The crude product was acidified, lyophilized, and desalted on Sephadex G-25 in 0.1 *M* acetic acid. It was then fractionated on IRC-50 in 0.2 *M* sodium phosphate buffer, pH 6.47.[11] Five fractions were detected at 280 mμ. The largest one, fraction I, eluted at the same volume as reduced and reoxidized natural RNase. It was desalted on Sephadex G-25 in 0.1 *M* acetic acid and was lyophilized (yield, 85 mg). This synthetic ribonuclease was indistinguishable from the native enzyme by paper electrophoresis at pH 2.5 (R_{His} 0.58). Peptide maps from tryptic digests[12] of performic acid oxidized samples showed the 14 expected[12] ninhydrin-positive spots in the same relative positions as the natural ribonuclease control. There was one small additional spot in the synthetic preparation near the position of free lysine. Amino acid analyses of acid hydrolysates compared well with those of natural RNase which had been treated with HF and TFA (Table I). Enzymic digestion (papain followed by aminopeptidase M)[13] was complete. It also showed that 79% of the methionine sulfoxide residues had been converted to methionine during the mercaptoethanol reduction of the RNase$(SSO_3^-)_8$. The remainder was recovered as methionine sulfone.

The synthetic enzyme (fraction I) showed a specific activity of 13% by two methods[14,15] with yeast RNA as substrate, and 24% with 2′,3′-cyclic cytidine phosphate[16] as substrate when compared with pure natural bovine pancreatic ribonuclease A. Fraction II contained some activity, but the other fractions were inactive. The synthetic enzyme was completely inert toward DNA, 2′,3′-cyclic guanosine phosphate, or 5′-(3′-guanylyl)cytidylic acid (GpCp)[17] under conditions where DNase[18] or RNase T_1[19] were active and also was without effect on 5′-(3′-adenylyl)adenylic acid (ApAp), demonstrating the high substrate specificity to be expected of RNase A. A further indication that the synthetic material contained the same active enzyme species as natural RNase was obtained from the Michaelis constant. Initial velocities were measured spectrophotometrically[14] and the K_m values, calculated from Eadie plots, were found to be 2.4 mg/ml for natural RNase and 2.5 mg/ml for the synthetic product. These results provide direct evidence for the hypothesis[10a,20] that the linear amino acid sequence of a protein contains all the information necessary to direct the formation of an active enzyme.

Although the physical and chemical methods so far applied to the synthetic protein indicate a good degree of homogeneity, the presence of very closely related molecules cannot be excluded, and the failure to obtain a fully active enzyme means that our product is not yet pure. Assembly of the 124 amino acid residues into the protected, resin-bound straight-chain precursor of RNase required 369 chemical reactions and 11,931 steps of the automated peptide synthesis machine without any intermediate isolation steps. Deficiencies in these peptide-forming reactions, in the work-up conditions, and in the final oxidation and refolding of the protein probably all contributed to the decreased activity, but an assessment of the relative effects of each step much await the completion of further work.

These experiments demonstrate for the first time that a protein molecule with true enzymic activity toward its natural substrate can be totally synthesized from the component amino acids.

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Studies on the Total Synthesis of an Enzyme. I. Objective and Strategy

Sir:

The remarkable advances in polypeptide synthesis during the past 15 years have made the synthesis of an enzyme a feasible objective. From a number of considerations the synthesis of RNase S′ appeared to be an attractive goal.

The studies of Richards[1] and his collaborators have shown that RNase A may be cleaved at a single bond without impairment of enzymatic activity to produce RNase S, which may be separated into a tetrahectapeptide (S-protein) and an eicosapeptide (S-peptide). Recombination of these two enzymatically inactive fragments in equimolar ratio restored full enzymatic activity (RNase S′).

Because the work of Smyth, Stein, and Moore[2] had established the complete primary structure of RNase A, the amino acid sequence of S-protein is thus known. Furthermore, since S-peptide had been synthesized by Hofmann, Smithers, and Finn,[3] S-protein, a tetrahectapeptide, appeared to be the smallest protein whose synthesis would constitute a total synthesis of an enzyme. Finally the observation by Haber and Anfinsen[4] that the oxidation of reduced S-protein (eight cysteine residues) regenerates enzymatic activity when the resulting protein is assayed in the presence of S-peptide allowed these workers to infer that "information determining secondary and tertiary structure of RNase" is contained in the amino acid sequence of S-protein.

The molecular weight of S-protein and especially the

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presence of both eight cysteine and three methionine residues in the molecule argued against the use of a strategy which entails catalytic hydrogenation for the removal of protecting groups. Furthermore our investigations and those of Sarid and Patchornik[6] made unattractive the use of sodium–liquid ammonia to remove the frequently used S-benzyl blocking group for cysteine. For the protection of the sulfhydryls of the eight cysteines, we had developed[6] the acetamidomethyl blocking group which is stable, *e.g.*, to trifluoroacetic acid at 25°, to anhydrous HF at 0°, and to hydrazine and which may be selectively removed with Hg(II) under mild conditions. Moreover, use of the NCA's[7] of arginine and glutamic and aspartic acids and the NTA of histidine[8] in peptide bond formation permitted the development of a strategy[9] in which the third functionality needed to be protected only for the amino acids lysine and cysteine. Furthermore, the recent reports[10] that the benzyloxycarbonyl protecting group may be smoothly removed with liquid HF, in conjunction with fact that we had found S-protein to be stable in this solvent at 0°, led us to select this protecting group for the ε-amino function of lysine. This choice of the "permanent" protecting groups permitted the use of the butyloxycarbonyl group as the acid-labile, temporary blocking group of the growing peptide chains. In addition this combination of protecting groups enabled us to remove all of the N-blocking groups of the tetrahectapeptide while leaving the cysteines protected. The formation of the four disulfide bridges subsequent to the liberation of the ε-amino groups of the eight lysine residues was considered to be a desirable feature of our strategy. Finally the synthesis of this tetrahectapeptide—with only the sulfhydryl groups protected—from natural S-protein appeared feasible. Indeed the availability of the acetamidomethylated reduced natural S-protein enabled us to work out conditions for the final steps before committing any of our synthetic intermediates.

To synthesize S-protein we relied on the fragment condensation method. A total of 19 fragments were prepared. About 40% of the bonds in the peptide fragments were formed through the use of NCA's[11] and NTA's,[8] the remainder, with the Boc-hydroxysuccinimide esters of Anderson.[12] The latter were routinely employed for the incorporation of the amino-terminal amino acid of all fragments and also for the introductions of asparagine, serine, or threonine.

To permit the use of the unprotected ω-carboxy groups of aspartic and glutamic acids, we relied on the azide method to couple fragments. As a consequence, the carboxy-terminal amino acids of all fragments but one had to be converted to esters prior to the introdution of dibasic acids.[9] Those esters then served as precursors of the hydrazides. In this manner it was possible to synthesize fragments 65–124 and 21–64.

The preparation of these protected hexaconta- and tetratetracontapeptides and their coupling will be described in separate communications.

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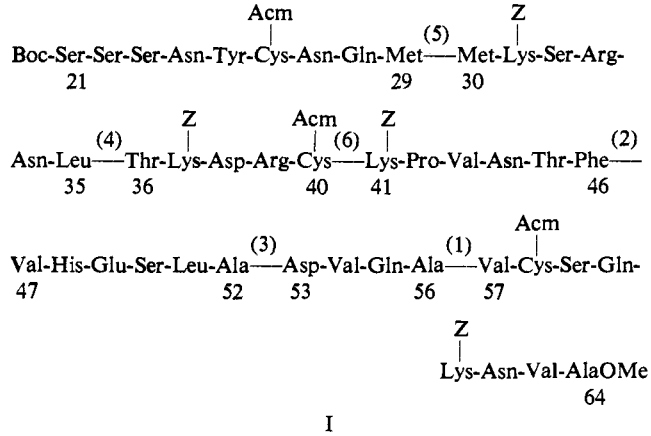
Studies on the Total Synthesis of an Enzyme. II. Synthesis of a Protected Tetratetracontapeptide Corresponding to the 21–64 Sequence of Ribonuclease A

Sir:

This communication describes the preparation of the protected tetratetracontapeptide fragment 21–64 (I) of RNase A. The fragments were prepared by the



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general scheme outlined in the preceding communication.[1] The azide couplings of the fragments were performed in the order indicated by the numerals in parentheses above the appropriate bonds in I. The azide components, blocked at the amino-terminal end with the Boc protecting group, were prepared and allowed to react *in situ* under anhydrous conditions,[2] at temperatures ranging from −40 to +5°. Stabilities of the azide components and reactivities of the nucleophiles determined the choice of reaction temperatures. Removal of the Boc protecting groups was carried out either with anhydrous HCl in ethyl acetate at 0° or with anhydrous trifluoroacetic acid at room temperature.

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