Chem. Pharm. Bull. 25(9)2369—2377(1977)

UDC 547.269.4.08 : 543.426.061

Spectrophotometric Determination of Bunte Salts as Bisulfite with Acid-bleached Basic Fuchsine or N-(*p*-Dimethylaminophenyl)-1,4-naphthoquinoneimine after Reduction with Dithiothreitol

Hiroshi Nakamura and Zenzo Tamura

Faculty of Pharmaceutical Sciences, University of Tokyo[1]

(Received January 20, 1977)

Colorimetric and fluorometric methods for the determination of Bunte salts have been developed. Bunte salts are reduced with dithiothreitol (DTT) to monothiols and bisulfite. The latter is determined colorimetrically with a basic fuchsine reagent in the presence of mercuric chloride or fluorometrically with N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine in the presence of a mixture of *p*-chloromercuribenzoic acid and sodium arsenite. The sulfhydryl reagents and arsenite mask the thiols by forming mercaptides and a DTT-arsenite complex, respectively. The colorimetric and the fluorometric methods can determine Bunte salts in a range of 5×10^{-9} to 1×10^{-7} mol and 2×10^{-8} to 1.5×10^{-7} mol, respectively.

Keywords——Bunte salt; dithiothreitol; bisulfite; reduction; colorimetry; fluorometry; basic fuchsine; thiol; addition reaction; 1,4-naphthoquinoneimine

Since first reported by Bunte[2] in 1874, the so-called "Bunte salts" or organic thiosulfates have attracted wide interest in the textile industry as dyes[3] and in the pharmaceutical industry as intermediates[4] in the synthesis of unsymmetrical disulfides, such as thiamine.[5] Some of the Bunte salts exhibit radioprotective actions,[6,7] a synergic effect on the bacteriostatic action of 2-mercaptobenzothiazole[8] and a preventative and curative action against fowl coccidiosis.[9] Naturally occurring Bunte salts include cysteine-S-sulfate,[10] glutathione-S-sulfate,[11] pantetheine-S-sulfate,[12] 4′-phosphopantetheine-S-sulfate[12,13] and 3′-dephosphocoenzyme A-S-sulfate.[14] Recently, Bunte salts have been recognized as detoxification products of inhaled sulfur dioxide.[15] Their latent capacity to release bisulfite *in vivo*[16] is significant in view of the mutagenecity and carcinogenecity of bisulfite.[17] Several reviews on Bunte salts have appeared.[18–21]

1) Location: *Hongo-7-3-1, Bunkyo-ku, Tokyo, 113, Japan.*
2) H. Bunte, *Chem. Ber.*, **7**, 646 (1874).
3) K. Schimmelschmidt, H. Hoffmann, and E. Baier, *Angew. Chem.*, **74**, 975 (1962).
4) H.B. Footner and S. Smiles, *J. Chem. Soc.* (London), **1925**, 2887.
5) T. Matsukawa and S. Yurugi, "Review of Japanese Literature on Beriberi and Thiamine" ed. by N. Shimazono and E. Katsura, Vitamin B Research Committee of Japan, Kyoto, 1965, p. 110.
6) L. Eldjarm and A. Pihl, *J. Biol. Chem.*, **223**, 341 (1956).
7) B. Holmberg and B. Sörbo, *Nature*, **183**, 832 (1959).
8) G. Bargellini and E. Del Pinto, *Chem. Abstr.*, **42**, 8867 (1948).
9) H.Z. Lecher and E.M. Hardy, *J. Org. Chem.*, **20**, 475 (1955).
10) T. Nakamura and R. Sato, *Nature*, **185**, 163 (1960).
11) S.G. Waley, *Biochem. J.*, **71**, 132 (1959).
12) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **20**, 2008 (1972).
13) M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 178 (1971).
14) M. Yoshioka and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo). **19**, 186 (1971).
15) A.F. Gunnison and A.W. Benton, *Arch. Environ. Health*, **22**, 381 (1971).
16) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1632 (1974).
17) H. Hayatsu and A. Miura, *Biochem. Biophys. Res. Commun.*, **39**, 156 (1970).
18) B. Milligan and J.M. Swan, *Review of Pure and Applied Chemistry*, **12**, 72 (1962).
19) H. Distler, *Angew. Chem. Int. Ed. Engl.*, **6**, 544 (1967).
20) S. Oae, G. Tsukamoto, and T. Kurusu, *Kagaku* (Kyoto), **26**, 1066 (1971).
21) D.L. Klayman and R.J. Shine, *Quarterly Reports on Sulfur Chemistry*, **3**, No. 3, 188 (1968).

Despite their significance a specific assay for Bunte salts has not been reported. Possible assays could be based on their cyanolysis (Eq. 1), acid hydrolysis (Eq. 2) or reduction (Eq. 3).

$$RSSO_3Na + NaCN \longrightarrow RSCN + Na_2SO_3 \quad \text{(Eq. 1)}$$

$$RSSO_3Na + H_2O \longrightarrow RSH + NaHSO_4 \quad \text{(Eq. 2)}$$

$$RSSO_3Na + 2H \longrightarrow RSH + NaHSO_3 \quad \text{(Eq. 3)}$$

Determination of sulfite released by reduction of Bunte salts seems most likely to be free of interfering side reactions since thiosulfate ions give sulfite upon cyanolysis; disulfides and the sulfate esters give thiols and sulfate, respectively, after acid hydrolysis and, finally, disulfides also give thiols through reduction.

In the present investigation, spectrophotometric methods for determination of Bunte salts have been developed. They are based on reduction of the salts with dithiothreitol (DTT) and measurement of the bisulfite with either acid-bleached basic fuchsine[22] or N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine.[23] A preliminary report has appeared.[24]

Experimental

Apparatus——The following were used: a Hitachi MPF-2A spectrofluorometer and 1 cm quartz cells, a photoelectric spectrophotometer (Type 6B, Hirama Rika Kenkyujo, Kawasaki, Japan), a Hitachi Recording Spectrophotometer Model EPS3-T, a Toa HM-5A pH meter (Toa Denpa Kogyo, Tokyo, Japan), Mitsumi SJ-1050 (A) (Mitsumi Scientific Co., Tokyo, Japan) for paper electrophoresis (1 M HCOOH, pH 1.75, 2 hr, 13.3 V/cm) and a Packard radiochromatogram scanner model 7200 operated with a time constant, 10 sec; linear scale, 3×10^3 cpm; chart speed, 5 cm/min; flow rate of He, 300 ml/min.

Materials——Cysteine-S-sulfate monohydrate ($CySSO_3H$), cysteamine-S-sulfate ($CyNSSO_3H$), penicillamine-S-sulfate ($PenSSO_3H$), dimethylaminoethanethiol-S-sulfate ($DMCyNSSO_3H$), *p*-aminothiophenol-S-sulfate ($PATPSSO_3H$) and benzylmercaptan-S-sulfate sodium salt ($BzSSO_3Na$) were synthesized from the corresponding thiols and chlorosulfonic acid as previously described.[25] The first four are internal salts, "Zwitter ions." Other Bunte salts, pantetheine-S-sulfate calcium salt [$(PaSSO_3)_2Ca$] and 4′-phosphopantetheine-S-sulfate calcium salt [$(P\text{-}PaSSO_3)_2Ca$] were provided by Daiichi Seiyaku, Tokyo, Japan. The following were purchased from commercial sources: dithiothreitol (DTT), Seikagaku Kogyo, Tokyo; basic fuchsine (extra pure reagent), formalin (GR, minimum assay 37.0%), mercuric chloride ($HgCl_2$, GR, 99.5%), sulfuric acid (GR), ethylenediaminetetraacetic acid disodium salt (EDTA, GR), sodium thiosulfate pentahydrate (GR), sodium arsenite ($NaAsO_2$, GR, 97%) and sodium bisulfite (GR), Kanto, Tokyo; N-ethylmaleimide (NEM, specially prepared reagent), Nakarai, Kyoto; N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine (indophenol blue), L-cysteine (GR) and *p*-chloromercuribenzoic acid (PCMB, GR), Tokyo Kasei, Tokyo; radioactive NEM (ethyl-2-^{3}H, specific activity, 150—300 mCi/mmol), New England Nuclear; Toyo Filter Paper No. 514, Toyo Roshi, Tokyo.

Preparation of Reagents and Buffers——a) Bunte Salts: Stock solutions of 0.5 mM of each Bunte salt were prepared with distilled water. They were stable for at least six months when stored at −20° in the dark.

b) Bisulfite: A 0.5 mM solution was prepared daily by dissolving 10.47 mg of $NaHSO_3$ in 200 ml of distilled water just before use. This solution was standardized with 0.01 N iodine solution.

c) Dithiothreitol (DTT): A 5 mM solution was prepared by dissolving 7.71 mg of DTT in 10 ml of distilled water just before use.

d) Mercuric chloride: A 0.1 M solution was prepared by dissolving 271.52 mg of $HgCl_2$ in 10 ml of distilled water.

e) Solution A: 0.05 M Tris–HCl buffer (pH 9.20) containing 5 mM EDTA.

f) Indicator Solution: Acid-bleached basic fuchsine was mixed with formaldehyde as described by Urone and Boggs[22] except that 37% formaldehyde was used instead of 40% formaldehyde.

g) Sodium Arsenite: A 10 mM solution was prepared by dissolving 12.99 mg of $NaAsO_2$ in 10 ml of distilled water.

h) SH Blocker: 10 mM PCMB and 10 mM $NaAsO_2$ were dissolved in Solution A.

i) HCl-Citrate Buffer (pH 3.48): One to one mixture of 1.0 M HCl and 1.0 M dipotassium hydrogen citrate.

22) P.F. Urone and W.E. Boggs, *Anal. Chem.*, **23**, 1517 (1951).
23) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1950 (1974).
24) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 2208 (1974).
25) T. Tanaka, H. Nakamura, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 2725 (1974).

j) N-(*p*-Dimethylaminophenyl)-1,4-naphthoquinoneimine: A 0.1 mM solution was prepared by dissolving 2.76 mg of N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine in 100 ml of absolute ethanol.

Procedure

a) Colorimetric Method

To 1.0 ml of sample, blank (water) or standard solution containing 5×10^{-9} to 1×10^{-7} mol of a Bunte salts, add 1.0 ml of 5 mM DTT in 0.05 M Tris-HCl buffer (pH 9.20) containing 5 mM EDTA (solution A) and incubate at 37° for 5 min. Then add 1.5 ml of 0.1 M $HgCl_2$, and centrifuge at 4000 rpm for 5 min to remove the mercaptides.

Add 1.0 ml of the indicator solution to 3.0 ml of the clear supernatant, let stand at room temperature for 10 min and measure the absorbance at 580 nm against water. The value thus obtained corresponds to a sum of the preexistent bisulfite and the bisulfite liberated from the Bunte salt. The amount of the Bunte salt is determined by subtracting from the total amount of bisulfite the amount of preexistent bisulfite determined by reversing the order of addition of DTT and $HgCl_2$.

b) Fluorometric Method

One ml of the sample containing 2×10^{-8} to 1.5×10^{-7} mol of a Bunte salt is reduced as described above. To 2.0 ml of the reduction mixture, add 1.0 ml of a mixture of 10 mM PCMB and 10 mM $NaAsO_2$ in solution A (SH blocker), incubate at 37° for 10 min, and add 1.0 ml of 1.0 M HCl-citrate buffer (pH 3.48). Remove the mercaptide precipitates by centrifugation at 4000 rpm for 5 min, add 1.0 ml of 0.1 mM N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine in ethanol to 3.0 ml of the resultant clear supernatant; incubate at 37° for 30 min. Add 1.0 ml of 2.0 N NaOH, mix, and measure the fluorescence at λ_{ex} 340 nm and λ_{em} 435 nm.

The amount of the preexistent bisulfite to be subtracted was obtained by adding 1.0 ml of SH blocker and 1.0 ml of solution A to 1.0 ml of the sample and incubating at 37° for 10 min.

Determination of Reaction Products of $CySSO_3H$ and DTT

One ml each of 0.5 mM $CySSO_3H$, 0.5 mM DTT and 0.05 M Tris-HCl buffer (pH 7.98) containing 5 mM EDTA was mixed, incubated at 40° for 45 min and the reaction products were analyzed as follows:

a) Cysteine——Thiols in the presence of bisulfite were determined by a modification of Ellis's method.[26] To the reaction mixture was added 1 ml of 0.2 M NEM in isopropanol and the solution was incubated at 40° for 70 min. Then 1 ml of 2.0 M Na_2CO_3 was added, the solution was allowed to stand at room temperature for 20 min, and the absorbance at 520 nm was measured against water in 1 cm cuvettes.

b) Bisulfite——Bisulfite in the presence of thiols was determined fluorometrically by our previous method[27] using 1 ml aliquots of the reaction mixture.

c) Oxidized DTT——The absorbance at 283 nm of the reaction mixture was measured and the amount of oxidized DTT was calculated using a molar extinction coefficient of 273 at 283 nm for oxidized DTT.[28]

Results

Reduction of Bunte Salts

We used Cleland's reagent DTT[29] which quickly reduces disulfide bonds for the reduction of Bunte salts. As Figure 1 illustrates, two representative Bunte salts, $CySSO_3H$ and

26) R.J. Ellis, *Biochim. Biophys. Acta*, **85**, 335 (1964).
27) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **23**, 1261 (1975).
28) K.S. Iyer and W.A. Klee, *J. Biol. Chem.*, **248**, 707 (1973).
29) W.W. Cleland, *Biochemistry*, **3**, 480 (1964).

$(PaSSO_3)_2Ca$, were completely reduced within a few min at 37° and pH 9.20 with 1.67 mM DTT. With the exception of $PenSSO_3H$, the reduction of other Bunte salts, including $CyNSSO_3H$, $DMCyNSSO_3H$, $PATPSSO_3H$, $BzSSO_3Na$ and $(P\text{–}PaSSO_3)_2Ca$, was also quantitative under these conditions (Fig. 1). At a lower temperature (20°) or pH 7.98 the rare of reduction was decreased. The reaction of Bunte salts was incomplete when the concentration of DTT was decreased to 0.167 mM. Inorganic thiosulfate gave no bisulfite with DTT. Bisulfite was stable in the reaction mixture for at least 2 hr.

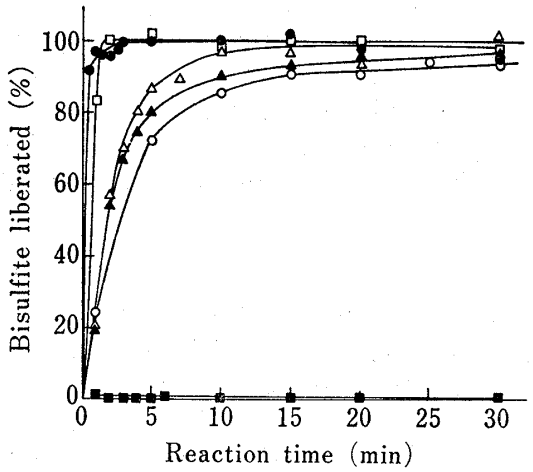


Fig. 1. Reduction of Bunte Salts as a Function of DTT Concentration, pH and Temperature

One ml each of a Bunte salt and DTT solutions was mixed with 1.0 ml of 0.05 M Tris-HCl buffer containing 5 mM EDTA, and the reaction mixture was incubated at 37° or at 20°. The reaction was followed by measuring the amount of bisulfite formed by using the fluorometric method described in the text. The relative fluorescence intensities to the fluorescence intensity of 2×10^{-7} mol $NaHSO_3$ were plotted.

—○—: 2×10^{-7} mol $CySSO_3H$, 5×10^{-7} mol DTT, pH 9.20, 37°.
—●—: 2×10^{-7} mol $CySSO_3H$, 5×10^{-6} mol DTT, pH 9.20, 37°.
—△—: 2×10^{-7} mol $CySSO_3H$, 5×10^{-6} mol DTT, pH 7.98, 37°.
—▲—: 2×10^{-7} mol $CySSO_3H$, 5×10^{-6} mol DTT, pH 7.98, 20°.
—□—: 1×10^{-7} mol $(PaSSO_3)_2Ca$, 5×10^{-6} mol DTT, pH 9.20, 37°.
—■—: 2×10^{-7} mol $PenSSO_3H$, 5×10^{-6} mol DTT, pH 9.20, 37°.

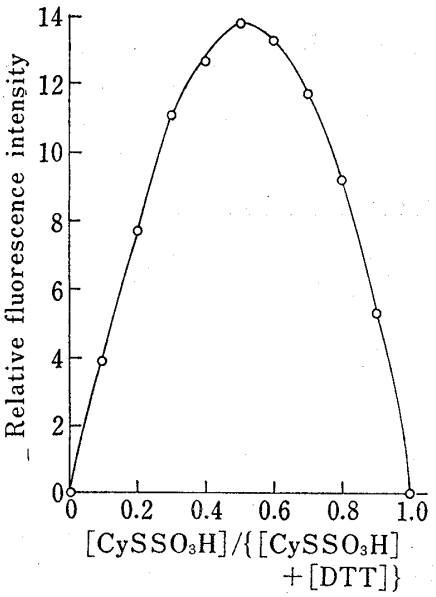


Fig. 2. Estimation of the Stoichiometry of the Reaction of $CySSO_3H$ with DTT by the Continuous Variation Method

To one ml of various combinations of 200 μM of $CySSO_3H$ and 200 μM of DTT was added 1 ml of solutions A, and the mixture was incubated for 35 min at 37°. The assay was the fluorometric method given in the text.

$[CySSO_3H]+[DTT]=1\times10^{-4}$ M.

Stoichiometry of the Reaction

The stoichiometry of the reduction of Bunte salts with DTT was investigated using $CySSO_3H$ as a model compound. An equimolar reaction of $CySSO_3H$ with DTT was indicated by both the continuous variation method (Fig. 2) and the molar ratio method (Fig. 3). When the reaction mixture was treated with ^3H–NEM at pH 7 and 37° for 45 min followed by paper electrophoresis in 1 M HCOOH, pH 1.75 for 2 hr, NEM adducts of cysteine, bisulfite and DTT were apparent (Fig. 4). Under the analytical procedures employed, ^3H–NEM was not detected in the paper electrophoretogram, probably because it was lost during the *in vacuo* evaporation. Analytical data of the reaction mixture obtained by a modification of Ellis's method,[26] which permits the selective determination of CySH in the presence of bisulfite by the color reaction with NEM (Table I,a) indicates that 4.61×10^{-7} mol of thiol (as CySH) was formed from 5×10^{-7} mol each of $CySSO_3H$ and DTT. Furthermore, 4.80×10^{-7}

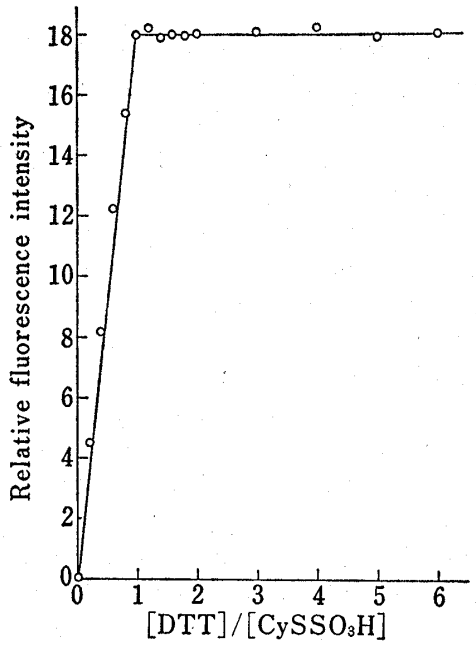


Fig. 3. Estimation of the Stoichiometry of the Reaction of $CySSO_3H$ with DTT by the Molar Ratio Method

One ml of 100 μM (or 0 μM) of $CySSO_3H$ was mixed with 0 to 3.0 ml of 200 μM DTT and then the volume was adjusted to 4.0 ml with distilled water. After addition of 1 ml of solution A, the mixture was incubated for 16 hr at 37° and liberated bisulfite was determined by the fluorometric method described in the text.
$[CySSO_3H]=2\times10^{-5}$ M.

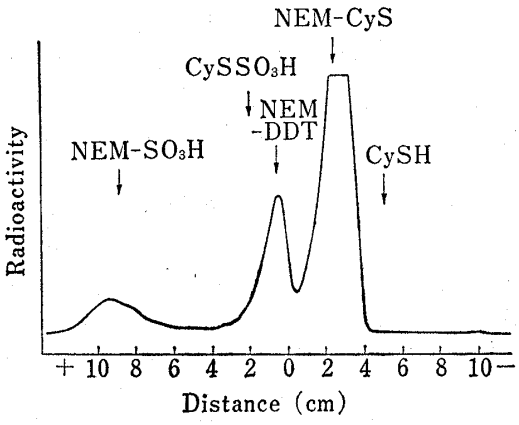


Fig. 4. Electrophoretogram of the Reaction Products

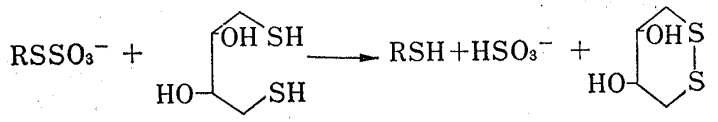
One ml of 0.5 mM $CySSO_3H$, 0.1 ml of 5 mM DTT and 10 μl of 2 M Na_2CO_3 were mixed and incubated for 40 min at 37°. The mixture was adjusted to pH 7 with 5% acetic acid and 0.2 ml of ^{3}H-NEM (4.57×10^6 dpm, 8.96×10^{-9} mol) and then 50 μl of 10 mM NEM in isopropanol were added. After incubation for 45 min at 37°, the reaction mixture was evaporated to dryness *in vacuo* at 40°, dissolved in 50 μl of methanol and repeatedly spotted on Toyo Filter Paper No. 514 under a cold stream of air. Electrophoresis was performed for 2 hr at 400 V/30 cm in 1 M HCOOH, pH 1.75. The arrows show the migration positions of standards. NEM–SO_3H, NEM–DTT and NEM–CyS were synthesized by reacting ^{3}H-NEM with $NaHSO_3$, DTT and CySH respectively. $CySSO_3H$ and CySH were detected by ninhydrin spray.

Table I. Analytical Data for Thiol, Bisulfite and Oxidized DTT in the Reaction Mixture

Compound	10^{-7} mol added	a) Thiol[a] absorbance at 520 nm	b) Bisulfite[b] relative fluorescence intensity	c) Oxidized DTT[c] absorbance at 283 nm
Authentic				
None	—	0	0	0
$CySSO_3H$	5.00	0	0	0
$NaHSO_3$	5.00	0.001	95.0	0
L-CySH	5.00	0.077	0	0
DTT	5.00	0.133	0	0.113
$NaHSO_3$+L-CySH	5.00 each	0.078	95.1	0
Reaction product				
($CySSO_3H$+DTT)	5.00 each	0.071	91.2	0.159

a) Ellis method.[26]
b) Nakamura and Tamura method.[24,27]
c) Iyer and Klee method.[28]

mol of bisulfite (Table I,b) and 5.05×10^{-7} mol of oxidized DTT (Table I,c) were found in the same reaction mixture by our fluorometric method for bisulfite[24,27] and the UV method,[28] respectively. These results suggest that the reaction between Bunte salts and DTT proceeds in the following scheme:



Determination of Bunte Salt

Attempts to directly determine bisulfite in the reduction mixture with the acid-bleached basic fuchsine or N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine were unsuccessful due to liberated monothiols and the interference of excess DTT. Monothiols and DTT reacted with the color reagent to yield colors similar to that obtained with bisulfite. The interfering thiols were easily removed before reaction with the color reagent by treating with 0.1 M mercuric chloride to form water-insoluble mercaptides which were removed by centrifugation. N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine also reacted with thiols to give bluish green fluorescence as previously reported.[27] In general, thiols reduced the fluorescence because they consumed the reagent. Mercuric chloride could not be used as a masking agent of thiols because it quenches the fluorescence.[27] A mixture of 10 mM $NaAsO_2$ and 10 mM PCMB in a buffered solution of pH 9.20 completely masked the excess amount of DTT and monothiols. Although 10 mM $NaAsO_2$ and 10 mM PCMB were effective in removing DTT and thiols respectively, a slightly higher fluorescence was always obtained with the mixture than when they were used individually. Incubation of the reduction mixture with the sulfhydryl reagents at 37° for 10 min yielded a bluish white colloidal solution due to mercaptides of PCMB. The colloid was removed by adding 1.0 M HCl-citrate buffer (pH 3.48) to induce precipitation. Reaction of N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine with bisulfite in the presence of the precipitates often gave non-reproducible results, probably owing to adsorption of the fluorogenic reagent to the colloid.

Based on these observations, two procedures were established for the determination of Bunte salts as described in Experimental. The working curves for $CySSO_3H$, $CyNSSO_3H$ and $BzSSO_3Na$ by the colorimetric method are linear in the range of 5×10^{-9} to 1×10^{-7} mol (Fig. 5). In the fluorometric method, the lower limit of determination is approximately 2×10^{-8} mol of a Bunte salt (Fig. 6) and the working curves are linear up to 1.5×10^{-7} mol. The reproducibilities of the colorimetric (Table II) and the fluorometric (Table III) methods were

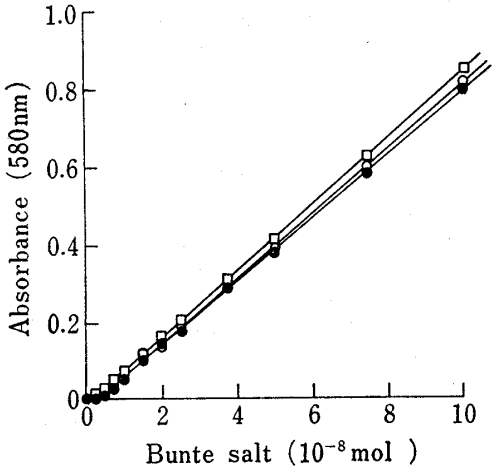


Fig. 5. Working Curves for $CySSO_3H$, $CyNSSO_3H$ and $BzSSO_3Na$ using the Colorimetric Method

—○—, $CySSO_3H$.
—●—, $CyNSSO_3H$.
—□—, $BzSSO_3Na$.

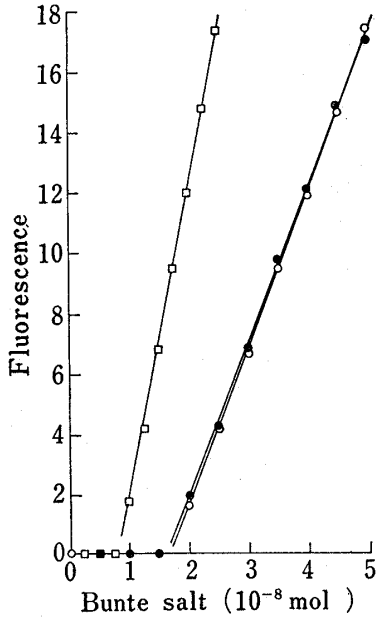


Fig. 6. Working Curves for $CySSO_3H$, $DMCyNSSO_3H$ and $(PaSSO_3)_2Ca$ using the Fluorometric Method

—○—, $CySSO_3H$.
—●—, $DMCyNSSO_3H$.
—□—, $(PaSSO_3)_2Ca$.

Table II. Reproducibility of the Colorimetric Method

$CySSO_3H$ (added)	Found (10^{-8} mol)											
	1	2	3	4	5	6	7	8	9	10	Mean	σ
5.00×10^{-8} mol	5.06	4.91	5.05	5.13	5.12	5.01	4.98	4.88	4.85	4.88	5.00	0.09
1.00×10^{-7} mol	9.86	10.8	10.4	10.1	9.93	9.61	9.96	10.0	9.59	9.75	10.0	0.35

Table III. Reproducibility of the Fluorometric Method

$CySSO_3H$ (added)	Found (10^{-8} mol)											
	1	2	3	4	5	6	7	8	9	10	Mean	σ
5.00×10^{-8} mol	5.27	4.85	4.67	4.59	4.98	4.88	5.23	5.08	5.01	4.99	4.97	0.21
1.00×10^{-7} mol	9.86	10.7	9.08	9.43	10.3	10.1	10.1	10.4	10.1	10.3	10.0	0.45

Table IV. Determination of $CySSO_3H$ in the Presence of Bisulfite by the Colorimetric Method

Added (10^{-8} mol)		Found (10^{-8} mol)[a]			
$CySSO_3H$	$NaHSO_3$	$CySSO_3H$		$NaHSO_3$	
1.00	0	1.09	(109)	0	—
1.00	1.00	0.92	(92.0)	1.07	(107)
1.00	5.00	0.89	(89.0)	4.82	(96.4)
1.00	10.0	1.05	(105)	9.78	(97.8)
1.00	25.0	1.20	(120)	24.4	(97.6)
5.00	0	4.82	(96.4)	0	—
5.00	1.00	4.70	(94.0)	0.98	(98.0)
5.00	5.00	4.98	(99.6)	4.85	(97.0)
5.00	10.0	4.89	(97.8)	9.51	(95.1)
5.00	25.0	5.53	(111)	24.8	(99.2)

a) Values in parentheses are recovery (%).

Table V. Determination of $CySSO_3H$ in the Presence of Bisulfite by the Fluorometric Method

Added (10^{-8} mol)		Found (10^{-8} mol)[a]			
$CySSO_3H$	$NaHSO_3$	$CySSO_3H$		$NaHSO_3$	
5.00	0	4.89	(97.8)	0	—
5.00	1.00	4.84	(96.8)	1.02	(102)
5.00	2.00	5.08	(102)	1.95	(97.5)
5.00	3.00	5.16	(103)	2.98	(99.3)
5.00	4.00	4.92	(98.4)	3.80	(95.0)
5.00	5.00	4.87	(97.4)	5.00	(100)
10.0	0	10.2	(102)	0	—
10.0	1.00	9.99	(99.9)	1.01	(101)
10.0	2.00	9.98	(99.8)	1.88	(94.5)
10.0	3.00	10.4	(104)	2.94	(98.0)
10.0	4.00	9.82	(98.2)	3.83	(95.6)
10.0	5.00	9.97	(99.7)	4.76	(95.2)

a) Values in parentheses are recovery (%).

satisfactory. Moreover, $CySSO_3H$ was successfully determined in the presence of varying amount of bisulfite by both methods (Tables IV and V). However, large excesses of bisulfite produced errors.

Discussion

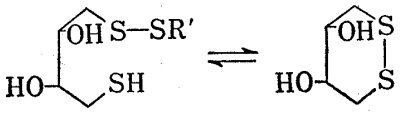
Bunte salts have been reduced with conventional agents such as sodium amalgam,[30] zinc and acid[31–35] and sodium arsenite.[36,37] However, these methods require rather drastic conditions and their yields are generally low. This makes them unsuitable for microanalysis.

It is well known that Bunte salts react with thiols in alkaline media to form disulfides and sulfite[4] according to Equation 4. When R and R′ were dissimilar the product in most

$$RSSO_3^- + R'S^- \rightleftarrows RSSR' + SO_3^{2-} \quad \text{(Eq. 4)}$$

$$RSSR' + SO_3^{2-} \rightleftarrows R'SSO_3^- + RS^- \quad \text{(Eq. 5)}$$

cases was not the expected unsymmetrical disulfides, but an equimolar mixture of the two corresponding symmetrical disulfides.[18–20] This result has been explained only in terms of disulfide interchange.[18–20] However, auto-oxidation to symmetrical disulfides of $R'S^-$ (Eq. 4) and RS^- formed by the reversible reaction (Eq. 5) of Eq. 4 must be considered. Thus, the reversibility of the reaction between Bunte salts and thiols usually leads to the appearance of a complex mixture during the reaction. This situation is similar to the disulfide-thiol interchange reaction. By analogy with the reduction of disulfides with DTT,[29] the use of DTT or dithioerithritol as a thiol reagent in Eq. 4 should make the reaction practically irreversible, because the equilibrium constant for the cyclization reaction of the thiol-DTT mixed disulfide (Eq. 6) is reported[29] to be about 10^4.



(Eq. 6)

This assumption was, in fact, verified by analysis of the reaction of Bunte salts with DTT. Reduction of Bunte salts in alkaline media proved to be a stoichiometric reaction and complete at 37° within a few min, yielding equimolar amounts of the corresponding monothiols, bisulfite and oxidized DTT. The bisulfite liberated was stable for 2 hr owing to the excess DTT which protected bisulfite from auto-oxidation to sulfate. This rapid and quantitative reduction of Bunte salts with DTT seems to be much superior to those with the conventional reducing agents[30–37] and cyanolysis of Bunte salts.[4,38] It is noteworthy that, unlike cyanide, DTT does not give sulfite from thiosulfate.

Among several Bunte salts tested, only $PenSSO_3H$ was not reduced with DTT under the conditions employed. This lower reactivity of DTT with $PenSSO_3H$ is thought[39] to be due to the steric hindrance around the "inner" sulfur atom of $PenSSO_3H$ which is bonded to a carbon atom with two methyl groups. The reaction between Bunte salts and DTT is believed to be initiated by a nucleophilic attack of the thiol group of DTT to the inner sulfur atom of

30) W. Spring, *Chem. Ber.*, **7**, 1157 (1874).
31) A. Bernthsen, *Liebigs Ann. Chem.*, **251**, 1 (1889).
32) A. Bernthsen, *Chem. Ber.*, **25**, 3128 (1892).
33) A.G. Green and A.G. Perkin, *J. Chem. Soc.* (London), **83**, 1201 (1903).
34) W. Alcalay, *Helv. Chim. Acta*, **30**, 578 (1947).
35) A.P. Tiselskii, *Chem. Abstr.*, **53**, 20039 (1959).
36) A. Gutmann, *Chem. Ber.*, **40**, 2818 (1907).
37) A. Gutmann, *Z. Analyt. Chem.*, **66**, 225 (1910).
38) E.W. McClelland and L.A. Warren, *J. Chem. Soc.* (London), **1930**, 2690.
39) H. Nakamura and Z. Tamura, *J. Chromatogr.*, **104**, 389 (1975).

the Bunte salt from the rear with displacement of sulfite ion, analogous to reactions between Bunte salts and other nucleophiles.[40,41] A spray procedure essentially the same as the colorimetric method described here for thin-layer chromatographic detection of Bunte salts at the nanomole level has bee developed recently.[39]

Acknowledgement The authors thank Drs. John J. Pisano and Lauren M. Cagen, National Institutes of Health, Bethesda, Maryland, U.S.A., for their interest in this work and generous advice to the preparation of the manuscript.

40) O. Foss, in N. Kharasch (Editor), "Organic Sulfur Compounds", Vol. I, Pergamon Press, London, 1961, p. 83.
41) A. Fava and A. Iliceto, *J. Am. Chem. Soc.*, **80**, 3478 (1958).