Table I. Amino Acid Analyses of Fragments

Fragment[a]	Spinco amino acid analysis
H(118–124)OH	$Val_{2.00}His_{0.98}Phe_{0.98}Asp_{1.00}Ala_{1.00}Ser_{0.99}$
H(118–124)OH[b]	$Val_{2.00}His_{0.99}Phe_{0.98}Asp_{0.98}Ala_{0.99}Ser_{0.97}$
Boc(113–117)$NHNH_2$	$Asp_{1.05}Pro_{2.04}Tyr_{0.98}Val_{0.97}$
Boc(113–124)OH	$Asp_{2.11}Pro_{1.99}Tyr_{0.99}Val_{2.97}His_{0.94}Phe_{1.02}Ala_{1.02}Ser_{0.97}$
Boc(110–112)OEt	$Glu_{1.01}Gly_{1.00}$
Boc(110–124)OH	$^1/_2$-$Cys_{0.83}Glu_{1.13}Gly_{1.06}Asp_{2.02}Pro_{2.08}Tyr_{0.99}Val_{2.82}His_{0.90}Phe_{0.94}Ala_{0.94}Ser_{1.04}$
Boc(103–109)OMe[c]	$Asp_{0.99}Lys_{0.99}His_{0.99}Ile_{1.95}$[d]$Val_{1.01}Ala_{1.00}$
Boc(103–124)OH	$Asp_{3.16}Lys_{0.97}His_{1.86}Ile_{1.09}Val_{4.11}Ala_{2.06}$ $^1/_2$-$Cys_{0.75}Glu_{0.97}Gly_{0.94}Pro_{2.07}Tyr_{0.99}Phe_{0.96}Ser_{0.98}$
Boc(103–124)OH[k]	$Asp_{3.04}Lys_{0.98}His_{2.00}Ile_{1.94}Val_{4.04}Ala_{2.05}$ $^1/_2$-$Cys_{0.19}Glu_{0.97}Gly_{0.66}Pro_{2.26}Tyr_{0.98}Phe_{1.01}Ser_{0.66}$
Boc(97–102)OMe	$Tyr_{0.98}Lys_{1.00}Thr_{1.99}Glu_{1.03}Ala_{1.01}$
Boc(91–96)OMe	$Lys_{0.98}Tyr_{0.98}Pro_{1.85}$[e]$Asn_{1.05}Ala_{1.01}$
	Acm
	|
H(91–96)OMe[f,g]	$Lys_{1.08}Tyr_{0.98}Pro_{1.04}Asn_{0.84}$[i]$Cys_{1.08}$[h,i]$Ala_{0.99}$
Boc(91–96)$NHNH_2$	$Lys_{1.00}Tyr_{0.99}Pro_{1.22}$[e]$Asp_{1.00}$ $^1/_2$-$Cys_{0.72}Ala_{1.01}$
Boc(91–102)OMe	$Lys_{2.02}Tyr_{2.02}Pro_{1.02}Asp_{1.04}$ $^1/_2$-$Cys_{0.95}Ala_{1.96}Thr_{1.96}Glu_{1.02}$
Boc(86–90)$NHNH_2$	$Glu_{1.02}Thr_{1.03}Gly_{1.00}Ser_{1.95}$
Boc(86–102)$NHNH_2$	$Glu_{1.97}Thr_{3.03}Gly_{0.97}Ser_{2.02}Lys_{1.97}Tyr_{0.93}Pro_{0.88}Asp_{1.01}Ala_{2.02}$
Boc(86–124)OH	$Glu_{3.10}Thr_{3.15}Gly_{2.00}Ser_{3.04}Lys_{2.94}Tyr_{2.89}Pro_{3.29}Asp_{3.96}Ala_{4.10}His_{1.86}Ile_{1.13}Val_{3.87}Phe_{0.98}$
Boc(86–124)OH[k]	$Glu_{3.11}Thr_{2.87}Gly_{2.00}Ser_{2.19}Lys_{3.07}Tyr_{2.96}Pro_{3.24}$[e]$Asp_{4.00}Ala_{4.13}His_{1.85}Ile_{1.91}$[d]$Val_{3.88}Phe_{0.98}$
Boc(82–85)OEt	$Thr_{1.02}Asp_{1.00}Arg_{0.99}$
Boc(77–81)$NHNH_2$	$Ser_{2.03}Thr_{1.05}Met_{0.97}Ile_{0.95}$
Boc(77–85)OEt	$Ser_{2.01}Thr_{2.03}Met_{0.91}Ile_{0.99}Asp_{1.01}Arg_{1.00}$
Boc(73–76)OEt	$Tyr_{2.05}Glu_{1.00}Ser_{0.98}$
Boc(69–72)OEt	$Glu_{1.00}Thr_{1.00}Asp_{1.00}Cys_{0.86}$
Boc(69–76)$NHNH_2$	$Glu_{2.09}Thr_{0.99}Asp_{1.00}Tyr_{1.90}Ser_{1.01}$
Boc(65–68)OH	$Cys_{0.84}$[j]$Lys_{1.00}Asp_{1.01}Gly_{1.01}$
Boc(65–76)$NHNH_2$	$Lys_{1.00}Asp_{2.07}Gly_{1.00}Glu_{1.99}Thr_{1.01}Tyr_{1.78}Ser_{0.94}$
Boc(65–85)OEt	$Lys_{0.99}Asp_{2.98}Gly_{1.00}Glu_{2.02}Thr_{3.07}Tyr_{1.75}Ser_{2.91}Met_{0.96}Ile_{1.00}Arg_{1.04}$
Boc(65–85)$NHNH_2$	$Cys_{1.74}Lys_{1.06}Asp_{3.10}Gly_{0.94}Glu_{1.85}Thr_{3.12}Ser_{3.00}Met_{0.90}Ile_{1.00}Arg_{0.91}$
Boc(69–85)OEt	$Glu_{1.91}Thr_{3.19}Asp_{2.04}Tyr_{1.96}Ser_{2.90}Met_{1.01}Ile_{1.01}Arg_{0.96}$
Boc(65–124)OH[l]	$Asp_{7.02}Gly_{2.99}Glu_{5.03}Thr_{6.24}Tyr_{4.82}Ser_{6.03}Met_{0.99}Ile_{2.09}Pro_{3.16}Ala_{3.78}Val_{3.45}Phe_{0.93}$
Boc(65–124)OH[k]	$Lys_{4.29}Asp_{7.24}Gly_{3.43}Glu_{5.15}Thr_{5.72}Tyr_{4.95}Ser_{5.24}Met_{1.14}Ile_{2.95}Arg_{0.86}Pro_{2.67}Ala_{4.09}His_{2.57}$-$Val_{3.62}Phe_{0.86}$

[a] Fragments refer to formula I and carry the substituents shown therein, unless otherwise indicated. Except as noted in the table, analyses were carried out after 20-hr acid hydrolysis. [b] Submitted to enzymatic degradation with leucine aminopeptidase. [c] 100-hr acid hydrolysis. [d] Plus 0.07 alloisoleucine. [e] The proline value can be variably high, because of the presence of unoxidized cysteine. [f] Blocking groups removed with anhydrous HF. [g] Submitted to enzymatic degradation with aminopeptidase M. [h] Acetamidomethylcysteine emerges with aspartic acid on the amino acid analyzer. [i] No aspartic acid was seen by tlc after enzymic digestion. [j] As cysteic acid after performic acid oxidation. [k] 70-hr hydrolysis. [l] Satisfactory values for the basic amino acids were obtained on a separate specimen.

involved the addition of 2 equiv of the azide of the protected fragment 86–102 to the nucleophile 103–124 at 5°. The yield (based on the latter) was about 50% after purification on Sephadex G-50. The azide couplings (indicated in formula I) which were required for the preparation of fragment 86–102 proceeded without difficulty. The docosapeptide 103–124 was prepared *via* three azide couplings as shown in formula I. Because of the low solubility in dimethylformamide, the nucleophile 118–124[7] was dissolved in hexamethylphosphoramide for the azide couplings. Only the docosapeptide required gel filtration for purification.

In the final coupling reaction the nucleophilic nonatriacontapeptide 86–124 in hexamethylphosphoramide was added to a fourfold excess of the protected azide of fragment 65–85 in dimethylformamide. About 40% of the nonatriacontapeptide was converted to product at −20°. An aliquot of the product was treated with a large excess of hydrazine in dimethylformamide at room temperature for 3 min. After removal of solvents, the recovered polypeptide appeared to be unchanged and did not react with Tollens reagent. This result supports the view that the hexacontapeptide contains neither ester linkages nor amide bonds involving the imidazole ring of histidine and that the pyrimary amide groups in asparagine and glutamine are stable under these conditions.

(7) J. E. Shields and H. Renner, *J. Am. Chem. Soc.*, **88**, 2304 (1966), have already described the synthesis of this heptapeptide.

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Studies on the Total Synthesis of an Enzyme. IV. Some Factors Affecting the Conversion of Protected S-Protein to Ribonuclease S′

Sir:

In two of the three preceding communications we described the preparation of the protected tetratetracontapeptide fragment 21–64[1] of RNase A as well as the

(1) R. G. Strachan, W. J. Paleveda, Jr., R. F. Nutt, R. A. Vitali, D. F. Veber, M. J. Dickinson, V. Garsky, J. E. Deak, E. Walton, S. R. Jenkins, F. W. Holly, and R. Hirschmann, *J. Am. Chem. Soc.*, **91**, 503 (1969).

hexacontapeptide fragment 65–124.[2] In the subsequent paper[3] we will report the coupling of these two fragments to yield a tetrahectapeptide in which all ϵ-amino functions of lysine are protected, as the benzyloxycarbonyl derivatives, the ω-amino group on serine-21 as the butyloxycarbonyl derivative, and all eight sulfhydryls as the acetamidomethyl derivatives.

To pave the way for the conversion of blocked synthetic S-protein to RNase S′, the following studies were carried out with natural tetrahectapeptide.

We found S-protein to be stable in liquid HF at 0° even in the presence of an excess of added benzyloxycarbonyl-blocked amino acid, provided anisole was added to trap benzyl fluoride or benzyl carbonium ions formed in anhydrous HF. No problem was, therefore, expected in the removal of benzyloxycarbonyl blocking groups of the eight lysine residues. It was found, however, that addition of even 1 equiv of a butyloxycarbonyl derivative of an amino acid to the HF reaction mixture greatly reduced the subsequent regeneration of enzymatic activity. This side reaction, which is presumed to result from the attack of the *t*-butylium ion on methionyl residues in the protein, could be circumvented by the addition of a large excess of methionine to the HF reaction mixture.

In order to study the removal of the eight sulfhydryl acetamidomethyl blocking groups we attempted to acetamidomethylate reduced natural S-protein. Using the aqueous conditions which we had previously described[4] for the preparation of acetamidomethylcysteine itself, we were unable to regenerate enzymatically active protein. However, S-alkylation in anhydrous HF, using a 20% excess of acetamidomethanol, gave a mixture from which a monomeric species could be isolated which gave a negative Ellman test[5] and from which about 90% of the theoretical amount of acetamidomethylcysteine could be obtained by total enzymatic digestion. Cleavage of the sulfhydryl blocking groups with Hg(II) in 50% acetic acid for 5 hr followed by removal of mercury with mercaptoethanol and desalting on Sephadex G-25 afforded reduced S-protein which was satisfactorily converted to enzymatically active material.

The regeneration of enzyme activity by air oxidation of reduced RNase-S or S-protein had already been studied by Haber and Anfinsen.[6] Those workers observed that at pH 8 a mean of 33% of the original enzymatic activity could be regenerated in experiments in which both phosphate and S-peptide were present during the oxidation step.

Using these conditions we found on CG-50 chromatography that more than one enzymatically active component was present in the oxidation mixture. On the other hand, when the oxidation was carried out at pH 6.5 in the presence of mercaptoethanol the reaction was considerably slower but most of the enzymatically active material corresponded to RNase-S in chromatographic behavior. Under these conditions we obtained essentially quantitative recovery of enzymatic activity corresponding to RNase-S in chromatographic behavior, using concentrations of reduced S-protein of 0.2 mg/ml. However, at lower concentrations of reduced S-protein (0.03 mg/ml) enzymatic recovery yields have generally been in the range of 10% after 5 days of oxidation. Thus, we have been able to carry S-protein and acetamidomethylated S-protein through the final steps required in the synthetic approach[7] to yield material having the same properties as observed for RNase S′.

(2) S. R. Jenkins, F. W. Holly, R. S. Dewey, D. F. Veber, R. F. Nutt, W. J. Paleveda, Jr., T. Lanza, Jr., R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, M. J. Dickinson, J. Sondey, R. Hirschmann, and E. Walton, *J. Am. Chem. Soc.*, **91**, 505 (1969).

(3) R. Hirschmann, R. F. Nutt, D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *ibid.*, **91**, 507 (1969).

(4) D. F. Veber, J. D. Milkowski, R. G. Denkewalter, and R. Hirschmann, *Tetrahedron Letters*, 3057 (1968).

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(6) E. Haber and C. B. Anfinsen, *J. Biol. Chem.*, **236**, 422 (1961).

(7) R. G. Denkewalter, D. F. Veber, F. W. Holly, and R. Hirschmann, *J. Am. Chem. Soc.*, **91**, 000 (1969).

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Studies on the Total Synthesis of an Enzyme. V. The Preparation of Enzymatically Active Material

Sir:

In previous communications we described the preparation of the carboxy-terminal hydrazide of the protected tetratetracontapeptide fragment 21–64[1] of RNase A and also the preparation of the similarly protected hexacontapeptide fragment 65–124[2] of RNase A in which the carboxy-terminal amino acid (valine-124) is free.

About 5 mg of the tetratetracontapeptide hydrazide was converted to the azide in DMF in the usual manner,[1,2] and to this solution was added a hexamethylphosphoramide solution of 1.6 mg of the hexacontapeptide from which the butyloxycarbonyl blocking group on cysteine-65 had been removed with trifluoroacetic acid. Triethylamine was added to neutralize excess acid, and the coupling reaction was allowed to proceed at 5° for 4 days. To the crude product (about 5.5 mg), precipitated by the addition of ethyl acetate, was added 139 mg of methionine and 0.1 ml of anisole. This mixture was dissolved in 1 ml of anhydrous HF at 0° and allowed to stand for 45 min. After evaporation of HF the mixture was washed with ethanol and chromatographed on Sephadex G-50 (50% aqueous acetic acid) to remove the bulk of the methionine. The reaction product was then chromatographed on a freshly prepared column of Sephadex G-75 to obtain a fraction enriched in the desired tetrahectapeptide. The column was subsequently calibrated with natural acetamidomethylated S-protein[3] and appropriate fractions of the synthetic material were

(1) R. G. Strachan, W. J. Paleveda, Jr., R. F. Nutt, R. A. Vitali, D. F. Veber, M. J. Dickinson, V. Garsky, J. E. Deak, E. Walton, S. R. Jenkins, F. W. Holly, and R. Hirschmann, *J. Am. Chem. Soc.*, **91**, 503 (1969).

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