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Dithiothreitol, a New Protective Reagent for SH Groups*

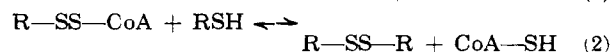
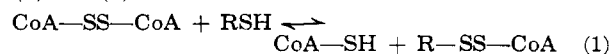
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Received November 4, 1963

Because of its low redox potential (-0.33 volts at pH 7), dithiothreitol (and its isomer, dithioerythritol) is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively. Since this compound is a highly water-soluble solid with little odor and little tendency to be oxidized directly by air, it should prove much superior to the thiols now used as protective reagents for sulfhydryl groups.

Thiol groups such as those of coenzyme A and of some enzymes are readily oxidized in air to disulfides. To maintain these groups in the reduced state, another thiol such as cysteine, glutathione, mercaptoethanol, 2,3-dimercaptopropanol, or thioglycolate is often added so that interchange takes place according to reactions (1) and (2):

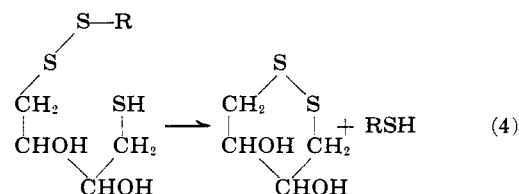
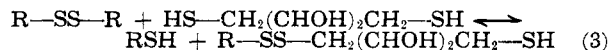


However, the equilibrium constants of these reactions are near unity, so that a sizable excess of the second thiol must be used. It occurred to this author that if reaction (2) were intramolecular and RSSR were a sterically favorable cyclic disulfide, there would be two products produced from one reactant, so that the equilibrium should be displaced to the right, particularly in dilute solutions. It appeared that a 1,4-dithiolbutane structure would produce the most sterically favorable cyclic disulfide, and that addition of hydroxy groups on the middle carbons should make the compound water soluble and reduce the stench of the thiol groups.

Dithiothreitol (DTT)¹ and dithioerythritol (DTE), the threo and erythro isomers of 2,3-dihydroxy-1,4-dithiolbutane, were therefore prepared as described by Evans *et al.* (1949) and found to have the desired properties. Reaction with a disulfide takes place according to reactions (3) and (4), and is complete in several minutes at pH 8.

* Supported in part by a grant (HE-05095) from the National Institutes of Health, U. S. Public Health Service.

¹ Abbreviations used in this work: DTT, dithiothreitol; DTE, dithioerythritol; oxidized DTT, cyclic disulfide of DTT (*trans*-4,5-dihydroxy-*o*-dithiane); oxidized DTE, cyclic disulfide of DTE (*cis*-4,5-dihydroxy-*o*-dithiane).



Attempts were made to determine the over-all equilibrium constant for reactions (3) and (4) by following the reduction of cystine by DTT or DTE, which can be conveniently measured because the thiol groups of DTT and DTE give only 4% as much color as cysteine in the nitroprusside assay of Grunert and Phillips (1951). Within experimental error, reaction between cystine and DTT or DTE went to completion, even when concentrations of the cyclic oxidized form of DTT or DTE (prepared by ferricyanide oxidation of DTT or DTE) ten times those of DTT or DTE were added.

The actual redox potential of DTT was measured by equilibrating the DTT-oxidized DTT system with the DPN⁺-DPNH system in the presence of lipoamide and dihydrolipoic dehydrogenase, and measuring the amount of DPNH at equilibrium at 340 m μ (making suitable corrections for the absorption of lipoamide and oxidized DTT at this wavelength). The equilibrium constant for reaction of DTT with DPN⁺ to give oxidized DTT and DPNH was about 2.5 at pH 7.0 and 35 at pH 8.1. Assuming the redox potential of DPN⁺ to be -0.330 v at pH 7.0 (Burton and Wilson, 1953), the redox potential of DTT is -0.332 v at pH 7.0, and -0.366 v at pH 8.1. This is about 0.044 v more negative than the potential of lipoamide (Massey, 1960), corresponding to an equilibrium constant for reaction of lipoamide and DTT of 31.

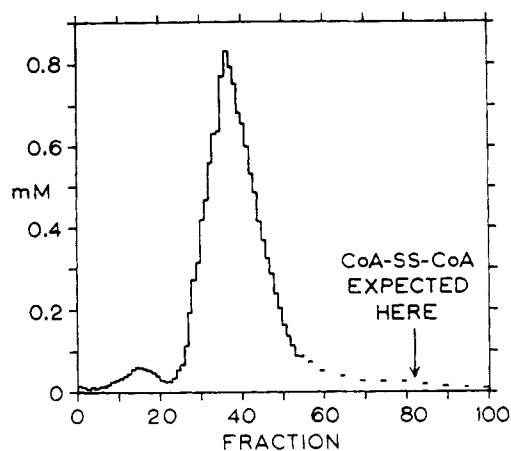


FIG. 1.—Chromatography of coenzyme A on diethylaminoethyl-cellulose-bicarbonate in the presence of DTT. A solution containing 135 μ moles of coenzyme A (Pabst) and 50 mg DTT in 200 ml was absorbed on a 1.8×20 -cm column, and eluted with a 2-liter linear gradient of triethylammonium bicarbonate, pH 7.4, running from 0.1 to 1 M. All eluting solutions contained 0.1 mg/ml DTT. Fraction size, 10 ml. Concentrations are based on OD₂₆₀. No optical density was found in fractions 100–200.

Considerable uncertainty exists concerning the redox potentials of thiols such as cysteine, glutathione, and coenzyme A, with reported values at pH 7 ranging from -0.22 to -0.35 v (Clark, 1960). An attempt was made to determine the potential of the L-cysteine–L-cystine system by equilibration with the DPN⁺–DPNH system in the presence of lipoamide and dihydrolipoic dehydrogenase. At pH 7.9 the equilibrium constant for the reduction of DPN⁺ by cysteine was estimated to be 0.0013, which corresponds to a potential for cysteine of -0.21 v at pH 7.0. This value is close to that reported by Fruton and Clarke (1934) (-0.22), but more positive than the values obtained by others. Accepting this value, we can calculate an equilibrium constant of 1.3×10^4 for the reduction of cystine by DTT (reactions 3 and 4). Since the initial reaction of DTT and cystine (reaction 3) should have an equilibrium constant not far from unity, the equilibrium constant for the cyclization reaction (reaction 4) would be about 10^4 . It is interesting to note that formation of the dithiane ring of oxidized DTT occurs more readily than formation of the dithiolane ring of lipoamide, as shown by the equilibrium constant of 31 for reduction of lipoamide by DTT.

The ability of DTT to keep a monothiol reduced can be seen from Figure 1, which shows the results of chromatography of commercial coenzyme A (Pabst) on diethylaminoethyl-cellulose-bicarbonate in the presence of DTT. Only one small ultraviolet-absorbing impurity is present, and there is no oxidized coenzyme A peak at all (commercial coenzyme A is only 75% reduced). Chromatography of the same preparation of coenzyme A in the absence of a reducing agent gives several small peaks (presumably mixed disulfides) in addition to the major peaks of reduced and oxidized coenzyme A.

In addition to the favorable equilibrium for reduction of disulfides, DTT and DTE possess other convenient properties. Both the reduced and oxidized forms are solids which are quite soluble in water and alcohols. Water solutions of the reduced forms are surprisingly stable to air oxidation (more stable than glutathione, for example), but their oxidation is catalyzed by the presence of a monothiol such as cysteine. The amount of DTT or DTE present can be determined by assaying

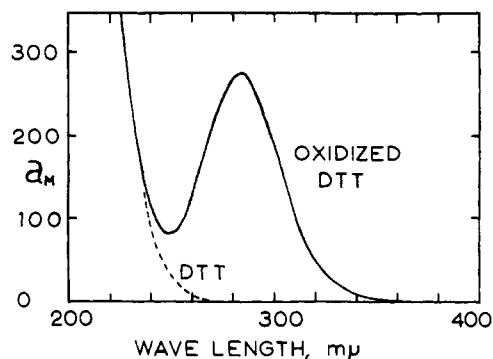


FIG. 2.—Ultraviolet spectrum of oxidized DTT and DTT in water.

for thiol groups both by a method which determines all thiol groups, and by the nitroprusside method (Grunert and Phillips, 1951), in which DTT and DTE give low color yields. The amount of the oxidized forms present can be determined from their ultraviolet spectrum (Fig. 2). Although the solid reduced forms and their concentrated solutions have the characteristic thiol odor, this is apparent only at close range, and these compounds do not need to be used in a hood.

DTT and DTE thus seem to do very admirably the job they were designed to do. Because of their low redox potential and other convenient properties, they are obviously the reagents of choice for protecting thiol groups. So far no real difference between the isomers has been noted, and the more easily prepared DTT has been routinely used in this laboratory.

EXPERIMENTAL

Dithiothreitol and Dithioerythritol.—The tetraacetyl derivatives of DTT and DTE were prepared by oxidation of *trans*-1,4-dibromobutene-2 to the corresponding dibromoglycol, acetylation, and reaction with potassium thioacetate as described by Evans *et al.* (1949). DTT and DTE were prepared from the tetraacetates by refluxing in 1 N methanolic HCl under N₂ for 5 hours, taking to dryness in a rotary flash evaporator, and storage over P₂O₅ and KOH *in vacuo* for several days. DTT prepared in this way melted at 40° (reported by Evans *et al.*, 1949, 43°) and was 97–100% pure by assay for SH groups. It can be sublimed at 37° (0.005 mm) onto a cold finger for further purification. DTE recrystallized from ether-hexane, mp 83° (reported by Evans *et al.*, 1949, 83°), was 100% pure by SH assay. Both DTT and DTE give full color yield when assayed for SH groups using *N*-ethyl maleimide (Roberts and Rouser, 1958; Alexander, 1958) or 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman, 1959), but only 4% of the color given by cysteine in the nitroprusside reaction (Grunert and Phillips, 1951).

Oxidized DTT and DTE.—The previously unreported cyclic disulfides of DTT and DTE (the *trans* and *cis* isomers, respectively, of 4,5-dihydroxy-*o*-dithiane) were prepared by ferricyanide oxidation. A solution of 1 g (6.5 mmoles) of DTT in 25 ml water was titrated with 0.8 M ferricyanide (with the pH kept at 7 by addition of 2 N KOH) until a yellow color persisted. Exactly 13 mmoles each of ferricyanide and base were required. The solution was evaporated to 10 ml and 200 ml ethanol was added. After filtration the clear filtrate was taken to dryness, and the crude product was crystallized by addition of hexane to a solution in ethyl acetate. The final product, mp 132°, was obtained by sublimation at 80° (0.005 mm) onto a cold finger.

Anal. Calcd. for $C_4H_8O_2S_2$: C, 31.56; H, 5.30; S, 42.13. Found: C, 31.58; H, 5.27; S, 42.27.

Oxidized DTE prepared similarly had the same melting point as oxidized DTT, but a mixture of the two melted over the range 110–120°.

Anal. Found: C, 31.84; H, 5.35; S, 42.15.

The ultraviolet spectrum of oxidized DTT is shown in Figure 2. The maximum of the disulfide peak is at 283 $m\mu$ ($a_M = 273$). The spectrum of oxidized DTE is essentially the same.

Redox Potentials of DTT and Cysteine.—Reactions were carried out at room temperature in 3 ml volume in silica cuvetts containing 0.2 M buffer (phosphate, pH 7.0, or Tris, pH 7.9 or 8.1), 0.18 mg/ml crystalline dihydrolipoic dehydrogenase (Massey, 1960) and from 0.4 to 1.2 mM lipoamide. Either DPN^+ or $DPNH$ (0.13–0.2 mM) was present initially. For the DTT experiments, the combined concentration of DTT and oxidized DTT was kept constant at 6.7 mM, and ratios of DTT to oxidized DTT of 4:1, 1:1, and 1:4 were used. The optical density of all components except enzyme was read, the enzyme was added, and the optical density was followed until equilibrium was reached. The amount of dihydrolipoic dehydrogenase used catalyzed a nearly instantaneous equilibration of DPN^+ and lipoamide, so that approach to equilibrium in the over-all reaction was limited by thiol interchange, equilibrium being reached in 1 minute at pH 8, and 10 minutes at pH 7 with DTT. The measured change in optical density was considered as a first approxima-

tion to be caused by $DPNH$ formation or disappearance; corrections were later made for the small optical density changes resulting from changes in the concentrations of lipoamide and oxidized DTT.

In order to observe DPN^+ reduction by cysteine, it was necessary to use 42 mM cysteine and no cystine present initially. The observed reduction of DPN^+ was small, but was completely reversed by addition of 0.83 mM cystine.

ACKNOWLEDGMENT

The author wishes to thank Vicki Lonngren and Alan Bayless for technical assistance, and Dr. Peter Jurtshuk for a generous gift of dihydrolipoic dehydrogenase and lipoamide.

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The Enzymic Condensation of a Thiol Ester-Type Carboxyl-activated Acylamino Acid with an Amino Acid Amide to Form a Peptide*

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Received December 30, 1963

Papain is capable of catalyzing a transfer reaction in which the thiol ester group of benzoylglycine thiol ethyl ester is replaced by glycinamide. This is the first known example of a proteolytic enzyme catalyzing a replacement reaction involving a thiol ester-type activated amino acid carboxyl group. The effect of glycinamide concentration upon this reaction has been studied. These results indicate that a two-step reaction occurs. The first step, the hydrolysis of the thiol ester, is rate determining. The reaction shows a pH optimum at pH 7.0.

The first indication that proteolytic enzymes can effect the hydrolysis of a thiol ester was reported by Goldenberg *et al.* (1950). These investigators observed, upon incubating acetyl DL-phenylalanine thiol ester with chymotrypsin, the strong odor of ethyl mercaptan. Strecker *et al.* (1955) have shown that ox brain and ox liver contain thioesterases. In 1956 in this laboratory it was demonstrated that the plant proteolytic enzyme papain possessed powerful thioesterase activity (Johnston, 1956). Papain was shown to catalyze the hydrolysis of benzoylglycine thiol

ethyl ester (BGTEE)¹ and benzoylglycine thiol isopropyl ester as well as benzoylglycine ethyl ester. Previously Bergmann *et al.* (1935) reported that benzoylglycinamide is an active substrate for papain.

Cysteine-activated papain is known to catalyze the transamidation reaction involving the replacement of the amide of benzoylglycinamide by ¹⁵N ammonia introduced as isotopic diammonium hydrogen citrate or ammonia (Fruton, 1950). Papain also catalyzes the replacement of amide nitrogen of a number of acylamino acid amides by the —NHOH group of hydroxylamine or by amino acids to form peptides (Johnston *et al.*, 1950a,b; Fruton *et al.*, 1950).

The discovery of the thioesterase action of this proteolytic enzyme suggested the possibility that the enzyme might be capable of catalyzing the transfer of the acyl group of a thiol ester to an amino compound to form a peptidic structure. Previous to the work reported here, the catalysis by proteolytic enzymes of a transfer reaction of this general type has not been reported. In this report the papain-catalyzed synthesis of BGGA from BGTEE and glycinamide is described.

* This work has been supported in part by a grant (HE-2970) from the National Institutes of Health.

† Part of this work was presented in a thesis by Robert M. Metrione submitted to the Graduate College of the University of Nebraska in partial fulfillment of the requirements of the degree of Master of Science. Present address: Department of Biochemistry, Yale University School of Medicine, New Haven, Conn.

¹ The following abbreviations will be used: BGTEE = benzoylglycine thiol ethyl ester; BGGA = benzoylglycylglycinamide; BG = benzoylglycine.