to discriminate accurately among acetazolamide tablets. That considerable refinement would be necessary is accentuated by the fact that the *in vivo* data show statistical differences among tablets tested in only one bioequivalence parameter. Detection of this inequivalence by *in vitro* testing would require highly sensitive and statistically validated methodology.

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NOTES

Hydrolytic Degradation of 2,6-Dichlorobenzylthiopseudourea Hydrochloride

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Received October 18, 1976, from the *Analytical and Physical Chemistry Department, Research Division, William H. Rorer, Inc., Fort Washington, PA 19034.* Accepted for publication April 12, 1977.

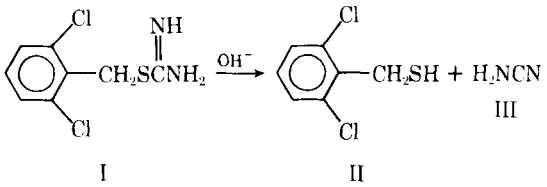
Abstract □ Degradation of 2,6-dichlorobenzylthiopseudourea hydrochloride was followed in basic medium (pH 7.5) to isolate and characterize all possible degradation products. IR, Raman, and NMR spectroscopy, TLC, and elemental analysis were used to identify the products. Degradation of base-hydrolyzed 2,6-dichlorobenzylthiopseudourea hydrochloride produced 2,6-dichlorobenzylthiol and cyanamide and was followed by oxidation (air) to produce bis(2,6-dichlorobenzyl) disulfide, dimerization to give cyanoguanidine, and hydrolysis to yield urea. The kinetics of hydrolysis at 22.5° (pH 7.0 and 7.5) and at 37° (pH 7.0) revealed a pseudo-first-order reaction with respect to the substrate. Apparent first-order rate constants and energy of activation, entropy of activation, and half-life values were determined.

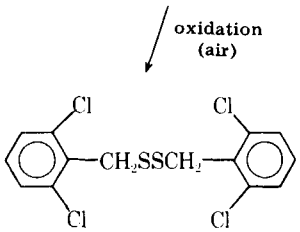
Keyphrases □ 2,6-Dichlorobenzylthiopseudourea—hydrolytic degradation in basic medium, products isolated and identified □ Degradation, hydrolytic—2,6-dichlorobenzylthiopseudourea in basic medium, products isolated and identified □ Hydrolysis—2,6-dichlorobenzylthiopseudourea in basic medium, products isolated and identified □ Thiopseudourea, substituted—hydrolytic degradation in basic medium, products isolated and identified

The novel thiopseudourea analogs exhibited gastric, antisecretory, spasmolytic, and antiulcerogenic activities in laboratory animals. 2,6-Dichlorobenzylthiopseudourea (I) was selected as a candidate for exhaustive pharmacological and chemical studies.

Thiopseudourea is chemically susceptible to hydrolytic degradation and scission of the sulfur–carbon bond in strongly basic solutions (1). Limited information is available on solution stability and on the degradation products resulting from base hydrolysis. Zuman and Fedoreňko (2) investigated the kinetic hydrolysis of several substituted thiopseudoureas at pH 11, using a polarographic method to detect thiols as degradation products. No reference was made of the formation of the disulfide as one major resultant product. The homogeneous base-catalyzed oxidation of *n*-butylthiol to form disulfide with molecular oxygen has been studied in aprotic (3) and aqueous (4) solutions.

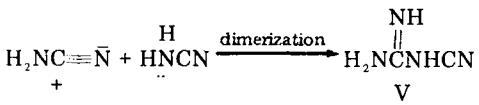
The objectives of this investigation were to isolate all possible degradation products resulting from the base hydrolysis of I at pH 7.5 and to follow the hydrolysis kinetics of this entity in a neutral medium and a weakly basic buffered medium at pH 7.0 and 7.5 at 22.5° and at pH 7.0 at 37°. This study was also conducted to characterize the



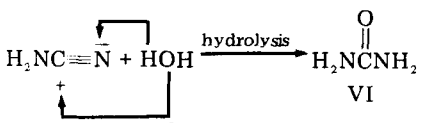


IV

Scheme I



Scheme II



Scheme III

structures of the major degradation products by IR, Raman, and NMR spectroscopy, melting points, TLC, and elemental analysis.

The methods of isolation of the degradation products were liquid–liquid extraction and column chromatography. The hydrolysis kinetics of I were followed by the UV method. In addition, a limited study was carried out on solution stability of I at pH 1.5 and 4.0 in water and methanol.

EXPERIMENTAL

Materials—All chemicals were reagent grade. Compound I was synthesized by a reported method (5). The identity and purity of I were confirmed by IR[1], UV[2], and NMR[3] spectra, potentiometric titration[4], melting-point[5] (132–134°) determination, elemental analysis[6] for $C_8H_9Cl_3N_2S$ (calc.: C, 35.37; H, 3.34; Cl, 39.16; N, 10.31; S, 11.81; found: C, 35.77; H, 3.35; Cl, 39.48; N, 10.30; S, 11.58), and TLC using 250-μm aluminum oxide[7] plates (Type T) eluted with methanol–chloroform (50:50) (R_f 0.72). Cyanoguanidine[8] and urea[9] were obtained commercially.

Base Hydrolysis Degradation Products—A solution of I, 300 mg in 100 ml of pH 7.5 phosphate buffer, was incubated for 3 days at 37° in a constant-temperature water bath. During incubation, a white precipitate was formed and some needle-like crystals accumulated in the flask neck. After incubation, the contents were analyzed as follows.

The long, colorless needles were removed and dried over phosphorus pentoxide. IR spectroscopy, melting-point determination, and elemental analysis were carried out to identify the sublimed crystals. The entire contents of the flask were transferred to a 250-ml separator to which 20 ml of chloroform was added to extract insoluble material. The chloroform extract, after evaporation of the solvent, yielded a white solid, which was crystallized two times with ethanol and dried at 55° for 5 hr under vacuum. IR, UV, NMR, and Raman spectra, melting-point determination, elemental analysis, and TLC [250-μm alumina plate (acidic) using cyclohexane–chloroform (90:10) and methanol–chloroform (30:70) and visualized under UV light] were used to identify the product (R_f 0.63 and 0.75, respectively).

The aqueous phase was evaporated to dryness. The resultant solid was triturated with 2 ml of ethanol, and the ethanol solution was passed through an alumina column (neutral, 10 × 1 cm). The ethanol-eluted fractions (~4 ml) were collected. The fourth fraction contained most of the solid after solvent evaporation. The solid was examined by IR spectroscopy, melting-point determination, and TLC[10] [alumina plate (acidic) using methanol–chloroform–ammonia (40:60:5 drops)]. Similar analyses were carried out on authentic cyanoguanidine.

The ethanol-insoluble solid was mixed with a few milliliters of methanol, and this solution was passed through an alumina column (a methanol–chloroform mixture was used to elute fractions). A small amount of solid was obtained on solvent evaporation. IR spectra and TLC, using an alumina acidic plate and solvents consisting of ethanol–chloroform (30:70) and methanol–chloroform (40:60), were obtained to identify the product. The spot was visualized using bromcresol green spray reagent. Similar analyses were performed on authentic urea.

Kinetics—Three solutions (10^{-3} *M*) of I were prepared in phosphate buffers at an ionic strength of 0.1 with potassium chloride in a 100-ml

Table I—Apparent Rate Constants and Activation Parameters for the Hydrolysis of I

Temperature	pH	$10^{-4}k$, hr^{-1}	E_a, kcal	$\Delta S^{\ddagger}$, eu	$t_{1/2}$, days
22.5°	7.0	4.55	19.0	−25.7	63.4
22.5°	7.5	8.79	—	—	32.8
37.0°	7.0	20.72	—	—	14.0

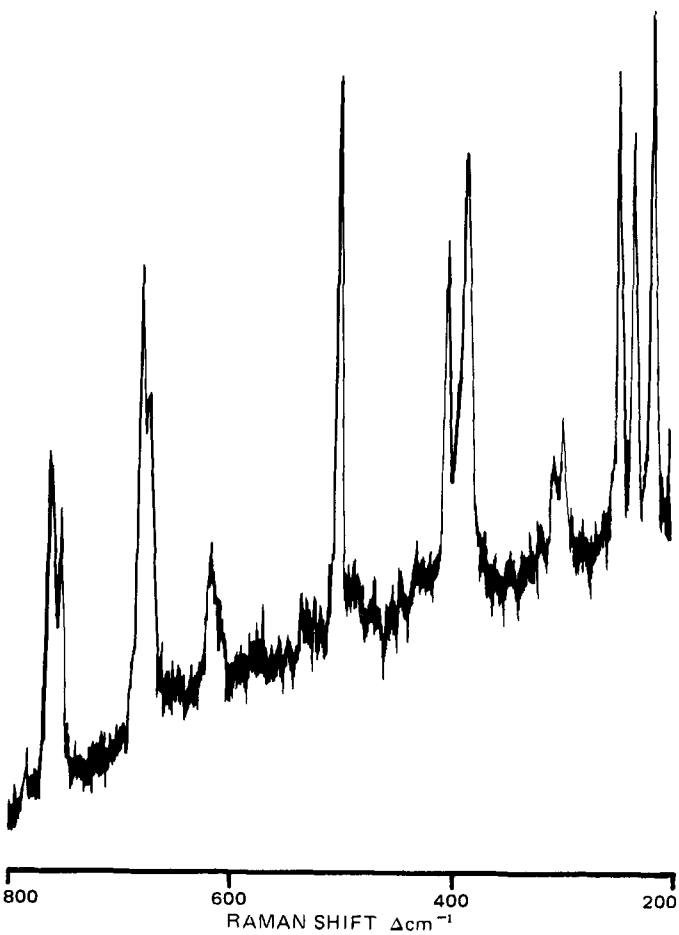


Figure 1—*Raman spectrum of bis(2,6-dichlorobenzyl) disulfide.*

[1] Perkin-Elmer model 221 spectrophotometer (potassium bromide disk).
[2] Cary model 14 spectrophotometer.
[3] Varian spectrometer model T-60.
[4] Brinkmann instruments pH meter.
[5] Thomas-Hoover capillary melting-point apparatus.
[6] Micro-Analysis, Wilmington, Del.
[7] Brinkmann Instruments, Westbury, N.Y.
[8] Eastman Kodak Co., Rochester, N.Y.
[9] J. T. Baker Chemical Co., Phillipsburg, N.J.

[10] Spray reagent was 10% aqueous sodium hydroxide, 10% aqueous sodium nitroprusside, 10% aqueous potassium ferricyanide, and water (1:1:1:3). The mixture is mixed with an equal volume of acetone before use.

volumetric flask at pH 7.0 and 7.5. Two solutions at pH 7.0 and 7.5 were equilibrated at a reaction temperature of 22.5°; the third solution at pH 7.0 was equilibrated at a reaction temperature of 37°. An aliquot was withdrawn periodically and filtered[11] (5-μm pore size), and the maximum absorbance at λ 275 nm was measured by running a UV spectrum from 400 to 260 nm. A plot of log concentration I remaining in solution *versus* time was constructed.

RESULTS AND DISCUSSION

Degradation Products—This study shows that I undergoes degradation in an aqueous medium (pH 7.0 and 7.5). The degradation of I and the formation of the degradation products are depicted in Schemes I–III. The major degradation product isolated by chloroform extraction from the reaction was bis(2,6-dichlorobenzyl) disulfide (IV). The identity of this new compound, mp 107–108°, was confirmed by IR spectroscopy: 3060, 2950, 1580, 1439, 1090, 897, 885, 781, and 759 cm^{-1}; NMR (carbon tetrachloride plus tetramethylsilane): δ 4.3 (CH_2) and 7.42 (aromaticity) ppm; and TLC, one spot visualized under UV light after fluorescein spray. In addition, a Raman spectrum[12] was obtained to confirm the S–S stretching vibration at 502 cm^{-1} of IV (Fig. 1).

Anal.—Calc. for $C_{14}H_{10}Cl_4S_2$: C, 43.77; H, 2.62; Cl, 36.91; S, 16.69. Found: C, 43.56; H, 2.52; Cl, 37.37; S, 17.27.

The crystals that accumulated above the solution at pH 7.5 were identified as 2,6-dichlorobenzylthiol (II). The identity of this new product, mp 38°, was confirmed by IR spectroscopy (2550, 1580, 1560, 1437, 1090, 975, 775, and 755 cm^{-1}).

Anal.—Calc. for $C_7H_6Cl_2S$: C, 43.54; H, 3.13. Found: C, 43.43; H, 2.90.

This compound had a very disagreeable odor.

[11] Millipore.
[12] Model 82 laser-Raman spectrophotometer, Cary Instruments.

Cyanoguanidine (V) was isolated as the ethanol-soluble fraction and was shown to be comparable with the authentic compound by IR, melting point (206–208°), and TLC (R_f 0.57). The presence of urea (VI) as the methanol-soluble fraction was confirmed by IR and TLC and was comparable with authentic urea (R_f 0.16 and 0.43). The schemes thus indicate the formation path of the degradation products as evidenced by the experimental data.

Kinetics—The hydrolytic degradation of I in solution at pH 7.0 and 7.5 at 22.5° and at pH 7.0 at 37° has been shown to be a pseudo-first-order reaction with respect to substrate. A linear relationship was obtained when the log concentration of I was plotted against time. Apparent first-order rate constants were calculated from the slope by least-squares regression, with a correlation coefficient of 0.99. Table I shows apparent rate constants and energy of activation, entropy of activation, and half-life values of I under the conditions studied. Increasing the temperature from 22.5 to 37° accelerated the degradation rate of I 4.5-fold, as expected.

Limited experimental data also were obtained on I stability in water (pH 5.86) and in solutions at pH 1.5 and 4 at 22.5°. The compound was stable up to 76, 39, and 17 days, respectively. A methanolic solution of I (9.48×10^{-4} *M*) showed degradation of 15.10 and 49.82% in 2 and 10 days, respectively.

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Polynitro Aromatic Compounds in Analytical Chemistry II: Reaction of Menadione with 2,4-Dinitrophenylhydrazine

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Abstract □ The intense blue color formed in the official assay of menadione injection by treatment of the sample with 2,4-dinitrophenylhydrazine and ammonia is shown by data from visible light spectra and mass spectra and by comparison of the pKa value with that of the corresponding reaction product of acetone to be due to proton abstraction from a monohydrazone.

Keyphrases □ Menadione—blue-colored product of reaction with 2,4-dinitrophenylhydrazine identified □ 2,4-Dinitrophenylhydrazine—blue-colored product of reaction with menadione identified □ Spectrophotometry—analysis, menadione, blue-colored product of reaction with 2,4-dinitrophenylhydrazine identified □ Vitamins—menadione, blue-colored product of reaction with 2,4-dinitrophenylhydrazine identified

The NF (1) assay for menadione (I) consists of heating menadione in alcohol–ether with an acidic solution of 2,4-dinitrophenylhydrazine to give an orange precipitate, followed by the addition of alcoholic ammonia to produce an intense blue color. The absorbance in the visible at 635 nm is proportional to the menadione concentration. The orange precipitate has been assigned Structure II, in which hydrazone formation has taken place at the less hindered carbonyl (2), but no definitive information seems to be available about the blue material.

In the present work, the composition of the precipitate II was confirmed by mass spectrometry. Since menadione contains a methyl group capable of losing a proton to form an anion, it is possible that Meisenheimer complex formation with the nitro aromatic ring might be responsible for the blue color formation. However, spectrophotometric comparison of the pKa value of menadione 2,4-dinitrophenylhydrazone with that of a simple model compound, acetone 2,4-dinitrophenylhydrazone, suggested that *N*-deprotonation rather than Meisenheimer complexation was taking place.

EXPERIMENTAL[1]

Menadione 2,4-Dinitrophenylhydrazone (II)—This compound was obtained as orange crystals according to the NF procedure (1). The ma-

[1] Mass spectral data were obtained on a Hitachi Perkin-Elmer RMU 6E instrument. UV–visible spectra were run on a Cary model 15 spectrophotometer. Measurements of pH were made with an ionalyzer, Orion Research model 801 digital pH meter, using a glass–silver–silver chloride combination electrode.