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CHROMATOGRAPHIC DETECTION OF BUNTE SALTS AND DISULPHIDES*

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SUMMARY

A spray method for selective detection of Bunte salts was developed. Bunte salts were cleaved with dithiothreitol (DTT) to hydrogen sulphite and the corresponding thiols, and the former was selectively detected with basic fuchsine reagent as violet-red spots with the aid of mercury(II) chloride, which reacts with the thiols liberated and the excess amount of DTT to form mercaptides. The limit of detection of Bunte salts was $5 \cdot 10^{-9}$ mole after thin-layer chromatography. Methods for the detection of disulphides were also developed. Based on these methods, a stepwise method and a simultaneous method were devised for the detection of thiols, disulphides, Bunte salts and sulphite.

INTRODUCTION

There has so far been no selective method for the detection of S-alkylthiosulphates and S-arylthiosulphates, the so-called "Bunte salts" ($RSSO_3^-$), on chromatograms. Recently, we developed spectrophotometric procedures for the determination of Bunte salts[1]. As an extension of this work, the development of a selective chromatographic method for the detection of Bunte salts in the presence of thiols, disulphides and sulphite was undertaken in the present investigation.

Bunte salts on paper or thin layers were reduced with dithiothreitol (DTT) to hydrogen sulphite and the corresponding thiols, and the hydrogen sulphite was coloured with acid-bleached basic fuchsine and formaldehyde after masking the thiols and the excess amount of DDT with mercury(II) chloride.

Several methods have been reported for the chromatographic detection of disulphides[2–6]. In this work, spray methods that permit the selective detection of

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[*] The abbreviations used in this paper are: DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; $CySSO_3H$, L-cysteine-S-sulphate monohydrate; $CyNSSO_3H$, cysteamine-S-sulphate; $DMCyNSSO_3H$, dimethylaminoethanethiol-S-sulphate; $PenSSO_3H$, penicillamine-S-sulphate; $BzSSO_3Na$, benzylmercaptan-S-sulphate sodium salt; $PATPSSO_3H$, *p*-aminothiophenol-S-sulphate; $(PaSSO_3)_2Ca$, calcium salt of pantetheine-S-sulphonic acid monohydrate; $(P\text{-}PaSSO_3)_2Ca$, calcium salt of 4′-phosphopantetheine-S-sulphonic acid; EDTA, ethylenediaminetetraacetic acid disodium salt; $GSSO_3Na$, glutathione-S-sulphate sodium salt.

disulphides were developed by using DTT or potassium cyanide as a reducing agent and successive combination with colour reagents.

EXPERIMENTAL

Reagents and solvents

Dithiothreitol (DTT) was purchased from Seikagaku Kogyo, Tokyo, Japan. Basic fuchsine (extra pure reagent), formalin (GR, minimum assay 37.0%), sulphuric acid (GR), sodium arsenite ($NaAsO_2$, GR, > 97%), mercury(II) chloride ($HgCl_2$, GR, 99.5%), sodium nitroprusside ($Na_2Fe(CN)_5NO\cdot 2H_2O$, GR), potassium cyanide (KCN, GR) and methyl red (GR) were purchased from Kanto, Tokyo, Japan. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, specially prepared reagent), cystamine dihydrochloride, cysteamine hydrochloride and N-ethylmaleimide (NEM, specially prepared reagent) were obtained from Nakarai, Kyoto, Japan. $CySSO_3H$, $CyNSSO_3H$, $DMCyNSSO_3H$, $PenSSO_3H$, $BzSSO_3Na$, $PATPSSO_3H$ and $GSSO_3Na$ were synthesized by our method[7] from the corresponding thiols and chlorosulphonic acid. Pantethine, $(PaSSO_3)_2Ca$ and $(P\text{-}PaSSO_3)_2Ca$ were kindly provided by Daiichi Seiyaku, Tokyo, Japan. Other reagents and solvents used were obtained from other commercial sources.

Filter-paper

Spot tests on filter-paper and paper chromatography (PC) were performed with Toyo Filter-Papers No. 2 and No. 514, respectively.

Thin-layer chromatographic plates

The following commercially available chromatoplates were used without any treatment. Spotfilm (20 × 5 cm or 20 × 20 cm), which consists of an activated silica gel layer (250 μm) on a synthetic resin film was purchased from Tokyo Kasei Kogyo, Tokyo, Japan. Wakogel FM plates (10 × 5 cm), consisting of a silica gel layer containing mixed fluorescent material[5], were purchased from Wako, Osaka, Japan. Avisel SF plates (microcrystalline cellulose, 10 × 10 cm) were obtained from Funakoshi, Tokyo, Japan.

Solvent systems for separation

Solvent system 1: *n*-butanol–acetic acid–water (5:2:3).
Solvent system 2: ethyl acetate–2.8% ammonia solution–ethanol (6:2:3).
Solvent system 3: acetone–diethyl ether–methanol (5:2:3).

Spray reagents

(1) DTT solution. A 20 m*M* solution of DTT in 0.05 *M* Tris–hydrochloric acid buffer (pH 9.20) containing 5 m*M* EDTA was prepared by dissolving 154.2 mg of DTT and 93.1 mg of EDTA in 50 ml of the buffer. The solution was freshly prepared before use.

(2) 0.1 M mercury(II) chloride solution. A 2.715-g amount of mercury(II) chloride was dissolved in 100 ml of water.

(3) Acid-bleached basic fuchsine solution. A 0.2-g amount of basic fuchsine was dissolved in a mixture of 8 ml of concentrated sulphuric acid and 92 ml of water.

(4) 1.2% formaldehyde solution. A 3.24-ml volume of 37% formaldehyde was mixed with 96.76 ml of water.

(5) Basic fuchsine reagent. Spray reagents 3 and 4 were mixed 1:1 (v/v) and used immediately for spraying.

(6) DTNB reagent. A 10 m*M* solution of DTNB in 0.067 *M* phosphate buffer (pH 7.0) was prepared by dissolving 396.4 mg of DTNB in 100 ml of 0.067 *M* phosphate buffer (pH 7.0).

(7) Arsenite solution. A 0.1 *M* solution of sodium arsenite in 0.1 *M* hydrochloric acid–citrate buffer (pH 3.5) was prepared by dissolving 1.299 g of the arsenite in 100 ml of the buffer.

(8) NEM reagent. A 0.2 *M* solution of NEM in isopropanol was prepared by dissolving 2.5 g of NEM in 100 ml of isopropanol.

(9) Potassium cyanide solution. A 2% solution of potassium cyanide in 95% methanol was prepared by dissolving 2 g of the cyanide in 100 ml of the alcohol. The solution was freshly prepared before use.

(10) Nitroprusside reagent. This reagent was substantially that of Toennies and Kolb[2], but the precipitates formed were removed by filtration and the resultant clear supernatant was used.

(11) 2 M sodium carbonate solution. A 21-g amount of sodium carbonate was dissolved in 100 ml of water.

Preparation of authentic solutions

The usual Bunte salts were dissolved in water to 0.1 *M*, except for PATPSSO$_3$H, which was dissolved in 0.01 *M* sodium carbonate solution because of its insolubility in water. These stock solutions were diluted as required with the same solvents. Volumetric micro-pipettes ("Microcaps", Drummond, Broomall, Pa., U.S.A.) were used for all of the spotting of stock solutions and their dilutions.

RESULTS

Selective detection of Bunte salts

The established spraying procedures for the selective detection of Bunte salts are as follows.

Spot an appropriate amount of 0.2% alcoholic methyl red solution on part of a paper or thin layer dipped in the developing solvent, and dry the chromatogram. If the colour of methyl red is red or reddish because of acidity of the solvent, fume the chromatogram with ammonia until the colour becomes persistent yellow. Spray with DTT solution, allow to stand at room temperature for at least 5 min, and spray with 0.1 *M* mercury(II) chloride solution. Usually the colour of the indicator is turned red by the procedure; if not, fume the chromatogram with the vapour of concentrated hydrochloric acid until a red colour appears. After a few minutes, spray with basic fuchsine reagent.

The usual Bunte salts yielded violet-red spots on a colourless or faint pink background with reasonable limits of detection, as shown in Table I. PATPSSO$_3$H gave a yellowish spot after spraying with 0.1 *M* mercury(II) chloride solution, probably due to the mercaptide of *p*-aminothiophenol. PenSSO$_3$H was not detected.

Sulphite and hydrogen sulphite, of course, gave the same violet-red colour unless

TABLE I

LIMITS OF DETECTION OF BUNTE SALTS AND HYDROGEN SULPHITE

Compound	*Spot test** (10^{-9} *mole*)	*TLC*** (10^{-9} *mole*)
$CySSO_3H$	1.5	5
$CyNSSO_3H$	1	5
$DMCyNSSO_3H$	1	5
$PenSSO_3H$	>100	>100
$PATPSSO_3H$	1	5
$BzSSO_3Na$	2	5
$(PaSSO_3)_2Ca$	1	5
$(P\text{-}PaSSO_3)_2Ca$	1	5
$GSSO_3Na$	2	5
$NaHSO_3$	1	>100

* One microlitre of a stock solution or its dilution was spotted on to Toyo Filter-Paper No. 2.
** One microlitre of a stock solution or its dilution was spotted on to Spotfilm, air-dried and developed for a distance of 17 cm with solvent system 1.

they were developed with the acidic solvent system 1. Thiols, disulphides and sulphide ion yielded no colour.

Table II summarizes the results of the separation of nine Bunte salts and hydrogen sulphite by paper chromatography (PC) and thin-layer chromatography (TLC). Bunte salts were well separated in general with solvent system 2 or 1, the latter growing compact spots, as shown in Fig. 1. Tailing spots were observed when the neutral solvent system 3 was used.

Detection of Bunte salts and disulphides

Twelve spray methods (Table III) were examined for the detection of Bunte

TABLE II

R_F VALUES OF BUNTE SALTS AND HYDROGEN SULPHITE

I, Solvent system 1, paper chromatography; II, solvent system 1, Spotfilm; III, solvent system 1, Wakogel FM plate; IV, solvent system 2, Wakogel FM plate; V, solvent system 2, Avisel SF plate; VI, solvent system 2, Spotfilm; VII, solvent system 3, Spotfilm.

Compound	$R_F \times 100$						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
$CySSO_3H$	39	34	39	15	13	13	28
$CyNSSO_3H$	50	45	51	48	61	33	55
$DMCyNSSO_3H$	56	34	41	55	89	45	38
$PenSSO_3H$*	50	38	48	23	40	20	46
$PATPSSO_3H$	60	57	63	74	90	58	79
$BzSSO_3Na$	73	72	74	82	99	81	76
$(PaSSO_3)_2Ca$	57	47	59	66	88	63	75
$(P\text{-}PaSSO_3)_2Ca$	33	21	34	0	0	3	11
$GSSO_3Na$	57	—	—	0	0	—	0
$NaHSO_3$	ND**	ND**	ND**	0	4	3	66

* Detected with ninhydrin.
** ND = not detected.

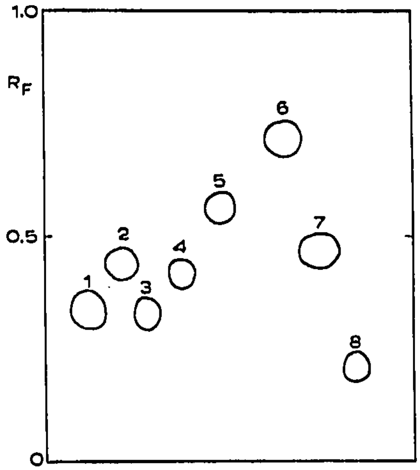


Fig. 1. Separation of Bunte salts. 1, CySSO$_3$H; 2, CyNSSO$_3$H; 3, DMCyNSSO$_3$H; 4, PenSSO$_3$H; 5, PATPSSO$_3$H; 6, BzSSO$_3$Na; 7, (PaSSO$_3$)$_2$Ca; 8, (P-PaSSO$_3$)$_2$Ca. Bunte salts were developed with solvent system 1 on Spotfilm and detected by the procedure for the selective detection of Bunte salts described in the text. PenSSO$_3$H was detected with ninhydrin.

salts and disulphides. Of these methods, one (Method I) was essentially identical with that proposed by Toennies and Kolb[2] for the detection of disulphides.

Method A. Spray the DTT solution on the sample, allow to stand for more than 5 min, then spray with arsenite solution. After a few minutes, spray with nitroprusside reagent. Bunte salts and disulphides were detected as reddish pink spots on a colourless background. Thiols yielded the same colour. A large amount of sulphite also showed a positive response, but the colour was orange. Usually, the nitroprusside colour did not appear or was transient on silica gel layers.

Method B. The DTNB reagent was used instead of the nitroprusside reagent in Method A. Bunte salts, disulphides, thiols and sulphite yielded yellow spots on a colourless or faint yellow background.

Method C. The basic fuchsine reagent was used instead of the nitroprusside reagent in Method A. Bunte salts, disulphides, thiols and sulphite yielded violet-red spots on a colourless or faint pink background.

Method D. The NEM reagent was sprayed instead of the nitroprusside reagent in Method A, and after 5 min or later 2 *M* sodium carbonate solution was sprayed. Disulphides and thiols appeared red. By prolonged reaction with the NEM reagent at room temperature or by performance of the reaction at higher temperature (above 60°), Bunte salts and sulphite also gave a red colour.

Method E. Spray the DTT solution on the sample, allow to stand for more than 5 min, then spray with 0.1 *M* mercury(II) chloride solution. After a few minutes, spray with the nitroprusside reagent. Bunte salts, disulphides, thiols and sulphite were not detected. The background was also colourless.

Method F. The DTNB reagent was used instead of the nitroprusside reagent in Method E. Bunte salts, disulphides, thiols and sulphite were not detected. The background was also colourless.

Method G. The basic fuchsine reagent was used instead of the nitroprusside reagent in Method E. This method was used for the selective detection of Bunte salts as described above.

Method H. After spraying with 2 *M* sodium carbonate solution to neutralize the acidity of mercury(II) chloride, the NEM reagent was sprayed instead of the nitroprusside reagent in Method E. After 5 min or later at room temperature, 2 *M* sodium carbonate solution was resprayed. Bunte salts, disulphides, thiols and sulphite gave no colour. The background was colourless.

Method I. Spray the potassium cyanide solution on the sample, allow to stand for 5 min, then spray with the nitroprusside reagent. Disulphides and thiols yielded reddish pink spots on a colourless background. The presence of large amounts of sulphite yielded an orange spot. Bunte salts were not detected with this procedure; however, by performing the cyanolysis at 100° for 5 min, Bunte salts also gave reddish pink spots.

Method J. The DTNB reagent was used instead of the nitroprusside reagent in Method I. Bunte salts, disulphides, thiols and sulphite were not detectable owing to a strong yellow background.

Method K. The basic fuchsine reagent was used instead of the nitroprusside reagent in Method I. Bunte salts, disulphides, thiols and sulphite yielded violet-red spots on a colourless or faint pink background.

Method L. The NEM reagent was sprayed instead of the nitroprusside reagent in Method I. After 5 min, 2 *M* sodium carbonate solution was sprayed. Disulphides and thiols appeared red. By performing the reaction with NEM at 100° for 5 min, Bunte salts were also detected.

As can be seen from Table III, Methods A, B, C and K were effective for the simultaneous detection of Bunte salts and disulphides. On the other hand, Methods G

TABLE III

LIMITS OF DETECTION OF DISULPHIDES AND BUNTE SALTS WITH VARIOUS SPOT TESTS ON TOYO FILTER-PAPER NO. 2

Method	*Reducing agent*	*Masking agent for DTT*	*Colour reagent**	*Limit of detection (10^{-9} mole)***			
				Cystamine	*Pantethine*	*CyNSSO$_3$H*	*(PaSSO$_3$)$_2$Ca*
A	DTT	NaAsO$_2$	NP	2	0.2	2	0.7
B	DTT	NaAsO$_2$	DTNB	0.5	0.3	5	2.5
C	DTT	NaAsO$_2$	BF	1	0.2	2	0.5
D	DTT	NaAsO$_2$	NEM	100	100	>100	>100
E	DTT	HgCl$_2$	NP	>100	>100	>100	>100
F	DTT	HgCl$_2$	DTNB	>100	>100	>100	>100
G	DTT	HgCl$_2$	BF	>100	>100	1	1
H	DTT	HgCl$_2$	NEM	>100	>100	>100	>100
I[2]	KCN	—	NP	0.5(0.5)	1(0.5)	>100(10)	>100(2)
J	KCN	—	DTNB	>100	>100	>100	>100
K	KCN	—	BF	2(2)	2(2)	20(10)	10(5)
L	KCN	—	NEM	3(0.5)	3(0.5)	>100(25)	>100(3)

* NP = nitroprusside; BF = basic fuchsine.

** Values in parentheses were obtained by heating at 100° for 5 min after spraying with the potassium cyanide solution.

and I were suitable for the specific detection of Bunte salts and disulphides, respectively. Reductive cleavage of disulphide bonds by DTT occurred almost completely at room temperature in 5 min, but cyanolysis of Bunte salts at room temperature was incomplete and the reduction was markedly accelerated by heating at 100° for 5 min.

Disulphides were not detected when 0.1 *M* mercury(II) chloride solution was used as the masking agent for the excess amount of DTT, because the thiols liberated also reacted with mercury(II) chloride to give mercaptides (Methods E, F, G and H). In the case of Bunte salts, although hydrogen sulphite liberated together with thiols by DTT did not react with mercury(II) chloride, the detection of Bunte salts was almost impossible, except with Method G, owing either to the low responses of hydrogen sulphite to the colour reagents (Methods E and H) or to the formation of the mercaptide of the chromophore 2-nitro-5-thiolbenzoic acid resulting from the exchange reaction between DTNB and hydrogen sulphite (Method F).

Stepwise detection of thiols, disulphides, Bunte salts and sulphite

Based on the above findings, a spray method that permits the stepwise detection of some classes of sulphur compounds was developed as follows.

Spray the nitroprusside reagent on to a chromatogram, and thiols are detected as reddish pink spots. Spray with the potassium cyanide solution, and disulphides are detected within a few minutes as reddish pink spots. Spray with 0.1 *M* mercury(II) chloride solution and, after a few minutes, with the basic fuchsine reagent, and Bunte salts and sulphite yield violet-red colour.

Sulphite was usually not detectable when it was developed with acidic solvents such as solvent system 1. Bunte salts were easily distinguishable from sulphite by co-chromatographing authentic sulphite, even when a neutral or alkaline solvent system was used and sulphite was stably developed.

As shown in Fig. 2, $1 \cdot 10^{-7}$ mole each of sodium sulphite, cysteamine, cystamine and $CyNSSO_3H$ were detected consecutively, although cysteamine was not detected (Fig. 2A) owing to its auto-oxidation to cystamine in the alkaline solvent, nor sulphite (Fig. 2B) because of evaporation as sulphur dioxide in the acidic solvent during the development.

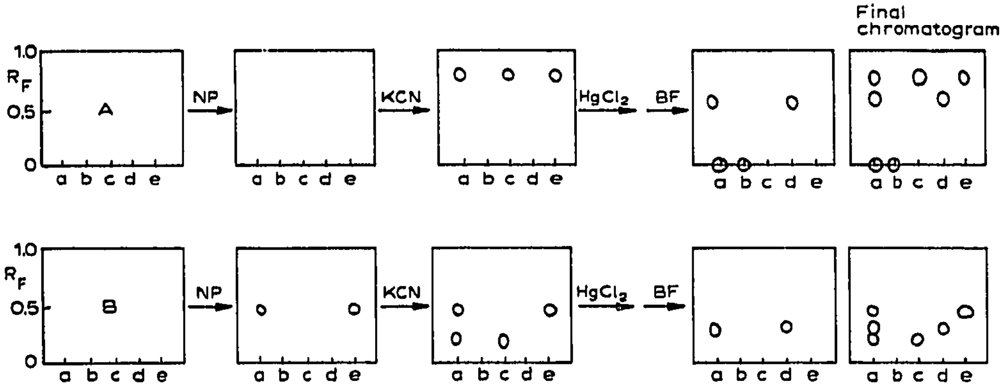


Fig. 2. Representative chromatograms of stepwise detection of compounds. The following compounds in amounts of $1 \cdot 10^{-7}$ mole each were developed on Avisel SF plates with solvent system 2 (A) or solvent system 1 (B). NP, nitroprusside reagent; BF, basic fuchsine reagent. a, Mixture; b, sodium sulphite; c, cystamine; d, $CyNSSO_3H$; e, cysteamine.

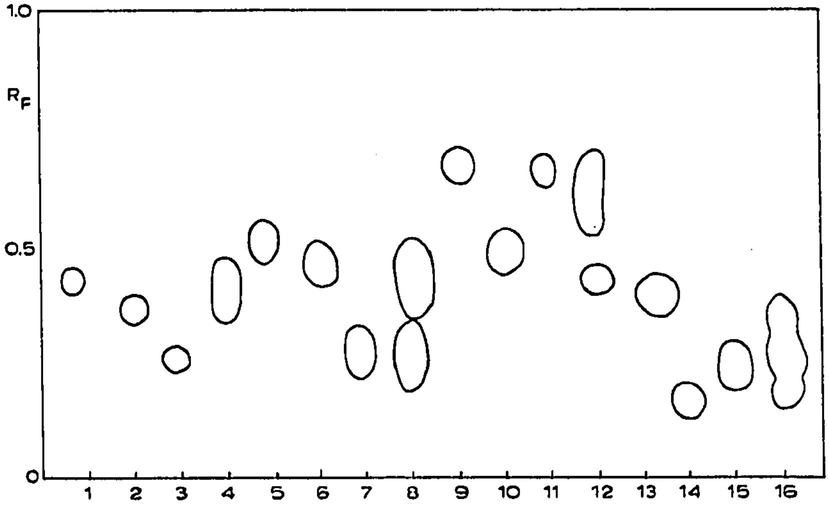


Fig. 3. Simultaneous detection of thiols, Bunte salts and disulphides by Method B. A mixture (1 ml) of 0.4 M disulphide and 0.4 M sodium sulphate was incubated at 37° for 30 min and a volume of 1 μl of the mixture, and 1 μl each of authentic thiols, Bunte salts and disulphides at a concentration of 0.1 M were separately applied on to Spotfilm and developed with solvent system 1. 1, L-Cysteine; 2, $CySSO_3H$; 3, L-cystine; 4, L-cystine + sodium sulphite; 5, cysteamine; 6, $CyNSSO_3H$; 7, cystamine; 8, cystamine + sodium sulphite; 9, pantetheine (pantethine + DTT); 10, $(PaSSO_3)_2Ca$; 11, pantethine; 12, pantethine + sodium sulphite; 13, reduced glutathione; 14, $GSSO_3Na$; 15, oxidized glutathione; 16, oxidized glutathione + sodium sulphite.

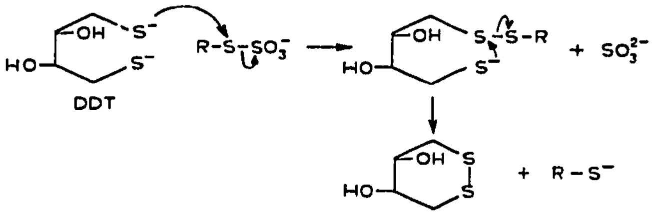
Simultaneous detection of reaction products of sulphitolysis

Methods A, B, C and K for Bunte salts and disulphides were also suitable for the detection of thiols and sulphite, and therefore the simultaneous detection of these classes of compounds could be achieved by any one of these methods. As an example, the thin-layer chromatograms of sulphitolysis products of L-cystine, cystamine, pantethine and oxidized glutathione together with authentic compounds detected by Method B are shown in Fig. 3.

DISCUSSION

Since Cleland reported the usefulness of DTT for the reduction of disulphides[8], this reagent has been conveniently and widely used for the reduction of low-molecular-weight disulphides and the disulphide bonds in proteins. In the present investigation, DTT was applied for the first time to the reduction of disulphides and Bunte salts on paper and thin-layer chromatograms and proved to be superior in terms of reactivity with them and rapidity of reaction to conventional reagents such as potassium cyanide and sodium borohydride; unlike potassium cyanide, DTT does not release sulphite from thiosulphate[1], and disulphides and Bunte salts, except for $PenSSO_3H$, were completely reduced with DTT at room temperature within 5 min (Tables I and III). In contrast, cyanolysis of them under the same reaction conditions resulted in incomplete reduction (Methods I, K and L in Table III) and the chromatographic procedure of Glaser *et al.*[3] using sodium borohydride requires a period of 30 min for the reduction of disulphides.

Among the Bunte salts examined, only PenSSO$_3$H, whose "inner" sulphur atom is sterically hindered, was scarcely reduced with DTT, which indicates that the reactions between Bunte salts and DTT, as well as those between Bunte salts and nucleophiles[9,10], are initiated by a nucleophilic attack of the thiol group of DTT to the "inner" sulphur atom of the Bunte salt from the rear with displacement of sulphite ion as follows:



Only a few methods have been reported for the selective detection of disulphides on chromatograms: the potassium cyanide–sodium nitroprusside method[2], the sodium borohydride–DTNB method[3] and the fluorescein–mercury(II) acetate method[4]. In the present investigation, three methods (Methods A, B and C) were developed for the detection of disulphides by using DTT as the reducing agent and successively chelating it with sodium arsenite[11], together with two methods (Methods K and L) utilizing cyanolysis. The limits of detection of these methods are less than a few nanomoles of disulphide in all instances, thus having almost the same sensitivity as reported colorimetric methods[2,3], but being less sensitive than the fluorescence quenching method with fluorescein–mercury(II) acetate[4] ($1 \cdot 10^{-10}$ mole of disulphide).

Disulphides and Bunte salts are also detectable by the mixed fluorescent material methods[5,6], the sensitivities of detection being $5 \cdot 10^{-11}$–$1 \cdot 10^{-8}$ mole and $5 \cdot 10^{-8}$ mole, respectively. On the other hand, thiols[5] and sulphite[12] are not detected in amounts of $1 \cdot 10^{-7}$ mole. Therefore, the use of the mixed fluorescent material method prior to the present spray methods will provide a useful technique for the detection of compounds.

The simultaneous and stepwise detection methods for thiols, disulphides, Bunte salts and sulphite presented in this paper would be useful for the resolution of reactions associated with these classes of compounds, especially for sulphitolysis reactions, thiol–disulphide exchange reactions and the syntheses of mixed disulphides.

In order to obtain distinct chromatograms with the methods described here, the most important factor is the prior adjustment of the pH of the chromatograms to the optimal for the spray reagents to be used. In particular, the reduction of Bunte salts and disulphides with DTT occurrs effectively only under alkaline conditions. On the other hand, spraying the basic fuchsine reagent and the DTNB reagent on to alkaline samples causes the development of colour on the background. The other important step is to dry the sample before spraying of next reagent, in order to prevent compounds from diffusing.

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