S-Acylcysteine Peptides. Synthesis and Kinetics of Hydrolysis[1]

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A series of *S*-acylcysteines, seryl-*S*-acylcysteines, and *S*-acylcysteinylthreonines was synthesized and characterized. Both *N,S*-diacetylcysteinamide and *N*-acetyl-*S*-benzoylcysteinamide are more reactive toward hydroxide ion than expected on the basis of the reactivity of simpler thiol esters: second-order rate constants for alkaline hydrolysis in water at 39° are 390 M^{-1} min^{-1} and 154 M^{-1} min^{-1}, respectively. *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester was found to be 5–6 times more reactive than *N,S*-diacetylcysteinamide; k_2 for reaction with hydroxide ion at 39° is 2300 M^{-1} min^{-1}. No S → O acyl transfer reaction was detectable during the hydrolysis of this *S*-acetyldipeptide. The magnitude of the solvent deuterium isotope effect for hydrolysis of this substrate, $k_{H_2O}/k_{D_2O} = 2.7$, suggests that the enhanced reactivity may reflect general acid–base catalysis involving the threonyl hydroxyl function. Imidazole catalyzes the hydrolysis of both *N,S*-diacetylcysteinamide and *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester by a general base mechanism; these two substrates are about equally reactive toward imidazole. *N*-Cbz-L-serinyl-*S*-benzoyl-L-cysteine methyl ester has a second-order rate constant for alkaline hydrolysis in 40% dioxane at 39° of 480 M^{-1} min^{-1}, some 40 times greater than that for *N*-acetyl-*S*-benzoylcysteinamide under the same conditions. Implications of these results for understanding the reactivity of S-acylated glyceraldehyde 3-phosphate dehydrogenases are discussed.

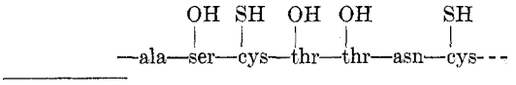
Glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate: NAD oxidoreductase (phosphorylating)], abbreviated G3PD hereafter, is a well-charactreized polyfunctional enzyme which catalyzes a crucial step in the glycolytic pathway.[3] It has been clearly established that the catalytic pathway involves the transient formation of a covalent substrate–enzyme intermediate involving acylation of a particular cysteine residue.[3,4]

One of the simplest reactions catalyzed by this versatile enzyme is the hydrolysis of *p*-nitrophenyl acetate,[3]

$$E + S \rightleftharpoons ES \xrightarrow{-P_1} ES' \longrightarrow E + P_2$$

in which S is *p*-nitrophenyl acetate, P_1 is *p*-nitrophenol, P_2 is acetate, ES is the Michaelis–Menten complex, and ES′ is the acyl enzyme intermediate. It has proved possible to account for the rate of the acylation reaction in terms of the reactivity of the sulfhydryl anion at the active site of the enzyme and the local concentrations of ester and this anion in the Michaelis–Menten complex.[5] On the other hand, the rate of enzyme deacylation, hydrolysis of a thiol ester bond, is about one million times faster than the rate of hydrolysis of simple thiol esters under similar conditions, and this rate difference has not been satisfactorily explained.[5]

The complete sequence of G3PD has been established, and, specifically, the sequence of amino acids near the active site cysteine is known.[6] There are four nucleophilic amino acids in the sequence including this particular cysteine, and a fifth is not far waay.



The occurrence of four consecutive nucleophilic groups at the enzyme active site suggests a variety of possible modes of catalysis for hydrolysis of a thiol ester involving the crucial cysteine residue. In an effort to judge the importance of the immediate neighbors of this cysteine as potentiators for the hydrolysis of such thiol esters, it was decided to synthesize some related *S*-acylcysteines and to determine their reactivity toward nucleophilic reagents. Results of these studies are detailed herein.

Experimental Section

Materials.—All inorganic chemicals and mineral acids were reagent grade. All water employed was distilled and that used in kinetic measurements was redistilled in a Corning AG-1a glass still and degassed before use. Silica gel used in column chromatography was 80–200 mesh from the Fisher Chemical Co. Silica gel G for thin layer chromatography was obtained from Merck and Sephadex LH-20 from Pharmacia, Inc. Organic materials employed were the highest grade commercially available. *N*-Acetylcysteinamide was the generous gift of Mead Johnson Co., Evansville, Ind. Hydroxylamine hydrochloride was recrystallized twice from ethanol–water; imidazole was recrystallized twice from ethanol–water; imidazole was recrystallized twice from benzene; triethylamine hydrochloride was recrystallized twice from ethanol. Triethylamine was redistilled prior to use. Dioxane was purified according to the method of Fieser[7] and was stored frozen, layered with argon. Analytical analyses were performed by Alfred Bernhardt, Mulheim, West Germany, and Midwest Microlabs, Indianapolis, Ind. Infrared spectra were taken in KBr pellets unless otherwise noted using Perkin-Elmer 137 and 137G infrared spectrophotometers. Ultraviolet and visible absorption spectra were recorded on a Cary-14 recording spectrophotometer. Proton magnetic resonance spectra were obtained with Varian A-60, HA-100, and HR-220 MHz nuclear magnetic resonance spectrophotometers. Melting points were taken on a Thomas-Hoover Uni-Melt apparatus and are uncorrected.

Synthesis. ***N,N′*-Dicarbobenzoxy-L-cystine** was prepared according to the method of Bergmann and Zervas.[8]

***N*-Carbobenzoxy-*S*-acetyl-L-cysteine** (1) was prepared by a modification of the method of Zervas, *et al.*[9,10] *N,N′*-Dicarbobenzoxy-L-cystine (0.01 mol, 5.1 g) was dissolved in 30.0 ml of methanol and the solution was cooled to 0–5°. With continual stirring, 3.0 g of zinc dust was slowly added over a period of 20

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(4) J. H. Park, B. P. Meriwether, P. Clodfelder, and L. W. Cunningham, *J. Biol. Chem.*, **236**, 136 (1961); O. P. Malhotra and S. A. Bernhard, *ibid.*, **243**, 1243 (1968); E. L. Taylor, B. P. Meriwether, and J. H. Park, *ibid.*, **238**, 734 (1963); W. F. Harrington and G. M. Karr, *J. Mol. Biol.*, **13**, 885 (1965), and references cited therein.

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(7) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath, Boston, Mass., 1957, p 284.

(8) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(9) L. Zervas and I. Photaki, *J. Amer. Chem. Soc.*, **84**, 3887 (1962).

(10) L. Zervas, I. Photaki, and N. Ghelis, *ibid.*, **85**, 1337 (1963).

min at this temperature. The solution was filtered and the filtrate was reduced in volume by two-thirds under reduced pressure, maintaining the temperature of the solution below 40°. Several volumes of water were added to the concentrated methanolic solution, which was then extracted several times with benzene. The benzene solution was dried over $MgSO_4$, filtered, and taken to dryness under reduced pressure. The resulting viscous *N*-carbobenzoxy-L-cysteine was used directly in the next reaction without further purification.

N-Carbobenzoxy-L-cysteine (0.02 mol) was dissolved with vigorous stirring in 30.0 ml of 3.5 *N* NaOH and 200 ml of saturated $NaHCO_3$. To this was slowly added with stirring 24 ml of acetic anhydride and vigorous stirring was continued for 30 min, at which time the solution was acidified with 9 *N* H_2SO_4 until precipitation was completed. The resulting gummy residue crystallized upon cooling to yield **1**, 4.54 g (74%), mp 113°. Recrystallization from carbon tetrachloride raised the melting point to 116° (lit.[10] mp 116–117°).

***N*-Carbobenzoxy-*S*-acetyl-L-cysteinyl-L-threonine Ethyl Ester (2). Method 1.**—Threonine ethyl ester hydrochloride[11] (3.5×10^{-3} mol, 0.641 g) was dissolved in 10 ml of dimethylformamide and to this was added 30.0 ml of tetrahydrofuran and 5 ml of triethylamine. This mixture was stirred for 30 min at room temperature and filtered, and the filtrate was washed with tetrahydrofuran and taken to dryness under high vacuum. The oily residue was taken up in 40 ml of tetrahydrofuran, the solution was cooled to 0°, and 1.04 g of **1** was added. A cold solution of 0.72 g of dicyclohexycarbodiimide in 10 ml of tetrahydrofuran was then added and the resulting reaction mixture was stirred at 5° for 20 hr. Insoluble dicyclohexylurea was removed by filtration and the filtrate was taken to dryness under reduced pressure. Repeated recrystallization of the residue (0.64 g, 43%) yielded a product with a melting point of 140–141°. Chromatography of the recrystallized material on silica gel eluting with 1:1 chloroform–ethyl acetate and recrystallization of the chromatographed material from acetone–water yielded pure **2**, mp 141°. Infrared absorption spectra exhibited major peaks at 2.9, 3.05, 5.85 (sh), 5.91, 5.95 (sh), 6.06, 6.08 (sh), 6.54, 7.90, 9.20, 9.70, 13.3, and 14.4 μ. Ultraviolet absorption spectra had a λ_{max} 230 nm (ϵ 4630). Nmr (acetone-d_6) showed the following major absorption peaks: τ 8.80 (m, 6, $-CHOHCH_3$, $-CH_2CH_3$), 7.70 (s, 3, $-SCOCH_3$), 6.88 (s, unassigned), 5.85 (q, 2, $-CH_2CH_3$), 4.87 (s, 2, $-CH_2Ph$), and 2.61 (s, 5, $-CH_2Ph$). *Anal.* Calcd for $C_{19}H_{26}N_2O_7S$: C, 53.51; H, 6.15; N, 6.57; S, 7.50. Found: C, 53.44; H, 6.10; N, 6.47; S, 7.57.

Method 2.—**1** (0.01 mol, 2.97 g) was dissolved in 75 ml of chloroform and 50 ml of tetrahydrofuran and cooled to −5°. Isobutyl chloroformate (0.01 mol, 1.37 g) and 1.4 ml of triethylamine were added to this solution and stirred at −5° for 5 min. Threonine ethyl ester hydrochloride (0.01 mol, 1.84 g) in tetrahydrofuran was added and 1.4 ml of triethylamine was slowly dripped in over a 5-min period. The reaction mixture was then stirred at −5° for 30 min followed by stirring at room temperature for 3 hr. After it was allowed to sit overnight, the reaction mixture was filtered and the filtrate was taken to dryness under reduced pressure. Once recrystallized product from acetone–water gave 3.34 g (78%) yield. Purification, as in method 1, gave pure **2**, mp 141°.

***N*,*S*-Diacetylcysteinamide (3).**—*N*-Acetylcysteinamide (0.011 mol, 2.043 g) was dissolved in 50 ml of water containing 6 g of $KHCO_3$. Acetic anhydride (12 ml) was added dropwise with stirring; upon completion of the addition, the reaction mixture was stirred for an additional 30 min, acidified with 9 *N* H_2SO_4, and repeatedly extracted with ethyl acetate. The extract was dried over $MgSO_4$, filtered, and taken to dryness under reduced pressure, water was added to the resulting residue, and the solution was again taken to dryness. The resulting white residue was recrystallized from ethanol–ligroin (bp 60–90°) to yield 0.90 g (36%) of **3**, mp 146–147°. Infrared absorption spectra showed major absorption peaks at 2.98, 3.06, 3.15, 5.92, 6.18, 6.50, 7.10, 7.90, 8.90, 10.5, and 13.6 μ. An ultraviolet absorption spectrum revealed a λ_{max} at 226 nm (ϵ 5440) in water. The nmr spectrum (D_2O) exhibited the following major absorption peaks: τ 8.00 (s, 3, $-CONCH_3$), 7.63 (s, 3, $-SCOCH_3$), 6.73 (m, 1, 2, $-CH-$, CH_2-). *Anal.* Calcd for $C_7H_{12}N_2O_3S$: C, 41.16; H, 5.92; N, 13.72; S, 15.71. Found: C, 41.20; H, 6.09; N, 13.72; S, 15.71.

(11) K. Podiska and J. Rudinger, *Collect. Czech. Chem. Commun.*, **24**, 3449 (1959).

***S*-Benzoyl-L-cysteine and *S*-benzoyl-L-cysteine methyl ester hydrochloride (4)** were synthesized according to the method of Zervas, *et al.*[10] ***N*-Carbobenzoxy-*S*-benzoyl-L-cysteine (5)** was made by the method of these authors[10] except that a purification step of chromatography on silica gel eluting with 5:1 chloroform–ethyl acetate was added prior to recrystallization.

***N*-Carbobenzoxy-L-seryl-*S*-benzoyl-L-cysteine Methyl Ester (6).**—Cbz-serine (1×10^{-3} mol, 0.239 g) was dissolved in a mixture of 80 ml of tetrahydrofuran and 20 ml of dimethylformamide at −5°. To this solution was added triethylamine (0.14 ml) and isobutyl chloroformate (0.14 ml) at −5° and the mixture was stirred for 15 min. At the end of this time, **4** (1.1×10^{-3} mol, 0.303 g) was added and triethylamine (0.14 ml) was dripped into the reaction mixture over a 5-min period. The reaction mixture was stirred at room temperature for 3 hr, kept at 0° overnight, and filtered. The filtrate was taken to dryness under reduced pressure, the oily residue was again taken up in tetrahydrofuran and filtered, and the filtrate was taken to dryness. The resulting yellow oil, when washed with ethyl acetate, gave a white precipitate, mp 173–175°. The white precipitate was washed with methanol, removing a trace of yellow color. Chromatography on silica gel eluting with 19:1 chloroform–methanol removed the remaining impurities. Recrystallization of the chromatographed material from chloroform gave 75 mg of product, mp 179.5–181.5°. An infrared absorption spectrum showed the following major absorption peaks: 2.9 (sh), 3.04, 5.80, 5.83 (sh), 5.95 (sh), 6.02 (sh), 6.10, 7.60, 8.00 (sh) 8.10, 8.30, 8.50, 9.90, 11.00, and 14.80 μ. Ultraviolet absorption spectrum showed major absorption peaks at λ_{max} 264 nm (ϵ 7.68×10^3) in ethanol containing 0.3% (v/v) dimethyl sulfoxide. Nmr spectrum ($CDCl_3$) showed the following assigned absorption peaks: τ 6.23 (s, 3, $-CO_2CH_3$), 4.95 (s, 2, 2H's of Cbz), 2.70 (s, 5, 5 H's of Cbz), 2.67 (m, 3, 3,4,5 H's of *S*-benzoyl), and 2.06 (d of d, 2, J = 6.2 Hz, 2,6 H's of *S*-benzoyl). *Anal.* Calcd for $C_{22}H_{24}N_2SO_7$: C, 57.38; H, 5.25; N, 6.08; S, 6.96. Found: C, 56.93; H, 5.38; N, 6.07; S, 7.13.

***N*-Carbobenzoxy-*S*-benzoyl-L-cysteinyl-L-threonine Methyl Ester (7).**—L-Threonine methyl ester hydrochloride[12] (4.5×10^{-3} mol, 0.763 g) was dissolved in a mixture of 10 ml of dimethylformamide and 40 ml of tetrahydrofuran. To this was added with stirring triethylamine (0.7 ml) and, after 15 min, the solution was filtered and the filtrate was taken to dryness under vacuum. The resulting residue was taken up in 100 ml of tetrahydrofuran and to this was added **5** (1.6 g) and dicyclohexylcarbodiimide (0.928 g). The reaction mixture was stirred for 24 hr at 5° and filtered, and the filtrate was taken to dryness under reduced pressure. The residue was dissolved in ethyl acetate and washed with dilute hydrochloric acid and water. The ethyl acetate layer was dried over $MgSO_4$, filtered, and taken to dryness under reduced pressure. The residue was dissolved in acetone and cooled, and additional dicyclohexylurea was filtered off. The filtrate was again taken to dryness and the residue was chromatographed on silica gel eluting with 2:1 chloroform–ethyl acetate. The chromatographed sample was crystallized from ethyl acetate to yield 170 mg of **7**, mp 133–140°. The sample showed one spot on tlc (iodine vapor stain) in three solvent systems: 2:1 chloroform–ethyl acetate, 1:5 ethyl acetate–chloroform, and 10:1:3 1-butanol–acetic acid–water. The infrared absorption spectrum exhibited major absorption peaks at 2.88, 3.02, 5.81, 5.92, 5.95 (sh), 6.03 (sh), 6.08, 6.11 (sh), 6.56, 8.05, 8.30, 9.90, 11.05, 13.65, and 14.70 μ. Ultraviolet absorption spectra showed major absorption peaks at λ_{max} 238 nm (ϵ 1.07×10^4) and 264 (ϵ 8.55×10^3) in ethanol containing 3.3% (v/v) dimethyl sulfoxide. Nmr spectra ($CDCl_3$) exhibited assignable peaks at τ 8.83 (d, 3, J = 6 Hz, $-CHOHCH_3$), 6.31 (s, 3, $-CO_2CH_3$), 4.95 (s, 3, 2 H's of Cbz), 2.74 (s, 5, 5 H's of Cbz), 2.66 (m, 3, 3,4,5 H's of *S*-benzoyl), and 2.07 (d, 2, 2,6 H's of *S*-benzoyl). *Anal.* Calcd for $C_{23}H_{26}N_2SO_7$: C, 58.21; H, 5.52; N, 5.90; S, 6.76. Found: C, 58.54; H, 5.91; N, 6.23; S, 6.98.

***N*-Acetyl-*S*-benzoyl-L-cysteinamide (8).**—*N*-Acetylcysteinamide (0.111 mol, 2.04 g) was dissolved in 50 ml of water. To this was added 20 ml of ethyl ether and the solution was cooled to 5°. Benzoyl chloride (0.01 mol) was added followed by 6 g of $KHCO_3$ which was added slowly with stirring over a 15-min period. After an additional 20 min of stirring at room temperature, the reaction mixture was neutralized by the addition of 15 ml of 6 *N* hydrochloric acid to precipitate 2.1 g (75%) of a white

(12) T. Tanaka and N. Sugimoto, *Yakugaku Kenkyu*, **33**, 428 (1961).

solid which upon recrystallization from tetrahydrofuran yielded a product of melting point 195–198°. The infrared absorption spectrum exhibited major absorption peaks at 2.98, 3.05, 3.15, 5.92, 5.99, 6.12 (sh), 6.18, 6.50, 7.10, 7.70, 8.28, 10.95, 12.95, and 14.60 μ. Ultraviolet absorption spectra showed two major peaks at 266 nm (ϵ 1.15 × 10^4) and 241 (1.22 × 10^4) in water containing 3.3% (v/v) acetonitrile. *Anal.* Calcd for $C_{12}H_{14}N_2O_3S$: C, 54.12; H, 5.30; N, 10.52; S, 12.04. Found: C, 54.14; H, 5.46; N, 10.51; S, 11.96. Silica gel thin layer chromatography of the purified *S*-acylcysteines **2**, **6**, and **7** gave single spots with iodine vapor stain substantiating their homogeneity.

Kinetics.—A Zeiss PMQII spectrophotometer with thermostated cell holder through which water from a constant-temperature bath was circulated was used throughout this work. Values of pH were either taken at the beginning and end of a kinetic run or the pH of the blank was taken and compared to that of the reaction mixture at infinite time. All values of pH were taken on a Radiometer PHM4c pH meter employing a glass electrode or a combination glass-KCl electrode, and were taken at the temperature at which the kinetics were run. Kinetic runs in which the pH of the buffered solution fluctuated more than 0.10 pH unit between the infinite time pH and the initial pH value were discarded.

Hydrolysis of the thiol esters described in this study was performed at 39.0 ±0.1° by incubating the buffered reaction solutions, which had been layered with argon prior to introduction of the substrate, in a constant-temperature bath; the reactions were initiated by the addition of the substrate. The rate of hydrolysis was followed by monitoring the disappearance of the thiol ester as measured by the hydroxylamine–ferric chloride method;[13] the reactions were followed through a minimum of 2 half-lives.

The kinetics of the hydrolysis of the thiol acetates **2** and **3** were followed by the neutral hydroxylamine–ferric chloride method of Jencks, *et al.*[14] The hydrolysis of dipeptide **2** was also monitored in a separate series of kinetic runs by the alkaline hydroxylamine method of these workers.[14] For those kinetic runs monitored by the neutral hydroxylamine method, 0.05 *M* borate, tricine, and triethylamine buffers were used, each containing 10^{-4} *M* EDTA and maintained at ionic strength 0.25 by the addition of lithium chloride; the concentration of the tricine buffer was varied in the case of the hydrolysis of **2**. For those kinetic runs monitored by the alkaline hydroxylamine assay, a 0.05 *M* borate buffer containing 10^{-4} *M* EDTA at ionic strength 0.25 was used exclusively. For all the kinetic runs with the acetyl thiol esters, the final concentration (v/v) of organic solvent (acetonitrile) was 3.3%.

The hydrolysis of the thiol ester **3** was also studied titrimetrically, using a Radiometer pH-Stat apparatus equipped with a G-202B glass electrode and a calomel electrode. The hydrolytic reactions were run at 39° in a chamber which was continually flushed with nitrogen and stirred with a magnetic stirrer. Into a solution which was 0.25 *M* in lithium chloride was injected the thiol ester (final concentration 10^{-3} *M*) and pH-Stat recording was initiated at the desired pH. Several values of pH were studied.

Because the aromatic thiol esters **6**, **7**, and **8** were in general less soluble in water than the corresponding acetates and because they were much less reactive, higher concentrations of organic solvent and more alkaline buffer solutions were employed in the kinetic studies. For study of the kinetics of the hydrolysis of the dipeptide **6**, 0.15 *M* triethylamine buffer containing 10^{-4} *M* EDTA and a final concentration of organic solvent (dioxane) of 40% were found to be appropriate; for the dipeptide **7**, 10% (v/v) dimethyl sulfoxide in 0.05 *M* borate buffer containing 10^{-4} *M* EDTA sufficed. *N*-Acetyl-*S*-carbobenzoxycysteinamide (**8**) was hydrolyzed in both these solvent-buffer systems. For the hydrolysis of all the aromatic thiol esters, the ionic strength of the buffer system was maintained at 0.30 with lithium chloride. As was the case with the thiol acetates, hydrolysis of the aromatic thiol esters was performed at 39 ±0.1 and all buffer solutions were layered with argon prior to addition of substrate. The rate of hydrolysis of the aromatic thiol esters **6**, **7**, and **8** was followed by monitoring the loss of ester as measured by the hydroxylamine–ferric chloride method. A reproducible neutral hydroxylamine assay was not obtained and consequently a modification of the previous[14] alkaline hydroxylamine–ferric chloride method was used. Aliquots (2.0 ml) of the buffered reaction solution were withdrawn at given time intervals and pipetted into 0.5 ml of the alkaline hydroxylamine solution prepared as described previously. The resulting mixture was incubated for 2 min at room temperature and then 1.0 ml of the ferric chloride solution was added, shaken vigorously, and read at 540 nm against a blank prepared as previously described.

Calculation of the pseudo-first-order rate constants for the hydrolysis of thiol esters as monitored by the hydroxylamine–ferric chloride and pH-Stat methods was performed by determination of the half-life for each reaction from semilog plots of the differences in the optical density (or the equivalents of base consumed as in the titrimetric assay) at time *t* and at infinite time *vs.* time. Infinite time optical density readings were taken at approximately 10 half-lives and were taken repeatedly until the readings were constant. Infinite time values of equivalents of base consumed in the titrimetric assay were those values at which base consumption had ceased and agreed well with the theoretical amount calculated for the reaction.

Solvent Deuterium Isotope Effect.—The effect of D_2O upon the hydrolytic rate of the thiol ester **2** was studied. Separate kinetic runs, one in D_2O and the other in aqueous solution, were performed simultaneously at 39° using the neutral hydroxylamine–ferric chloride assay method. For the run in D_2O, tricine buffer (0.10 *M*) was used in which the tricine had been deuterated by equilibration in D_2O followed by lyophilization to dryness. The buffer was brought to the appropriate alkaline pH with 2 *M* NaOD such that the pD of this run was equal to the pH of the first kinetic run which was performed as previously described. The relationship pD = pH (reading of the pH meter) + 0.4 was used to calculate the pD of the second run.[15,16] Ionic strength was maintained at 0.25 with the use of 4 *M* LiCl in D_2O. The data were plotted in the fashion already described for the hydrolysis of dipeptide **2**.

Product Analysis.—For the thiol acetates **2** and **3**, the alkaline reaction solutions were assayed by the method of Ellman utilizing the reagent 5,5′-dithiobis(2-nitrobenzoic acid), DTNB.[17] At times approaching and/or identical with the infinite time of the hydrolytic reaction, 2.5 ml of a freshly prepared solution of 1.0 *M* tris, pH 8.0, containing 10^{-3} *M* DTNB was added to 0.5 ml of the reaction solution and this solution was read at 412 nm against a blank prepared by adding 2.5 ml of the buffered DTNB solution to 0.5 ml of the appropriate buffer. The production of the chromophoric 4-nitro-2-carboxyphenyl thiolate, λ_{max} 412 nm (ϵ 1.36 × 10^4)[17] was usually completed within 5 min after mixing the buffered DTNB reagent with the reaction solution. In addition, for the thiol acetates, the Cary 14 ultraviolet absorption spectrum of the reaction solutions at infinite time were recorded.

For the aromatic thiol esters **6**, **7**, and **8**, only the DTNB assays were performed on the infinite time hydrolysis solutions.

Kinetics of Aminolysis.—The reaction of imidazole with the thiol acetates **2** and **3** was studied as a function of imidazole concentration and pH. Solutions of imidazole containing 0.05, 0.10, 0.15, 0.20, and 0.25 *M* total imidazole at different values of pH, ionic strength maintained at 0.25 with lithium chloride, were treated with 1.33 × 10^{-4} *M* substrate. The rate of disappearance of thiol ester was followed spectrophotometrically at 232 nm in stoppered quartz cuvettes. The temperature was maintained at 25 ±0.1° by a circulating constant-temperature bath. Pseudo-first-order rate constants were obtained as described above.

Determination of pK_a Values.—The pK_a of *N*-acetylcysteinamide, mp 147–149°, was determined titrimetrically at ionic strength 0.25 (LiCl) and 25° and was found to be 8.50. To prevent air oxidation, the titrations were either performed under nitrogen or were done rapidly to minimize oxidation. Attempts to measure the value of the pK_a for *N*-Cbz-L-cysteine methyl ester by this same method failed owing to an intermolecular reaction of the thiol anion with the oxygen ester at basic values of pH resulting in a uv spectrum with a maximum at 230 nm.

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(14) W. P. Jencks, S. Cordes, and J. Carriuolo, *ibid.*, **235**, 3608 (1960), and references cited therein.

(15) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

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(17) G. C. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

TABLE I

REACTION CONDITIONS AND THE SECOND-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF A SERIES OF THIOL ESTERS[a]

Thiol ester	Buffer	pH Range[b]	k_{OH}, M^{-1} min^{-1}	Assay
N,*S*-diacetylcysteinamide (**3**)	0.25 *M* LiCl 1.5% CH_3CN	9.5–10.0	4.6×10^2	T
	0.05 *M* Borate 3.3% CH_3CN	9.46–9.83	3.9×10^2	NN
N-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**)	0.05 *M* Borate 3.3% THF	8.21–9.07	3.2×10^3	NN
	0.05 *M* Borate 3.3% CH_3CN	8.37–9.12	2.0×10^3	NN
	0.05 *M* Et_3N 3.3% CH_3CN	9.50–10.13	1.7×10^3	NN
	0.05 *M* Tricine 3.3% CH_3CN	8.35–8.87	2.5×10^3	NN
	0.10 *M* Tricine 3.3% CH_3CN	8.32–8.82	2.3×10^3	NN
	0.05 *M* Borate 3.3% CH_3CN	8.44–9.00	2.5×10^3	AN
N-acetyl-*S*-benzoyl-L-cysteinamide (**8**)	0.05 *M* Borate 3.3% THF	9.62–10.09	1.54×10^2	AN
	0.05 *M* Borate 10% DMSO	9.64–9.98	1.22×10^2	AN
	0.15 *M* Et_2N 40% Dioxane	10.54–10.98	11.0	AN
N-Cbz-*S*-benzoyl-L-cysteinyl-L-threonine methyl ester (**7**)	0.05 *M* Borate	9.30–9.92	2.5×10^2	AN
N-Cbz-L-seryl-*S*-benzoyl-L-cysteine methyl ester (**6**)	0.15 *M* Et_3N 40% Dioxane	9.99–10.62	4.8×10^2	AN

[a] Abbreviations used: T, titrimetric assay; NN, neutral hydroxylamine–ferric chloride assay; AN, alkaline hydroxylamine–ferric chloride assay (see Experimental Section); THF, tetrahydrofuran. [b] Measured (glass electrode) pH.

Results

The hydroxylamine–ferric chloride methods described above sufficed to yield satisfactory kinetic data for the hydrolysis of the series of thiol esters studied. A typical plot of first-order rate data, that for hydrolysis of the dipeptide **2** at pH 8.37 in 0.05 *M* borate buffer, is shown in Figure 1. A titrimetric assay was also employed in one case and proved satisfactory. In contrast, efforts to follow the reactions employing ultraviolet spectrophotometry at the absorption maxima of the substrates failed, yielding first-order rate plots of continuously decreasing slope. This probably reflects the small changes in total optical density observed and, perhaps, the occurrence of side reactions.

In Table I, second-order rate constants for the alkaline hydrolysis of the series of thiol esters studied, together with the reaction conditions under which they were measured, are collected. In each case, the second-order rate constants were evaluated from slopes of plots of first-order rate constants against the activity of hydroxide ion; satisfactory plots were obtained in all cases. A typical example for the hydrolysis of **2** at six values of pH at 39° is provided in Figure 2. In the cases of all substrates studied, the intercept at zero hydroxide ion concentration was not detectably different from zero, indicating the unimportance of a neutral (water-catalyzed) reaction under the conditions employed.

A number of minor complications were observed in the study of the hydrolysis of these thiol esters. Values of pH above 10 could not be employed in studies of the hydrolysis of **3** and several of the other substrates, owing to the occurrence of β-elimination reactions under more alkaline conditions. Moreover, **6** was insufficiently soluble in water, necessitating the use of appreciable quantities of dioxane as solvent in this case. For comparative purposes, this solvent was also employed for the kinetics of hydrolysis of **8** (Table I). The rate of hydrolysis of **2** exhibited small variation as a function of the nature of the buffer or organic component of the solvent; buffer catalysis by tricine was not observed, however. Note that the measured second-order rate constant employing the neutral and alkaline hydroxylamine–ferric chloride assays yielded identical results, a matter to which we return later. The lower reactivity of the *S*-benzoyl substrates, **6**, **7**, and **8**, compared to the *S*-acetyl substrates necessitated the use of the alkaline

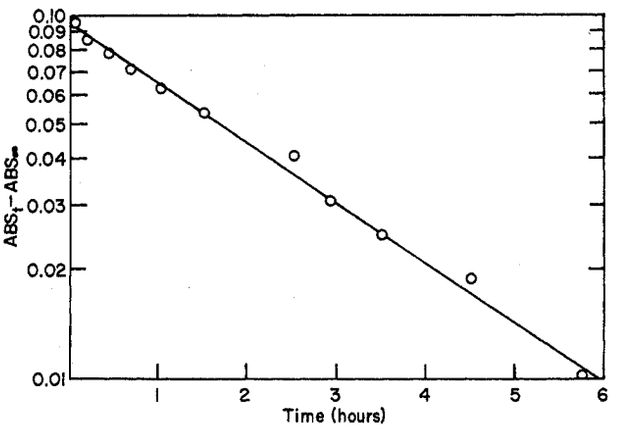


Figure 1.—Semilogarithmic plot of the difference of the optical density at time *t* and the optical density at infinite time as a function of time for the hydrolysis of *N*-Cbz-S-acetyl-L-cysteinyl-L-threonine ethyl ester at 39° (**2**) as followed by the neutral hydroxylamine–ferric chloride method. Initial substrate concentration was 2×10^{-4} *M* in 0.05 *M* borate buffer containing 10^{-4} *M* EDTA at pH 8.37 and maintained at ionic strength 0.25 with lithium chloride. Final concentration of organic solvent (acetonitrile) was 3.3%.

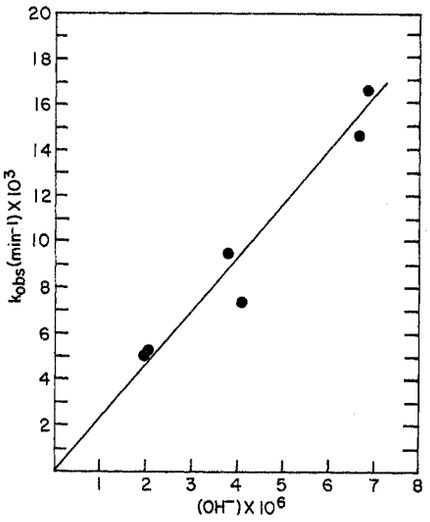


Figure 2.—Second-order rate constants for the alkaline hydrolysis of *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**) at 39° plotted against the activity of hydroxide ion. Initial concentration of substrate was 2×10^{-4} *M* in 0.10 *M* tricine buffers at ionic strength 0.25. Final concentration of organic solvent (acetonitrile) was 3.3%.

hydroxylamine–ferric chloride assay for the former group of compounds.

The alkaline hydrolysis of *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**) was found to be subject to a solvent deuterium effect, the value of k_{H_2O}/k_{D_2O} being 2.68 ± 0.40 under conditions in which pH equaled pD at 39°.

Analysis of the Products of Thiol Ester Hydrolysis.—Two methods were used to analyze the products of the hydrolysis of the thiol esters: assay for free thiol using the Ellman reagent DTNB,[17] and analysis of the uv spectrum with emphasis on the absorbance value at 241 nm. The last assay is a sensitive one for dehydroalanine type compounds which result upon β elimination of an amino acid. Consequently, if β elimination were to occur, either prior to or following thiol ester hydrolysis, it could be easily detected; Riley, *et al.*, have reported a λ_{max} of 241 nm (ε 5300) for α-*N*-Cbz-aminoacrylic acid.[18]

Upon hydrolysis of the thiol ester *N*,*S*-diacetylcysteinamide (**3**), at the conclusion of reactions monitored titrimetrically or by neutral hydroxylamine, the absorbance at 241 nm using the molar extinction coefficient for α-*N*-Cbz-aminoacrylic acid indicated that less than 3% β elimination had occurred, while analysis of free thiol by the DTNB method accounted for 93% of that expected theoretically for complete hydrolysis of the thiol ester. It would appear, therefore, that for this thiol ester, conditions of pH 10 and below resulted only in hydrolysis of the thiol ester.

On the other hand, analysis of the products of hydrolysis of the dipeptide *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**) by the DTNB method accounted at most for only 44% of the theoretical amount of thiol (0.10 *M* tricine buffer) and the average value was 20–30% of the theoretical quantity in the pH range 8.2–9.1 (0.05 *M* borate buffer). However, uv analysis at times considerably greater than 10 half-lives of the hydrolysis reaction revealed that less than 9% dehydroalanine had been formed. The possibility that thiol had been oxidized to sulfenic acid, or higher oxidation states, was explored by attempting to reduce with arsenate any oxidation product which may have been formed; several reports in the literature[19,20] indicate that large molar excesses of arsenate are necessary. Consequently, aliquots of the hydrolysis reaction mixture at infinite time were analyzed for thiol content by the DTNB method; one sample was treated, prior to reaction with DTNB, with a 2000:1 molar excess of sodium arsenate at room temperature for 30 min while a second sample was analyzed directly with DTNB. The amount of thiol accounted for in the arsenate-treated sample was 24% of the theoretical, while that for the untreated sample was 20%, ruling out oxidation of the thiol to the sulfenic acid as responsible for the loss of thiol at infinite time of the hydrolysis of **2**.

Analysis of the hydrolysis products of the aromatic thiol esters **6**, **7**, and **8** could only be performed for free thiol, since benzoic acid, which is liberated in the hydrolysis reaction, absorbs in the region of the uv at which α-*N*-Cbz-aminoacrylic acid absorbs.

DTNB assay for free thiol after hydrolysis of *N*-acetyl-*S*-benzoylcysteinamide (**8**) had been completed accounted for 75% of the theoretical amount of thiol released upon hydrolysis under conditions of hydrolysis identical with those used for the dipeptides **6** and **7**. However, analysis of the amount of thiol present at the conclusion of the hydrolysis of **6** and **7** accounted for only 17 and 27%, respectively, of the theoretical amounts. A study of the production of thiol with respect to time for the dipeptide **6** revealed that at a calculated 5 half-lives of the hydrolysis reaction, 75% of the theoretical thiol released at that point in the reaction was present; this value slowly decreased to that found (17%) for the hydrolysis solution at infinite time (10 half-lives).

Kinetics of Aminolysis.—The susceptibility to attack by imidazole was examined for the thiol acetates **2** and **3**, these thiol esters being more soluble in aqueous solutions than any of the others studied. In Figure 3 is illustrated the second-order rate plots for the attack of imidazole (free base) upon the thiol ester **3** at three different values of pH; Figure 4 shows similar data for the dipeptide **2**. As can be seen from these figures, the slopes of second-order rate plots are identical within the error of the experiments, indicating that imidazole free base is the reactive species. In Table II are collected the individual second-order rate constants for the attack of imidazole on the substrates **2** and **3** at various values of pH.

TABLE II

SECOND-ORDER RATE CONSTANTS FOR IMIDAZOLE-CATALYZED DISAPPEARANCE OF THIOL ESTERS **2** AND **3** AT 25°

Thiol ester	pH	k_{Im}, M^{-1} min^{-1} × 10^2
N-Cbz-S-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**)	8.23	6.20
	7.70	5.10
	7.53	5.10
N,*S*-diacetylcysteinamide (**3**)	8.02	4.56
	7.77	4.31
	7.50	3.95

(18) G. Riley, J. H. Turnbull, and W. Wilson, *J. Chem. Soc.*, 1373 (1957).

(19) J. Parker and W. S. Allison, *J. Biol. Chem.*, **244**, 180 (1967).

(20) A. Gutmann, *Ber.*, **41**, 1650 (1908).

TABLE III

VALUES OF pK_a OF THE CONJUGATE ACID OF THE LEAVING GROUP AND THE SECOND-ORDER RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS AND THE ATTACK OF IMIDAZOLE UPON A SERIES OF THIOL ESTERS

Registry no.	R	pK_a[a]	Temp, °C	k_{OH}, M^{-1} min^{-1}	k_{Im}, M^{-1} min^{-1}	Ref
		CH_3COSR				
928-47-2	*n*-Bu	11.05	20	0.22	0.04	*b*
926-73-8	*i*-Pr	10.86	20	0.82		*b*
625-60-5	C_2H_5	10.50	20	1.54	0.996	*c*
32362-99-5	$CH_2C_6H_5$	9.43	0	3.80		*d*
36914-44-0	$CH_2CH(CONH_2)$-N-$COCH_3$	8.50	39	390–460	0.046	
14897-48-4	CH_2CF_3	7.30	30	64.5	6.85	*e*
		C_6H_5COSR				
7269-35-4	*n*-Bu	11.05	0	0.07		*d*
13402-51-2	$CH_2C_6H_5$	9.43	0	0.22		*d*
36914-48-4	$CH_2CH(CONH_2)$-N-$COCH_3$	8.50	39	154		

[a] pK_a data for the conjugate acids was taken either from the indicated reference or from W. P. Jencks, in H. A. Sober, Ed., "Handbook of Biochemistry," Chemical Rubber Publishing Co., Cleveland, Ohio, 1968, p J-186. [b] T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, New York, N. Y., 1966, pp 259–298. [c] M. L. Bender and B. W. Turnquist, *J. Amer. Chem. Soc.*, 79, 1656 (1957). [d] G. Losse, R. Mayer, and K. Kuntze, *Z. Chem.*, 7, 104 (1967). [e] M. L. Gregory and T. C. Bruice, *J. Amer. Chem. Soc.*, 89, 2121 (1967).

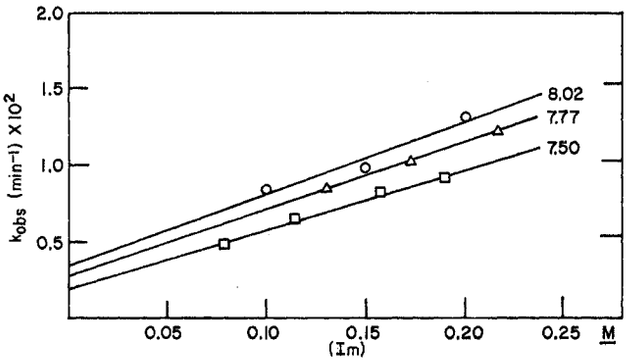


Figure 3.—Second-order rate constants for the attack of imidazole on *N*,*S*-diacetylcysteinamide (**3**) plotted against the concentration of imidazole over the pH range 7.50–8.02 at 25°. Initial concentration of substrate was 1.3×10^{-4} *M*. Ionic strength was maintained at 0.25 with LiCl and the final concentration of organic solvent (acetonitrile) was 3.3%.

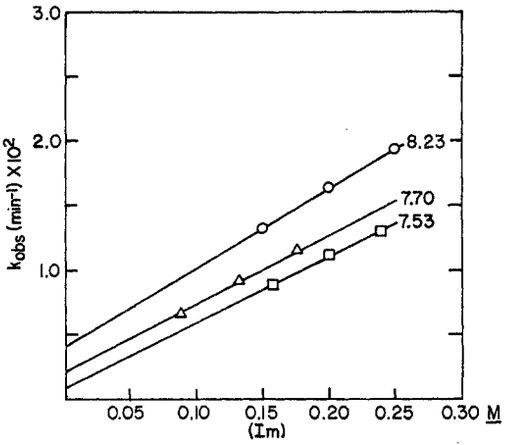


Figure 4.—Second-order rate constants for the attack of imidazole on the dipeptide *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**) plotted against the concentration of imidazole over the pH range 7.53–8.23 at 25°. Initial concentration of substrate was 1.3×10^{-4} *M*. Ionic strength was maintained at 0.25 with LiCl and the final concentration of organic solvent (acetonitrile) was 3.3%.

Discussion

The main thrust of the experimental work described above deals with reactivity and catalysis for the hydrolysis of thiol esters, a topic which has been extensively studied and thoroughly reviewed.[21–23] Special emphasis has been placed on possible roles of the nucleophilic groups which flank the crucial cysteine in the active site of G3PD in the process of thiol ester cleavage. Synthetic difficulties which precluded the synthesis of several desirable substrates (S → N acyl transfer reactions, the existence of which is most interesting for understanding the chemistry at the enzyme active site, were a particularly annoying plague in this respect) have left us with a less complete picture than might have been desired. Nevertheless, a number of new findings have come out of these studies. To begin with, let us consider the reactivity of the simplest substrates studied, the blocked *S*-acetyl- and *S*-benzoylcysteinamides, **3** and **8**, in comparison to that of ordinary thiol esters. In Table III, second-order rate constants for alkaline hydrolysis and for reaction with imidazole of several thiol esters are collected as a function of the pK_a of the conjugate acid of the leaving thiolate anion. Qualitatively, the expected trend of greater reactivity toward hydroxide ion with increasing acidity of the conjugate acid of the leaving group is observed for both *S*-acetyl and *S*-benzoyl thiol esters. Evident from this table is the abnormal reactivity of the *S*-acylcysteines. Thus, making reasonable estimates of rate differences reflecting differences in the temperatures at which the reactions were run, it is found that the acylcysteines are about 20-fold more reactive than expected on the basis of the pK_a of the leaving group and the reactivity of simple thiol esters. This finding contrasts with that for hydrolysis of acylserines, which, though more reactive than simple oxygen esters, are not particularly more reactive than expected on the basis of the relatively high

(21) L. Zervas, I. Photaki, A. Cosmatos, and D. Borovas, *J. Amer. Chem. Soc.*, **87**, 4922 (1965).

(22) T. C. Bruice and S. Benkovic, "Bioorganic Mechanisms," W. A. Benjamin, New York, N. Y., 1966.

(23) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969.

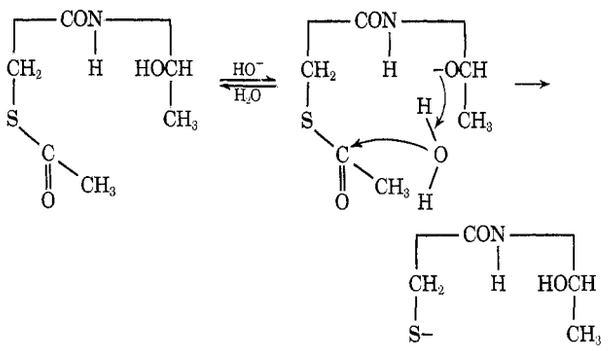
acidity of the leaving group.[24,25] Clearly, this is not the case for hydrolysis of the corresponding thiol esters. One may consider the possibility that the electron-withdrawing inductive effects of the substituted cysteine moiety are more important for the hydrolysis of derived thiol esters, perhaps reflecting the degree of polarization of the thiol ester bond, than for the ionization of the thiol itself. It is also possible that the amide substituents may facilitate the reaction through intramolecular general acid–base catalysis.

The observed greater reactivity of acetyl compared to benzoyl esters, Tables I and III, is expected; similar results have been previously obtained with oxygen esters as well.[26]

In contrast to the results obtained with hydroxide ion, acylcysteines are not abnormally reactive toward imidazole (Table III). If any trend can be discerned, just the opposite is true. This distinct behavior probably reflects the fact that imidazole facilitates the hydrolysis of thiol esters by general base catalysis of the attack of a water molecule rather than by direct nucleophilic attack.[22]

The reactivity of *S*-acylcysteines is appreciably enhanced by the presence of neighboring hydroxylic amino acids. For example, *N*-Cbz-*S*-acetyl-L-cysteinyl-L-threonine ethyl ester (**2**) is about five times as reactive toward hydroxide ion as *N*,*S*-diacetylcysteinamide (**3**), a suitable model compound (Table I). The presence of the neighboring threonine residue suggests the possibility of an S → O acyl transfer reaction, leading to an increased rate of loss of thiol ester. Assuming that the oxygen ester formed from such an acyl transfer reaction would be more stable than the initial thiol ester,[14,24] it is possible to examine this possibility by comparing the kinetic behavior using the neutral hydroxylamine test, which detects only the thiol ester, and the alkaline hydroxylamine test, which detects both thiol and oxygen esters. If the acyl transfer reaction was one of importance, one would expect to observe a lag phase in the alkaline hydroxylamine assay; moreover, the rate constant measured by this assay should be smaller than that measured using the neutral hydroxylamine assay. In fact, the two assay methods yield identical rate constants (Table I), ruling out an appreciable contribution to the rate of thiol ester disappearance from an S → O acyl transfer reaction. This is not surprising. Such reactions have been observed for those cases involving formation of tetrahedral intermediates having not more than six atoms.[27,28] In the case of the dipeptide, **2**, formation of a nine-membered-ring intermediate would be required.

A possible mode of facilitation of thiol ester hydrolysis by the neighboring threonine residue is suggested by a study of the kinetics of hydrolysis of a series of diol monoacetates.[29] It was observed that a vicinal hydroxyl function has a small catalytic effect on the alkaline hydrolysis of the neighboring acetate moiety. In part because of a solvent deuterium isotope effect, k_{H_2O}/k_{D_2O} near 0.5, it was suggested that the rate increase reflected "internal solvation of the transition state for the attack of hydroxide at the ester carbonyl."[29] However, the solvent deuterium isotope effect for hydrolysis of the dipeptide **2** is 2.7, suggesting that this explanation will not suffice for the present case. On the whole, it seems most reasonable to assign the facilitation of the cleavage of the thiol ester bond by the neighboring threonine hydroxyl group to one of several possible mechanisms of the general acid–base type, such as the one shown below.



It is not clear why an adjacent threonine does not enhance the reactivity of an *S*-benzoylcysteine as it does for the corresponding *S*-acetyl compound (Table I).

Returning to the data in Table I, it is clear that introduction of a serine residue adjacent to the *S*-acylcysteine moiety also increases the reactivity of the thiol ester toward hydroxide ion. In fact, the rate increase elicited by serine is substantially greater than that observed for threonine. By analogy with the above discussion, one may tentatively conclude that a similar catalytic mechanism is involved in the two cases. The hydroxyl group of serine is, of course, less sterically hindered than that of threonine, which may increase its effectiveness as a general base catalyst.

An alternate mechanism for the acceleration of thiol ester hydrolysis by neighboring alcoholic functions is that proposed by Bernhard and coworkers[30] for the hydrolysis of some aspartyl serine derivatives. This mechanism postulates that the amide nitrogen of the peptide bond is ionized by abstraction of the amino proton by the anion of the alcoholic amino acid. Subsequent to this, the anion of the amide nitrogen attacks an adjacent ester carbonyl carbon, forming an imide. Several observations argue against such catalysis in the present case. First, increasing reactivity for the hydrolysis of neighboring thiol esters by serine and threonine derivatives exhibited small rate increases over that for the *N*-acetyl-*S*-acylcysteinamides, while hydrolysis of the β-benzylaspartylserines of Bernhard exhibited rate increases of 10^7. Secondly, the relatively small rate increases exhibited by the threonine and serine thiol esters are inconsistent with participation of the amide nitrogen in view of the well-known high susceptibility of thiol esters to attack by nitrogen nucleophiles.[22,23]

Isolation of the products of the reaction of imidazole or of hydroxide ion with the dipeptides **2**, **6**, or **7** was not attempted owing to the small concentrations of sub-

(24) B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.*, **236**, 455 (1961).
(25) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. G. Brandon, *Biochemistry*, **1**, 7 (1962).
(26) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill, New York, N. Y., 1940, p 212.
(27) J. S. Harding and L. N. Owen, *J. Chem. Soc.*, 1528 (1954).
(28) J. S. Harding and L. N. Owen, *ibid.*, 1536 (1954).
(29) T. C. Bruice and T. H. Fife, *J. Amer. Chem. Soc.*, **84**, 1973 (1962).
(30) S. A. Bernhard, A. Berger, J. C. Carter, E. Katchalski, M. Sela, and Y. Shalitin, *J. Amer. Chem. Soc.*, **84**, 2421 (1962).

strate used. However, analyses for the products of alkaline hydrolysis were performed and several conclusions were drawn. First, it appears that little or no β elimination occurs in alkaline solution up to approximately pH 10.0. Secondly, the release of thiol as the ester hydrolyzes can, in the case of simple *N*-acetyl-*S*-acylcysteinamides, be quantitatively accounted for by the DTNB method. The picture which emerges for the dipeptides, however, is different; only modest fractions of total thiol released at infinite time can be accounted for by this method. However, the time course for production of thiol, as measured during the hydrolysis of dipeptide **6**, reveals that nearly quantitative amounts of thiol are released during the first 5 half-lives of the reaction, following which the amount of thiol diminishes. It would appear that thiol liberated is converted to some secondary product the nature of which, with the exception that oxidation to sulfenic acid is ruled out (see Experimental Section), is not known.

What are the implications of the modest rate facilitations observed in this study for the understanding of the reactivity of S-acyl-G3PD? First, the reactivity of simple *S*-acylcysteines toward hydroxide ion is about 20-fold greater than would have been expected on the basis of the valus of pK_a for the conjugate acid of the leaving group (Table III). Second, both the flanking serine and threonine residues do lead to appreciable increases in the reactivity of the adjacent thiol ester. Having both serine and threonine present in the same molecule might lead to either addition or multiplication of the catalytic effects, depending on whether the catalytic mechanisms are different or the same. At best, one might anticipate a total increase in reactivity toward hydroxide ion of some 200-fold in the presence of both serine and threonine functions. Combining this rate increase with that observed for the simple *S*-acylcysteines leads to a maximal total factor of perhaps 4000. While such a reactivity increase certainly partially bridges the gap between the reactivity of the acyl enzyme and that of simple thiol esters, it would still fall at least three orders of magnitude short of accounting for all of the difference. Hence the reactivity of the acyl enzyme must depend to a major extent on catalytic mechanisms involving residues not in the primary sequence of amino acids at the active site.

Registry No.—**2**, 36914-96-2; **3**, 16820-83-0; **6**, 36912-46-6; **7**, 36914-97-3; **8**, 36912-47-7.

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Studies on 3,3-Diaryltricyclo[3.2.1.0^{2,4}]octanes. I. Synthesis and Reactions of *exo*-3,3-Diphenyltricyclo[3.2.1.0^{2,4}]oct-6-ene and Its Derivatives[1]

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The thermal reaction of diphenyldiazomethane and norbornadiene affords mono and bis adducts. These pyrazolines can be thermally transformed to polycyclic hydrocarbons with stereospecific loss of nitrogen. Comparison is made to similar reactions reported in the literature. The title hydrocarbon so obtained has been characterized by its nmr spectrum, most notably by the singlet resonance of its endo H-2,4 protons. Reactions in this system that have been studied include the reaction of the hydrocarbon with bromine and the solvolysis of the exo- and endo-6 tosylates. Both processes proceed *via* the same rearrangement solely to nortricyclyl derivatives, presumably because the phenyl groups present stabilize overwhelmingly the cation precursor to these derivatives. An exo/endo rate difference of over 4000 in aqueous dioxane implies that anchimeric assistance is well developed in the exo isomer. Possible mechanistic pathways are discussed. Other transformations of the title hydrocarbon to unrearranged dibromides and to its exo epoxide are also mentioned.

Although the unsubstituted tricyclo[3.2.1.0^{2,4}]-ocyl system has had its chemistry explored in a variety of processes,[3] much less is known about the 3,3-disubstituted cases. Two of the interesting results from our early studies on the exo-3,3-diphenyl-substituted system were the solvolytic rearrangements undergone by the *syn*- and *anti*-8-tosylates.[4] To understand more of the chemistry associated with the tricycle, the investigation of the title compound itself, as well as its 6 derivatives, was undertaken.

Discussion

Synthesis and Characterization of *exo*-3,3-Diphenyltricyclo[3.2.1.0^{2,4}]oct-6-ene.—Addition of diphenyldiazomethane to norbornadiene afforded pyrazoline **1** which in turn was converted thermally into the title

(1) Taken from portions of the dissertation of T. P. M., Loyola University of Chicago, 1970; Abstracts, 5th Great Lakes Regional Meeting of the American Chemical Society, Bradley University, Peoria, Ill., June 1971, Paper 55.

(2) National Science Foundation Trainee, 1969–1970.

(3) Of the many studies extant, the following are of special interest to the present article. (a) Synthesis of exo tricyclic 6-ene: H. E. Simmons, E. P. Blanchard, and R. D. Smith, *J. Amer. Chem. Soc.*, **86**, 1347 (1964). This preparation leads to 18% bicyclo[3.2.1]octadiene as well: T. J. Katz and S. A. Cerefice, *ibid.*, **93**, 1049 (1971). This fact must be considered when one peruses earlier studies in this area. (b) Synthesis of endo tricyclic 6-ene: K. B. Wiberg and W. Bartley, *ibid.*, **82**, 6375 (1960). (c) Solvolysis of arenesulfonates of exo and endo tricyclic 8-alcohols: H. Tanida, T. Tsuji, and T. Irie, *ibid.*, **89**, 1953 (1967); M. A. Battiste, C. L. Deyrup, R. E. Pincock, and J. Haywood-Farmer, *ibid.*, **89**, 1954 (1967); J. S. Haywood-Farmer and R. E. Pincock, *ibid.*, **91**, 3020 (1969). (d) Solvolysis of arenesulfonates of endo and exo tricyclic 6-alcohols: K. B. Wiberg and G. R. Wenzinger, *J. Org. Chem.*, **30**, 2278 (1965). (e) Hydrogenation of endo and exo tricyclic 6-enes: P. K. Freeman and K. B. Desai, *ibid.*, **36**, 1554 (1971). (f) Photochemical isomerization of endo and exo tricyclic 6-enes: H. Prinzbach and W. Eberbach, *Chem. Ber.*, **101**, 4083 (1968). (g) Reduction of endo and exo 6-oxides: B. C. Henshaw, D. W. Rome, and B. L. Johnson, *Tetrahedron*, **27**, 2255 (1971); D. W. Rome and B. L. Johnson, *ibid.*, **27**, 2271 (1971).

(4) J. W. Wilt and T. P. Malloy, *J. Amer. Chem. Soc.*, **92**, 4747 (1970).