Japan. The 2-hydroxy-4-methylthiobutyric acid is a commercial product of the Monsanto Company, St. Louis, Mo. 63166, U.S.A. and was donated through the courtesy of Mr. N. L. REDING.

This research was supported, in part, by Louisiana State University Research Grant No. FR-5376, awarded to the Medical Center by the National Institutes of Health.

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1 T. E. MCCARTHY AND M. X. SULLIVAN, *J. Biol. Chem.*, 141 (1941) 871.
2 A. G. NEWCOMBE AND S. G. REID, *Nature*, 172 (1953) 455.

Received March 9th, 1970

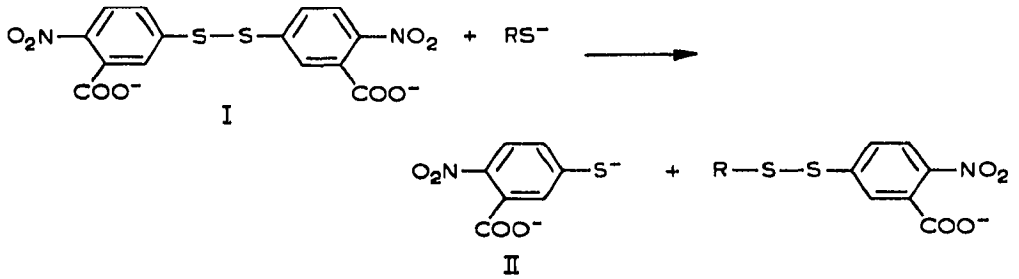
J. Chromatog., 50 (1970) 150–151

CHROM. 4738

Chromatographic detection of thiols, disulfides, and thioesters with 5,5′-dithiobis(2-nitrobenzoic acid)

Few reagents are available for the detection of sulfhydryl containing compounds on chromatograms. Sodium nitroprusside[1], platinic iodide[2], or various quinones[3], which are not specific for sulfhydryl, have been used. Recently GRASSETTI AND MURRAY[4] reported the use of 2,2′-dithiobis(5-nitropyridine) as a selective reagent for the detection of thiols.

We have used alcohol–buffer solutions of 5,5′-dithiobis(2-nitrobenzoic acid)[5] (I, DTNB, ELLMAN reagent) as a convenient and sensitive spray reagent for the visualization of thiols on chromatograms as yellow spots*. The reagent reacts specifically with sulfhydryl groups by a disulfide exchange reaction to give the yellow thioanion (II) of 2-nitro-5-mercaptobenzoate[5].



I

II

We have extended this method to the chromatographic detection of disulfides and thioesters, which are both easily converted to their thiol derivatives: the former by reduction with sodium borohydride and the latter by alkaline hydrolysis.

* GRASSETTI AND MURRAY[4] reported that DTNB "does not appear to be suited as a spray reagent in organic media" for the chromatographic detection of thiols.

Experimental

Reagents

(a) DTNB reagent: 0.1% solution of 5,5'-dithiobis(2-nitrobenzoic acid) (Calbiochem, Los Angeles, Calif., U.S.A.) in a 1:1 mixture of ethanol and 0.45 *M* tris-(hydroxymethyl)aminomethane hydrochloride buffer at pH 8.2.

(b) Borohydride reagent: freshly prepared 0.4% solution of sodium borohydride (Metal Hydrides, Inc., Beverly, Mass., U.S.A.) in 95% ethanol.

(c) Acid solution: a mixture of glacial acetic acid, 6 *N* hydrogen chloride and acetone (8:2:90).

(d) Hydrolysis reagent: 0.2 *N* sodium hydroxide solution in 50% aqueous ethanol.

(e) pH indicator: 0.1 g phenol red (phenolsulfonephthalein) in 28.2 ml 0.01 *N* NaOH, diluted with water to 250 ml.

Procedures

All reagents were applied by aerosol spray (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.) in a ventilated hood. Approximately 10^{-6} mole of each compound to be tested was spotted on filter paper or on thin-layer plates [silica, aluminum oxide, cellulose, polyamide (Eastman Kodak, Rochester, N.Y., U.S.A.)]. Development of a yellow color within a few seconds after spraying the DTNB reagent was called a "positive result". The color persisted for at least 15 h.

Thiols. The DTNB reagent was applied.

Disulfides. The borohydride reagent (one volume) was applied to the chromatogram. 15–20 min later the borohydride was destroyed by spraying with the acid solution (three volumes)*. The chromatogram was air dried for 1 h. pH indicator (Phenol Red) was spotted in several positions along the edges of the chromatogram which was then exposed to an ammonia atmosphere in a glass cylinder containing a dish with concentrated ammonia until the indicator spots turned red. After air drying for 4–5 min the DTNB reagent was applied.

Thioesters. The chromatogram was sprayed with the hydrolysis reagent followed 2 to 3 min later by the DTNB reagent. A yellow background gradually developed on standing.

Results and discussion

Compounds (thiols) which gave yellow spots on chromatograms within a few seconds after spraying with a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB reagent) are listed in Table I. Cysteine was easily detected at 2×10^{-9} mole. 2-Mercaptouracil and 6-mercaptopurine gave negative results, probably due to the double bond character of the thiol function.

The specificity of DTNB for sulfhydryl was further corroborated by negative results obtained with S-protected cysteine derivatives, thioethers, disulfides, and thioesters (Table I). None of the common amino acids gave a positive test except cysteine. The high sensitivity and specificity of this procedure equals that obtained

* Complete decomposition of the reducing agent is essential. Residual sodium borohydride would interfere with the thiol test due to reduction of DTNB. Other reducing agents such as sodium bisulfite and sodium thiosulfate interfere similarly.

TABLE I

RESULTS OF DTNB SPRAY ON CHROMATOGRAMS

+, Appearance of yellow color within a few seconds. —, No color within 15 h.

Compound	*DTNB reagent alone*	*After reduction*	*After alkaline hydrolysis*
Cysteine	+		
N-*tert.*-Butyloxycarbonylcysteine methyl ester	+		
2-Mercaptopyridine	+		
Dithiothreitol	+		
Glutathione (reduced)	+		
Coenzyme A	+		
S-blocked cysteine derivatives[a]	—		
Methionine	—	—	
Thiourea	—	—	
Biotin	—	—	
Cystine	—	+	
Glutathione (oxidized)	—	+	
Vasopressin	—	+	
Neocarzinostatin	—	+	
Lysozyme	—	+	
Acetyl coenzyme A	—		+
N-Acetylhomocysteine thiolactone	[b]		+
Ethyl thioacetate	—		+[b]
n-Propyl thiopropionate	—		+
n-Butyl thioacetate	—		+
N-Benzyloxycarbonyl-S-acetylthiothreonine methyl ester	—		+
Butyryl thiocholine iodide	—		+

[a] Blocking groups used were: methyl, benzyl, diphenylmethyl, triphenylmethyl, benzylthiomethyl, benzyloxycarbonyl, tetrahydropyranyl, and ethylcarbamoyl.

[b] See discussion.

with 2,2'-dithiobis(5-nitropyridine) which recently has been recommended[4] for the chromatographic detection of thiols.

Disulfides were detected by DTNB reagent after reduction to thiols (Table I). An efficient reducing agent for this purpose was sodium borohydride in 95% ethanol. This test should be particularly useful for the detection of disulfide-containing amino acids and peptides on two-dimensional chromatograms. A peptide map of a peptic–chymotryptic hydrolysate of lysozyme gave seven yellow spots of disulfide-containing peptides*. The proteins lysozyme and neocarzinostatin gave positive results for disulfide without denaturation prior to application on filter paper.

The DTNB reagent was also used for the detection of thioesters on chromatograms after alkaline hydrolysis by spraying with aqueous ethanolic sodium hydroxide (Table I). N-Acetylhomocysteine thiolactone gave a positive result after 5 to 10 min with the DTNB reagent alone, probably due to hydrolysis at pH 8.2.

Highly volatile thiols generated on disulfide reduction or thioester hydrolysis may escape detection. However, by applying the reagents within 1 min, it was possible to detect ethyl mercaptan obtained from ethyl thioacetate.

* From the amino acid composition of the spots the positions of all four disulfide bonds of lysozyme could be independently assigned. This rapid determination of the positions of disulfide bridges in proteins will be reported elsewhere[6,7].

In conclusion, a rapid and sensitive technique has been developed for the identification and differentiation of thiols, disulfides, and thioesters on thin-layer and paper chromatograms. Thiols are detected as yellow spots after spraying chromatograms with an alcohol-buffer solution of the readily available 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman reagent, DTNB). Disulfides can be detected after reduction to thiols with sodium borohydride and application of the DTNB spray reagent. Thioesters are hydrolyzed by alkali and the resulting thiols are treated with DTNB.

The authors wish to thank Dr. S. FARBER for his support, Dr. C. H. LI and Mr. R. COTTON for helpful discussions, and Dr. H. ZAHN (Aachen, G.F.R.) for samples of many S-protected cysteine derivatives.

This work was supported by Public Health Service Research Grants C-6516, National Cancer Institute, and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health, by A. and M. Lasker Foundation, New York, and A. T. and V. D. Fuller Cancer Research Unit Grant, American Cancer Society, Inc., Massachusetts Division.

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1 F. P. CHINARD AND L. HELLERMAN, *Methods Biochem. Anal.*, 1 (1954) 1.
2 C. W. EASLEY, B. J. M. ZEGERS AND M. DE VIJLDER, *Biochim. Biophys. Acta*, 175 (1969) 211.
3 K. HOFMANN, *Naturwiss.*, 52 (1965) 428.
4 D. R. GRASSETTI AND J. F. MURRAY, Jr., *J. Chromatog.*, 41 (1969) 121.
5 G. E. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
6 H. MAEDA, C. B. GLASER AND J. MEIENHOFER, *Fed. Proc.*, 29 (1970) 2725.
7 H. MAEDA, C. B. GLASER AND J. MEIENHOFER, *Biochem. Biophys. Res. Commun.*, (1970) in press.

First received February 19th, 1970; revised manuscript received March 23rd, 1970

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