PAPER CHROMATOGRAPHY OF MERCAPTIDES*

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The rather general occurrence of cysteine residues in proteins and enzymes is well established. Evidence for the participation of thiols in protein structure and in the chemical events relevant to enzyme catalysis is very meager[1] though speculation on these points has been popular. Recently published comparative structural studies of hydrolytic enzymes have shown that similar amino acid sequences are found in the active centers of these enzymes although they have different substrate specificities[2]. If this "conservation of structure" is a general phenomenon among sulfhydryl containing enzymes, examination of the structure of these enzymes in greater detail could conceivably contribute appreciably to knowledge of the structure-function relationship. Studies of the role of the sulfhydryl group in these enzymes would be facilitated by convenient techniques of separation and identification of the sulfhydryl-containing peptides. To this end the application of the chromophoric sulfhydryl reagent N-(4-dimethylamino-3,5-dinitrophenyl)-maleimide in the study of protein sulfhydryl groups by WITTER AND TUPPY[3] appears very promising. The chief disadvantage of this reagent appears to be its limited solubility in water[4]. The phenylmercurials are attractive as sulfhydryl reagents because of their relatively high specificity and also because they react rapidly and reversibly with sulfhydryl compounds under appropriate conditions. *p*-Mercuriphenylsulfonate has the advantage of relatively high solubility in aqueous solutions. The reversible reaction of the phenylmercurials with mercaptans may be advantageous in that the unaltered sulfhydryl compound may be reclaimed and subjected to study.

The apparent need for suitable analytical methods for study of cysteine-containing peptides led to the preliminary studies reported in this communication.

EXPERIMENTAL

Reagents

L-Cysteine hydrochloride; Nutritional Biochemicals.
Diphenylthiocarbazone; Commercial Solvents Corporation.
Iodoacetamide; Mann Research Laboratories.

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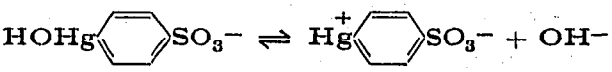
Ninhydrin; Matheson, Coleman, and Bell.
p-Chloromercuribenzoate; Sigma Chemical Company.
Pinakryptol Yellow; K and K Laboratories.
Reduced glutathione; Schwarz Laboratories Incorporated.
Thiomalic acid; National Aniline Division, Allied Chemical and Dye Corporation.
Whatman filter paper No. 1 and No. 3 MM.

All other chemicals were reagent grade unless otherwise noted.

p-Mercuriphenylsulfonate was synthesized according to the method of DUNKER *et al.*[5] with modifications by BOYER[6]. The purity, after recrystallization, was examined by filter paper chromatography using two solvent systems. A single spot was observed with dithizone (to indicate the position of the mercury) and pinakryptol yellow (to indicate the position of the sulfonate group). The first solvent contained *n*-butanol–acetic acid–water (41:10:41) and the second solvent contained propanol–ammonia (2:1). The R_F's are 0.39 and 0.20 respectively. The *p*-mercuriphenylsulfonate was further characterized by ultraviolet spectrophotometry, infrared spectrophotometry, titration, and elementary analysis. The last three analyses indicated that the phenylmercurial was purified as a mixture of the zwitterion and the double salt. The titration studies indicated that the *p*-mercuriphenylsulfonate exists in aqueous solution as the undissociated hydroxide salt at alkaline pH values[7]. The apparent dissociation constant for the following equilibrium:



was observed to be approximately $2 \cdot 10^{-8}$ at 37°.

Chromatography

Filter paper was prepared for chromatography by washing with a 0.1% aqueous solution of ethylenediaminetetraacetic acid (EDTA). The EDTA treatment was followed by washing with distilled water, 1.0 *N* hydrochloric acid, and distilled water again until the pH of the effluent was that of pure distilled water. Washing was facilitated by descending flow through ten sheets of filter paper, supported on a lucite rack inclined at about 30° to the horizontal. The purpose of this washing was to remove any metal ions and other troublesome contaminants.

A number of solvent systems were examined for their suitability in the separation of various sulfhydryl adducts. The most satisfactory was that of WOIWOD[8] which was composed of redistilled *n*-butanol (b.p. 116–118°), distilled water, and glacial acetic acid (42:42:10). Two phases appeared after mixing the above solvents. The less dense phase, which was employed only if it accounted for approximately 68% of the total volume, was used as the chromatography solvent. The more dense phase was used to saturate the chromatography jar. The descending technique was employed in all cases, and fresh solvent was prepared for each chromatography run in order to assure reproducible R_F values.

The oxidation of sulfhydryl compounds by oxygen is catalyzed by metal ions[1]. Therefore, distilled water was used in the preparation of all solvents and buffers, and the spots applied to the chromatograms were dried in a stream of nitrogen gas. Mercaptides were formed by mixing equimolar amounts of sulfhydryl compound and sulfhydryl reagent. The reaction mixture was allowed to stand approximately one hour at room temperature. Salt-free solvents or solvents of low ionic strength were used to avoid streaking of spots in chromatography.

Individual chromatograms, 15 × 57 cm, were run in the machine direction. The solutions were spotted on the chromatograms from micropipettes in volumes of 2 to 10 μl. Spotted chromatograms were allowed to equilibrate for five hours in the chromatography jar before being run in a constant temperature cabinet at 29°. The completed chromatograms were dried for one hour in a fume hood before spraying or dipping with the desired color-developing reagent.

The developing reagents were:

(a) Ninhydrin (0.20–0.25 %) in dry acetone, for general amino acid and peptide detection[8].

(b) Dithizone (0.002 % diphenylthiocarbazone) in carbon tetrachloride, for the detection of the mercury-containing spots[9]. *p*-Mercuribenzoate and *p*-mercuriphenylsulfonate in the free form as well as their mercaptides produced gold to peach colored spots. The color was intensified when the paper was sprayed with 0.2 *M* acetic acid. The dithizone is a sensitive color reagent for many heavy metal ions; therefore, such ions in the paper due to incomplete washing give interfering spots.

(c) Pinakryptol yellow (0.05 % in water), for the detection of the sulfonate group of *p*-mercuriphenylsulfonate[10]. The spots were detected under ultraviolet light and were orange to deep brown on a blue fluorescing background.

RESULTS

Table I lists the R_F values obtained for various mercaptans and their adducts with sulfhydryl reagents.

Ninhydrin was found to be the most sensitive detecting reagent. A minimum of $2 \cdot 10^{-8}$ moles of amino acid or peptide per cm^2 is detectable. The mercury compounds could easily be detected in quantities of $3–5 \cdot 10^{-8}$ moles/cm^2 with dithizone; whereas $6–8 \cdot 10^{-8}$ moles/cm^2 were required for detection of the mercury mercaptide by the dithizone reagent. The dithizone reacting spots could be detected more easily on chromatograms sprayed with acid. This was attributed to two effects. The color of the mercury-dithizone complex is intensified in acid and the mercaptide dissociation at acid pH makes the mercury more available for complex formation.

Pinakryptol yellow was less sensitive than the dithizone reagent. If the developing reagents were applied by spraying, all three could be used on the same chromatogram. No interference of the reagents occurred if dithizone and ninhydrin were applied to the opposite sides of the chromatogram. Pinakryptol was subsequently applied to the same side as the dithizone.

TABLE I

R_F VALUES FOR VARIOUS MERCAPTANS AND THEIR ADDUCTS

Compound	R_F* × 10²
Cystine	8 ± 1.2
Cysteine	28 ± 1.8
Cysteine-*p*-mercuribenzoate	47 ± 2.4
Cysteine-*p*-mercuriphenylsulfonate	20 ± 1.0
Cysteine-iodoacetamide	17 ± 2.0
Glutathione	26 ± 1.5
Oxidized glutathione	6 ± 1.0
Glutathione-*p*-mercuriphenylsulfonate	19 ± 1.0
Glutathione-*p*-mercuribenzoate	35 ± 2.0
Glutathione-iodoacetamide	14 ± 1.5
p-Mercuribenzoate	49 ± 2.5
p-Mercuriphenylsulfonate	39 ± 1.0
Thiomalic-*p*-mercuriphenylsulfonate	70 ± 1.0

* Whatman No. 1 paper, 29°, *n*-butanol–acetic acid–water (42:10:42) descending solvent.

Preliminary experiments indicate that this technique can be applied to the study of proteins. Tryptic hydrolysates of two milligrams of yeast alcohol dehydrogenase were treated with *p*-mercuriphenylsulfonate. The peptide products of this reaction were separated by the INGRAM "fingerprint" method[11] modified by the use of alkaline buffers (propanol–ammonia 2:1 for chromatography and pH 8.5 ammonium bicarbonate, 0.05 *M* with respect to ammonia, for the electrophoresis). The dithizone spray indicated the position of several mercury-containing spots on the "fingerprint" pattern.

SUMMARY

The chromatographic separation and identification of several sulfhydryl compounds with iodoacetamide, *p*-mercuriphenylsulfonate, and *p*-mercuribenzoate is described. The possibility of applying this technique to structural studies of cysteine-containing proteins is indicated.

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