differentiation of cysteic acid and cysteine-S-sulfonate was possible on the amino acid analyzer even though they cochromatograph by using a comparison of the 570 nm/440 nm ratio as empirically demonstrated by DeMarco et al, (11)]. No other amino acids coeluted from the column. A typical GC chromatogram of the TMS derivatives of the S-BSA digest before and after the cation-exchange chromatography is presented in Fig. 6 (A) and 6 (B) respectively.

Method Application: Confirmation of Cysteine-S-sulfonate in Plasma of Rabbits Treated With Sulfite.

To demonstrate the utility of the described method, cysteine-S-sulfonate was determined in plasma samples obtained from a sulfite-treated rabbit. The results, presented in Table 2, show that 158 nmoles of cysteine-S-sulfonate was formed in a rabbit treated with sulfite as hypothesized and indirectly demonstrated by Gunnison and Benton (4).

Plasma samples were dialyzed against normal saline buffered with Tris at pH 7.4 to determine the proportion of cysteine-S-sulfonate not associated with plasma proteins. As indicated in Table 2, 30% of the cysteine-S-sulfonate was dialyzable and determined directly in the dialyzate. For comparison, Gunnison's spectrophotometric method was run on plasma samples obtained from the same sulfite-treated rabbit. As shown in Table 2, the total S-sulfonate level was lower than the S-sulfonate level determined as cysteine-S-sulfonate by the proposed GC method. The S-sulfonate levels generated by the spectrophotometric method was in very close agreement to values reported

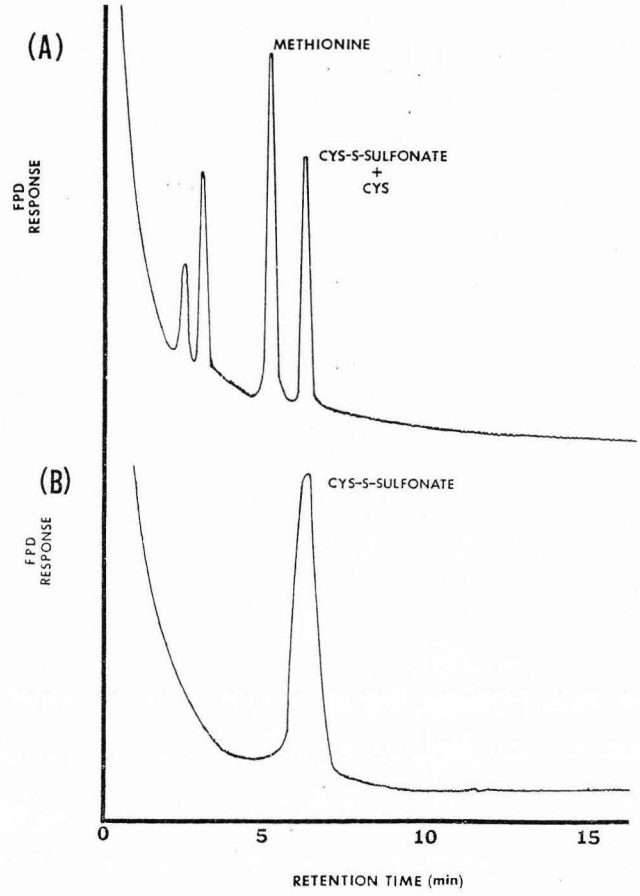


Figure 6. GLC analysis of enzymatic hydrolysate of S BSA before(A) and after cation exchange chromatography(B). Injection volume: 2ul(A) and 3ul(B). Column and conditions are the same as Figure 4.

by Gunnison and Palmes (6). This indicates that the sulfite-treatment technique reproduced S-sulfonate levels previously generated by Gunnison and Palmes; they reported 56% dialyzable low molecular weight S-sulfonate as compared to 53% reported here.

Table 2. Cysteine-S-sulfonate and total S-sulfonate levels in rabbit plasma following treatment with sulfite (0.7 mmole/kg BW) as determined by the proposed GLC method and the spectrophotometric method of Gunnison (4).

	GLC method	Spectrophotometric method
	Cysteine-S-sulfonate nmole/ml plasma	Total S-sulfonate nmole/ml plasma
undialyzed	110	46
dialyzed	77	21
fraction dialyzed	(30%)	(53%)

To assess the efficiency of the spectrophotometric method for detecting cysteine-S-sulfonate residues as cyanolytic sulfite in sulfonated protein, the method was applied to intact S-BSA and enzymatically hydrolysed S-BSA. The data presented in Table 3 indicate that about 37% of the cysteinyl-S-sulfonate residues were not accessible to alkaline cyanolysis prior to enzymatic hydrolysis.

Table 3. Total S-sulfonate levels of intact S-BSA and enzymatically hydrolysed S-BSA as determined by the spectrophotometric method of Gunnison (4).

	Total S-sulfonate fraction of theoretical
Intact S-BSA	60%
Enzymatically hydrolysed S-BSA	97%

[a]theoretical is based on amino acid analysis of sulfonated bovine serum albumin

DISCUSSION

By use of the described method, which combines the selectivity of cation-exchange chromatography and the sensitivity of the FPD-GLC, cysteine-S-sulfonate was directly shown to occur in sulfonated bovine serum albumin and rabbit plasma proteins following treatment with sulfite. Heretofore, the presence of cysteinyl-S-sulfonate residues in plasma proteins of sulfite-treated mammals had been indirectly demonstrated via sulfite displacement and radiolabeled sulfur experiments (3,4). Confirmation of this sulfite adduct is important to future attempts to monitor systemic exposure to sulfite.

An essential feature of the proposed method was the use of enzymatic hydrolysis to insure the stability of the acid-labile S-sulfonate moiety. As presented in Table 1, the enzymatic hydrolysis was effective in quantitatively releasing the cysteine-S-sulfonate from S-BSA. It is interesting that the hydrolysis of S-BSA occured more rapidly than the hydrolysis of bovine serum albumin. A possible explanation is that sulfonation permits better access by the proteolytic enzymes to the sites of hydrolysis by breaking disulfide bonds and thus unfolding the albumin molecule. While the described use of proteolytic enzymes is costly and cumbersome for routine analytical work, it is likely that this may be improved upon through the use of matrix-bound proteinases (14).

Quantitation of cysteine-S-sulfonate by gas chromatography with the sulfur-specific detector offers increased sensitivity over conventional colorimetric detection methods. The calibration curves shown in Fig. 3 indicate that the gas chromatographic method was sensitive

to a minimum of 6 nmole of cysteine-S-sulfonate with the optimum linear range from 10-100 nmole. Preliminary experiments indicate that the potential for greater sensitivity by manipulation of the cysteinyl-S-sulfonate residues prior to derivatization is possible. This would utilize the acid lability of the S-sulfonate group, making it possible to analyze cysteine-S-sulfonate as the cysteine derivative which we showed had at least a 10-fold greater sensitivity. Further work is necessary to verify the accuracy and utility of such an alternative for improving the sensitivity of this method.

The cysteine-S-sulfonate level in undialyzed rabbit plasma was shown to be 2.4 times higher by the described GC method than the total S-sulfonate determined by the spectrophotometric method. In searching for an explanation to this discrepancy, the spectrophotometric method was run on the model protein, S-BSA. The data presented in Table 3 indicated that there were "buried" S-sulfonate groups uncovered by the enzymatic hydrolysis and thus made available to cyanolytic attack. This finding, however, does not fully account for the 2-fold difference in values. The contribution of exogenous cysteine-S-sulfonate by the protease preparations was ignored because no cysteine-S-sulfonate was detected in dialyzates of the protease preparations. Further application of the two methods will have to be conducted to determine if this discrepancy is statistically significant. Thirty percent or 33 nmoles of the total cysteine-S-sulfonate was detected by the GC method in the dialyzate of the sulfite-treated plasma. Since there is approximately 40 nmoles cystine/ml plasma in rabbits, it is most likely that this cysteine-S-sulfonate was derived from free cystine that was sulfonated

during treatment of the rabbit (6). The level of low molecular weight S-sulfonates found by the spectrophotometric method would be higher because glutathione-S-sulfonate would also be dialyzable. The 30% dialyzable sulfonate obtained with the GC method was determined by direct analysis of the dialyzate and would be specific for cysteine-S-sulfonate, therefore this value would be less than that determined for the total low molecular weight S-sulfonates.

In conclusion, the data presented in Table 2 are significant in that they confirm the presence of cysteine-S-sulfonate as a primary adduct formed upon treatment of rabbits with sulfite. Previous attempts to follow the distribution of bisulfite have lacked specificity for the particular adducts formed when bisulfite is absorbed by the upper airways or through the G.I. tract, enters the circulatory system, and is deposited in peripheral tissues (21,9). This method allows investigators to selectively probe the distribution of bisulfite for physiologically significant molecular interactions. It is hoped that by judicious employment of basic research techniques such as the method proposed herein, the primary toxic agent(s) associated with sulfur oxides will be determined.

LITERATURE CITED

1. R. Shapiro. Genetic effects of bisulfite (sulfur dioxide). *Mutation Res.* **39**: 149-176 (1977).

2. W.B. Gibson and F.M. Strong. Metabolism and elimination of sulfite by rats, mice, and monkeys. *Food Cosmet. Toxicol.* **11**: 185-189 (1973).

3. E. Yokoyama, R.E. Yoder, and N.R. Frank. Distribution of ^{35}S in the blood and it's excretion in urine of dogs exposed to $^{35}SO_2$. *Arch. Environ. Health* **22**: 389-395 (1971).

4. A.F. Gunnison and A.W. Benton. Sulfur dioxide: sulfite interaction with mammalian serum and plasma. *Arch. Environ. Health* **22**: 381-388 (1971).

5. J.L. Bailey and R.D. Cole. Studies on the reaction of sulfite with proteins. *J. Biol. Chem.* **234**: 1733-1739 (1959).

6. A.F. Gunnison and E.D. Palmes. Persistence of plasma S-sulfonates following exposure of rabbits to sulfite and sulfur dioxide. *Toxicol. Appl. Pharmacol.* **24**: 266-278 (1973).

7. A.F. Gunnison and E.D. Palmes. S-sulfonates in human plasma following inhalation of SO_2. *Am. Ind. Hyg. Assoc. Journal* **35**: 288-291 (1974).

8. A.F. Gunnison and E.D. Palmes. A model for the metabolism of sulfite in mammals. *Toxicol. Appl. Pharmacol.* **38**: 111-126 (1976).

9. K.A. Caldwell and A.L. Tappel. Separation by GLC of silylated derivatives of some sulfo- and selenoamino acids and their oxidation products. *J. Chromatog.* **32**: 635-640 (1968).

10. F. Shahrokhi and C.W. Gehrke. Quantitative GLC of sulfur-containing amino acids. *J. Chromatog.* **36**: 31-41 (1968).

11. C. DeMarco, R. Mosti, and D. Cavallini. Column chromatography of some sulfur-containing amino acids. *J. Chromatog.* **18**: 492-497 (1965).

12. P. Ruffin and G. Biserte. Column chromatography of cysteine sulfinic acid, cysteic acid, and S-sulfocysteine. *Anal. Biochem.* **76**: 233-238 (1976).

13. R.L. Hill and W.R. Schmidt. The complete enzymatic hydrolysis of proteins. *J. Biol. Chem.* **237**: 389-396 (1962).

14. C.C.Q. Chin, and F. Wold. The preparation of matrix-bound proteases and their use in the hydrolysis of proteins. *Anal. Biochem.* **61**: 379-391 (1974).

15. Y. Narahashi. Pronase. *Methods in Enzymology* **19**: 651-664 (1970).

16. A. Gillman, F.S. Philips, E.S. Koelle, R.P. Allen, and E. St. John. The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulfhydryl compounds. *Am. J. Physiol.* **147**: 115-126 (1946).

17. I.H. Segel and M.J. Johnson. Synthesis and characterization of sodium cysteine-S-sulfonate monohydrate. *Anal. Biochem.* 5: 330-337 (1963).

18. S. Moore and W.H. Steine. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**: 907-913 (1954).

19. J. Leibrand and L.L Dunham. Preparing high efficiency pack GC columns. *Research/Development* **24**: 32-36 (1973).

20. W.M. Lamkin, N.S. Jones, T. Pan, D.N. Ward. Application of the flame photometric sulfur detector to the gas-chromatographic analysis of trimethylsilylated methylthiohydantions of amino acids. *Anal. Biochem.* **58**: 549-558 (1974).

21. D.P. Rall. Review of the health effects of sulfur oxides. *Environ. Health Perspectives* **8**: 97-121 (1974).

APPENDIX

General Review of Literature
on Sulfite Exposure and Cysteine-S-sulfonate Biochemistry

Exposure to oxides of sulfur

Man is exposed to the hydrate bisulfite principally through the inhalation of the gaseous air pollutants, sulfur oxides, and by ingestion of sulfite used in the processing of food and beverages.

Atmospheric exposure

Sulfur oxides (SO_x) are a combination of two gases--sulfur dioxide (SO_2) and sulfur trioxide (SO_3)--and particulate sulfates (RSO_4). Most of these pollutants are emitted as sulfur dioxide but are chemically converted in the atmosphere to SO_3 and sulfates. The major sources of SO_2 emissions are the burning of sulfur containing fuels, primarily coal and residual oil, and such industrial processing as ore smelting and petroleum refining (22). The industrial processing produces 1/4 the SO_x produced by burning fossil fuels (22). However, these industrial emissions are less consistant and thus more difficult to control than the general combustion of fossil fuels. Fuel combustion emissions are the heaviest in the Midwest and Northeast. Factors contributing to this higher emission level are (22):

1. greater urbanization and industrialization density
2. less natural gas supplies and thus greater dependence on coal and fuel oil
3. use of regionally available high sulfur coal and imported fuel oil.

The sulfur content of coals used as fuel determines the final concentrations of sulfur oxides emitted at the exhaust stack. Coals mined in the eastern U.S. are primarily bituminous and of high sulfur

content where as coal mined west of the Mississippi River are subtuminous and lignite types of low sulfur content (22). Together, these conditions mentioned have resulted in average annual concentrations at downtown sites of 364μg/m^3 for New York City, 232μg/m^3 for Chicago, and 152μg/m^3 for Jersey City (23). When compared to Western cities such as Denver with 26μg/m^3 one gains an appreciation for contribution these conditions make to the SO_x pollution seen in the urban areas of the East. The main SO_2 standards for the world are presented in Table 4 (24).

Table 4. Atmospheric standards presently in use

COUNTRY	STANDARD
USA 1974	80 μg/m^3 (mean, year), 365 μg/m^3 (highest day in year) 1300 μg/m^3 (3 hour maximum)
USSR 1973	50 μg/m^3 (24 hrs), 500 μg/m^3 (20 min)
Sweden 1965	150 μg/m^3 (highest month), 300 μg/m^3 (highest day in month) 750 μg/m^3 (30 min)
West Germany 1974	140 μg/m^3 (24 hrs), 400 μg/m^3 (30 min)
Japan 1973	120 μg/m^3 (24 hrs), 300 μg/m^3 (1 hr)

Dietary Exposure

We must consider another important source of sulfur oxides exposure to man. Sulfites as food additives have been in common for centuries with the earliest recorded use dating to Roman times where

they treated wines with SO_2 (25). Various forms of sulfite such as sodium sulfite, bisulfite, metabisulfite, and SO_2 have been used to prevent browning of light colored foods during the processing of dehydrated fruits and vegetables such as dried apples and instant potatoes (26). In addition one of the least known but none the less major contributors of dietary sulfite is its use as a selective antibacterial agent allowing yeast development during wine making (26, 27). When these compounds are dissolved in H_2O they dissociate into the sulfite, SO_3^{-2}, or bisulfite, HSO_3^-, as described by the following equilibrium: (28)

$$SO_2 + H_2O \rightleftharpoons H_2SO_3 \overset{-H^+}{\rightleftharpoons} HSO_3^- \overset{-H^+}{\rightleftharpoons} SO_3^{=}$$

at pH 7.2, 25°C, and low salt concentration, bisulfite and sulfite will appear in equal concentration (28).

In the presence of water and the organic constituents of foods or beverages, the possibilities for their chemical reaction are numerous, the three most common being oxidation to sulfate (29, 30) addition across carbonyl groups (31, 32) and addition to disulfide linkages in proteins (33).

This type of reactivity of sulfite with other compounds makes it difficult to determine the exact amount of sulfite ingested in the "normal diet" of humans. Gibson and Strong attempted to estimate the average dietary intake of sulfite in the USA by basing their per capita estimates on a population of 200 million with the beer and wine drinking population estimated at 75% of the total. Solid foods and non-alcoholic beverages contributed approximately 2mg SO_2/day to the total, while alcoholic beverages accounted for the remainder (beer 1.2

and wine 3.0 mg SO_2/day). Because of the inclusion of alcoholic beverages, children are not included in these per capita estimates presented in Table 5 (34).

Table 5. Dietary sulfite levels.

Levels of intake	mg/kg*	mg/70 kg man*
US Average	0.10	7.2
US Maximum	1.7	120
FAO/WHO "acceptable"	0.35	25
FAO/WHO "conditionally acceptable"	1.5	105

*Expressed as SO_2

This estimate is in close agreement with an estimate by Bigwood for the daily SO_2 consumption in Belgium. The major difference is the estimate for alcoholic beverages with Bigwood at 16.3 mg/day vs Gibson at 5.2 mg/day (35).

Exposure estimate

By bringing these two routes of exposure together, one can calculate a maximum exposure level for oxides of sulfur in the U.S. The maximum concentration for SO_2 in the workplace is 4.1 ppm. If a worker was exposed at this concentration for 8 hours of light work, he would absorb 140 mg of SO_2. In addition if he were to consume the U.S. max dietary sulfite level of 120 mg that would be a total of 260 mg of

sulfite/day. As one can see, this is a significant exposure to a reactive chemical moiety for which the molecular targets have not been fully elucidated.

Metabolism

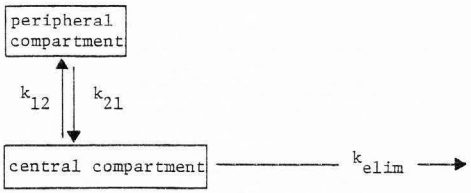
Sulfite is rapidly absorbed into the blood of mammals either by the nasotrachea region or by the digestive tract depending on the exposure (34, 36). Once in the blood the sulfite has been shown to react very rapidly with disulfide bonds of cystine, protein, and other low molecular weight disulfide-containing molecules (37). Using a spectrophotometric color reaction that follows the uptake of inorganic sulfite, Gunnison and Benton were able to follow the uptake of sulfite by plasma proteins, cysteine, cystine, oxidized glutathione, and insulin. They weren't able to identify the product species directly, but offered evidence that it was the S-sulfonate of cysteine that was being formed by the following reaction:

$$RSSR + SO_3^{2-} \rightleftharpoons RSSO_3^- + RS^-$$

This reaction is not without precedent. It has been used as a gentle method for breaking disulfide bonds in vitro and was suggested by many workers as part of normal sulfur metabolism in molds and mammals (38-42).

Expanding the use of their indirect method, Gunnison and coworkers have accumulated a wealth of kinetic data on the absorbtion, distribution, and elimination of both inhaled SO_2 and dietary sulfite (43-

46). The evidence indicates that sulfite pharmacokinetics in mammals fits a two-compartmental, open-system model when administered by rapid i.v. injection. It has been suggested that the sulfite rapidly distributes between a central compartment from which it is eliminated and a peripheral compartment. A graphic representation would look like this:



and described by the following equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_p=plasma concentration, A,B=coefficients (nmole/ml) α, β,=hybrid rate constants.

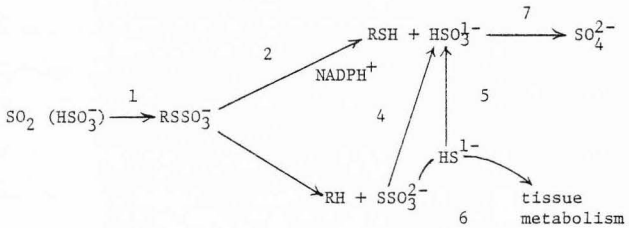
Supporting evidence for a slower equilibrating peripheral compartment has recently been presented by Gunnison and Farrugella (47). They demonstrated the formation of stable S-sulfonates in microfibrillar proteins of the lung and aorta of rabbits with clearance half lives of 2-3 days. Gibson and Strong also suggested that radio-labeled sulfite not eliminated from rats might be bound to body components with a slow turnover rate (34). Balchum et al were able to detect ^{35}S in the airway tissues of a dog one week after the animal had been exposed to $^{35}SO_2$ through a tracheal cannula (48).

The clearance of absorbed sulfite from the central compartment is quite rapid with 70% of an absorbed dose appearing in the urine within 24 hours (34). The elimination rate constants for i.v.-administered sulfite have been determined in rabbits and rhesus monkey (45). Gunnison and Palmes have shown that sulfite is cleared almost exclusively by oxidation to sulfate (45). This oxidation of sulfite to sulfate is catalyzed by the enzyme sulfite oxidase (sulfite: O_2 oxidoreductase, EC 1.8.3.1) (49). This enzyme has been found in all mammals investigated and in a variety of tissues but primarily in the liver and kidney (49). The essential nature of this enzyme in humans was observed in a two year old patient born with an apparent deficiency in this enzyme. The child was highly abnormal and died at 2 1/2 years of age. The patient's metabolism was such that it excreted inorganic sulfite and S-sulfonates but no sulfate (50).

Supporting evidence indicating the essential nature of sulfite oxidase in protecting against sulfite exposure was performed by Cohen et al. (51). Sulfite oxidase is known to contain molybdenum. Cohen et al. were able to produce a sulfite oxidase deficient condition by feeding rats a low molybdenum diet (30 µg/kg of diet) and allowing the animals free access of water supplemented with 100 ppm tungsten. Tungsten has been shown by Higgins et al. to be a competitive inhibitor of Mo in animal systems (52). Animals treated with tungsten and low Mo diet for 3 weeks and 5 weeks showed a LD_{50} to sodium bisulfite (I.P. inj.) of 271 and 181 mg/kg, respectively. The LD_{50} for those on rat chow was 551 mg/kg and those on low Mo without tungsten, 475 mg/kg. Animal exposed to SO_2 showed no difference in survival rate of

control animals but did show difference in survival time. In addition the symptoms accompanying death were different. Those animals deficient in sulfite oxidase exhibited central nervous system disorders while those on normal diet showed symptoms largely associated with respiratory difficulties.

It has been proposed that the suspected S-sulfonates formed by the reaction of sulfite with disulfide bonds is a buffer system which gives sulfite oxidase time to oxidize the sulfite to sulfate by binding the sulfite and rendering it inactive. The following metabolic scheme has been suggested (53):



1, occurs in blood and lung tissue

2-7, occur in liver

2, $NADPH^+$ dependent reduction

3, reductive cleavage

4, catalysis by rhodanase or thiosulfate reductase

5, oxidation

6, tissue metabolism

7, catalysis by sulfite oxidase

Reactions with Nucleic Acids

A review of the biochemical effects of sulfite would not be complete without a brief summary of the reaction of sulfite with nucleic acids. The principal reactions were reviewed by Shapiro (28):

1) deamination of cytosine
2) transamination; crosslinking of proteins
3) addition of bisulfite to uracil and thymine
4) free radical reactions.

The concern surrounding the interaction of sulfite with nucleic acids is the increasing awareness of the correlation between mutagenesis and carcinogenesis (54). Since sulfite has been shown to cause deamination of cytosine the potential for a mutant transition could result in a new arrangement of bases during the replication process (55). It is evidence of this nature that has heightened the concern over systemic exposure to sulfite and raised the question of dietary tolerances. For a good review of the biochemistry of sulfite see Shapiro and Petering (28, 52).

Literature Cited for
General Review of Literature

1. Lynn, D.A. 1976. Air pollution: threat and response. Addison-Wesley Publishing Co., Reading, Mass. 388 pp.

2. U.S. Environmental Protection Agency. 1974. Health consequences of sulfur oxides: a report from CHESS, 1970-1971. May. EPA-650/1-74-004.

3. Biersteker, K. 1976. Sulfur dioxide and suspended particulate matter. Where do we stand? Environmental Research 11: 287-304.

4. Institute of Food Technologist. 1976. Sulfites as food additives. Nutr. Rev. 34: 58.

5. Burroughs, L.F. 1974. Browning control in fruit juices and wines, Chem. Ind. (London) 718-719.

6. Chichester, D.F. and F.W. Tanner, Jr. 1972. Antimicrobial food additives in T.E. Furia (ed.) Handbook of food additives, 2nd ed., CRC Press, Cleveland. pp 115-184.

7. Shapiro, R. 1977. Genetic effects of bisulfite (SO_2) Mut. Res. 39: 149-176.

8. Postgate, J.R. 1963. The examination of sulfur auxotrophs: a warning. J. Gen. Microbiol. 30: 481-490.

9. Schroeter, L.C. 1963. Kinetics of air oxidation of sulfurous acid salts. J. Pharm. Sci. 52: 559-563.

10. Brauerman, J.B.S. 1953. The mechanism of the interaction of SO_2 with certain sugars. J. Sci. Fd. Agric. 4: 540-548.

11. Ingles, D.L. 1959. Chemistry and non-enzymatic browning. The preparation of aldose-potassium bisulfite addition compounds and some amine derivatives. Aust. J. Chem. 12: 97-108.

12. Milligan, B. and Swan, J.M. 1962. Bunte salts ($RSSO_3Na$). Rev. Pure Appl. Chem. 12: 72-89.

13. Gibson, W.B. and Strong, F.M. 1973. Metabolism and elimination of sulfite by rats, mice, and monkeys. Fd. Cosmet. Toxicol. 11: 185-198.

14. Bigwood, E.J. 1968. Consommation probable de SO_2 en Belgique. Arch. Belg. Med. Soc. 26: 473-479.

15. Rall, D.P. 1974. Review of the health effects of sulfur oxides. Environ. Hlth. Perspectives 8: 97-121.

16. Gunnison, A.F. and A.W. Benton. 1971. Sulfur dioxide: sulfite interaction with mammalian serum and plasma. Arch. Environ. Health 22: 381-388.

17. Leach, S.J., J.M. Swann, and L.A. Holt. 1963. Separation and characterization of the products of oxidative sulfitolysis of insulin. BBA 78: 196-205.

18. Waley, S.G. 1959. Acidic peptides of the lens 5. S-sulfoglutathione. Biochem. J. 71: 132-137.

19. Bailey, J.L. and R.D. Cole. 1959. Studies on the reaction of sulfite with proteins. J. Biol. Chem: 234 (7): 1733-1739.

20. Shepherd, C.J. 1956. Pathways of cysteine synthesis in Aspergillus nidulans. J. Gen. Microbiol. 15: 29-34.

21. Sorbo, B. 1958. On the metabolism of thiosulfate esters. Acta. Chemica Scandinavica 12: 1990-1996.

22. Gunnison, A.F. and E.D. Palmes. 1973. Persistance of plasma S-sulfonates following exposure of rabbits to sulfite and sulfur dioxide. Toxicol. Appl. Pharmacol. 24: 266-278.

23. Gunnison, A.F. and E.D. Palmes. 1974. S-sulfonates in human plasma following inhalation of SO_2. Am. Ind. Hyg. Assoc. J. 35: 288-291.

24. Gunnison, A.F. and E.D. Palmes. 1976. A model for the metabolism of sulfite in mammals. Toxicol. Appl. Pharmacol. 38: 111-126.

25. Gunnison, A.F. and E.D. Palmes. 1978. Species variability in plasma S-sulfonate levels during and following sulfite administration. In press.

26. Gunnison, A.F. and T.J. Farruggella. 1979. Preferential S-sulfonate formation in lung and aorta. Chemico-biological Interactions 25 (2-3): 271-278.

27. Balchum, O.J., J. Dybicci, G.R. Meneely. 1960. Pulmonary resistance and compliance with concurrent radioactive sulfur distribution in dogs breathing $^{35}SO_2$. J. Appl. Physiol. 15: 62-66.

28. Cohen, H.J. and I. Fridovich. 1971. Hepatic sulfite oxidase. J. Biol. Chem. 246 (2): 359-366.

29. Irreverre, F., S.H. Mudd, W.D. Heizer, and L. Laster. 1967. Sulfite oxidase deficiency: studies of a patient with mental retardation, dislocated ocular lenses, and abnormal urinary excretion of S-sulfo-cysteine, sulfite, and thiosulfate. Biochem. Med. 1: 187-217.

30. Cohen, H.J., Drew, R.T., Johnson, J.L., Rajagopalan, K.V. 1973. Molecular basis of the biological function of molybdenum. The relationship between sulfite oxidase and the acute toxicity of bisulfite and SO_2. Proc. Nat. Acad. Sci. 70 (12): 3655-3659.

31. Higgins, E.S., D.A. Richert, W.W. Westerfield. 1956. Molybdenum deficiency and tungstate inhibition studies. J. Nutr. 59: 539-559.

32. Petering, D.H. and N.T. Shih. 1975. Biochemistry of bisulfite-SO_2. Environ. Res. 9: 55-65.

33. Miller, E.C. and J.A. Miller. 1971. The mutagenicity of chemical carcinogens: Correlations, problems, and interpretations. In A. Hollander, (ed.): Chemical Mutagens. Plenum Press, New York. pp. 81-120.

34. Shapiro, R., V. Difate, M. Welcher. 1974. Deamination of cytosine derivatives by bisulfite: Mechanism of the reaction. J. Amer. Chem. Soc. 96: 906-912.