fact that the compounds VII, VIII and IX were essentially inactive in comparison with the ED_{50} values for the cytotoxicities of III and IV (*cf.* Table I).

Acknowledgements We thank Professor T.A. Geissman, Department of Chemistry, University of California at Los Angeles for his interest in this work as well as the specimen of deacetoxymatricarin. This investigation was supported by a U.S. Public Health Service Research Grant CA12360 from the National Cancer Institute to K.H. Lee and a Fellowship from the National Science Council of the Republic of China to R.Y. Wu.

Department of Medicinal Chemistry
School of Pharmacy
University of North Carolina
Chapel Hill, North Carolina 27514
U.S.A.

Kuo-Hsiung Lee
Toshiro Ibuka

Institute of Botany
Academia Sinica
Nankang, Taipei, Taiwan
Republic of China

Rong-Yang Wu

Received July 18, 1974

[Chem. Pharm. Bull.
22(9)2208—2210(1974)]

UDC 547.269.5.04.08 : 547.269.1.04.09

Spectrophotometric Determination of Bunte Salts[1]

S-Alkylthiosulfates and S-arylthiosulfates, known as "Bunte salts," have attracted particularly wide interest in the textile and pharmaceutical industries.[2] Some of them are also reported to exhibit various types of biological activity.[2] In spite of the industrial and biological significance, any general method of determination of Bunte salts has not been reported.

During the course of our investigation on the bacterial metabolism of Bunte salts, development of a microanalytical method of Bunte salts in biological materials was required. In this communication, a colorimetric and a fluorometric methods of determination of Bunte salts are described.

We first applied the Cleland's reagent (DTT),[3] which is known to reduce disulfide bonds, to the reduction of Bunte salts. The reduction of $CySSO_3H$ and $(PaSSO_3)_2Ca$ (Fig. 1) as well as $CyNSSO_3H$, $DMCyNSSO_3H$, $BzSSO_3Na$, $PATPSSO_3H$ and $(P\text{-}PaSSO_3)_2Ca$ proceeded rapidly and quantitatively in alkaline media. $PeSSO_3H$ was not reduced under the same condition probably because of its steric hindrance around the $-S-SO_3^-$ group. An equimolar reaction of $CySSO_3H$ with DTT was confirmed by the continuous variation method (Fig. 2) and the molar ratio method.

---

[1] The abbreviations used are: DTT, dithiothreitol; $CySSO_3H$, S-sulphocysteine; $(PaSSO_3)_2Ca$, calcium salt of pantetheine-S-sulfonic acid; $(P\text{-}PaSSO_3)_2Ca$, calcium salt of 4′-phosphopantetheine-S-sulfonic acid; $CyNSSO_3H$, cysteamine-S-sulfate; $DMCyNSSO_3H$, dimethylaminoethanethiol-S-sulfate; $PeSSO_3H$, penicillamine-S-sulfate; $BzSSO_3Na$, benzylmercaptan-S-sulfate sodium salt, $PATPSSO_3H$, *p*-aminothiophenol-S-sulfate; NEM, N-ethylmaleimide; CySH, L-cysteine; EDTA, ethylenediaminetetraacetic acid disodium salt; PCMB, *p*-chloromercuribenzoic acid.

[2] H. Distler, *Angew. Chem. Int. Ed. Engl.*, 6, 544 (1967); S. Oae, G. Tsukamoto, and T. Kurusu, *Kagaku* (Kyoto), 26, 1066 (1971).

[3] W.W. Cleland, *Biochemistry*, 3, 480 (1964).

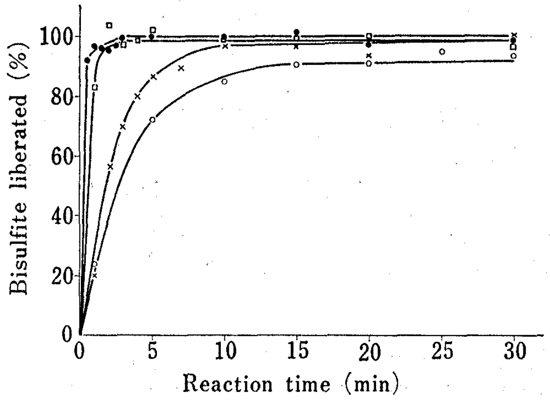


Fig. 1. Reduction of Bunte Salts with DTT

One ml each of Bunte salt and DTT solutions was mixed with 1.0ml of 0.05 M Tris-HCl buffer containing 5 mM EDTA, and the reaction mixture was incubated at 37°.

—○—: 2×10^{-7} mole CySSO$_3$H, 5×10^{-7} mole DTT, pH 9.20

—●—: 2×10^{-7} mole CySSO$_3$H, 5×10^{-6} mole DTT, pH 9.20

—×—: 2×10^{-7} mole CySSO$_3$H, 5×10^{-6} mole DTT, pH 7.98

—□—: 1×10^{-7} mole (PaSSO$_3$)$_2$Ca, 5×10^{-6} mole DTT, pH 9.20

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} mole NaHSO$_3$ were plotted.

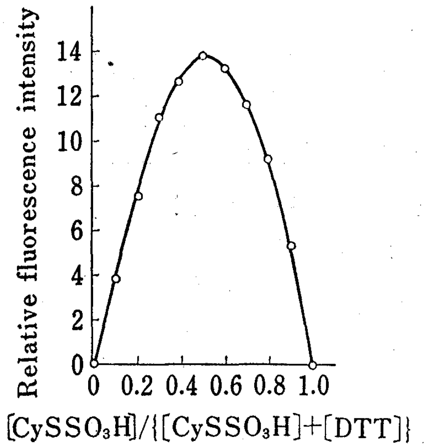
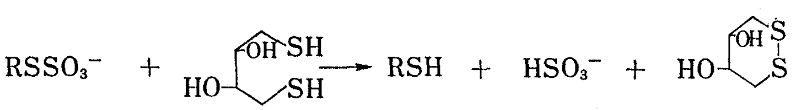


Fig. 2. The Estimation of the Stoichiometry of the Reaction of CySSO$_3$H and DTT by the Continuous Variation Method

[CySSO$_3$H]+[DTT]$=1\times10^{-4}$M

Bisulfite and CySH were identified in the reaction mixture of CySSO$_3$H and DTT as their radioactive NEM (ethyl-2-^{3}H, New England Nuclear) adducts by paper electrophoresis (1 M HCOOH, pH 1.75) followed by scanning the electrophoretogram. Any additional radioactive compounds other than NEM adduct of DTT were not detected. Furthermore, it was found that one mole of CySH and one mole of bisulfite were formed from one mole of CySSO$_3$H by determining the amounts of CySH and bisulfite in the reaction mixture of CySSO$_3$H and DTT by a modified Ellis' method[4] and our fluorometric method[5] respectively. These data indicate that the reaction between Bunte salts and DTT proceeds in the following manner:



DTT

oxidized DTT

On the other hand, DTT gave no bisulfite or sulfite from thiosulfate ($S_2O_3^{2-}$) which is usually the most serious interfering compound in the analysis of Bunte salts in biological materials.

Based upon these observations, the following two procedures for determination of Bunte salts were established.

a) Colorimetric Method

To 1.0 ml of sample, blank (water), or standard solution, 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 (reduction mixture). To the reduction mixture, 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[6] (a

---

[4] R.J. Ellis, *Biochim. Biophys. Acta*, **85**, 335 (1964).

[5] H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1950 (1974).

[6] P.F. Urone and W.E. Boggs, *Anal. Chem.*, **23**, 1517 (1951).

mixture of the acid-bleached basic fuchsin and formaldehyde) to 3.0 ml of the clear supernatant, allow to stand at room temperature for 10 min and measure the optical density 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, therefore, by subtracting the total amount with that of the preexistent bisulfite which is obtained by exchanging the order of addition of DTT and $HgCl_2$. Under these procedures, the working curves of usual Bunte salts are linear in the range of 5×10^{-9} to 1×10^{-7} mole.

b) Fluorometric Method

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[5] in ethanol to 3.0 ml of the resultant clear supernatant, and incubate at 37° for 30 min. Add 1.0 ml of 2.0N 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 by incubating at 37° for 10 min followed by performance of the same procedure described above.

The lower limit of determination is 2×10^{-8} mole of a Bunte salt and the working curves are linear up to 1.5×10^{-7} mole in this procedure.

Faculty of Pharmaceutical Sciences,
University of Tokyo
Hongo, Bunkyo-ku, Tokyo, 113 Japan

HIROSHI NAKAMURA
ZENZO TAMURA

Received June 29, 1974