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′.

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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

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combined and rechromatographed on a second, freshly prepared column of Sephadex G-75 which, in turn, was subsequently calibrated with natural material as described above. After combination of the appropriate fractions, amino acid analysis indicated the presence of about 75 μg of the desired tetrahectapeptide. This protein fraction was allowed to react with 142 μg of $Hg(OAc)_2$ in aqueous acetic acid at 25° for 6 hr to remove the cysteinyl blocking groups. After the addition of 0.1 ml of a 50% aqueous solution of mercaptoethanol, the mixture was kept at 25° for 17 hr and the solution was then desalted by passage through Sephadex G-25. Fractions containing excluded protein were combined (2.6 ml).

An aliquot (2.0 ml) was converted into enzymatically active material as described below, and a 0.6-ml aliquot was retained as a control. The former was treated with 0.07 ml of a 1% solution of aqueous mercaptoethanol and then with 26 μg of S-peptide. The pH was adjusted to 6.5 and the phosphate ion concentration to 0.2 *M*, two drops of $CHCl_3$ was added, and the mixture was held at 25° for 5 days. In an assay[4] using RNA as substrate, Mr. I. Putter found this solution to contain about 2 μg of RNase-S activity. By contrast, the 0.6-ml aliquot, which had been processed in a completely analogous manner, but to which no S-peptide had been added, was devoid of enzymatic activity (less than 0.02 μg). Similarly, Dr. M. Zimmerman, using an assay[5] involving polycytidilic acid as the substrate, found the 2.0-ml solution to contain 1.2 μg of RNase-S activity and the 0.6-ml aliquot to be inactive (less than 0.01 μg). As expected, none of the reagents employed, including S-peptide, showed any enzymatic activity.

Simultaneously with the above experiments parallel experiments were carried out on 130 μg of natural acetamidomethylated S-protein.[3] The aliquot (2.0 ml) to which S-peptide had been added yielded about 10 μg of enzymatic activity. Oxidation without added S-peptide (0.6-ml aliquot) again served as a negative control.

It may be concluded therefore that, under conditions where a 100-μg aliquot of natural acetamidomethylated S-protein gave 8–10 μg of RNase-S activity, an aliquot of about 60 μg of our synthetic protected protein gave 1.2–2 μg of RNase-S activity. It is also apparent that enzymatic activity is obtainable from our synthetic protein only when S-peptide is added.

The data presented herein represent a repeat of our first synthesis of the protected tetrahectapeptide. This earlier experiment had also led to enzymatically active material after deblocking and oxidation in the presence of S-peptide.

We are now attempting to prepare sufficient quantities of protein to permit us to carry out the oxidation step at more favorable concentrations and to permit a more complete purification and characterization of the enzymatically active material.

Acknowledgment. The authors are deeply grateful to Mr. I. Putter and Dr. M. Zimmerman of these laboratories for carrying out the assays reported herein. We are indebted to them as well as to Professor S. Beychok of Columbia University and to Messrs. F. Kahan and P. Cassidy of these laboratories for numerous discussions concerning assay problems.

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Conductances of Potassium Perchlorate in the Plastic Phase of Sulfolane

Sir:

For the last few years we have been studying the physicochemical properties of sulfolane.[1] This solvent belongs to a family of substances (so-called "globulaire"[2]) which have one or more transitions below the freezing point.

Thus, in the temperature range 28.45–15.45°, the sulfolane molecules are able to freely rotate on their own axes, and, in some respects, they resemble a liquid.[3] Evidence for this is given by several studies on this solvent.[1,4]

We have measured the resistance of a potassium perchlorate solution in the plastic phase of sulfolane in order to obtain information on the transport properties of electrolytes in this new phase of matter.

During the solidification the potassium perchlorate solution was maintained at reduced pressure (10^{-5} torr) in order to avoid fractures between the electrode surfaces. Resistances were measured by a Jones and Josephs bridge; 30,000 ohms of the bridge resistance was shunted in parallel with the cell. A cell with a constant of 0.2706 cm^{-1} was used. The cell was calibrated by comparison with another cell, which in turn was calibrated with potassium chloride solutions at 25° using the Jones and Bradshaw[5] standards. In the calculations of the conductivities of the solution, no allowance was made for the variation of the cell constant with the temperature. The concentration of the solution was deduced *a posteriori* from the known conductivity values of potassium perchlorate in sulfolane solutions at 30°.[6] The temperature of the oil bath was controlled within ±0.01° by a series of calibrated thermometers. With the same solution the measurements were performed several times, and each time the entire cycle, as depicted in Figure 1, was explored. In the plastic phase the conductance values from several runs were reproducible within ±2%; this is probably due to small fractures in the solid invisible with the naked eye.

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