SYNTHESIS, PURIFICATION AND STABILITY IN SOLUTION OF DITHIOBIURET-2,4-^{35}S

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SUMMARY

2,4-Dithiobiuret (dithioimidodicarbonic diamide) can be labelled with ^{35}S by isotopic exchange with hydrogen sulfide-^{35}S in boiling 0.01N hydrochloric acid. The product obtained by crystallization from the reaction medium contains more than 93% of the radioactivity as 2,4-dithiobiuret and less than 3% as thiuret (3,5-diimino-1,2,4-dithiazoline). Up to 40% of the initial radioactivity can be recovered as solid products. Solutions containing dithiobiuret must be stored under refrigeration to prevent decomposition to thiuret, monothiobiuret, and other products. Solutions prepared in this way are stable for 8 days. Dithiobiuret may be separated from thiuret, monothiobiuret, biuret and unidentified decomposition products by thin-layer chromatography on silica gel.

Key Words: 2,4-Dithiobiuret, 3,5-Diimino-1,2,4-dithiazoline, Sulfur-35.

INTRODUCTION

2,4-Dithiobiuret has strong reducing properties (1). After its synthesis on a commercial scale (2), it was proposed for use as a rodenticide (3), plant root-growth promoter (4) or polymer stabilizer (5).

2,4-Dithiobiuret is highly toxic to rats when administered chronically (6). Lethality is preceded by the development of skeletal muscle weakness, commencing with a flaccid paralysis of the hindlimbs (6,7). The muscle weakness appears to involve decreased neuronal acetylcholine release in response to nerve stimulation (8,9).

2,4-Dithiobiuret, suitably labelled with a radioisotope, was required for studies of its absorption, distribution, metabolism, excretion and pharmacodynamic properties. Furthermore, the purity and stability of solutions of labelled dithiobiuret had to be assured, as preliminary studies showed marked differences in the toxicity of dithiobiuret and some of its suspected degradation products.

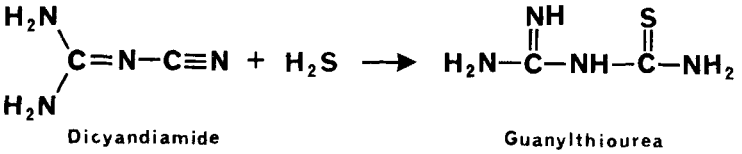
0362-4803/82/070881-09$01.00

Received December 21, 1981
Revised March 22, 1982

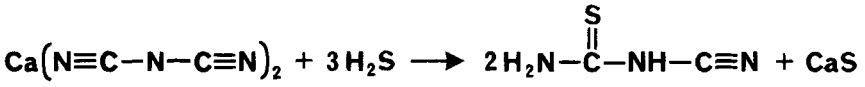
RESULTS AND DISCUSSION

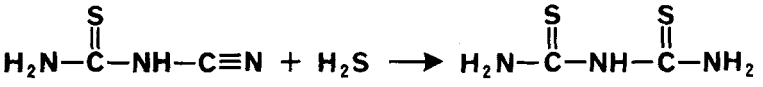
Dithiobiuret may be prepared by the addition of hydrogen sulfide to dicyandiamide (Scheme I) (10) or to calcium dicyanamide (Scheme II) (11,12).

Scheme I:



Scheme II:

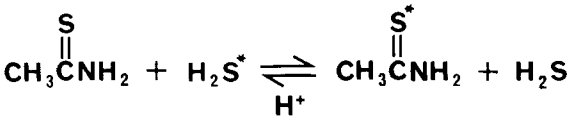




Although the first reaction requires pressure to give adequate yields of dithiobiuret, the second reaction gives 44-64% yields without the use of pressure.

The facile addition of hydrogen sulfide to cyanothiourea (Scheme II) at normal atmospheric pressure to give dithiobiuret suggested to us that labelling of dithiobiuret with ^{35}S might be accomplished by acid-catalyzed exchange with hydrogen sulfide-^{35}S, in a manner analogous to the preparation of thioacetamide-^{35}S (Scheme III) (13). Treatment of dithiobiuret in hot 0.01N hydrochloric acid

Scheme III:

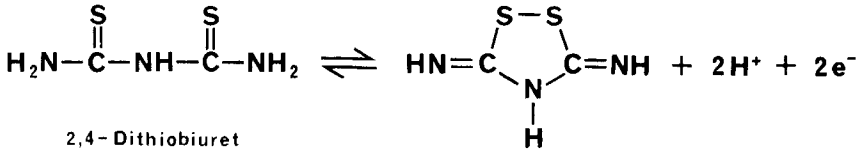


Thioacetamide

with hydrogen sulfide-^{35}S for two hours, followed by recrystallization of the product in a nitrogen atmosphere, gave 36-44% yields of dithiobiuret-^{35}S. Between 6% and 40% of the radioactivity initially present in the hydrogen sulfide-^{35}S could be recovered in the product.

Dithiobiuret is reversibly oxidized (14) to 3,5-diimino-1,2,4-dithiazoline (thiuret) by hydrogen peroxide, iodine, ceric salts, thallic salts, or dithioformamidine salts (Scheme IV). Thiuret is slowly reduced to dithiobiuret by

Scheme IV:



2,4-Dithiobiuret

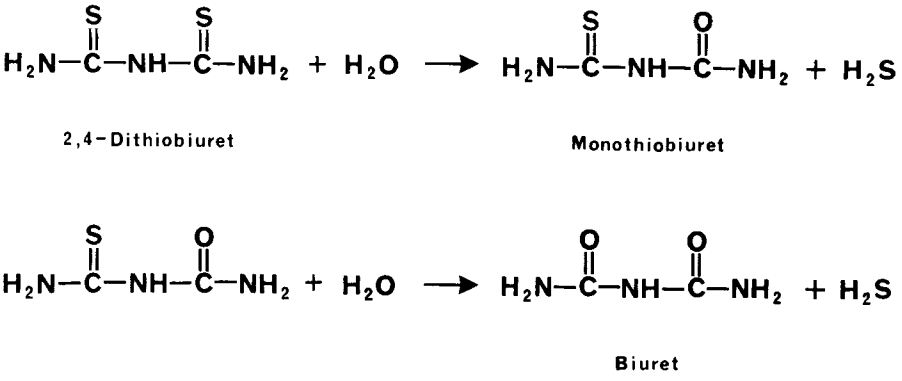
Thiuret

sulfhydryl compounds (15) such as cysteine, 2-aminoethanethiol, and glutathione. We have found that dithiobiuret readily undergoes air oxidation to thiuret when spotted on silica gel as shown by two dimensional chromatography and cochromatography with authentic thiuret. This facile oxidation hinders the determination of the radiochemical purity of dithiobiuret-^{35}S and severely limits the conditions under which the labelled product may be stored and used.

Oxidation of dithiobiuret may occur during recrystallization of the labelled compound, during the preparation and storage of solutions containing dithiobiuret, or during the sample application and development stages of thin-layer chromatographic analysis. Suppression of oxidation requires exclusion of oxygen or other oxidizing agents during all of these procedures.

Dithiobiuret can also undergo hydrolysis (16) to monothiobiuret, biuret, and their degradation products (Scheme V). The rate of hydrolysis may be expected to

Scheme V:



be affected by the pH, temperature, and water content of dithiobiuret solutions. The potential hydrolysis of dithiobiuret also affects the determination of the radiochemical purity of the labelled product as well as restricts the conditions under which it may be stored or used.

Recrystallization methods developed for the purification of unlabelled dithiobiuret (8) yield a product free of hydrolysis products but occasionally contaminated with thiuret. Minimization of oxidation during recrystallization was found to be best accomplished by using Schlenk-type glassware to maintain a nitrogen atmosphere throughout the operation. Solvents had to be degassed before use. Filtration, washing, and drying of the crystals were performed under positive nitrogen pressure. This procedure yielded dithiobiuret of constant melting point and ultraviolet absorption spectrum (1). The crystalline product is stable for at least six months when stored in an amber glass bottle in a dessicator at -10°. The solid may be stored in air. The same procedures were used in the preparation of the labelled product as well.

The radiochemical purity of dithiobiuret-^{35}S, prepared as described above, was determined by thin-layer chromatography on silica gel. All chromatographic procedures were performed in a nitrogen atmosphere at 4°. Using acetonitrile: carbon tetrachloride:80% formic acid (60:5:2) as a mobile phase, more than 93% of the spotted radioactivity was recovered as a single peak (R_f = 0.82), comigrating

with authentic dithiobiuret. A second peak (R_f = 0.24), comigrating with authentic thiuret, contained less than 3% of the initial radioactivity.

A solution containing 1 mg/ml dithiobiuret-^{35}S in 0.9% aqueous sodium chloride was saturated with air and stored for 8 days at room temperature. An aliquot was analyzed using the thin-layer chromatographic system described above. The dithiobiuret was extensively decomposed. In addition to thiuret (R_f = 0.24), radioactive peaks were observed at the origin, in the region R_f = 0.05-0.20, in the region R_f = 0.43-0.64, comigrating with monothiobiuret (R_f = 0.67), and in the region R_f = 0.88-0.97. It was noted that biuret (R_f = 0.77), a non-sulfur containing decomposition product, was not well resolved from dithiobiuret (R_f = 0.82) and monothiobiuret (R_f = 0.67). This chromatographic system therefore is not very satisfactory for determining the radiochemical purity of dithiobiuret labelled with ^{14}C.

In an attempt to reduce the extensive decomposition of dithiobiuret, when stored at room temperature in aerated, aqueous sodium chloride, we investigated the stability of dithiobiuret in two aqueous solvents under different temperature and aeration conditions. Solutions containing 1 mg/ml unlabelled dithiobiuret in 0.9% sodium chloride solution or 50% propylene glycol in water were prepared. Solutions either were degassed by boiling the vehicle prior to adding the dithiobiuret or were vigorously aerated. All solutions were protected from light and were stored either at room temperature (20°) or under refrigeration (4°). The samples were assayed for dithiobiuret by monitoring their ultraviolet absorbance at 282 nm immediately after preparation or after 1, 2, 4 or 8 days of storage. The absorbance results were statistically analyzed by analysis of variance methods.

All samples stored at 4° behaved similarly and the dithiobiuret in these solutions did not degrade appreciably. However, samples stored at 20° displayed uniquely different behavior and varying degress of dithiobiuret decomposition was observed. More specifically, all samples stored for up to 8 days at 4° had dithiobiuret concentrations that were 95-105% (95% confidence interval) of their initial concentration. Likewise, samples stored for 1 day at 20° had 91-104%

(95% confidence interval) of their initial dithiobiuret concentration. During the 8 day storage period at 4° and the first 24 hours at 20° there was no significant change in the ultraviolet spectra of the samples over the range 230-350 nm. However, the dithiobiuret in all solutions stored at 20° began to decompose by the second day after preparation, as evidenced by decreases in absorbance at 282 nm. By the eighth day of storage the concentration of dithiobiuret in these solutions was 25-80% of its initial concentration.

Dithiobiuret can be labelled with ^{35}S and formulated for use in biological experiments with adequate radiochemical purity and chemical stability provided that precautions to maintain a nitrogen atmosphere or cool storage temperatures are observed during handling of the bulk material or prepared solutions. Dithiobiuret solutions stored at room temperature are not stable for more than 24 hours.

EXPERIMENTAL

Materials. 2,4-Dithiobiuret (Pfaltz and Bauer, Stamford, CT) was recrystallized from 0.01N HCl (50 ml/g) prior to use (mp 189-190°). 2,4-Dithiobiuret (Ash Stevens, Detroit, MI) was used without initial purification (mp 186-188°). Dithiobiuret melting points were obtained using an oil bath (Thomas-Hoover Uni-Melt, Thomas Co., Philadelphia, PA) preheated to 178°. Biuret (Aldrich, Milwaukee, WI), monothiobiuret (Fluka AG, Buchs, Switzerland) and hydrogen sulfide-^{35}S (Amersham, Arlington Heights, IL) were used as received. All other chemicals were reagent grade.

Thiuret (3,5-diimino-1,2,4-dithiazoline) hydrochloride. Dithiobiuret was oxidized with cold dilute hydrogen peroxide as described (14). UV λ_{max} 246 nm, mp > 310°.

2,4-Dithiobiuret-^{35}S. Dithiobiuret (0.5-1.0 g) was suspended in 50 ml/g 0.01N HCl in a 100 ml Schlenk flask (K-213100, Kontes, Inc., Vineland, NJ) equipped with a side-arm stopcock to permit evacuation and a ȝ 14/20 female joint. The suspension was frozen by immersing the flask in pulverized dry ice and a 25 x 8 mm magnetic stirring bar was placed in the flask. A break-seal ampul containing

hydrogen sulfide-^{35}S (5-6 mCi) and fitted with a ℔ 14/20 male joint was connected to the flask; the apparatus was evacuated through the side-arm. The ampul seal was broken using the stirring bar, and then the contents of the flask were thawed by immersing the apparatus in a water bath. After melting was complete, stirring was begun and the water bath was heated to boiling. After 2 hours the flask was removed from the boiling water bath and cooled. Crystals of dithiobiuret separated.

After standing overnight, the vacuum was released. The ampul was quickly removed and the apparatus head space was flushed with N_2. Exhaust gases were passed through the side-arm into a saturated solution of lead acetate to trap any remaining hydrogen sulfide-^{35}S. The flask was then reheated in a boiling water bath to dissolve the precipitate. The solution was filtered quickly through a pad of cotton into a Schlenk filter tube (K-215000, Kontes, Inc., Vineland, NJ) which was flushed with N_2. The filter tube was then sealed and cooled slowly. Needles of dithiobiuret separated overnight. The suspension was filtered under N_2 pressure, and the precipitate was washed with cold, deoxygenated 0.01N HCl (40 ml) followed by cold, deoxygenated 100% ethanol (5 ml). The crystals were dried in a stream of N_2. Yield: 36-44% of dithiobiuret-^{35}S containing 0.3-2.0 mCi/g. m.p. 189-190°.

Stability of dithiobiuret solutions. Solutions (250 ml) containing 0.9% NaCl or 50% propylene glycol in water were heated to boiling. Part of the boiling solution was poured into a 100 ml volumetric flask, filling to overflowing. The flask was stoppered and cooled to 20°. The solution in excess of 100 ml was then removed and 100 mg dithiobiuret was added. Dissolution was hastened by gentle stirring with care taken to avoid aeration. Samples (10 of each solvent) were prepared in 5 ml clear glass septum seal vials. The vials were filled to overflowing and sealed with rubber septa. The remaining 0.9% NaCl and 50% propylene glycol stock solutions were vigorously aerated by shaking. Dithiobiuret (100 mg) was dissolved in aerated solvent (100 ml) and 10 samples of ~ 3 ml were stored in 5 ml clear glass septum seal vials. 5 Vials from each solvent lot were stored at room temperature (20°) and the remaining 5 vials were refrigerated (4°). Both

sets of vials were protected from exposure to light. Vials were opened within 1 hour of preparation and at 1, 2, 4 and 8 days thereafter. A 0.300 ml aliquot was removed from each vial at the designated time and diluted to 50.0 ml with degassed deionized distilled water. The absorbance at 282 and 246 nm and the ultraviolet spectrum between 230-350 nm was determined using a double beam spectrophotometer (Cary 118, Varian, Inc., Palo Alto, CA) using degassed deionized distilled water as a reference.

Thin-layer chromatography. Solutions containing dithiobiuret-^{35}S or its decomposition products were spotted on silica gel thin-layer plates with preadsorbant spotting zones (Type LK-5D, Whatman Inc., Clifton, NJ). Spotting and all further manipulations were performed inside a Glove-Bag® (Instruments for Research and Industry, Cheltenham, PA) flushed with N_2. All operations were carried out in a cold room maintained at 4°. Solvents were degassed by boiling prior to mixing; solvents were cooled in a N_2 atmosphere. Failure to maintain either a N_2 atmosphere or low temperature resulted in extensive decomposition on the thin-layer chromatography plate, as evidenced by trailing spots and erratic results. Pilot studies showed that both sample application and plate development steps contributed to sample decomposition when either step was carried out in air or at room temperature.

Plates were developed in acetonitrile (60 parts)/carbon tetrachloride (5 parts)/80% formic acid (2 parts by volume). After development and drying, sections of the adsorbant were removed by scraping and transferred to scintillation vials. The vials were filled with 10 ml of toluene containing 4% POP and 0.4% POPOP scintillators and counted in a liquid scintillation counter (Model 460CD, Packard Instrument Co., Downers Grove, IL).

Samples of unlabelled dithiobiuret, monothiobiuret, and thiuret were separated by silica gel thin-layer chromatography as described above. The separated components were scraped from the plate, extracted, and analyzed by ultraviolet spectrophotometry. The recovered materials had ultraviolet spectra which agreed with those of authentic standards.

ACKNOWLEDGEMENTS

This investigation was supported by U.S.P.H.S. grant ES01906. The authors thank Dr. Joseph Gal and Mr. Matt Cheung for their contributions to the development of the chromatographic procedure reported herein.

REFERENCES

1. Preisler, P. W. - J. Amer. Chem. Soc. 71: 2849 (1949).
2. Sperry, R. L. - U.S. patent 2, 371, 112 (1945).
3. Dieke, S. H., Allen, G. S. and Richter, C. P. - J. Pharmacol. Exp. Ther. 90: 260 (1947).
4. Dufrenoy, J. and Pratt, R. - Science 108: 715 (1948).
5. Webb, M. Q. - U.S. patent 2, 560, 053 (1952).
6. Astwood, E. B., Hughes, A. M. Lubin, M., Van der Lann, W. P. and Adams, R. - Science 102: 196 (1945).
7. Atchison, W. D., and Peterson, R. E. - Toxicol. Appl. Pharmacol. 57: 63 (1981).
8. Atchison, W. D., Lalley, P. M., Cassens, R. G., and Peterson, R. E. - Neurotoxicology 2: 329 (1981).
9. Atchison, W. D., Mellon, W. S., Lalley, P. M. and Peterson, R. E. - Neurotoxicology (1982, In press).
10. Kurzer, F. - J. Chem. Soc.:1 (1955).
11. Lane, L. C. and Hamilton, R. W. - U.S. patent 2, 557, 980 (1952).
12. Marsh, N. H. and Hamilton, R. W. - U.S. patent 2, 557, 984 (1952).
13. Nygaard, O., Eldjarn, L. and Nakken, K. F. - Cancer Res. 14: 625 (1954).
14. Preisler, P. W. and Bateman, M. M. - J. Amer. Chem. Soc. 69: 2633 (1947).
15. Roesler, J. R., Leslie, J. and Gorin, G. - J. Org. Chem. 29: 1488 (1964).
16. Walter, W. and Voss, J., The Chemistry of Amides, Zabicky, J., Ed., Ch. 8 - Interscience Pub., London (1970).