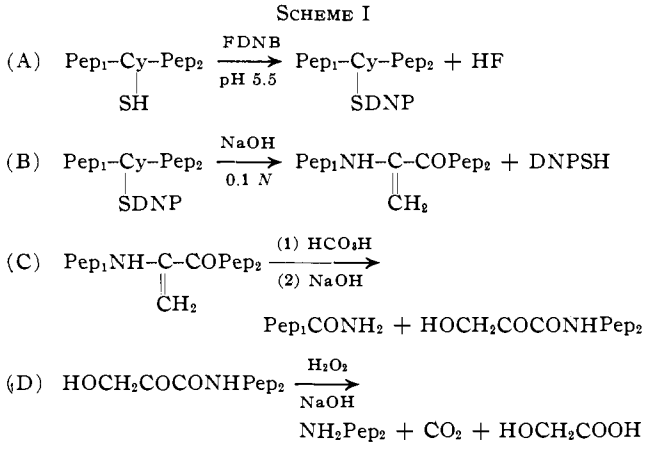
Nonenzymatic Cleavage of Peptide Bonds: The Half-cystine Residues in Bovine Pancreatic Ribonuclease[1]

Sir:

We wish to report the application of our nonenzymatic method for fission of cysteinyl peptide bonds[2] (Scheme 1) to bovine pancreatic ribonuclease, which in a chain of 124 amino acids contains eight cysteines. According to the published formula,[3] the cleavage of the peptide bonds adjacent to cysteine residues should release the following N-terminal residues[4]; asparagine–[27], lysine–[41], serine–[59], lysine–[66], tyrosine–[73], arginine–[85], alanine–[96], and glutamic acid–[111]. We were able to demonstrate the liberation of the above N-terminal amino acids with the exception of tyrosine–[73], instead of which we found glycine.

The reaction scheme for the cleavage of cysteinyl peptides is as follows[5]

SCHEME I



In model peptides, reactions A–C are almost quantitative, while reaction D proceeds generally with yields of 50–60%.[2]

Purified fully reduced RNase[6,7] was dinitrophenylated at pH 5.5 at room temperature, under which conditions the thiol groups are selectively dinitrophenylated.[2] The S-dinitrophenylated RNase (SDNP RNase, I) was separated from reagents by precipitation with acid acetone (1 ml. of 5 *N* H_2SO_4 per 100 ml. of acetone) and was acetylated with excess acetic anhydride at pH 9. The acetylated product, II, was precipitated with acid acetone and washed twice with distilled water to free the protein of salts. The acetylation was necessary to avoid side reactions.[8]

Analysis by the Van Slyke method revealed the existence of not more than 0.3 equiv. of free amino groups per mole of RNase (RNase contains 11 NH_2 groups). On treatment of II with 0.1 *N* sodium hydroxide at room temperature, for 30 min., the β-elimination reaction (step B) occurred with quantitative liberation of thiodinitrophenolate ion which was determined spectrophotometrically.[2,9] The resulting modified RNase (III) was purified by gel filtration on a Sephadex G-25 column developed with 0.1 *N* acetic acid, and the amount of dehydroalanine present in this derivative was determined by estimation of the pyruvic acid formed upon acid hydrolysis.[10] It was found to contain 7.6 dehydroalanine residues (in place of the eight cysteinyl residues originally present).

Compound III was oxidized with performic acid at 0° for 2 hr., lyophilized, and treated with hydrogen peroxide in 0.1 *N* sodium hydroxide at room temperature for 45 min. (steps C and D).

The end groups present after cleavage were determined by three methods: (a) dinitrophenylation and determination of the dinitrophenyl derivatives[11] after acid hydrolysis; (b) conversion of the dinitrophenyl derivatives obtained by method (a) into free amino acids with concentrated ammonia; (c) the cyanate method,[12] in which the terminal amino acids are converted *via* the corresponding hydantoins to free amino acids. Methods a and b were used only for determination of ratios of the terminal amino acids present and quantitative estimations were based on method c. Average results of repeated end-group determinations are shown in Table I. The yields of N-terminal groups released are of the order of 30–40%, apparently because step D of the cleavage procedure occurs with greater difficulty in proteins than in model compounds (where 50–60% over-all yields were obtained[2]).

TABLE I

YIELDS OF CLEAVAGE OF MODIFIED RNase[a]

	Mole/mole of III	Mole/mole of IV	Mole/mole of V
Lysine	0.95	...	
Arginine[b]	0.15	...	
Aspartic acid	0.34	0.03	0.96[d]
Serine	0.42	0.04	0.70
Threonine	0.03	0.03	..
Glutamic acid	0.52	0.04	1.2[e]
Proline	0.02	0.03	..
Alanine[c]	0.24	0.05	0.76
Glycine[c]	0.36	0.05	0.30
Tyrosine	...	...	...

[a] Quantitative amino acid analyses were performed on a Spinco automatic acid analyzer, by the method of Moore, Spackman and Stein.[13] [b] Arginine was found to give low yields by the cyanate method as found with arginine amide. [c] Values corrected for the nonspecific formation of additional quantities of these amino acids in the cyanate method.[12] [d] This value corresponds to a yield of 48% cleavage for each of 2 aspartic acid residues, [27] and [34]. [e] This value corresponds to a yield of 40% cleavage for each of 3 glutamic acid residues, [9], [86], and [111].

As a control experiment, we carried out the above procedure (steps B–D) with acetylated oxidized RNase, IV (Table I), in which no cleavage should be expected. The results obtained show the selectivity of the procedure of chemical cleavage at cysteine residues.

(1) This investigation was supported by Research Grant No. AM-5098 from the National Institutes of Health, U. S. Public Health Service.

(2) A. Patchornik and M. Sokolovsky, Fifth European Peptide Symposium, Oxford, England, 1962, Pergamon Press, New York, N. Y., 1963, p. 253; M. Sokolovsky, T. Sadeh, and A. Patchornik, *J. Am. Chem. Soc.*, **86**, 1212 (1964).

(3) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 227 (1963).

(4) The numbers in brackets denote the position of the residue in the peptide chain.

(5) The abbreviations used are FCNB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; Pep, peptide chain.

(6) Ribonuclease 5X crystallized, Sigma Chemical Company Lot No.: R-111B-240, R-12B-850, R-92B-205, R-23B-56.

(7) E. Haber and C. B. Anfinsen, *J. Biol. Chem.*, **237**, 1839 (1962).

(8) A. Patchornik and M. Sokolovsky, *J. Am. Chem. Soc.*, **86**, 1860 (1964).

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

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

(11) A. L. Levy, *Nature*, **174**, 126 (1954).

(12) G. H. Stark and D. G. Smyth, *J. Biol. Chem.*, **238**, 214 (1963).

(13) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1190 (1958).

The formation of N-terminal alanine is in agreement with the latest results of Smyth, Stein, and Moore.[3] However, no traces of N-terminal tyrosine could be detected in the cleavage products. The fact that in oxytocin the cysteinyl–tyrosine bond is cleaved in a 40% yield[2] demonstrates that such sequences can be detected by our procedure. The present studies suggest the presence in ribonuclease of the sequence (Cys–Gly), possibly derived from positions 72 and 73 in the polypeptide chain.

Supporting evidence for the suggested sequence was obtained by tryptic digestion[14] of acetylated S-(β-aminoethyl)RNase (V). End-group determination by the cyanate method gave all the amino acid residues following cysteine and arginine in the published formula, except for the appearance of glycine instead of tyrosine.

In view of the above findings, we are now engaged in the determination of the origin of the apparent sequence Cys–Gly in order to elucidate whether it corresponds to positions 72–73 in the ribonuclease polypeptide chain or whether it is the product of a side reaction.

Acknowledgments.—We thank Professor E. Katchalski and Professor A. Berger for their interest in this work.

(14) M. A. Rasterg and D. Cole, *Biochem. Biophys. Res. Commun.*, **10**, 467 (1963).

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Chemical Interactions between Lysine and Dehydroalanine in Modified Bovine Pancreatic Ribonuclease[1]

Sir:

When S-dinitrophenylated reduced RNase (I)[2] was treated with alkali, and the derivative thus obtained (II) subjected to acid hydrolysis, a new amino acid could be separated from the amino acids normally present in RNase, on the short column of the Spinco automatic amino acid analyzer[3] using citrate buffer (pH 5.28). This amino acid emerged before lysine as shown in Fig. 1.

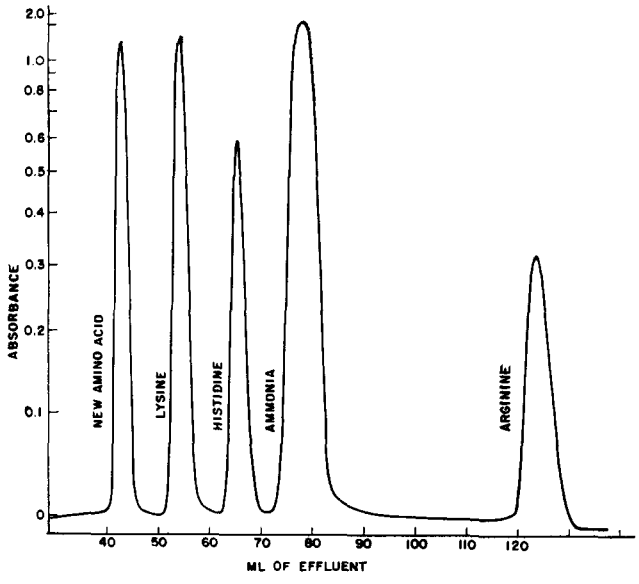
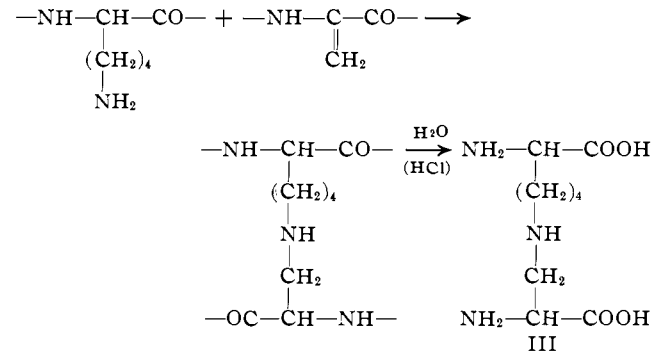


Fig. 1.—Peak position of the new amino acid on a 15-cm. ion-exchange column using citrate buffer (pH 5.28) on a Spinco automatic amino acid analyzer.

We propose that this amino acid[4] (III) is formed as a result of the reaction of ε-amino groups of lysine residues with dehydroalanine residues leading to DL-α-amino-β(-ε-N-L-lysine)propionic acid[5] according to the following scheme.



The following evidence is offered to support this proposal. When I was treated with alkali (0.1 *N* sodium hydroxide, 30 min. at room temperature), 3 moles of the new amino acid was formed on acid hydrolysis (assuming a ninhydrin color yield of twice that of leucine), and only 6.5 moles of lysine (out of the 10 moles initially present) and 5.5 moles of dehydroalanine residues (out of the 8 moles expected) were found.[6] However, fully acetylated S-DNP RNase[7] (IV), in which all the ε-amino groups of lysine residues are blocked, did not yield the new amino acid under the above conditions. Moreover, practically all the expected lysine residues (9.4 out of 10) and dehydroalanine residues (7.6 out of 8) were determined after acid hydrolysis.

It thus seems that 3 moles of the unprotected lysine residues reacted with 3 moles of dehydroalanine residues to yield 3 moles of the new amino acid.

The above reaction was also carried out on model compounds. N-Acetyl-S-DNP-L-cysteinyl-L-lysine methyl ester hydrobromide,[8] as well as a copolymer of S-DNP-cysteine and lysine (1:4),[8] yielded the new amino acid on treatment with alkali. The product obtained behaved in the automatic amino acid analyzer identically with that obtained from I, and its electrophoretic behavior was found to be as expected on the basis of the proposed structure.

The new amino acid (LAL) was also formed when S-DNP lysozyme and S-DNP cytochrome C were exposed to 0.1 *N* NaOH and totally hydrolyzed. Native RNase under these conditions also yielded LAL (see

(1) This investigation was supported by Research Grant AM-5098 from the National Institutes of Health, U. S. Public Health Service.

(2) M. Sokolovsky and A. Patchornik, *J. Am. Chem. Soc.*, **86**, 1859 (1964).

(3) Quantitative amino acid analyses were performed on a Spinco automatic amino acid analyzer.

(4) The same amino acid seems to have been isolated from alkali treated RNase by Z. Bohak, who also established its structure [private communication, Zvi Bohak at the Rockefeller Institute, on leave from the Hebrew University, Jerusalem.]

(5) We wish to propose the name Lysalanine (abbreviated LAL) for the new amino acid.

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

(7) The abbreviation used is DNP, 2,4-dinitrophenyl.

(8) M. Sokolovsky, Ph.D. Thesis submitted to the Hebrew University, Jerusalem, Israel, 1963.