The product was recrystallized from ethyl acetate; yield 0.7 g. (79%), m.p. 143–145°, $[\alpha]^{25}D$ −26.5° (*c* 1, methanol).

Anal. Calcd. for $C_{18}H_{23}N_3O_8S$: C, 48.98; H, 5.25; N, 9.52; neut. equiv., 220.5. Found: C, 49.17; H, 5.41; N, 9.51; neut. equiv., 223.

The neutral equivalent was obtained by titration with 0.1 *N* sodium methoxide using thymol blue in ethanol as indicator.[21] Iodometric titration revealed 97% free thiol groups. The same product was obtained by methanolysis of compound XII.

(b) **Glutathione.**—*N*-Carbobenzoxyglutathione (0.44 g., 0.001 mole) was suspended in 2 ml. of 45% hydrobromic acid in acetic acid. After 5 min. the compound had dissolved and the solution was kept for an additional 10 min. at room temperature. Anhydrous ether, 100 ml., was added, and the hydrobromide derivative then precipitated. The precipitate was repeatedly washed at the centrifuge with anhydrous ether and then dissolved in 3 ml. of oxygen-free water. The pH was adjusted to 2.9 with dilute triethylamine and was concentrated to 0.2 ml. at 25° *in vacuo*. Upon addition of ethanol the glutathione precipitated. The precipitate was repeatedly washed at the centrifuge with ethanol; yield 0.154 g. (50%), m.p. 189–190°.[22] On oxidation with iodine (80% of the theoretical value), the product behaved chromatographically and electrophoretically like an authentic sample. For further purification the method described by du Vigneaud, *et al.*,[22] was found to be necessary; m.p. 190°, $[\alpha]^{25}D$ −21.8° (reported[22] $[\alpha]^{27}D$ −21.3°).

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Acknowledgment.—This investigation was supported by Grant AM-5098 of the National Institutes of Health, United States Public Health Service.

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[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 into Dehydroalanine. I. Hydrolytic and Oxidative Cleavage of Dehydroalanine Residues[1,2]

BY ABRAHAM PATCHORNIK AND MORDECHAI SOKOLOVSKY

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In connection with the specific chemical fragmentation of peptides and proteins, the conditions for the cleavage of dehydroalanine residues, which can be formed from cysteine and serine residues, was investigated. Dehydroalanine residues are cleaved hydrolytically at the N-C_α bond with nearly quantitative yield by heating the compound to 100° at pH 2 for 1 hr. The cleavage can be facilitated by oxidative activation of the molecule. Oxidation by bromine or performic acid leads to a labile intermediate which is easily cleaved at alkaline pH at room temperature. Both methods lead to the formation of two fragments: $Pep_1CONHC(CH_2)CONHPep_2 \rightarrow Pep_1CONH_2 + XCH_2COCONHPep_2$ (X = H without oxidation, X = OH with oxidation). The nitrogen of the dehydroalanine residue appears as a new terminal amide group on one of the fragments and the rest of the molecule as a terminal pyruvoyl group on the other. The pyruvoyl group can be removed by oxidation with alkaline hydrogen peroxide, a step which liberates the new terminal amino group. A method for the quantitative estimation of dehydroalanine residues in peptides is described.

Introduction

A primary step in the elucidation of the chemical structure of proteins is the selective fragmentation of separated peptide chains. Such fragmentation can be achieved by enzymatic hydrolysis, but only few proteolytic enzymes in use are sufficiently selective.[3] Furthermore, the number of amino acids next to which proteolytic enzymes are known to cleave in a selective way is limited and side reactions sometimes complicate interpretation.[4]

Recently a number of selective nonenzymatic methods for the cleavage of peptide bonds involving a particular amino acid were reported[5–11] and reviewed by Witkop.[12] This new approach has been used recently in studies on the structure of several proteins[8,9,12–14] and natural peptides.[1c,7,8,14]

The methods described so far for the specific chemical cleavage of peptide bonds[12] have been based on interaction of the side chain of an amino acid residue (sometimes after specific chemical modification) with the adjacent peptide bond. This bond is labilized by the formation of intermediate cyclic structures which facilitate the hydrolysis of the peptide chain into two fragments.[12] A new approach to the labilization of peptide chains not involving a cyclic intermediate consists in the conversion of cysteine and serine[1c,15] residues into dehydroalanine residues. This can be achieved by the conversion of the cysteine or serine residue into a derivative which possesses a good "leaving group," *e.g.*, a sulfonium or thiodinitrophenyl group in the case of cysteine, and tosyl or phosphoryl group in the case of serine. These derivatives can be converted to the corresponding dehydroalanine derivative by an elimination reaction under basic conditions.

$$Pep_1CONH{-}CH(CH_2R){-}CONHPep_2 \longrightarrow Pep_1CONH{-}C(=CH_2){-}CONHPep_2 + RH$$

R = $-SC_6H_3(NO_2)_2$, $-S^+(CH_3)_2$, $-OSO_2C_6H_4CH_3$, $-OPO(OR_1)_2$

(1) Preliminary reports: (a) A. Patchornik, M. Sokolovsky, and T. Sadeh, Vth International Congress of Biochemistry, Moscow, 1961, 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 Grants A-3171 and AM-5098 of the National Institutes of Health, United States Public Health Service.

(3) F. Sanger, *J. Polymer Sci.*, **49**, 3 (1961).

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(14) S. Sarid and A. Patchornik, *Israel J. Chem.*, **1**, 63 (1963).

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In the present paper we describe cleavage experiments with synthetic dehydroalanyl peptides. Succeeding papers will describe the conversion of cysteine and serine residues into dehydroalanine residues susceptible to the method of cleavage described in the following.

Dehydroalanine residues are cleaved by hydrolysis, and the reaction can be facilitated by preliminary oxidation. The products obtained are amide derivatives and pyruvoyl derivatives.

$$\mathrm{Pep_1CONH{-}\underset{\displaystyle \underset{\displaystyle CH_2}{\|}}{C}{-}CONHPep_2} \longrightarrow \mathrm{Pep_1CONH_2 + XCH_2COCONHPep_2}$$
$$X = H, OH$$

The new terminal amino acid at the point of cleavage, which is the amino acid adjacent to the carboxyl function of the cysteine or serine residue before modification, is released through an additional step involving oxidative hydrolysis of the pyruvoyl derivative.[16]

$$\mathrm{XCH_2COCONHPep_2 \longrightarrow XCH_2COOH + CO_2 + NH_2Pep_2}$$

Results and Discussion

Hydrolytic Scission of Dehydroalanine Residue.—Peptides containing dehydroalanine residues have been cleaved by treatment with strong acid or base at room temperature.[17] We have observed that the hydrolysis of some dehydroalanine peptides can be achieved under less drastic conditions of pH at elevated temperatures. Thus, carbobenzoxyglycyldehydroalanine (I) yielded upon refluxing for 1 hr. in aqueous hydrochloric acid pH 2.2, carbobenzoxyglycine amide and pyruvic acid (Fig. 1) in 96% yield according to the equation

$$\mathrm{C_7H_7OCONHCH_2CONH{-}\underset{\displaystyle \underset{\displaystyle CH_2}{\|}}{C}{-}COOH} \xrightarrow{H_2O} \mathrm{C_7H_7OCONHCH_2CONH_2 + CH_3COCOOH}$$
I

The pyruvic acid was identified as the 2,4-dinitrophenylhydrazone, m.p. 218–219°.[18] The extent of cleavage was determined by the estimation of pyruvic acid by means of lactic dehydrogenase[19] as well as by titration of the unreacted dehydroalanine residues with iodine or bromine. The titration procedure is discussed in the section dealing with oxidative cleavage. In some runs the amount of carbobenzoxyglycine amide, m.p. 136–137°,[20] was determined by isolation. At pH 7 (aqueous phosphate buffer) hydrolysis is slower (see Fig. 1).

Essentially quantitative yields of cleavage were also observed with carbobenzoxydehydroalanine (II) which yielded benzyl carbamate and pyruvic acid, and with carbobenzoxydehydroalanylglycine (III) which yielded benzyl carbamate and pyruvoylglycine (IV). The latter was identified by paper chromatography.[21]

$$\mathrm{C_7H_7OCONH\underset{\displaystyle \underset{\displaystyle CH_2}{\|}}{C}{-}CONHCH_2COOH} \xrightarrow{H_2O} \mathrm{C_7H_7OCONH_2 + CH_3COCONHCH_2COOH}$$
III IV

A peptide chain of the formula $\mathrm{Pep_1CO{-}NH{-}\underset{\displaystyle \underset{\displaystyle CH_2}{\|}}{C}{-}CONHPep_2}$ will yield, on hydrolytic cleavage, the primary amide of the peptide fragment on the N-terminal side of the dehydroalanine residue, $\mathrm{Pep_1CONH_2}$, and the N-pyruvoyl derivative of the C-terminal peptide fragment, $\mathrm{CH_3COCONH\ Pep_2}$. A peptide chain with a C-terminal dehydroalanine residue $\mathrm{Pep_1CONH{-}\underset{\displaystyle \underset{\displaystyle CH_2}{\|}}{C}{-}COOH}$ will yield the amide $\mathrm{PepCONH_2}$ and pyruvic acid. It is suggested that an intermediate of the structure $\mathrm{Pep_1CONHC(OH)(CH_3)CONHPep_2}$ is formed during the hydrolytic step. This intermediate is analogous to the amides and esters of α-acylamino-α-hydroxy acids described by Chemiakine, *et al.*[22] These compounds are known to be hydrolyzed in base at room temperature and in acid at 100°.

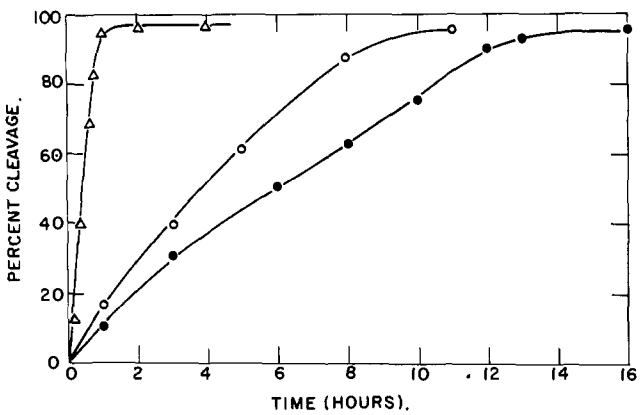

Fig. 1.—Hydrolytic cleavage of dehydroalanine peptides: Δ——Δ, carbobenzoxyglycyldehydroalanine (I) at pH 2.2; ●——●, carbobenzoxyglycyldehydroalanine (I) at pH 7 (0.05 *M* phosphate buffer). In both cases, the extent of cleavage was determined enzymatically by the amount of pyruvic acid formed. O——O, carbobenzoxydehydroalanylglycine (III) at pH 7. In this case the extent of cleavage was determined by bromine titration. All three cleavages were performed at 100°.

The pyruvoyl peptides, pyruvoyl amino acids, and free pyruvic acid formed in the above cleavage reaction can be detected on paper chromatograms by means of nitroprusside[21] or as their dinitrophenylhydrazones, but both methods of detection are not very sensitive. The possibility of removing the pyruvoyl group selectively with the release of a new amino group will be discussed below.

Hydrolytic Scission of Dehydroalanine Residues after Prior Oxidation with Iodine.—*p*-Nitrocarbobenzoxyglycyldehydroalanine (V) in aqueous solution (pH 1 to 6) reacted instantaneously at room temperature with 1 mole of iodine to yield *p*-nitrocarbobenzoxyglycine amide (VI) and 1 mole of iodide, which was determined by argentometric titration. The amide VI was extracted from the neutralized (pH > 7) reaction mixture with ethyl acetate, and its amount determined spectrophotometrically (ϵ_{270} 10,900 in ethyl acetate). By evaporation of the solvent, the amide was recovered in crystalline form and found to be identical with an authentic sample prepared as described below.

Almost quantitative yields of the amide VI were obtained when the pH was raised above 7 before the ethyl acetate extraction. Under these conditions no material absorbing at 270 mμ could be extracted from the organic solvent by means of aqueous bicarbonate. However, when the initial extraction was carried out under acidic conditions, an appreciable amount of acidic material absorbing at 270 mμ was carried into the bicarbonate layer. This finding indicates that the actual cleavage reaction proceeds much faster at high than at low pH.

(16) W. H. McGregor and F. H. Carpenter, *Biochem.*, **1**, 53 (1962).
(17) M. Bergman and K. Grafe, *Z. physiol. Chem.*, **187**, 187 (1930).
(18) E. A. Braude and E. R. H. Jones, *J. Chem. Soc.*, 498 (1945).
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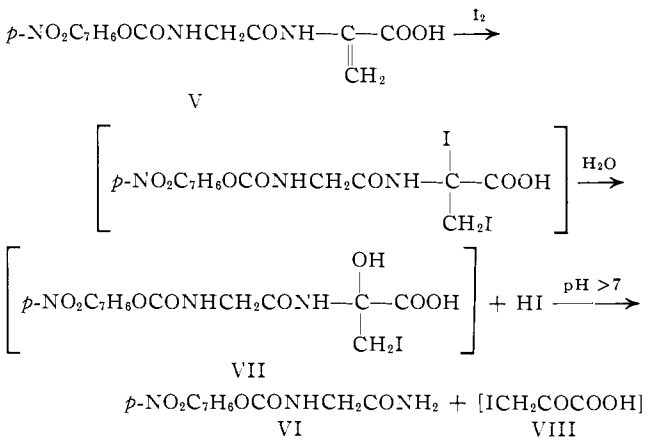
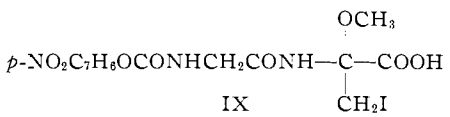
(20) J. S. Fruton, R. B. Johnston, and M. Fried, *ibid.*, **190**, 39 (1951).
(21) T. Wieland, K. H. Shin, and B. Heinke, *Chem. Ber.*, **91**, 483 (1958).
(22) M. M. Chemiakine, E. S. Tchaman, L. I. Denisova, G. A. Ravdel, and W. J. Rodionow, *Bull. soc. chim. France*, **26**, 530 (1959).

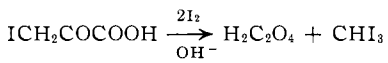
The following proposed mechanism of the cleavage reaction may explain the results obtained.



When the same reaction was carried out in water–methanol (3:2 v./v.) the yield of amide VI formed was only 48%, although 1 mole of iodine had been consumed and 1 mole of iodide had been liberated. This result might be due to the fact that the alcohol competes with water in the formation of the intermediate VII leading to the more stable compound[22] IX



An indication for the formation of iodopyruvic acid (VIII) according to the above scheme was obtained through the following observation: when, after the original uptake of 1 mole of iodine at low pH, excess of iodine was added and the pH of the reaction mixture was raised above pH 9, 2 more moles of iodine was absorbed to yield quantitatively the amide VI, iodoform, and oxalic acid. Iodoform was identified by isolation (m.p. 119°) and its amount (97%) was determined spectrophotometrically[23] after extraction into ethyl acetate. The amount of oxalic acid (98%) was estimated by permanganate titration after precipitation as calcium oxalate. It is suggested that the above compounds are formed from VIII as

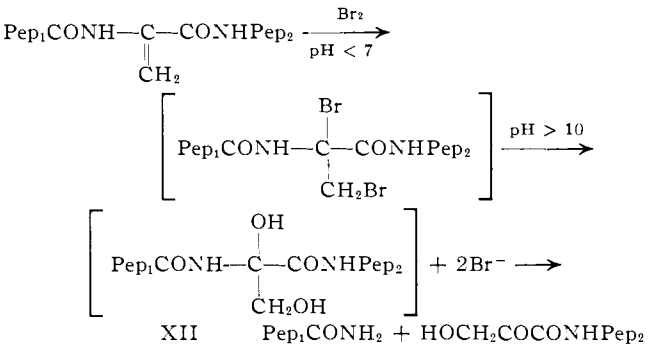


Iodopyruvic acid seems to be a rather unstable compound which, similarly to bromopyruvic acid,[24] is easily hydrolyzed to hydroxypyruvic acid. Indeed, when the above reaction mixture after uptake of 1 mole of iodine is exposed to high pH for a few minutes, the yields of iodoform, on addition of excess iodine, are markedly decreased (50%) probably owing to the fact that the hydroxypyruvic acid formed does not give the haloform reaction. Low yields of amide VI (48%) and of iodoform (45%) are also obtained in methanol–water (2:3 v./v.) even when excess iodine is added at low pH. This finding supports the assumption that under these conditions the methoxy derivative IX is formed to some extent.

Carbobenzoxydehydroalanine and carbobenzoxyglycyldehydroalanine reacted with iodine similarly to compound V. On oxidation with 1 mole of iodine the products were benzyl carbamate (94%) and carbobenzoxyglycine amide (93%), respectively. With excess iodine in alkali, iodoform and oxalic acid were obtained in almost quantitative yields.

p-Nitrocarbobenzoxyglycyldehydroalanine methyl ester (X), carbobenzoxydehydroalanylglycine (III), and γ-L-glutamyldehydroalanylglycine[25] (XI) do not react with iodine at a pH lower than 7. Iodination at high pH gave the corresponding amides in relatively low yields. It thus seems that oxidative cleavage by iodine is advantageous only for derivatives with C-terminal dehydroalanine.

Hydrolytic Scission of Dehydroalanine Residues after Prior Oxidation with Bromine.—Bromine, which is a more powerful oxidizing agent than iodine, reacts readily not only with compounds in which the dehydroalanine has a free carboxyl group (compounds I, II, and V) but also with derivatives in which the carboxyl of the dehydroalanine is blocked (compounds, III, X, and XI). One mole of bromine reacts instantaneously in acidic solution at room temperature with 1 mole of each of the above-mentioned dehydroalanine compounds, and on raising the pH, an amide and pyruvoyl peptide derivative are formed. The yield of the amide was found to be pH dependent and the highest yields (94%) were obtained when the reaction mixture, after addition of bromine, was kept at pH 11 for 10 min. Under these optimum conditions, 2 equivalents of bromide ions was formed, suggesting the reaction scheme



The intermediate XII is analogous to the amide of α-acylamino-α-hydroxy acid, known to be hydrolyzed in base.[22] Under the above conditions, III yielded benzyl carbamate (85%), V yielded *p*-nitrocarbobenzoxyglycine amide (92%), and XI yielded glutamine (89%).

The results show that the oxidative scission by bromine unlike that by iodine can be applied also to peptides in which the carboxyl group of the dehydroalanine is blocked.

Hydrolytic Scission of Dehydroalanine Residues After Prior Oxidation with Performic Acid.—Because of the chemical cleavage by brominating agents occurring at peptide bonds adjacent to tryptophan,[7] tyrosine,[6] and histidine,[8] the possibility of oxidation with performic acid was investigated. This reagent is used extensively in sequential analysis in proteins,[26] and it does not attack tyrosine and histidine, though it damages tryptophan residues.[27]

Dehydroalanine peptides react with 1 mole of performic acid in 2 hr. at 0° (Fig. 2), presumably yielding intermediate XII. At pH 10 this intermediate is hydrolyzed to an amide and hydroxypyruvoyl peptide. The latter, on treatment with alkaline hydrogen peroxide, yields glycolic acid, which was found by means

(23) S. Dal Nogare, T. O. Norris, and J. Mitchell, Jr., *Anal. Chem.*, **23**, 1473 (1951).

(24) D. B. Sprinson and E. Chargaff, *J. Biol. Chem.*, **164**, 417 (1946).

(25) M. Sokolovsky, T. Sadeh, and A. Patchornik, *J. Am. Chem. Soc.*, **86**, 1212 (1964).

(26) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).

(27) M. Sela, R. H. White, Jr., and C. B. Anfinsen, *Biochim. Biophys. Acta*, **31**, 417 (1959).

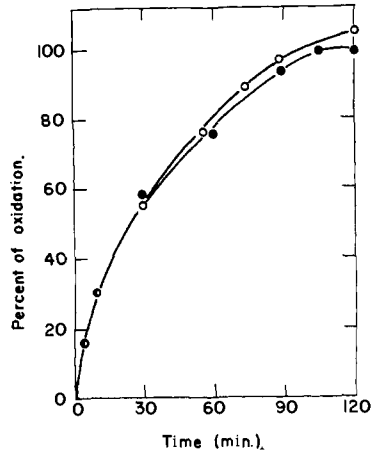


Fig. 2.—The rate of oxidation of carbobenzoxydehydroalanylglycine by performic acid at 0°: O——O, determined by titration of the unreacted performic acid[28]; ●——●, determined by following the decrease in extinction at 240 mμ.[29]

of the chromotropic acid method,[30] and a peptide with a free terminal amino group. The appearance of glycolic acid is an indirect proof for the existence of hydroxypyruvoyl derivative.

$$\mathrm{Pep_1CONH{-}C(=CH_2){-}CONHPep_2} \xrightarrow{HCO_3H} \left[\mathrm{Pep_1CONH{-}C(OH)(CH_2OH){-}CONHPep_2}\right] \xrightarrow{pH>10} \mathrm{Pep_1CONH_2 + HOCH_2COCONHPep_2}$$

XII

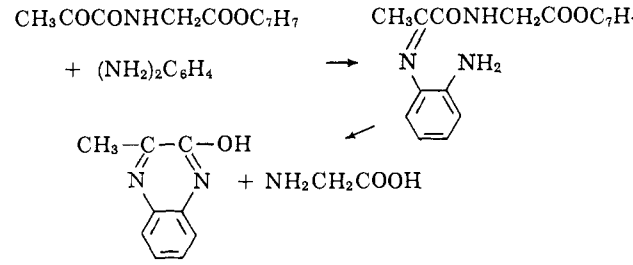
$$\mathrm{HOCH_2COCONHPep_2} \xrightarrow[OH^-]{H_2O_2} \mathrm{HOCH_2COOH + NH_2Pep_2 + CO_2}$$

Thus, compound V yielded the amide VI (94%) and glycolic acid (45%). Similarly, III yielded benzyl carbamate (89%), glycolic acid (43%), and glycine (53%).

It is important to avoid halide ions in the oxidation mixture in order not to cause cleavage of peptide bonds by free halogens.[12,26]

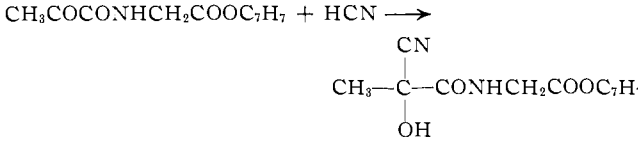
Determination of the New N-Terminal Amino Acid Formed on Cleavage.—The peptide fragment from the C-terminal side of the dehydroalanine peptides is obtained as its pyruvoyl or hydroxypyruvoyl derivative. In both cases, the amino group can be unmasked by the method of Carpenter.[16] In the case of oxidative cleavage, alkaline hydrogen peroxide is added to the oxidized compound to effect cleavage and unmasking of the amino group in one step. This avoids side reactions such as ring closure of the type described by Errera and Greenstein.[31] Under these conditions compounds III and XI yielded glycine (50–60%). It may be added that the treatment with performic acid not followed by alkaline released 20–28% of glycine.

The following possibilities of removing the pyruvoyl group and thus unblocking the amino group were investigated, but yields were found to be significantly lower than those reported above. Pyruvoylglycine benzyl ester, when heated at pH 7 at 100° with *o*-phenylenediamine for 1 hr., yielded 30% of the theoretical amount of glycine according to the scheme



Similarly, from the reaction mixture of the oxidative cleavage of XI (see above) 25% of free glycine was recovered by this method.

Alternatively, a pyruvoyl group can be removed by heating with 2% KCN in citrate buffer (pH 5.0, 0.2 *M*) for 1 hr. The yield of glycine from pyruvoylglycine benzyl ester was 15%. The cleavage observed may be explained by the scheme



It is probable that the amide bond of the 2-cyano-2-hydroxypropionic acid, a stronger acid than pyruvic acid, is hydrolyzed to some extent under the above conditions.

Pyruvoyl peptides can be quantitatively hydrolyzed by refluxing with 1.5 *N* hydrochloric acid for 90 min. to yield free pyruvic acid and the corresponding α-amino acid derivative.[31] However, this treatment causes nonspecific cleavage of peptide bonds. On the other hand, under milder conditions, *e.g.*, boiling for 20 hr. at pH 7.0, cleavage is completely specific, but the yield of pyruvic acid is of the order of 25%. Thus, pyruvoylglycine benzyl ester yielded pyruvic acid in 25% yield as determined enzymatically. It should be noted that the pyruvoylglycine benzyl ester is not reduced by lactic dehydrogenase and DPNH under the conditions which pyruvic acid is reduced completely.

Quantitative Estimation of Dehydroalanine Residues in Peptide Chains.—Dehydroalanine derivatives are hydrolyzed to an amide and a pyruvoyl compound as shown above. Under more drastic conditions, *e.g.*, heating for 1.5–2 hr. in 1 *N* hydrochloric acid, the amide bond of the pyruvoyl derivative is hydrolyzed and pyruvic acid is liberated and can be recovered quantitatively. It is easily estimated enzymatically in amounts of 0.1 μmole. Indeed, 1 mole of pyruvoylglycine benzyl ester yielded on such treatment 0.98 mole of pyruvic acid. Yields of pyruvic acid obtained with several dehydroalanyl compounds are summarized in Table I.

TABLE I

QUANTITATIVE ESTIMATION OF DEHYDROALANINE RESIDUES

Compound	Pyruvic acid found[19] —% of theory—	Bromine titration —% of theory—
Carbobenzoxyglycyldehydroalanine (I)	96	96
Carbobenzoxydehydroalanine (II)	97	97
Carbobenzoxydehydroalanylglycine (III)	97	97
p-Nitrocarbobenzoxyglycyldehydroalanine (V)	98	98
p-Nitrocarbobenzoxyglycyldehydroalanine methyl ester (X)	98	97

(28) G. Toennies and R. P. Homiller, *J. Am. Chem. Soc.*, **64**, 3054 (1942).
(29) V. E. Price and J. P. Greenstein, *J. Biol. Chem.*, **171**, 477 (1947).
(30) P. Fleury, J. Courtois, and U. R. Perl'es, *Mikrochim. Acta*, **36/37**, 863 (1952).
(31) M. Errera and J. P. Greenstein, *J. Natl. Cancer Inst.*, **8**, 39 (1947)

Alternatively, in the absence of interfering groups, dehydroalanine residues can be titrated with bromine in acid solution. Excess of aqueous bromine is added at a pH lower than 6 and the bromine absorbed is estimated iodometrically.

An additional quantitative check on the presence of dehydroalanine residues is based on the determination of amide nitrogen formed on hydrolytic or oxidative cleavage.

Concluding Remarks.—Both the hydrolytic and oxidative scission of dehydroalanine residues proceed almost quantitatively. In the hydrolytic scission, heating at 100° for 1 hr. at pH 2 is the most convenient method. It seems that even peptide bonds of aspartic acid, which are known to be most susceptible to acid hydrolysis, are resistant under these conditions.[10] In cases where elevated temperature must be avoided, the oxidative cleavage is to be preferred as it can be carried out either at room temperature (with bromine) or at 0° (with performic acid). However, oxidation with performic acid is known to attack only tryptophyl residues, while bromination may cause cleavage of tryptophyl,[7] tyrosyl,[6] and histidyl[8] bonds.

Since cysteine and serine residues are potential sources of dehydroalanine residues, the techniques described in this paper may be applicable to the study of peptide and protein structures.[1c] In addition to the specific fragmentation of the molecule under investigation, information as to the identity of the amino acid residue attached to the carboxyl of the susceptible cysteine and serine residue may be obtained.

Experimental

All melting points are uncorrected. Prior to analysis the compounds were dried at 50° under high vacuum over phosphorus pentoxide.

Synthesis of Model Compounds.—The following compounds were prepared according to published procedures: benzyl carbamate, m.p. 87°[32]; carbobenzoxydehydroalanine (II), m.p. 109–110°[33]; glycyldehydroalanine, m.p. 199–200° dec.[17]; carbobenzoxyglycine amide, m.p. 137–138°[20]; *p*-nitrocarbobenzoxyglycine, m.p. 121°[34]; and pyruvoylglycine benzyl ester, m.p. 99–100°.[21] Neutral equivalents were determined by titration with 0.1 *N* sodium methoxide using thymol blue in ethanol as indicator.[35]

Carbobenzoxyglycyldehydroalanine (I).—Carbobenzoxychloride (1.7 g., 0.01 mole) was added to 1 g. of glycyldehydroalanine (0.007 mole) dissolved in 20 ml. of 1 *N* sodium hydroxide. The reaction mixture was stirred for 0.5 hr. at 0°. The reaction mixture was extracted twice with ether and once with petroleum ether and the pH was adjusted to pH 2 with dilute hydrochloric acid. The solid compound which separated out weighed 1.75 g. (90%). After recrystallization from ethanol, the melting point was 195–196° (reported[36] 187°).

Anal. Calcd. for $C_{13}H_{14}N_2O_5$: C, 56.11; H, 5.07; N, 10.07; neut. equiv., 278. Found: C, 56.03; H, 5.01; N, 10.06; neut. equiv., 276.

Carbobenzoxydehydroalanylglycine Ethyl Ester.—*N,N'*-Dicyclohexylcarbodiimide (2.05 g., 0.01 mole) was added to a solution of 2.21 g. of carbobenzoxydehydroalanine (0.01 mole), 1.4 g. of glycine ethyl ester hydrochloride, (0.01 mole), and 1.44 ml. of triethylamine (0.01 moles) in 50 ml. of ethyl acetate–chloroform (1:1) at −5°. The reaction mixture was stirred 1 hr. at −5° and then overnight at room temperature. 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 ether–petroleum ether yielding long white needles, 1.54 g. (50%), m.p. 83–84°.[15e]

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

Carbobenzoxydehydroalanylglycine (III).—Carbobenzoxydehydroalanylglycine ethyl ester (1.53 g., 5 mmoles), in a mixture of 20 ml. of methanol, 20 ml. of water, and 5.5 ml. of 1 *N* sodium hydroxide (5.5 mmoles), was allowed to stand for 30 min. at room temperature. The aqueous solution was then extracted with ethyl acetate to remove any unreacted starting material, and the pH was adjusted to 2 with dilute HCl. The yield of colorless needles of carbobenzoxydehydroalanylglycine, m.p. 104–105°,[15e] after drying over P_2O_5, was 700 mg. (50%).

Anal. Calcd. for $C_{13}H_{14}N_2O_5$: C, 56.11; 5.07; N, 10.07; neut. equiv., 278. Found: C, 56.15; H, 5.03; N, 10.10; neut. equiv., 277.

***p*-Nitrocarbobenzoxyglycyldehydroalanine** (V).—*p*-Nitrocarbobenzoxy chloride[34] (2.16 g., 0.01 mole) dissolved in 6 ml. of dioxane was added to 1 g. of glycyldehydroalanine (0.007 mole) dissolved in 20 ml. of 1 *N* sodium hydroxide. The reaction mixture was stirred for 1 hr. at 0° and washed twice with ether and once with petroleum ether; the pH was adjusted to 2 with hydrochloric acid. The white crystals which separated out weighed 2.05 g. (90%); after recrystallization from ethanol, m.p. 197–198° dec.

Anal. Calcd. for $C_{13}H_{13}N_3O_7$: C, 48.30; H, 4.05; N, 13.00; neut. equiv., 323. Found: C, 48.42; H, 4.16; N, 12.94; neut. equiv., 324.

***p*-Nitrocarbobenzoxyglycine Methyl Ester.**—A solution of 2.54 g. (0.01 mole) of *p*-nitrocarbobenzoxyglycine in 50 ml. of absolute methanol and 5 ml. of concentrated sulfuric acid was heated to 60° for 2 min. Upon cooling, 2.5 g. (93%) of long needles of the ester separated. After recrystallizing from methanol, the compound melted at 121°.

Anal. Calcd. for $C_{11}H_{12}N_2O_6$: C, 49.25; H, 4.51; N, 10.45. Found: C, 49.31; H, 4.46; N, 10.61.

***p*-Nitrocarbobenzoxyglycine Amide** (VI).—A solution of 2.68 g. (0.01 mole) of *p*-nitrocarbobenzoxyglycine methyl ester in 25 ml. of dioxane was treated with 20 ml. of concentrated ammonia for 48 hr. The amide was precipitated upon distilling part of the solvent *in vacuo*. The yield after recrystallization from dioxane was 2 g. (78%), m.p. 198–199° (reported[34] 196–197°).

Anal. Calcd. for $C_{10}H_{11}N_3O_5$: C, 47.43; H, 4.38; N, 16.60. Found: C, 47.27; H, 4.34; N, 16.66.

***p*-Nitrocarbobenzoxyglycyldehydroalanine Methyl Ester** (X).—*p*-Nitrocarbobenzoxyglycyldehydroalanine (1.74 g., 0.0054 mole) dissolved in 15 ml. of dimethylformamide was esterified with an excess of diazomethane prepared from 4 g. of nitroso-N-methylurethan[37] dissolved in ether. After 20 min., the ether was distilled off and, on addition of water, white crystals formed. The yield was 1.64 g. (90%) and the material melted at 141°. By recrystallizing from ethanol the melting point was raised to 143–144°.

Anal. Calcd. for $C_{14}H_{15}N_3O_7$: C, 49.85; H, 4.48; N, 12.46; OCH_3, 9.19. Found: C, 49.63; H, 4.52; N, 12.40; OCH_3, 9.07.

Ultraviolet absorption spectra measurements were performed with an automatic recording Beckman DK 1 spectrophotometer.

Identification and Determination of Pyruvic Acid.—The pyruvic acid formed as a result of hydrolytic cleavage was isolated as the 2,4-dinitrophenylhydrazone, m.p. 218°.[18] For determination of small amounts of pyruvic acid, the enzymatic method[19] using lactic dehydrogenase and DPNH was used.

Spectrophotometric Determination of the *p*-Nitrobenzyl Group.—*p*-Nitrobenzyl compounds were estimated spectrophotometrically in ethyl acetate at 270 mμ. Authentic samples of *p*-nitrobenzyl alcohol, *p*-nitrocarbobenzoxyglycine amide, and *p*-nitrocarbobenzoxyglycine showed an extinction coefficient of ϵ 10,950 ± 200 in this solvent. These compounds gave ϵ 9550 in 95% ethanol at 270 mμ (reported[38] ϵ 9500).

Titration of Dehydroalanyl Residues with Iodine.—To an aqueous solution (pH 1–6) of a dehydroalanine derivative in which the dehydroalanine residue is C-terminal (0.1 mmole/20 ml.), excess of iodine was added, and the mixture back-titrated immediately with 0.02 *N* sodium thiosulfate. Calculations were based on the assumption that 1 mole of iodine reacts with 1 mole of dehydroalanine residue.

Titration of Dehydroalanyl Residues with Bromine.—To a solution of a dehydroalanine compound (0.1 mmole/20 ml.) dissolved in water or in dilute acetic acid, excess of aqueous bromine was added. After the addition of potassium iodide (5 ml. of a 10% solution) the liberated iodine was back-titrated with 0.02 *N* sodium thiosulfate. Calculations were based on the assumption that 1 mole of bromine reacts with 1 mole of dehydroalanine residue.

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

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apparatus described by Dreyer, *et al.*[39] Compounds with free amino groups were detected by developing with 0.5% ninhydrin in acetone.[40] 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.

Hydrolytic Cleavage.—(a) Carbobenzoxyglycyldehydroalanine (I, 55.6 mg., 0.2 mmole) was refluxed in 50 ml. of water. The compound went into solution after about 2 min. Aliquots of 5 ml. were withdrawn and titrated with 0.01 *N* iodine as described above, in order to determine the amount of unreacted compound. Aliquots of 1 ml. were tested after appropriate dilution for pyruvic acid, by means of lactic dehydrogenase. The course of hydrolysis is shown in Fig. 1.

In a parallel experiment, carbobenzoxyglycyldehydroalanine (I, 139 mg., 0.5 mmole), was refluxed in 50 ml. of water. After 1 hr., the solution was concentrated to a volume of 5 ml., and the pH was adjusted to 8 with 1 *N* potassium bicarbonate. The resultant solution was extracted with three 15-ml. portions of ethyl acetate and the combined extracts were washed twice with 15 ml. of 0.1 *N* potassium bicarbonate, dried over Na_2SO_4, and concentrated *in vacuo* to about 2 ml. On addition of petroleum ether, carbobenzoxyglycine amide, m.p. 136–137°,[20] was obtained as a colorless precipitate.

To the aqueous phase from the ethyl acetate extraction, 1 ml. of a solution of 158 mg. of dinitrophenylhydrazine (0.8 mmole) in 2 *N* hydrochloric acid was added. The precipitate was separated by centrifugation and recrystallized from ethyl acetate–petroleum ether; m.p. 218–219°.[18] The m.p. was not depressed on admixture with an authentic sample of the 2,4-dinitrophenylhydrazone of pyruvic acid.

(b) Carbobenzoxydehydroalanine (II, 221 mg., 1 mmole) in 10 ml. of water refluxed for 1 hr. yielded benzyl carbamate (136 g., 0.9 mmole), m.p. 87°.[32] The m.p. was not depressed on admixture with an authentic sample. The yield of pyruvic acid was found to be 0.97 mmole (97%), as determined enzymatically.

(c) Carbobenzoxydehydroalanylglycine (III, 55.6 mg., 0.2 mmole), was refluxed in dilute hydrochloric acid, pH 2.2 (50 ml.) for 1 hr. Aliquots of 5 ml. were withdrawn and titrated with 0.01 *N* bromine in order to determine the amount of unreacted compound. Results are shown in Fig. 1. After 1 hr., an aliquot of 10 ml. was concentrated to a volume of 1 ml. and aliquots of 10 and 20 λ were chromatographed on paper using the system butanol–formic acid–water (75:15:10 v./v.).[21] The product, pyruvoylglycine, was detected by spraying with a solution of sodium nitroprusside in an ammonia atmosphere and identified as a blue spot by comparison with an authentic marker.

Oxidation with Iodine.—*p*-Nitrocarbobenzoxyglycyldehydroalanine (V, 32.3 mg., 0.1 mmole) was dissolved in 2 ml. of 0.1 *N* sodium hydroxide and this solution diluted with water to 30 ml. The solution was acidified with 2.5 ml. of 0.1 *N* hydrochloric acid, and 5 ml. of 0.05 *N* iodine was added. The excess of iodine was back-titrated with 0.05 *N* sodium thiosulfate using starch as the indicator, showing that 4.0 ml. of iodine solution had been consumed. The solution was brought to pH 8 with 0.1 *N* potassium bicarbonate, and the *p*-nitrocarbobenzoxyglycine amide (VI) formed was separated by extracting with three 50-ml. portions of ethyl acetate. The combined extracts were washed twice with 30 ml. of 0.1 *N* potassium bicarbonate, twice with water, and dried over Na_2SO_4. The organic phase was diluted with ethyl acetate to 1000 ml. and its absorbancy at 270 mμ was 1.01 which is equivalent to 0.096 mmole (96%) of *p*-nitrocarbobenzoxyglycine amide. In another experiment, the ethyl acetate solution was concentrated *in vacuo* and the solid residue was recrystallized from dioxane, m.p. 196–197°.

The iodide formed on oxidation with iodine was determined in a separate experiment by titration with silver nitrate as follows: iodine (25.4 mg., 0.1 mmole) in 2 ml. of acetic acid was added to 50 ml. of an aqueous solution of carbobenzoxyglycyldehydroalanine (I, 27.8 mg., 0.1 mmole). The reaction mixture was shaken for 5 min. and titrated with 0.02 *N* silver nitrate with eosine as indicator.[41] Silver nitrate, 5.4 ml., was consumed, equivalent to 0.108 mmole of iodide formed.

To a 25-ml. aqueous solution of I (27.8 mg., 0.1 mmole) containing 12.0 ml. of 0.05 *N* aqueous iodine, 7 ml. of 0.1 *N* sodium hydroxide was added. The iodoform, which precipitated immediately, was extracted with three 50-ml. portions of ethyl acetate; the combined extracts were washed twice with water and dried over Na_2SO_4. The yield of iodoform was 38.1 mg. (0.097 mmole) as determined spectrophotometrically.[23] The ethyl acetate solution was concentrated *in vacuo* and the solid residue crystallized from methanol; m.p. 118°, not depressed on admixture with an authentic sample.

The aqueous solution was concentrated to a volume of 4 ml. and the pH was brought to 4.2 with dilute acetic acid. Calcium bromide (44 mg., 0.2 mmole) in 2 ml. of water was added to the warm reaction mixture. On cooling, calcium oxalate appeared as a white precipitate. After centrifugation, it was washed three times with water, dissolved in 2 *N* sulfuric acid, and titrated with 0.025 *N* potassium permanganate at 60° showing that 0.098 mmole (98%) of oxalic acid was formed in the alkaline oxidation. The above experiment was repeated with 32.3 mg. of V (0.1 mmole) in 50 ml. of water–methanol (3:2 v./v.); 3.95 ml. of iodine solution (0.05 *N*) had been consumed. The amide VI formed (0.048 mmole, 48%) was separated as described above.

In a parallel experiment, 32.3 mg. of V (0.1 mmole) dissolved in 50 ml. of water–methanol (3:2 v/v.) containing 7 ml. of 0.1 *N* sodium hydroxide was treated with 78.7 mg. of iodine (0.031 mmole) dissolved in 2 ml. of methanol–water solution (3:2 v./v.). The iodoform and amide VI which formed were extracted with 100 ml. of ethyl acetate and their quantity was estimated spectrophotometrically. The yield of the iodoform and amide VI was found to be 0.045 mmole (45%) and 0.048 mmole (48%), respectively.

Oxidation with Bromine.—*p*-Nitrocarbobenzoxyglycyldehydroalanine methyl ester (X, 33.7 mg., 0.1 mmole) was dissolved in 10 ml. of acetic acid, and 3.95 ml. of 0.05 *N* of aqueous bromine (0.098 mmole) was added. The pH was adjusted to 10.9, and the reaction mixture was incubated for 10 min. at room temperature and extracted with two 50-ml. portions of ethyl acetate. Evaporation to dryness of the extracts yielded *p*-nitrocarbobenzoxyglycine amide, m.p. 196°.[34] The amount of this amide was estimated by measuring the extinction at 270 mμ of the ethyl acetate solution before evaporation and was found to be 23.8 mg. (0.094 mmole).

A solution of 0.2 μmole of γ-L-glutamyldehydroalanylglycine[25] was treated with 0.2 μmole of aqueous bromine for 3 min. The pH of the reaction mixture was adjusted to 10.9 and incubated for 10 min. An aliquot, analyzed by paper electrophoresis, gave only one ninhydrin-positive spot which moved with the same rate as an authentic sample of L-glutamine. The product was obtained in a yield of 0.178 μmole (89%).

Oxidation with Performic Acid.—The performic acid used was prepared according to Hirs,[26] by incubating 9.5 ml. of 99% formic acid and 0.5 ml. of 30% hydrogen peroxide for 2 hr. at room temperature. The concentration of the performic acid was estimated iodometrically.[28] (a) Carbobenzoxydehydroalanylglycine (III) (27.8 mg., 0.1 mmole) was dissolved in 6 ml. of performic acid at 0° and kept at this temperature. The course of oxidation was determined by titration of the unreacted performic acid in aliquots of 0.6 ml. (see Fig. 2).

In a parallel experiment carbobenzoxydehydroalanylglycine (III) (13.9 mg., 0.05 mmole) was dissolved in 1 ml. of performic acid at 0°. Aliquots of 0.1 ml. were withdrawn and diluted to 25 ml. in order to determine spectrophotometrically the amount of unreacted compound. The absorption at 240 mμ[29] was measured, and it was found that on consumption of 1 mole of performic acid per mole of compound, the absorption had practically disappeared (see Fig. 2).

(b) *p*-Nitrocarbobenzoxyglycyldehydroalanine (V, 32.3 mg., 0.1 mmole) was dissolved in 1 ml. of formic acid by heating to 70° for 1 min. The solution was cooled to 0° and 0.6 ml. of performic acid was added. The reaction mixture was incubated for 2 hr. at 0° and lyophilyzed. The residue was dissolved in 10 ml. of water and the pH of the solution was adjusted to 11 with 0.1 *N* sodium hydroxide. The *p*-nitrocarbobenzoxyglycine amide formed was extracted with 100 ml. of ethyl acetate and estimated as described above. A maximum yield of this product, 23.3 mg. (92%), was obtained after incubation for 20 min. in the alkaline medium at room temperature.

Similarly, *p*-nitrocarbobenzoxyglycyldehydroalanine methyl ester (X, 33.7 mg., 0.1 mmole) yielded *p*-nitrocarbobenzoxyglycine amide, 23.8 mg. (94%); carbobenzoxydehydroalanylglycine (III), 278 mg. (1 mmole), yielded benzylcarbamate, 134 mg. (89%).

Oxidative Removal of the Pyruvoyl or Hydroxypyruvoyl Group with the Liberation of the New Terminal Amino Acid. (a) With Alkaline Hydrogen Peroxide.—Carbobenzoxydehydroalanylglycine (III, 27.8 mg., 0.1 mmole) was cleaved according to any of the methods described above, and the reaction mixture was treated with 1 ml. of 30% hydrogen peroxide. Sodium hydroxide was then added to a final concentration of 0.1 *N*; the solution was incubated for 45 min. at 37°. The pH of the solution was adjusted to 8 with dilute acetic acid and excess of hydrogen peroxide was destroyed by incubation with catalase (0.03 ml. of 5% solution). The final volume was 10 ml. On paper electrophoresis (2 hr.) of an aliquot, glycine in a yield of 53% was detected. To 0.5 ml. of the above solution obtained with one of the oxidative methods, 0.5 ml. of 5% 1,8-dihydroxynaphthalene-3,6-disulfonic acid disodium salt (chromotropic acid) in water and 5 ml. of concentrated sulfuric acid was added and the mixture was warmed at 100° for 20 min. The violet color produced was compared with that obtained with a standard solution of

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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.

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[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]

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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

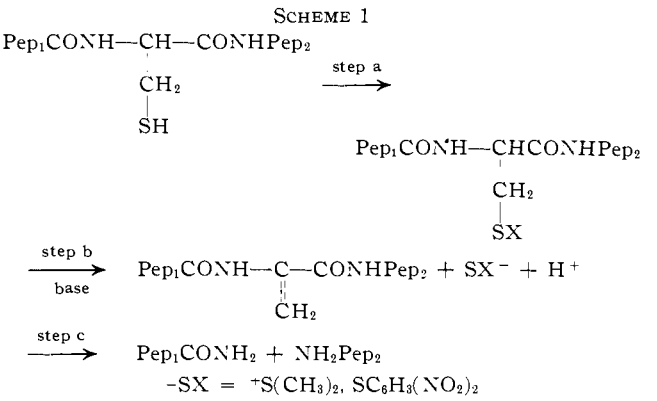
Cysteine residues play an important role in the biologically active site of a number of enzymes.[3] Moreover, disulfide bridges between two half-cysteines are often of crucial importance in keeping the protein molecule in its biologically active conformation. No direct enzymatic methods are as yet available 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

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