glycolic acid in a Klett–Summerson photoelectric colorimeter equipped with a No. 45 filter. It was found that 2.15 μmoles (43%) of glycolic acid had been released.

Similarly, 0.4 μmole of XI yielded glycine in a yield of 56% as determined by ninhydrin.

(b) **With *o*-Phenylenediamine.**—A solution of 0.4 μmole of XI was treated with bromine, as described above, the pH of the reaction mixture was adjusted to 7.0 with dilute acetic acid, and 2.5 mg. of recrystallized *o*-phenylenediamine was added. The final volume was 0.5 ml. and the reaction mixture was heated in a sealed tube to 100° for 1 hr. The solution was then concentrated to 0.1 ml. and glycine in a yield of 25% was determined on paper electrophoresis for 2 hr. In a control experiment, when *o*-phenylenediamine was omitted from the reaction mixture, no glycine was released. An authentic sample of pyruvoylglycine benzyl ester (11.8 mg., 0.05 mmole) was heated in a sealed tube to 100° for 1 hr. with *o*-phenylenediamine (16.2 mg., 0.15 mmole, twice recrystallized) in 3 ml. of 0.1 *M* phosphate buffer, pH 7.0. On paper electrophoresis of an aliquot for 2 hr., glycine (and small amounts of glycine benzyl ester) in a yield of 30% was determined. A control experiment without *o*-phenylenediamine was free of a detectable amount of glycine.

(c) **With Potassium Cyanide.**—Pyruvoylglycine benzyl ester (11.8 mg., 0.05 mmole) was heated in a sealed tube to 100° for 1 hr. with 2 ml. of a 2% KCN solution in 0.2 *M* citrate buffer, pH 5.0. On paper electrophoresis (2 hr.) of an aliquot, glycine in a yield of 15% was detected by spraying with ninhydrin containing 0.2% sodium hydroxide. A blank experiment without cyanide was free of a detectable amount of glycine.

Acknowledgments.—Many thanks are due to Professor E. Katchalski and Professor A. Berger for helpful discussions and to Mrs. H. Later for competent technical assistance.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL]

Nonenzymatic Cleavages of Peptide Chains at the Cysteine and Serine Residues through Their Conversion to Dehydroalanine (DHAL). II. The Specific Chemical Cleavage of Cysteinyl Peptides[1,2]

BY MORDECHAI SOKOLOVSKY, TSVI SADEH, AND ABRAHAM PATCHORNIK

RECEIVED AUGUST 14, 1963

Cleavage at the cysteine residue is effected by conversion of the cysteine residue to a dehydroalanine residue and subsequent scission of the dehydroalanine residue. The cleavage reaction proceeds in three stages: (a) conversion of the cysteine residue to its S-dinitrophenyl derivative or to a tertiary sulfonium salt; (b) formation of a dehydroalanine peptide by a β-elimination reaction under basic conditions; (c) hydrolytic or oxidative scission of the dehydroalanine derivative followed by oxidation with alkaline hydrogen peroxide. The cleavage reaction was carried out on dipeptides and tripeptides and also on oxytocin. The new NH_2 terminal amino acids released were identified and quantitatively estimated. Yields of 50–60% were obtained.

Introduction

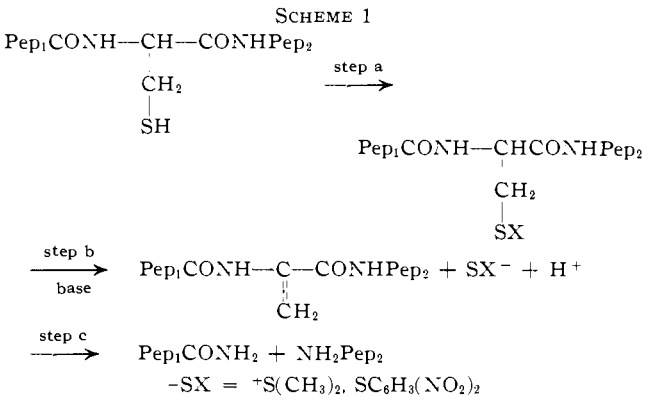
Cysteine residues play an important role in the biologically active site of a number of enzymes.[3] Moreover, disulfide bridges between two half-cysteines are often of crucial importance in keeping the protein molecule in its biologically active conformation. No direct enzymatic methods are as yet available for specific cleavage of peptide bonds adjacent to this residue. The present study was directed toward developing chemical nonenzymatic methods.

It is well known that cystine and cysteine peptides are labile at alkaline pH values, and it was assumed that their degradation proceeds through dehydroalanine,[4] β-elimination reaction being involved. The formation of lanthionine[5] in wool and the conversion of S-cyanocysteine derivatives to dehydroalanine derivatives[6] by the action of alkali are also believed to proceed through a β-elimination reaction.

One of the groups found to cause β-elimination reaction in sulfur compounds is the sulfonium group.[7,8] In the present work this group as well as the S-dinitrophenyl group were used to convert cysteine residues into dehydroalanine residues by β-elimination.

The chemical cleavage of cysteine residues proceeds thus through three steps: (a) conversion of the cysteine residue into its S-dinitrophenyl or dimethylsulfonium derivative; (b) formation of a dehydroalanine peptide by an elimination reaction; (c) hydrolytic or oxidative cleavage of the dehydroalanine residue[9] followed by treatment with alkaline hydrogen peroxide.

SCHEME 1



Results

Selective Conversion of Cysteine Residues into Dehydroalanine Residues.—The thiol groups of cysteine derivatives were dinitrophenylated by means of dinitrofluorobenzene (FDNB) at pH 5–6.[10] The reaction rate of thiols was compared with that of poly-L-lysine[11] by measuring the alkali uptake during the reaction in a pH-Stat. It was found that the thiol compounds reacted 30 times faster than poly-L-lysine and

(1) Preliminary reports: (a) A. Patchornik, M. Sokolovsky, and T. Sadeh, Vth International Congress of Biochemistry, Moscow, 1960, p. 11; (b) A. Patchornik and M. Sokolovsky, XXXth Meeting of the Israel Chemical Society, 1962, Vol. 11A, p. 80; and (c) A. Patchornik and M. Sokolovsky, "Vth European Peptide Symposium, Oxford, 1962," Pergamon Press, New York, N. Y., 1963, p. 253.

(2) This investigation was supported by Grant AM-5098 of National Institutes of Health, United States Public Health Service.

(3) P. D. Boyer, H. Lardy, and K. Myrback in "The Enzymes," Vol. 1, part II, Academic Press, Inc., New York, N. Y., 1959, p. 511; R. Cecil and J. R. McPhee, "Advances in Protein Chemistry," Vol. XIV, Academic Press, Inc., New York, N. Y., 1959, p. 255; P. D. Boyer, *Brookaven Symp. Biol.*, **13**, 1 (1960).

(4) H. T. Clarke and J. M. Inouye, *J. Biol. Chem.*, **94**, 541 (1931); B. H. Nicolet, *J. Am. Chem. Soc.*, **53**, 3066 (1931).

(5) H. Zahn, *Chimia*, **15**, 378 (1961).

(6) J. M. Swan, *Nature*, **180**, 643 (1957).

(7) C. K. Ingold in "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 420.

(8) R. P. Linstead, L. N. Owen, and R. F. Webb, *J. Chem. Soc.*, 1211 (1953); W. E. Parham and J. W. Wilbur, Jr., *J. Am. Chem. Soc.*, **81**, 6071 (1959); J. J. Roberts and G. P. Warwick, *Biochem. Pharm.*, **6**, 205 (1960); P. Mamalis and H. N. Rydon, *J. Chem. Soc.*, 1049 (1955).

(9) A. Patchornik and M. Sokolovsky, *J. Am. Chem. Soc.*, **86**, 1206 (1964).

(10) H. Zahn and K. Traumann, *Z. Naturforsch.*, **9b**, 578 (1954).

(11) Obtained from the departmental collection.

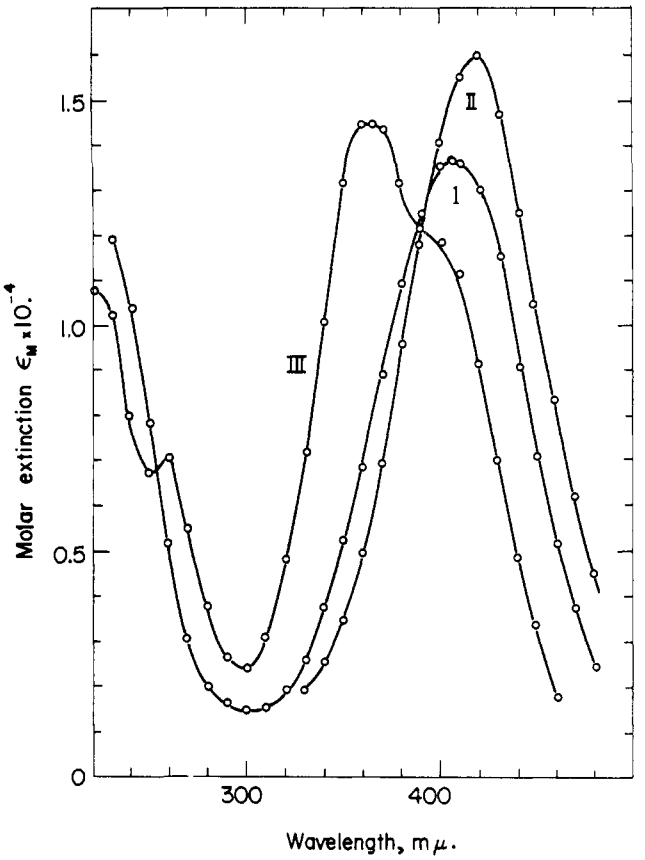


Fig. 1.—Absorption spectra of dinitrothiophenolate ion in water (I), absolute methanol (II), and dinitrophenolate ion in water (III).

carbobenzoxy-L-histidylglycine.[12] No reaction with the phenol function of tyrosine or hydroxyl group of serine was observed under these conditions.

Upon treating the S-dinitrophenylcysteinyl derivatives with base, either in aqueous or nonaqueous solutions, β-elimination of the dinitrothiophenolate ion occurs according to the above scheme (step b).

Since the dinitrothiophenolate ion has a characteristic absorption spectrum in the visible region, λ_{max} 408 mμ, ε 13,800 in aqueous media and λ_{max} 420 mμ, ε 16,100 in anhydrous methanol (see Fig. 1), the kinetics of elimination in alkaline solution could be measured spectrophotometrically. With all the S-dinitrophenylcysteine derivatives studied, this reaction proceeds almost quantitatively as shown in Table I.

TABLE I

EXTENT OF ELIMINATION REACTION IN S-DNP-CYSTEINYL DERIVATIVES

No.	S-DNP-cysteinyl derivative	Dinitrothiophenolate ion, %
I	N,S-Di-DNP-cysteine	95[a]
II	*p*-NO_2-Cbz-Gly-Cys(-S-DNP) methyl ester	98[b]
III	S-DNP-glutathione	97[b]
IV	N,S-Di-DNP-glutathione	96[a]
V	N,S-Di-DNP-glutathione dimethyl ester	97[a]
VI	*p*-NO_2-Cbz-Gly-Cys(-S-DNP)-Gly	98[a]

Abbreviations: *p*-NO_2-Cbz, *p*-nitrocarbobenzoxy; DNP, 2,4-dinitrophenyl. [a] The amount of dinitrothiophenolate ion was determined by means of the difference spectrum in methanol against a sample oxidized with iodine, assuming ϵ_{diff} 15,700 ± 300 at 420 mμ.[13] [b] The amount of dinitrothiophenolate ion was calculated from the optical density of the reaction mixture.

(12) Mr. Sh. Shaltiel kindly supplied us with a sample of this compound.

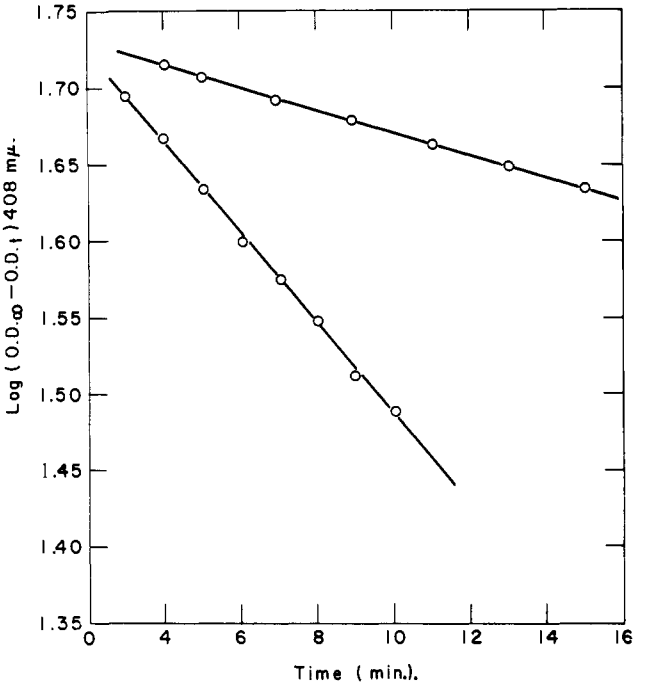
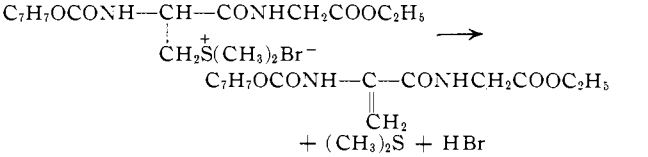


Fig. 2.—Rate of elimination of dinitrothiophenolate ion from S-DNP-glutathione as determined by measuring the increase of absorbancy at 408 mμ: O——O, in 0.1 *N* sodium hydroxide; ●——●, in 0.5 *N* sodium hydroxide.

The elimination reactions were performed in the presence of excess base at different concentrations. First-order kinetics were observed in all cases (Fig. 2). The fact that the β-elimination reaction of S-DNP-glutathione was five times more rapid in 0.5 *N* sodium hydroxide than in 0.1 *N* sodium hydroxide (Fig. 2), suggests a bimolecular mechanism for the elimination reaction.[7]

A comparison of elimination reactions of a number of compounds in 0.2 *N* sodium methoxide in methanol showed that the structure of the reacting molecule has a marked influence on the reaction rate. The reaction is fastest with compound II and the rate decreases in the order II > V > III > I (see Fig. 3). This finding can be explained by assuming that the elimination reaction involves ionization of the C_α–H bond. The strongest electron-withdrawing group in the above series is the ester carbonyl of compound II. The amide group of compound V is less effective in this respect, while the presence of a negatively charged carboxyl in compound I and that of the ionized carboxyls in compound III still further diminish the reaction rate.

Elimination Reactions of Sulfonium Derivatives.—The positively charged sulfonium group causes a β-elimination reaction at pH values higher than 8.5. The products of this reaction are a dehydroalanine peptide and a dialkyl sulfide. N-Carbobenzoxy-S-methyl-L-cysteinylglycine ethyl ester (IX) yielded, after methylation[14] and subsequent treatment with bicarbonate, carbobenzoxydehydroalanylglycine ethyl ester[9] (80%) and dimethyl sulfide.



Analogously, carbobenzoxydehydroalanine[9] (95%) and carbobenzoxyglycyldehydroalanine[9] (65%) were ob-

(13) A. Patchornik and M. Sokolovsky, *Bull. Res. Council Israel*, **11A**, 226 (1962).

(14) G. Toennies and J. J. Kolb, *J. Am. Chem. Soc.*, **67**, 849 (1945).

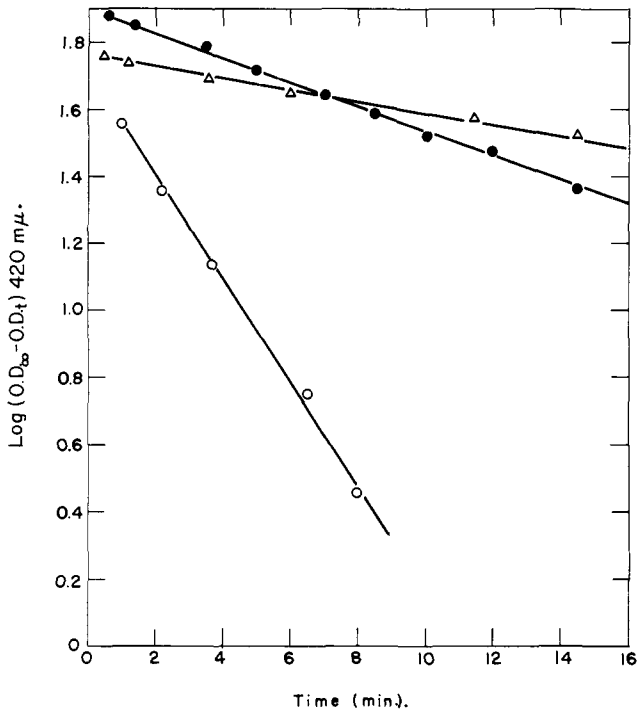
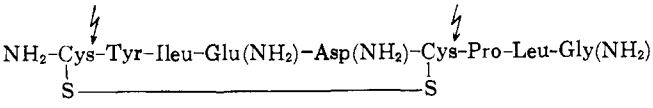


Fig. 3.—The rate of elimination of dinitrothiophenolate ion from S-DNP-cysteinyl derivatives in methanolic 0.2 *N* sodium methoxide: △——△, β-elimination reaction in N,S-Di-DNP-cysteine; ●——●, β-elimination reaction in S-DNP-glutathione; O——O, β-elimination reaction in N,S-Di-DNP-glutathione dimethyl ester.

tained from N-carbobenzoxy-S-methyl-L-cysteine (VIII) and N-carbobenzoxyglycyl-S-methyl-L-cysteine methyl ester (VII), respectively. Elimination reactions were followed by direct titration of the acid released.

Cleavage of Cysteinyl Peptides.—In order to determine the amino acid residue linked to the carboxyl of a cysteine residue in a peptide chain, the method of formation of dehydroalanine peptides described here is combined with the cleavage of dehydroalanine peptides described previously.[9] It is usually unnecessary to isolate the intermediate dehydroalanine derivative, but its existence can be verified by either bromine titration or by estimation of pyruvic acid released on acid hydrolysis.[9]

The feasability of this procedure was demonstrated in the experiments summarized in Table II, and with oxytocin. Reduced oxytocin was dinitrophenylated and treated with alcoholic 0.1 *N* sodium hydroxide. The reaction mixture was oxidized with performic acid and then with alkaline hydrogen peroxide. Two new amino terminal groups identified as tyrosine (40%) and proline (75%) were formed during the course of these reactions.



Discussion

Of the two "leaving groups" described above for studies of the β-elimination reaction with cysteine derivatives, the dinitrophenylthio group seems to be the more convenient one. Dinitrophenylation of the thiol group proceeds smoothly at room temperature at pH 5–6 and can be followed on the pH-Stat, whereas other functional groups, such as amino or hydroxyl groups, react very slowly or not at all under these conditions. The β-elimination reaction, which is carried out in alkaline medium, is quantitative and can be conveniently followed by spectrophotometric determination of the dinitrothiophenolate ion released. The disadvantage of this elimination reaction is the high pH necessary. Usually the elimination is carried out in 0.1 *N* sodium hydroxide for 20–30 min. at room temperature. Under these conditions, some hydrolysis of susceptible primary amide bonds may occur.

TABLE II

YIELDS OF AMINO ACIDS LIBERATED IN THE CLEAVAGE OF VARIOUS CYSTEINE PEPTIDES

Compound[a]	Amino acid released	Method of cleavage[b] a	b	c
Cbz-Cys-Gly-OEt	Glycine	45%	35%	56%
$Br^- + S(CH_3)_2$ γ-Glu-Cys-Gly	Glycine	64%	..	56%
S-DNP *p*-NO_2-Cbz-Gly-Cys-Gly	Glycine	58%	..	53%
S-DNP Cbz-Phe-Cys-Gly-OEt	Glycine	47%	45%	63%
S-DNP				

[a] Abbreviations: Cbz, carbobenzoxy; *p*-NO_2-Cbz, *p*-nitrocarbobenzoxy. [b] a, hydrolysis, by boiling at pH 2 for 1 hr. followed by oxidation with hydrogen peroxide in 0.1 *N* sodium hydroxide for 30 min. at 37°; b, oxidation with bromine followed by oxidation with alkaline hydrogen peroxide; c, oxidation with performic acid followed by oxidation with alkaline hydrogen peroxide.

One special feature of the cleavage reaction should be mentioned here. When a cysteine residue occupies the N-terminal position in a peptide chain, the dinitrophenyl group attached to the sulfur atom will shift to the terminal nitrogen[15] when the pH is raised above 7, and no elimination will occur. If it is also desired to cleave the terminal cysteine residue, the α-amino group must be blocked. In the experiment with oxytocin described here, this was done by dinitrophenylating the molecule completely at pH 8.6 before the elimination step. The S → N shift of the dinitrophenyl group can be easily recognized by a change in the absorption spectrum, the S-derivative having λ_{max} 330 mμ (ε 10,500) and the N-derivative λ_{max} 360 mμ (ε 14,500).

The other "leaving group" investigated, the sulfonium group, is easily eliminated at pH 8.5–9, 1–2 hr. at room temperature, but the extent of elimination was found to be only about 70–80%. Another drawback of the method is that tertiarization of the thiol group is slow (24 hr.) and is carried out in formic acid. Under these conditions, side reactions such as N → O shift[16] or formylation[17] may take place. A more serious disadvantage may be a loss in specificity, as methionine residues are cleaved under similar circumstances,[18] though it may be possible to perform the two reactions in two distinct stages.

In conclusion, it may be said that the high yields obtained in the elimination reaction, together with the high percentage of cleavage of the dehydroalanine residues formed, make this new method of cleavage at the cysteine residue a promising one for the specific fragmentation of proteins at the cysteine residues and for identifying the amino residue attached to the carboxyl group of the cysteine residue.

(15) H. P. Burchfield, *Nature*, **181**, 49 (1958).
(16) J. L. Rabinowitz, *Compt. rend. Trav. Lab. Carlsberg*, **31**, 483 (1961).
(17) L. Josefsson and P. Edman, *Biochim. Biophys. Acta*, **25**, 614 (1957).
(18) W. B. Lawson, E. Gross, C. N. Foltz, and B. Witkop, *J. Am. Chem. Soc.*, **83**, 1509 (1961).

Experimental

All melting points are uncorrected. Compounds were dried at 50° under high vacuum over phosphorus pentoxide, prior to analysis.

Synthesis of Model Compounds.—The following compounds were prepared according to the published procedures: S-methyl-L-cysteine, m.p. 247–248° dec.[19]; N-carbobenzoxyglycine, m.p. 119°[20]; S-DNP-cysteine, m.p. 146°[10]; *p*-nitrocarbobenzoxyglycine, m.p. 121°[21]; N-carbobenzoxy-L-phenylalanyl-L-cysteinylglycine ethyl ester.[22] Neutral equivalents were obtained by titration with 0.1 *N* sodium methoxide using thymol blue as indicator.[23]

N,S-Di-DNP-L-cysteine (I).—A solution of L-cysteine hydrochloride monohydrate (Schwarz Bioresearch Inc., 1.75 g., 0.01 mole) and 10 g.(0.1 mole) of potassium bicarbonate in 50 ml. of water was mixed with a solution of 3.65 g. of fluorodinitrobenzene (FDNB) in 30 ml. of methanol and stirred vigorously for 30 min. On acidification to pH 2 with dilute hydrochloric acid, the product precipitated as yellow crystals, 4 g. (88%). After recrystallization from acetic acid–water and drying *in vacuo* at 70°, the m.p. was 157–158°.[24]

Anal. Calcd. for $C_{15}H_{11}O_{10}N_5S$: C, 39.74; H, 2.43; N, 15.45; S, 7.06. Found: C, 39.65; H, 2.61; N, 15.59; S, 6.87.

S-DNP-L-cysteine Methyl Ester Hydrochloride.—A suspension of 2.87 g. of S-DNP-L-cysteine, (0.01 mole) in 50 ml. of anhydrous methanol was cooled to −5°, and 2.38 g. (0.02 mole) of thionyl chloride was added dropwise. The reaction mixture was kept for 4 hr. at room temperature, and then concentrated *in vacuo*. The product, 2 g. (60%), was precipitated by adding ether. After recrystallization from dimethylformamide–ether, the m.p. was 176° dec.

Anal. Calcd. for $C_{10}H_{12}N_3O_6SCl$: C, 35.60; H, 3.56; N, 12.46; OCH_3, 9.19; neut. equiv., 337. Found: C, 35.35; H, 3.65; N, 12.24; OCH_3, 9.13; neut. equiv., 329.

***p*-Nitrocarbobenzoxyglycyl-S-DNP-L-cysteine Methyl Ester (II).**—A solution of 1.35 g. of S-DNP-L-cysteine methyl ester hydrochloride (0.004 mole), 1.01 g. of *p*-nitrocarbobenzoxyglycine (0.004 mole), and 0.81 g. of N,N′-dicyclohexylcarbodiimide (0.004 mole) in 15 ml. of dimethylformamide was cooled to −5°, and 0.52 ml. of triethylamine (0.0036 mole) was added slowly to the reaction mixture at −5° during half an hour. The dicyclohexylurea which began to separate immediately was removed by filtration, after 12 hr. at 0°. The filtrate was diluted with 80 ml. of water and was then extracted three times with 25-ml. portions of ethyl acetate. The ethyl acetate extracts were washed twice with 0.1 *N* potassium bicarbonate and then with water, and then concentrated *in vacuo*. The residue was dissolved in 5 ml. of ethyl acetate and the compound was adsorbed on 10 g. of neutral alumina. The alumina column was washed with ether and petroleum ether, and the compound eluted with ethyl acetate in 10-ml. fractions. The absorption spectrum of each fraction was taken on a Beckman DK 1 recording spectrophotometer. The ratio between the absorbancy at 270 mμ (due to the absorbancy of the *p*-nitrobenzyl group[9] and the S-dinitrophenyl group) to the absorbancy at 330 mμ (due to the absorbancy of the S-dinitrophenyl group)[10] is approximately 1.6. The fractions exhibiting this ratio were combined and concentrated *in vacuo*, and the residue was recrystallized from ethyl acetate, yielding yellow crystals, 0.75 g. (38%), m.p. 162–163°.

Anal. Calcd. for $C_{20}H_{19}O_{11}N_5S$: C, 44.69; H, 3.53; N, 13.03; S, 5.95. Found: C, 44.54; H, 3.60; N, 13.14; S, 5.81.

S-Dinitrophenylglutathione (III).—To a solution of 3.06 g. of reduced glutathione (0.01 mole, Schwarz Bioresearch Inc.) in 40 ml. of 1 *N* potassium bicarbonate was added 1.96 g. (0.011 mole) of fluorodinitrobenzene in 5 ml. of methanol. The reaction mixture was stirred for 10 min., and acidified to pH 2 with dilute hydrochloric acid. The yellow product, 4.3 g. (91%), was washed with ether and acetone; m.p. 221°. When the compound was recrystallized from hot water, it contained one molecule of water and melted at 211°.[25]

Anal. Calcd. for $C_{16}H_{13}O_{10}N_3S$: C, 40.60; H, 4.04; N, 14.80; S, 6.77. Found: C, 40.69; H, 4.14; N, 14.84; S, 6.74.

N,S-Di-Dinitrophenylglutathione (IV).—A solution of 3.06 g. of reduced glutathione (0.01 mole) in 70 ml. (0.07 mole) of 1 *N* potassium bicarbonate was stirred for 2 hr. at room temperature with 5.58 g. of fluorodinitrobenzene (0.03 mole) in 15 ml. of methanol. Upon acidification with hydrochloric acid, the product separated as an oily residue which solidified when treated with ether. The compound was dissolved in dilute bicarbonate and precipitated by dilute HCl; 5.67 g. (88%) of the product was obtained, m.p. 139°, neut. equiv. 317 (calcd. 319.5). (As the compound is yellow, the actual color change observed is from yellow to green.)

Anal. Calcd. for $C_{22}H_{21}O_{14}N_7S$: C, 41.31; H, 3.28; N, 15.75; S, 5.01. Found: C, 40.98; H, 3.32; N, 15.10; S, 5.00.

N,S-Di-DNPGlutathione Dimethyl Ester (V).—Compound IV (639 mg., 1 mmole) was dissolved in 15 ml. of dimethylformamide and was esterified by addition of excess of diazomethane dissolved in ether which was prepared from 2 g. of nitroso-N-methylurethan.[26] After 20 min., the ether was distilled off and on addition of water, yellow crystals formed. The crystals were washed twice with 0.1 *N* potassium bicarbonate and twice with water. After recrystallization from ethanol, the m.p. was 131° and the yield was 0.6 g. (90%).

Anal. Calcd. for $C_{24}H_{25}O_{14}N_7S$: C, 43.17; H, 3.74; N, 14.69; OCH_3, 9.29. Found: C, 43.37; H, 3.66; N, 14.35; OCH_3, 9.32.

***p*-Nitrocarbobenzoxyglycyl-S-carbobenzoxy-L-cysteinylglycine Benzyl Ester (X).**—A solution of 2.54 g. of *p*-nitrocarbobenzoxyglycine (0.01 mole), 4.82 g. of S-carbobenzoxy-L-cysteinylglycine benzyl ester hydrobromide[22] (0.01 mole), and 1.44 ml. (0.01 mole) of triethylamine in 100 ml. of chloroform was cooled to 0° and 2.05 g. of N,N′-dicyclohexylcarbodiimide (0.01 mole) was added. The reaction mixture was stirred for 3 hr. and the precipitated dicyclohexylurea was removed by filtration. The filtrate was washed successively with 0.1 *N* hydrochloric acid, 0.1 *N* potassium bicarbonate, and water. After drying over Na_2SO_4, the solution was concentrated to a small volume. Light petroleum ether was added causing crystallization. The product was recrystallized from ethyl acetate yielding 5.43 g. (85%), m.p. 135–136°.

Anal. Calcd. for $C_{30}H_{30}N_4O_{10}S$: C, 56.42; H, 4.74; N, 8.77; S, 5.01. Found: C, 56.62; H, 4.76; N, 8.88; S, 4.85.

***p*-Nitrocarbobenzoxyglycyl-S-DNP-cysteinylglycine (VI).**—A solution of 1.59 g. (0.0025 mole) of X in 30 ml. of methanol was freed from oxygen by bubbling through nitrogen for 5 min. and 3 ml. of methanolic 2.5 *N* sodium methoxide was added. The reaction mixture was kept under nitrogen for 1 hr., and then the pH was adjusted to 8 with dilute acetic acid, and 930 mg. (0.005 mole) of fluorodinitrobenzene was added. The sodium salt of VI separated immediately. The reaction mixture was then stirred for 1 hr. at room temperature under nitrogen. Dilute hydrochloric acid (100 ml.) was added; the yellow precipitate was collected and washed with ether. The dry compound weighed 1.34 g. (90%), m.p. 113 after recrystallization from acetic acid–water.

Anal. Calcd. for $C_{21}H_{20}O_{12}N_6S$: C, 43.44; H, 3.44; N, 14.48; S, 5.51; neut. equiv., 580. Found: C, 43.52; H, 3.52; N, 14.34; S, 5.45; neut. equiv., 596.

N-Carbobenzoxyglycyl-S-methyl-L-cysteine Methyl Ester (VII).—A solution of 4.16 g. of carbobenzoxyglycine (0.02 mole) and 5.76 ml. of triethylamine (0.04 mole) in 100 ml. of ethyl acetate was cooled to −10° and 3.35 g. (0.02 mole) of *sec*-butyl chloroformate was added. After stirring for 15 min. at −10°, 3.66 g. (0.02 mole) of S-methyl-L-cysteine methyl ester hydrochloride in 100 ml. of ethyl acetate was added and the solution was stirred for 16 hr. at room temperature. The solution was washed successively with 0.1 *N* hydrochloric acid, 0.1 *N* sodium bicarbonate, and water. After drying over Na_2SO_4, the solution was concentrated *in vacuo* and the residue was crystallized from methanol. The yield was 2.76 g. (42%), m.p. 69–70°.

Anal. Calcd. for $C_{15}H_{20}O_5N_2S$: C, 52.93; H, 5.93; N, 8.23; S, 9.41. Found: C, 53.10; H, 5.81; N, 8.19; S, 9.52.

N-Carbobenzoxy-S-methyl-L-cysteine (VIII).—Carbobenzoxy chloride (8.5 g., 0.05 mole) was added to 4.0 g. of S-methyl-L-cysteine[20] (0.03 mole) in 50 ml. of 2 *N* sodium hydroxide. The reaction mixture was stirred for 0.5 hr. at 0°, and then extracted twice with ether and once with petroleum ether. The aqueous solution was adjusted to pH 2 with dilute hydrochloric acid and the acidified solution was extracted twice with ethyl acetate. The combined ethyl acetate extracts were dried over Na_2SO_4, and evaporated to an oily residue weighing 7.4 g. (91%).

Anal. Calcd. for $C_{12}H_{15}O_4NS$: S, 11.9; neut. equiv., 269. Found: S, 11.7; neut. equiv., 272.

N-Carbobenzoxy-L-cysteine Dimethylsulfonium Bromide (XI).—Methyl bromide (19 g., 0.2 mole) was added to a solution of 2.69 g. (0.01 mole) of VIII dissolved in 50 ml. of 98% formic acid and the solution was cooled to 0°. The reaction mixture was kept for 8 hr. at 0° and then for 16 hr. at room temperature. The solvent was then removed *in vacuo* at 50°, and the residue was dissolved in ethanol and precipitated by addition of ether. The oily residue weighed 3.5 g. (96%); 21.07% of bromide was

(19) V. du Vigneaud, H. S. Loring, and H. A. Craft, *J. Biol. Chem.*, **105**, 481 (1934).

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

(21) F. H. Carpenter and D. T. Gish, *J. Am. Chem. Soc.*, **74**, 3818 (1952).

(22) M. Sokolovsky, M. Wilchek, and A. Patchornik, *J. Am. Chem. Soc.*, **86**, 1202 (1964).

(23) J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, **23**, 589 (1951).

(24) A. L. Levy and D. Chung, *J. Am. Chem. Soc.*, **77**, 2899 (1955).

(25) B. C. Saunders, *Biochem. J.*, **28**, 1977 (1934).

(26) A. I. Vogel, "Textbook of Practical Organic Chemistry," Longmans, Green and Co., New York, N. Y., 1957, pp. 969–970.

found by argentometric titration (calcd. 21.97%). A neutral equivalent of 370 was obtained by titration with 0.1 *N* sodium hydroxide with phenolphthalein as indicator (calcd. 364).

S-Methyl-L-cysteine methyl ester hydrochloride was prepared according to the general procedure described by Fischer[27]; 4.05 g. (0.03 mole) of S-methyl-L-cysteine yielded 5.2 g. (95%) of the corresponding methyl ester. After recrystallization from methanol–ether, the m.p. was 135°.

Anal. Calcd. for $C_5H_{12}O_2NSCl$: S, 17.29; Cl, 19.19. Found: S, 17.05; Cl, 18.95.

N-Carbobenzoxy-S-methyl-L-cysteinylglycine Ethyl Ester (IX).—A solution of 2.69 g. of N-carbobenzoxy-S-methyl-L-cysteine (0.01 mole), 1.4 g. of glycine ethyl ester hydrochloride (0.01 mole), and 1.44 ml. of triethylamine (0.01 mole) in 50 ml. of ethyl acetate–chloroform (1:1) was cooled to −5° and 2.05 g. of N,N′-dicyclohexylcarbodiimide (0.01 mole) was added. The reaction mixture was stirred for 1 hr. at 0° and then overnight at room temperature. The precipitated dicyclohexylurea was removed by filtration and the filtrate was washed successively with 0.1 *N* hydrochloric acid, 0.1 *N* potassium bicarbonate, and water. After drying over Na_2SO_4, the solution was concentrated to dryness *in vacuo*. The oily residue was dissolved in benzene and triturated with light petroleum ether. On standing in the refrigerator, the compound crystallized in the form of short needles. Recrystallization from ethyl acetate–petroleum ether yielded 2.84 g. (80%), m.p. 86–87°.

Anal. Calcd. $C_{16}H_{22}O_5N_2S$: C, 54.32; H, 6.26; N, 7.91. Found: C, 54.30; H, 6.35; N, 7.90.

Dinitrophenylation.—Dinitrophenylation was followed titrimetrically in an automatic titrator (Radiometer, Model TTTIa, Copenhagen, Denmark). Glutathione (30.6 mg., 0.1 mmole) in 6 ml. water at pH 5.5 was treated with 74.4 mg. (0.4 mmole) of fluorodinitrobenzene dissolved in 2 ml. of methanol. The solution was stirred at room temperature and maintained at pH 5.5 during the reaction by addition of 0.1 *N* sodium hydroxide; 0.97 ml. of 0.1 *N* sodium hydroxide was consumed (97%).

The above procedure was applied to the following compounds: cysteine, thioglycolic acid, poly-L-lysine (average degree of polymerization 50), Nα-carbobenzoxy-L-histidylglycine (m.p. 237–239°),[12] N-acetyl-L-tyrosyl-L-alanine (m.p. 110°),[28] glycylglycine, and N-carbobenzoxy-L-serine (m.p. 121°).[29]

Alkylation of Thiol Group with Methyl Iodine.—The alkylation was followed titrimetrically with an automatic titrator. Glutathione (30.6 mg., 0.1 mmole) in 6 ml. of water at pH 6 was treated with 42.6 mg. (0.3 mmole) of methyl iodide. The solution was stirred at room temperature and maintained at pH 6 during the reaction by addition of 0.1 *N* sodium hydroxide; 0.97 ml. of 0.1 *N* sodium hydroxide (97%) was consumed.

Determination of Dinitrothiophenolate Ion.[13]—The concentration of dinitrothiophenolate ion was determined by measuring the difference spectrum at 420 mμ of an aliquot of nonoxidized methanolic solution (6×10^{-5} *M*) against an equal aliquot which had been oxidized with 50 λ of 0.1 *N* iodine solution (ϵ_{diff} 15,700 ± 300).

β-Elimination Reaction in S-Dinitrophenylcysteine Derivatives.—The kinetics of β-elimination in these compounds were followed in a Beckman DK 1 recording spectrophotometer. Solutions of 6×10^{-5} *M* of compounds I–VI in absolute methanol were prepared; 1.5 ml. of each solution was mixed with 1.5 ml. of base (sodium methoxide) in a cuvette of 4-ml. capacity. The rate of the reaction was followed by measuring the increase in absorbancy at 420 mμ. The amount of dinitrothiophenolate ion released from compounds I, IV, and V was determined by measuring the decrease in absorbancy at 420 mμ in the final reaction as described above. Results are shown in Table I and in Fig. 2.

Paper electrophoresis was carried out on Whatman No. 1 filter paper at 50 v. per cm. in pyridine acetate buffer, pH 3.6, in the apparatus described by Dreyer, *et al.*[30] Compounds with free amino groups were detected by developing with 0.5% ninhydrin in acetone. For quantitative determination of the amino acids, spots from the unknown as well as from markers were eluted with 80% aqueous ethanol, and their absorbancy at 570 mμ was measured.

Formation of Dehydroalanine Derivatives from the Corresponding Cysteinyl Derivatives. Conversion of II to *p*-Nitrocarbobenzoxyglycyldehydroalanine Methyl Ester.—A solution of 537 mg. (0.001 mole) of II in 1 ml. of dioxane was mixed with 2 ml. of methanolic 0.05 *N* sodium methoxide. The reaction mixture was stirred for 5 min., and then 20 ml. of ether and 50 ml. of water were added. The ether phase was washed successively with dilute potassium bicarbonate and water until the yellow color disappeared. The amount of dinitrothiophenolate ion in the aqueous solution was determined spectrophotometrically and the yield was found to be 0.93 mmole (93%). The ether phase was concentrated *in vacuo*. The solid residue was recrystallized from ethanol–water and melted at 141°.[9] The compound was identified as *p*-nitrocarbobenzoxyglycyldehydroalanine methyl ester and weighed 270 mg. (80%). The m.p. was not depressed on mixing with an authentic sample.

Conversion of III to γ-Glutamyldehydroalanylglycine.—To a solution of 50 μmoles of III in 0.5 ml. of 80% methanol containing 100 μmoles of triethylamine, 0.5 ml. of 1 *N* sodium methoxide was added and 10-λ aliquots were taken so as to determine the amount of dinitrothiophenolate ion formed. The aliquots were diluted to 10 ml. with water and the absorption was measured at 408 mμ. By this method it was found that the elimination reaction was 97% completed within 12 min. The solution was then adjusted to pH 4 with acetic acid, and most of the dinitrothiophenolate ion precipitated and was removed by centrifugation. Paper chromatography indicated the presence of several products. On high voltage electrophoresis (50 v./cm.) for 2 hr. at pH 3.6 in pyridine–acetate buffer, the main product which was to be the dehydroalanine derivative of glutathione moved toward the anode. It was extracted with 20 ml. of 0.1 *N* HCl. The amount of dehydroalanine in this derivative was determined as follows: 0.2 ml. of the above extract was added to 0.2 ml. of concentrated hydrochloric acid and incubated for 18 hr. at 110°. After evaporation to dryness, an aliquot was subjected to electrophoresis as described above. The glycine and glutamic acid spots were determined with ninhydrin, and it was found that the 0.2-ml. aliquot contained 0.198 μmole of glycine and 0.189 μmole of glutamic acid. On hydrolysis of another 0.2-ml. aliquot in 2 *N* hydrochloric acid for 2 hr., determination of pyruvic acid in the hydrolysate using lactic dehydrogenase and DPNH gave 0.196 μmole of pyruvic acid in the sample. The ratio of about 1:1:1 of glycine, glutamic acid, and pyruvic acid proves that the main product obtained was indeed the dehydroalanyl derivative of glutathione.

Conversion of VII to Carbobenzoxyglycyldehydroalanine.—One gram of VII (0.003 mole) in 20 ml. of 99% formic acid was cooled to 0° and 5 ml. of methyl bromide was added. The reaction mixture was incubated for 24 hr. at 0°. After evaporation at 50° *in vacuo*, an oily residue was obtained. This residue was stirred in 25 ml. of 0.25 *N* sodium hydroxide for 2 hr., and was then adjusted to pH 2 with dilute hydrochloric acid. The solid compound obtained melted at 191°. After recrystallization from ethanol the m.p. was 192–193°; 0.543 g. (65%) obtained was identified as carbobenzoxyglycyldehydroalanine. A mixture m.p. of 193° with authentic compound was obtained and the neutral equivalent was 276 (calcd. 278).

Conversion of XI to Carbobenzoxydehydroalanine.—A solution of 728 mg. (0.002 mole) of compound XI in 20 ml. of water was titrated with 19.8 ml. of 0.1 *N* sodium hydroxide using phenolphthalein as indicator. When the solution was boiled, dimethyl sulfide was evolved, and another equivalent of base (19.77 ml.) was needed in order to neutralize the acidity; when the solution was acidified with dilute hydrochloric acid, white crystals separated. The solid compound which was identified as carbobenzoxydehydroalanine melted at 108–109° and weighed 0.42 g. (95%). A mixture m.p., made using an authentic sample, gave m.p. 107–108°. The compound isolated was hydrolyzed for 1 hr. in 1 *N* hydrochloric acid and gave a quantitative yield of pyruvic acid as estimated enzymatically.[9]

In a parallel experiment, 20 ml. of ether was added and the dimethyl sulfide which formed passed into the ether layer. The ether layer was mixed with 60 ml. of 99% formic acid containing 10 ml. of methyl bromide. After 16 hr., the solvents were removed *in vacuo* and the oily residue was crystallized from methanol–ether. The white crystals melted at 200°. This compound was identified as trimethylsulfonium bromide.

Anal. Calcd. for C_3H_9SBr: S, 20.38; Br, 50.95. Found: S, 20.10; Br, 50.05.

Conversion of IX to Carbobenzoxydehydroalanylglycine Ethyl Ester.—Methyl bromide (5 ml.) was added to a solution of 1.06 g. of IX (0.003 mole) in 10 ml. of 99% formic acid. The reaction mixture was kept for 12 hr. at 0° and then for 12 hr. at room temperature. After evaporation *in vacuo* at 50°, an oily residue was obtained. This product was dissolved in 5 ml. of ethanol and 15 ml. of ether and was stirred for 2 hr. with a saturated solution of sodium bicarbonate. Ether (15 ml.) was added and the ether phase was then dried over Na_2SO_4. When the solvent was distilled *in vacuo*, white crystals separated. The 0.735 g. (80%) of material obtained was crystallized from ether–petroleum ether and was identified as carbobenzoxydehydroalanylglycine ethyl ester, m.p. 83°.[9]

Anal. Calcd. for $C_{15}H_{18}N_2O_5$: C, 58.81; H, 5.92; N, 9.15. Found: C, 58.43; H, 6.00; N, 9.11.

Cleavage of Cysteinyl Model Compounds.—The procedure for the chemical cleavage at cysteine residues is illustrated below by two typical examples: the cleavage of carbobenzoxy-L-phenyl-

(27) E. Fischer, *Chem. Ber.*, **34**, 433 (1901).

(28) This compound was a gift from Mr. M. Wilchek.

(29) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(30) A. M. Katz, W. J. Dreyer, and C. B. Anfinsen, *ibid.*, **234**, 2897 (1959).

alanyl-L-cysteinylglycine ethyl ester[22,31] (XII) and the cleavage of oxytocin. The dehydroalanine peptides resulting from the elimination reaction were not isolated.

Cleavage of XII.—A solution of 24.3 mg. of XII (50 μmoles) in 10 ml. of 60% ethanol was treated with 37.2 mg. (200 μmoles) of FDNB. The reaction was carried out at pH 6 and was followed with a pH-Stat, 0.492 ml. of 0.1 *N* sodium hydroxide being consumed. This is equivalent to a 98% yield. The excess of FDNB was allowed to react with 250 μmoles of thioglycolic acid at pH 8. Ethyl acetate (50 ml.) was then added and the mixture was washed with 0.1 *N* sodium bicarbonate to remove the S-dinitrophenylated thioglycolic acid. The ethyl acetate layer was dried over Na_2SO_4 and was concentrated to 25 ml.

The elimination reaction was carried out on a 4-ml. aliquot (8 μmoles). The sample was dried *in vacuo* at 40° and was treated with 1 ml. of 0.1 *N* sodium methoxide. After 3 min. the extent of elimination was 99% as determined spectrophotometrically. Aliquots of 0.25 ml. (2 μmoles) were taken for cleavage.

(a) **Hydrolytic Cleavage.**—The pH of the sample was adjusted to 7 with dilute hydrochloric acid and the mixture was lyophilized; 1 ml. of 0.01 *N* hydrochloric acid was added, and the tube containing the reaction mixture was closed and heated to 100° for 1 hr. The reaction mixture was lyophilized, and then 1 ml. of 0.1 *N* sodium hydroxide and 0.2 ml. of 30% hydrogen peroxide were added and the reaction mixture was incubated at 37° for 30 min. The reaction mixture was then neutralized with acetic acid and excess of hydrogen peroxide was destroyed by incubation with catalase (0.03 ml. of 5% solution). Aliquots were taken for high voltage electrophoresis. Only one amino acid glycine was detected and the yield was 0.94 μmole (47%).

(b) **Oxidative Cleavage with Bromine.**—The pH of the 0.25-ml. sample was adjusted to 5 with dilute acetic acid and 0.2 ml. of 0.02 *N* aqueous bromine (2 μmoles) was added. After 5-min. incubation at room temperature the pH was adjusted to 7 with dilute sodium hydroxide, and 0.2 ml. of 30% hydrogen peroxide in 0.5 ml. of 0.2 *N* sodium hydroxide was added. The reaction mixture was treated as described above, the yield of the liberated glycine being 0.9 μmole (45%).

(c) **Oxidative Cleavage with Performic Acid.**—The neutralized sample was lyophilized, and the residue was dissolved at 0° in 0.3 ml. of performic acid and kept at this temperature for 2 hr. The performic acid was removed by lyophilization and the residue was dissolved in 1 ml. of 0.1 *N* sodium hydroxide. Hydrogen peroxide 30% (0.2 ml.) was added. The reaction mixture was treated as described above; the yield of glycine detected after electrophoresis was 1.26 μmoles (63%).

Cleavage of Oxytocin.—A 1.8-mg. sample of lyophilized oxytocin (obtained from 90 ampoules of Syntocinon, Sandoz Ltd., Basel) was dissolved in 5 ml. of water. Redistilled thioglycolic acid (2.76 mg., 0.03 mmole) was added and the pH was adjusted to 8.6 with 5% trimethylamine. The container was flushed with nitrogen and the solution was allowed to stand overnight at room temperature. Dinitrophenylation was carried out at room temperature by adding 37.2 mg. of fluorodinitrobenzene (0.2 mmole), dissolved in 0.5 ml. of ethanol, and the pH was maintained at 8.6 for 1 hr. The excess of fluorodinitrobenzene was treated with an excess of thioglycolic acid (46 mg., 0.5 mmole) at the same pH. The reaction mixture was transferred to a separatory funnel and was extracted twice with 20-ml. portions of ethyl acetate. The dinitrophenylated reduced oxytocin which was suspended in the organic layer was washed with 1 *N* potassium bicarbonate in order to remove the excess thioglycolic acid and S-DNP-thioglycolic acid. The ethyl acetate solution was evaporated *in vacuo* at 40°. The yellow residue, found to be insoluble in the usual organic solvents, was suspended in 0.25 ml. of ethanol, and the elimination reaction was carried out by heating to 80° for 1 min., after adding 2 ml. of 0.1 *N* sodium hydroxide. A small amount of unreacted starting material was recovered by centrifugation and again treated as above. The amount of dinitrothiophenolate ion produced was estimated by measuring the difference spectrum at 408 mμ of an aliquot of nonoxidized solution against an equal aliquot which had been oxidized completely with 0.05 ml. of 0.1 *N* iodine. A total amount of 2.75 μmoles of dinitrothiophenolate ion was found corresponding to 1.37 μmoles of oxytocin. The pH of the combined reaction mixture was adjusted to 6.1 with dilute acetic acid and the solution was lyophilized.

The cleavage of the modified oxytocin was carried out by dissolving the lyophilized residue in 1 ml. of performic acid at 0°. A 0.1-ml. aliquot was removed and used as a control sample. The reaction mixture was incubated for 2 hr. at 0° and then the performic acid was removed by lyophilization. The oxidized product was dissolved in dilute alkali, and the reaction mixture was treated with 0.2 ml. of 30% hydrogen peroxide. Sodium hydroxide was then added to a final concentration of 0.1 *N*, and the solution was incubated for 30 min. at 37°. The reaction mixture was then neutralized with acetic acid. The excess of hydrogen peroxide was destroyed by incubation with catalase (0.03 ml. of a 5% solution). The final volume was 5.5 ml.

The amount of N-terminal proline released was determined on a 1-ml. aliquot using proline iminopeptidase as described by Sarid, *et al.*[32] By this method, 0.186 μmole (75%) of free proline was found. Under the same conditions, no free proline could be found in a control sample (which had undergone the same treatment).

Another 1-ml. aliquot was dinitrophenylated according to the method of Levy.[33] The dinitrophenylated product was subjected to acid hydrolysis in a sealed tube with constant boiling hydrochloric acid at 105° for 16 hr. An aliquot of the hydrolysate was developed by ascending chromatography in the "toluene" solvent in one dimension and by descending chromatography with 1.5 *M* phosphate buffer in the second dimension. Only two spots corresponding to di-DNP-tyrosine and 2,4-dinitroaniline could be detected on the chromatogram. The di-DNP-tyrosine spot was eluted from the chromatogram and estimated spectrophotometrically at 360 mμ; 0.098 μmole (40%) was found.

Acknowledgments.—Many thanks are due to Professor E. Katchalski and Professor A. Berger for helpful discussions and to Mrs. H. Later for competent technical assistance.

(31) Dr. I. Photaki, University of Athens, kindly supplied us with a sample of this compound.

(32) S. Sarid, A. Berger, and E. Katchalski, *J. Biol. Chem.*, **234**, 1740 (1959); **237**, 2207 (1962).

(33) H. Frankel-Conrat, J. I. Harris, and A. L. Levy in "Methods of Biochemical Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, pp. 359–425.

[CONTRIBUTION OF THE DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM 54, MASS.]

Kinetics of Hydrolysis of Diethyl Glutarate and β-Substituted Diethyl Glutarates by α-Chymotrypsin[1]

BY SAUL G. COHEN AND JOHN CROSSLEY

RECEIVED AUGUST 26, 1963

The kinetics of hydrolysis by α-chymotrypsin of four diethyl glutarates, $X-CH(CH_2CO_2Et)_2$, have been studied. The parameters at pH 7.8, 25.0°, 0.1 *M* NaCl, are: X = HO, K_m = 0.097 mole/l., k_3 = 0.032 sec.$^{-1}$; X = CH_3CONH, K_m = 0.065, k_3 = 0.033; X = CH_3COO, K_m = 0.037, k_3 = 0.20; X = H, K_m = 0.016, k_3 = 0.035. The slow stereospecific hydrolyses, X = HO, CH_3CONH, and the more rapid nonstereospecific hydrolyses, X = CH_3COO, H, have been interpreted in terms of associations of: (1) the β-acetamido and β-hydroxyl groups at the α-acylamido enzyme site; (2) the carbethoxyl groups at the β-aryl and hydrolytic sites; (3) the acetoxyl acyl group at the β-aryl site; and (4) hydrogen at a restricted volume site.

Introduction

The symmetric compound diethyl β-hydroxyglutarate, $HO-CH(CH_2CO_2C_2H_5)_2$ (I), was hydrolyzed by α-chymotrypsin,[2] slowly but with high stereospecificity in the L-sense, leading in high yield to the optically active half ester, (−)-$HO-CH(CH_2COOH)(CH_2CO_2C_2H_5)$. The related asymmetric compound ethyl *dl*-β-hydroxy-

(1) Requirements for stereospecificity in hydrolysis by α-chymotrypsin VI. For paper V see ref. 6. We are pleased to acknowledge generous support of this work by the Division of Research Grants, the National Institutes of Health, RG 4584.

(2) S. G. Cohen and E. Khedouri, *J. Am. Chem. Soc.*, **83**, 4228 (1961).