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Preparation of Cross-Linked Dimers of Pancreatic Ribonuclease†

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ABSTRACT: The cross-linking reaction between diimido esters and ribonuclease has been studied in terms of the yield of cross-linked dimer with optimum activity toward double-stranded RNA. With dimethyl suberimidate the most satisfactory conditions were condensation for 15 min at pH 7.5–8.0 at 21 °C with 1.25 mol equiv of the diimido ester and a protein concentration of 6%. The dimer (yield 20%) had 19 unmodified NH_2 groups out of a theoretical 20 for a molecule in which two such groups are involved in the cross-linkage; the activity toward poly(A)·poly(U) in 0.14 M salt solution by spectrophotometric assay was 8.5 times that of the monomeric enzyme toward the same substrate.

The reaction of dimethyl adipimidate with amino groups of bovine pancreatic ribonuclease to give a cross-linked dimer was first studied by Hartman and Wold (1967). Interest in the enzymic properties of dimeric RNases has been stimulated by the finding of D'Alessio and Leone and their colleagues (1972b, 1975) that the ribonuclease of bovine seminal plasma is a dimer in which the identical halves (each a homologue of the pancreatic enzyme) are linked by two disulfide bridges. Libonati and Floridi (1969) found that the seminal enzyme was more active than the pancreatic enzyme against double-stranded RNAs; they also noted that a non-cross-linked dimer prepared according to Crestfield et al. (1962) by aggregation of the pancreatic monomer had this property (Libonati, 1969). These observations prompted Bartholeyns and Moore (1974) to undertake a preliminary study of enzymic and physiological properties of a cross-linked dimer of the pancreatic enzyme. The desire to prepare such dimers in maximum yield and with optimum activity led to the present investigation of the chemistry of the cross-linking reaction with reagents of different lengths and of the activities of the products toward double-stranded ribonucleic acids.

Experimental Section

Materials and Methods

Bovine pancreatic ribonuclease A (Type IIA) was purchased from Sigma. Yeast RNA (Sigma VI) was dissolved in 0.025 M EDTA to give a 6% solution which was adjusted to pH 6.5–7.0 with NaOH and dialyzed twice for 6 h

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against 20 volumes of 0.025 M EDTA, twice against 0.15 M NaCl, and three times against distilled water, and lyophilized. Cyclic 2′,3′-cytidylic acid (Sigma) was purified by chromatography on a column (0.9 × 2.5 cm) of Dowex 1-X8 (Bio-Rad, AG1-X8 minus 400 mesh) according to the procedure of Fruchter and Crestfield (1965). The [^{14}C]poly(A)[1] was obtained from Miles; specific activity 2700 cpm/μg. The synthetic DNA-RNA hybrid, poly(dA)·poly(rU), and double-stranded poly(A)·poly(U) were obtained from P. L. Biochemicals. Gel filtration of poly(A)·poly(U) on Sephadex G-200 gave no retarded peak. Double-stranded viral RNA from a virus of *Penicillium chrysogenum* (cf. Robertson et al., 1968) was a gift from Hugh D. Robertson of the Rockefeller University; the tritiated sample had a specific activity of 50 000 cpm/μg.

Dimethyl pimelimidate (Brew et al., 1975) was a gift from Robert L. Hill of Duke University. The other diimido esters were obtained from Pierce Chemicals; the dimethyl suberimidate and adipimidate dihydrochlorides were checked for purity by elementary analysis.

Enzymic Assays. Activity toward yeast RNA was determined by the spectrophotometric assay of Kunitz (1946); the assays were performed at pH 7.5 in 0.1 M Tris-HCl buffer with 0.1–0.5 μg of enzyme/ml. The activity toward cyclic 2′,3′-cytidylic acid was determined at pH 6.0 by a spectrophotometric method similar to that described by Crook et al. (1960) and del Rosario and Hammes (1969) under the conditions defined by Lin (1970).

The activity toward poly(A)·poly(U) was usually determined spectrophotometrically under conditions similar to those described by Libonati and Floridi (1969); the increase in absorbance at 260 nm was measured as a function of time on a recording Zeiss PMQII spectrophotometer equipped with a linear scale-expander (0–0.2 A full scale). The substrate (40 μg/ml) was dissolved in a buffer which was 0.125 M in NaCl and 0.015 M in Tris-HCl at pH 7.5, and the assay was performed at 25°C. The cross-linked enzyme, usually dissolved in 10–20 μl of 0.9% NaCl, was added to give about 2–20 μg of RNase/ml. The increase in absorbance was linear over the first 2 min of the assay. Specific activity was expressed as ΔA_{260} min^{-1} mg of protein^{-1}. Under these conditions monomeric RNase A gives a slow change in the A_{260} and the measurement with the unmodified enzyme was used as a control in each series of assays. Since the secondary structure of the double-stranded complex is sensitive to the exact salt concentration (Michelson et al., 1967), the most reproducible expression of the activity of a dimeric RNase has been the ratio of the specific activty of the dimer to that of the unmodified monomer in the same series of assays.

The activity toward poly(A)·poly(U) was also measured by a precipitation assay based upon the procedure of Anfinsen et al. (1954). The digest contained 1 mg of substrate in 1 ml of 0.015 M Tris-HCl (pH 7.5), 0.125 M in NaCl; 20 μl of 0.5% bovine serum albumin and 1 μl of 0.1% enzyme solution were added. After incubation for 5–15 min at 37 °C, 1 ml of cold (0 °C) 10% perchloric acid, 0.25% in uranyl acetate, was added. The suspension was placed on ice for 30 min. The precipitate was spun down on a clinical centrifuge in the cold room at 4 °C (for 10 min). The supernatant was diluted 1:5 with water before measurement of the soluble nucleotides at 260 nm vs. an enzyme-free blank.

The activity toward the tritiated viral RNA was measured by a procedure similar to that described by Billeter and Weissmann (1966). The substrate solution contained 1000 cpm (20 ng of RNA) in 500 μl of 0.015 M sodium citrate (pH 7.0), 0.15 M in NaCl. After addition of the enzyme (20 μl of 0.1% solution), the mixture was incubated at 25 °C. At suitable intervals, 50 μl of 0.5% bovine serum albumin was added and followed by 500 μl of cold 5% trichloroacetic acid. The tubes were kept on ice for 30 min. Each solution was filtered through a Millipore HAWP-025-00 filter and the precipitate washed with 5 ml of the solution of trichloroacetic acid. The filters were dried under an infrared lamp and added to 10 ml of xylene-based scintillation fluid (Aquasol Universal LSC, New England Nuclear).

The activity toward [^{14}C]poly(A) was determined similarly; the substrate solution contained 16 500 cpm in 500 μl, and the enzyme solution added was 5 μl of a 0.1% solution.

Analytical Methods. The concentration of RNase was estimated spectrophotometrically (E_{280}(1%) 7.3, cf. Richards and Wyckoff, 1971) or by the method of Lowry et al. (1951) with RNase A as the standard. The number of free amino groups remaining in the dimer was estimated photometrically with 2,4,6-trinitrobenzenesulfonic acid by the method of Satake et al. (1960) as modified by Mokrasch (1967) and by Ferdinand et al. (1973). To a solution of protein (25–300 μg) in 1 ml of 0.1 M sodium tetraborate, 0.2 ml of 0.015 M 2,4,6-trinitrobenzenesulfonic acid was added and the mixture was incubated at 37 °C for 30 min in test tubes protected from light by aluminum foil. The reaction was stopped by the addition of 2 ml of 90% formic acid and the extinction was read at 340 nm. Under these conditions, RNase A (11 NH_2 groups per molecule) was used as the standard and gave the same (100 ± 5%) molar ϵ per amino group (1.2×10^4) as bovine serum albumin, and bovine chymotrypsinogen A. The temperature of 37 °C is important for obtaining this uniformity of reaction. The photometric method permitted estimation of the free NH_2 groups in ribonuclease derivatives with a reproducibility of ±1 group per dimer.

The number of modified lysine residues in the dimer was determined by ion-exchange chromatography (Spackman et al., 1958) performed on a Durrum D-500 analyzer, with pH 6.0 for the third buffer. Samples were hydrolyzed in 6 N HCl for periods of 23, 36, 48, and 72 h, at 110 °C. Calculation of the number of residues of lysine per molecule of dimer was determined by reference to the recoveries of stable amino acids (aspartic acid, glutamic acid, alanine, valine, and arginine) and after extrapolation to zero time to correct for the small percentage of hydrolysis of the ϵ-amidinated lysines (cf. Hartman and Wold, 1967).

Preparation of Dimers. For the initial derivatizations the protein concentration was the same as in the experiments of Hartman and Wold (1967); 20 mg of RNase A was dissolved in 2 ml of phosphate buffer of given molarity and pH, at about 21 °C. The bifunctional reagent was added as a solid in six approximately equal portions over a period of 5 min; the pH of the solution was maintained by titration with 0.1 M NaOH. After the prescribed reaction time, the unreacted reagent was quenched by the addition of 20 equiv of ammonium acetate (as 0.2 M solution) per initial imido ester group. The dimers in the reaction mixture were separated from the monomer by gel filtration on a column of Sephadex G-75 (0.9 × 158 cm) with 0.9% NaCl as eluent. The flow rate was about 18 ml/h. The separation of monomer, dimer, and higher molecular weight products was sim-

[1] Abbreviations used are: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid).

Table I: Conditions of Preparation and Properties of Cross-Linked Dimers of Ribonuclease.[a]

Bifunctional Reagent	Molar Ratio Reagent/RNase	pH	Reaction Time (min)	Ratio of Activity toward Poly(A)·Poly(U) Dimer/RNase Monomer	Free NH_2 Groups (± 1) per Molecule of Dimer
Dimethyl adipimidate[b]	3	10.5	90	2.3	9
$NH_2^+Cl^-$ $NH_2^+Cl^-$	3	10.5	90	2.2	9
$CH_3OC-(CH_2)_4-COCH_3$	3	7.8	30	6.6	17
	3	7.5	60	7.6	20
	1.4	7.8	30	8.2	
Dimethyl pimelimidate[c]	1.3	10.0	30	6.0	13
$NH_2^+Cl^-$ $NH_2^+Cl^-$	1.3	7.8	30	8.8	20
$CH_3OC-(CH_2)_5-COCH_3$					
Dimethyl submerimidate[d]	5	10.0	10	2.3	15
$NH_2^+Cl^-$ $NH_2^+Cl^-$	1	10.0	10	8.0	19
$CH_3OC-(CH_2)_6-COCH_3$	1.2[f]	7.7	15	8.8	19
	1.2[f]	7.7	15	8.6	19
Dimethyl 3,3'-dithiobispropioimidate[e]	2	8.0	30	3.4	17
$NH_2^+Cl^-$ $NH_2^+Cl^-$	2	7.5	30	7.0	20
$CH_3OCCH_2CH_2SSCH_2CH_2COCH_3$					

[a] The RNase concentration (except where indicated) was 1.46 μmol in 2 ml of 0.5 M phosphate buffer (except for the first two entries for which the buffer was 0.1 M and the reagent was added over 30 min). The cross-linking reagent was added in about six equal portions over 5 min. The yields of dimer in all instances were in the range from 12 to 22%. [b] Hartman and Wold (1967). [c] Davies and Kaplan (1972); Brew et al. (1975). [d] Davies and Stark (1970). [e] Wang and Richards (1974). [f] Preparation from 500 mg of RNase in 8 ml of phosphate buffer. Reagent was added over 10 min.

ilar to that shown in Figure 3 of Hartman and Wold (1967); the dimer was eluted between 57 and 65 ml.

The non-cross-linked dimer of RNase was formed by lyophilization of a solution of the enzyme in 50% acetic acid as described by Crestfield et al. (1962). The dimeric product was isolated by gel filtration on the Sephadex column referred to above equilibrated with 0.2 M phosphate buffer at pH 6.46. When the dissociable dimer was assayed against poly(A)·poly(U), an equal volume (usually 5 μl) of the phosphate buffer was added to the control with the monomeric RNase.

Results

In order to establish the optimum conditions for the reaction between diimido esters and RNase, the variables in the process were studied in terms of the yield of the cross-linked dimer, its activity toward poly(A)·poly(U), and the number of free amino groups in the product. Since monomeric RNase contains 11 free amino groups (Smyth et al., 1963), from 10 lysine residues one of which has its α-NH_2 group free, the maximum number of free amino groups in a dimer with one cross-link formed by a diimido ester is 20. In 0.1 M phosphate buffer at pH 10.5 with 3 mol of dimethyl adipimidate/mol of ribonuclease and a reaction time of 60 min, which were the conditions used by Hartman and Wold (1967), we obtained products that varied in the degree of amidination and in activity toward poly(A)·poly(U). Consequently, progressively milder conditions were tried by lowering the pH, decreasing the molar excess of reagent, and shortening the reaction time. In this way (Table I) it was established that when prepared at pH 7.5–8.0, with 1.2–1.3 mol equiv of diimido ester and a reaction time of 30 min, the cross-linked dimer invariably had close to optimum activity and minimum coverage of NH_2 groups. The four diimido esters tested gave similar results. On the basis of the data in Figure 1, a reaction time of 15 min was adopted for dimethyl suberimidate.

Phosphate was initially included in the reaction medium to protect Lys-41 (cf. Hirs et al., 1962; Cooke et al., 1963) from derivatization. In the present experiments the concentration has been increased to 0.5 M to facilitate maintenance of constant pH during the reaction; at pH 8.0 the molarity of phosphate, in the range from 0.1 to 0.5 M, does not have a significant effect upon the results.

The yield of cross-linked dimer obtainable in the above experiments did not vary much with pH. With increase in the protein concentration (Table II) the yield approached 20%, which is similar to that obtained by Hartman and Wold (1967). Attempts to raise the yield of dimer by more extensive amidination resulted in a higher conversion of the dimer to oligomers, and led to products with lowered activity toward poly(A)·poly(U), a result which could be correlated with a decrease in free NH_2 groups. Attempts to increase the yield by starting with the non-cross-linked dimer did not give more than 20% yield of undissociable (in 30 min at 60 °C) dimeric product with optimum activity.

From the above results, the following conditions were adopted for the preparation of the dimer with dimethyl suberimidate: 500 mg of RNase was dissolved in 8 ml of 0.5 M phosphate buffer at pH 7.7. The solution was stirred continuously with a magnetic stirrer as rapidly as possible without foaming. To this solution 12.5 mg of dimethyl suberimidate (molar ratio of diimido ester to protein = 1.25) was added as the solid in small aliquots over a period of 10 min at a rate of one portion about every 30 s. The pH was maintained by addition of 0.1 N NaOH. After five additional minutes, 8 ml of 0.2 M ammonium acetate was added and the solution was applied to a column (2.8 × 177 cm) of Sephadex G-75 equilibrated with physiological saline. The fractions (5 ml each) containing the dimer, eluted between 560 and 635 ml, could be used for chemical or physiological

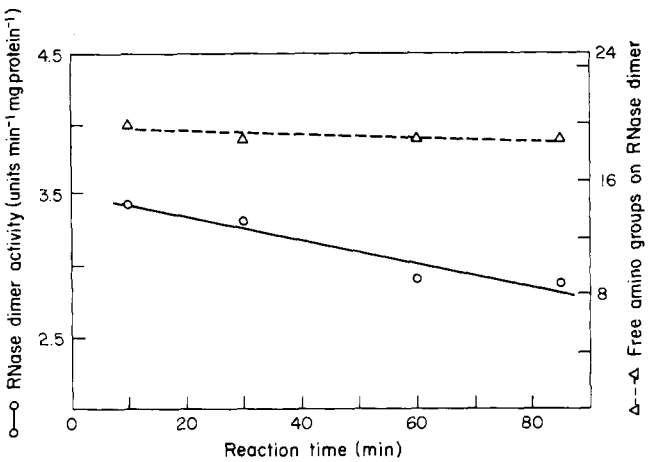

FIGURE 1: Specific activities of RNase dimeric products toward poly(A)·poly(U) and the number of free NH_2 groups on the dimer as a function of time of coupling reaction. To RNase (1.46 μmol) in 2 ml of 0.5 M phosphate at pH 8.0, dimethyl suberimidate (1.83 μmol) was added in portions during the first 5 min. Activity was measured spectrophotometrically.

tests or they were dialyzed against distilled water and the final solution was lyophilized to yield the salt-free dimer.

The product thus prepared with dimethyl suberimidate gave 19 free amino groups by colorimetric analysis. The modification of three lysine residues per molecule of dimer was confirmed by amino acid analysis. After extrapolation to zero time of hydrolysis, 17 free lysine residues out of the 20 per molecule of dimer were found. This result indicates that two lysines were involved in the cross-linking reaction and that on the average one lysine was monofunctionally amidinated by dimethyl suberimidate. The bis(amidine) derivative which chromatographs after arginine on the amino acid analyzer (cf. Hartman and Wold, 1967) is not detected using the normal buffer program for a protein hydrolysate; however, a small peak (not found in hydrolysates of RNase A) which chromatographed just before histidine, is presumably N^{ϵ}-suberimido-L-lysine, since it disappeared upon treatment of the hydrolyzed sample with 2 N NaOH (18 h at room temperature). After the alkali treatment, 19.6 residues of lysine per molecule of dimer was found. This result answers the question of whether the α-NH_2 groups of the NH_2-terminal lysine residues had reacted. If they had, the trinitrobenzenesulfonic acid reaction would have shown a greater coverage of NH_2 groups than amino acid analysis after acid hydrolysis, since α-amidinated groups are completely hydrolyzed in 6 N HCl at 110 °C for 20 h (Hunter and Ludwig, 1972). Both methods of analysis give the same value—three covered NH_2 groups per dimer. The activity of the dimer toward poly(A)·poly(U) was 8.5 times that of the monomer; the activities of the dimer toward cyclic 2′,3′-cytidylic acid and yeast RNA were 109 and 68%, respectively, of those of the monomer. The cross-linked products which were eluted from Sephadex G-75 just ahead of the dimer, and which can be expected to be a mixture of trimers and tetramers (in about 5% yield), generally had about twice the specific activity of the dimer against poly(A)·poly(U). The non-cross-linked dimer prepared according to Crestfield et al. (1962) and also studied by Libonati and Floridi (1969) and Libonati et al. (1975b) had an activity against the double-stranded substrate in our assays very close to that found for the cross-linked dimer, but it is subject to dissociation in aqueous solution in the absence of an appreciable phosphate concentration. The three other diimido esters tested (Table I) gave results closely paralleling those obtained with dimethyl suberimidate.

Table II: Effect of Concentration of RNase on Yield of Dimer.[a]

Amount of RNase (mg/ml)	Yield of RNase Dimers (%)	Amount of RNase (mg/ml)	Yield of RNase Dimers (%)
10	11.8	60	18.3
40	14.7	100	21.3
50	18.4		

[a] The amount of RNase indicated was dissolved in 1 ml of 0.5 M phosphate buffer (pH 7.7). Dimethyl suberimidate (molar ratio reagent/RNase = 1.25) was added in small approximately equal portions in 10 min at a rate of one portion per 30 s. Total reaction time was 15 min.

Discussion

The Cross-Linking Reaction. In applying dimethyl suberimidate to the study of the subunit structure of oligomeric proteins, Davies and Stark (1970) worked with dilute protein solutions (about 1%) in order to minimize cross-linking between oligomers. In the present experiments, the protein concentration is increased to near to 6% in order to favor linkage between molecules of the monomer of RNase in solution. We had hoped that the yield of cross-linked dimer might be increased by starting with the non-cross-linked dimer of RNase, but the maximum yield again was about 20%; the limiting factor is the need to keep the reagent/protein ratio low in order to limit the amidination reaction to preferably one NH_2 group per monomer unit in order to attain maximum activity of the product toward double-stranded RNA.

The Free NH_2 Groups of the Dimer. When the activity of dimeric RNases toward double-stranded RNAs was first observed (Libonati and Floridi, 1969), the initial thought was that the presence of two active sites in the enzyme might be important for the process (D'Alessio et al., 1974). Libonati and associates (1975a) have now shown that if the -S-S- bonds that hold the units of the seminal ribonuclease together are reduced and carboxymethylated as described by D'Alessio et al. (1975), the resulting monomeric protein retains much of its activity toward double-stranded RNAs. This result indicates that one of the important factors in determining activity toward double-stranded substrates may be the density of positive charges in the molecule; the seminal enzyme has three more lysine residues per monomeric unit than the pancreatic ribonuclease and an isoionic point (D'Alessio et al., 1972a) of 10.3 in Tris-HCl buffer compared to 9.5 for the pancreatic enzyme. The amidination reaction per se (Hunter and Ludwig, 1962; Ludwig and Hunter, 1967) which preserves a positive charge at the site of the modified NH_2 group should not markedly affect the activity of the enzyme when only a few groups are modified; Klee and Richards (1957) found that half of the NH_2 groups of RNase could be guanidinated with retention of about 90% of the activity toward yeast RNA. Stark et al. (1960) showed that when the derivatization of NH_2 groups abolishes the charge (as in carbamylation by cyanate in the presence of phosphate) the modification of the first few lysine residues markedly affects the enzymic activity. The reason for the lowered activity from modification of addi-

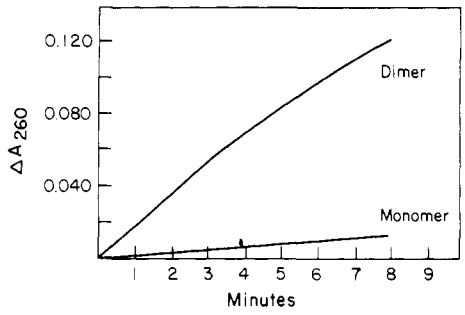

FIGURE 2: Spectrophotometric assay of RNase A and the cross-linked dimer with poly(A)·poly(U) as substrate in 0.140 M salt solution at pH 7.5. Conditions in text.

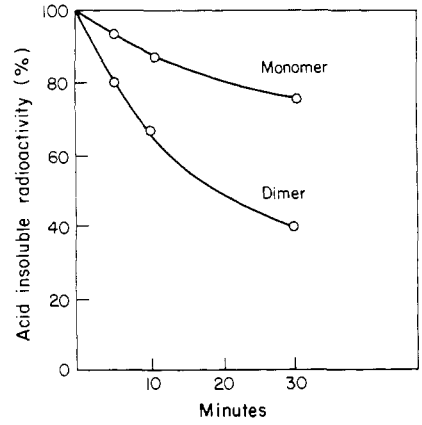

FIGURE 3: RNase A and the cross-linked dimer assayed toward [^{3}H]RNA from a virus of *Penicillium chrysogenum* in 0.165 M salt at pH 7.0 by measurement of acid precipitable nucleotides. Conditions in text.

tional NH_2 groups in the present experiments is open to study.

Specific Activity of the Cross-Linked Dimer. Bartholeyns and Moore (1974) reported a very much higher ratio for the activities of the dimer and the monomer toward poly(A)·poly(U) in a precipitation assay (Bartholeyns, 1973) than we obtain by the present spectrophotometric assay of Libonati and Floridi (1969). We have therefore tested the present product extensively by both spectrophotometric and precipitation assays.

The results of assay toward poly(A)·poly(U) by the spectrophotometric measurement are illustrated in Figure 2. The initial rates are linear for the dimer and the monomer for the first 3 min and give an activity ratio of 8.5. When poly(A) and poly(U) are mixed to form the homoribopolymer duplex, there is a decrease in the A_{260} (Warner, 1957); the assay is measuring both the spectral change as the double-stranded structure is disrupted and the smaller change that accompanies the hydrolysis of poly(U) or poly(A) alone.

In this assay, the non-cross-linked dimer prepared by aggregation of the monomer (Crestfield et al., 1962) had an activity equal to or measurably higher than that of the cross-linked dimer.

The precipitation assay toward poly(A)·poly(U) which we have used has given only two to three times as much acid-soluble nucleotides with the cross-linked dimer as with the monomer. A similar ratio was obtained when the double-stranded viral RNA was the substrate (Figure 3).

With poly(dA)·poly(rU) as substrate in the spectrophotometric assay the activity ratio of dimer/monomer was also 8.5 in 0.125 M NaCl–0.015 M Tris-HCl (pH 7.5). Libonati et al. (1975b) have shown that aggregates of RNase A and the cross-linked dimer will degrade DNA–RNA hybrids.

Whereas the cross-linked dimers maintained full activity against cyclic 2′,3′-cytidylic acid they showed reduced activity (in units/mg) against single-stranded yeast RNA. However, the relatively resistant [^{14}C]poly(A) was hydrolyzed two to three times as rapidly by the dimers as by the monomer. These enzymic properties are very similar to those observed with the aggregated dimer of RNase A by Libonati (1969, 1971) and the naturally occurring dimer from bovine seminal plasma (Libonati and Floridi, 1969). Libonati (1969) also observed that aggregates of RNase A larger than dimers degraded poly(A)·poly(U) at a higher rate than the dimer.

Assays of ribonucleases toward double-stranded substrates present a number of problems that are not fully obviated by any of the methods currently available. In all instances, the salt concentration is crucial since it determines the rigidity of the double-stranded structure (Michelson et al., 1967). For the present purposes an ionic strength near the physiological range has been selected; at higher ionic strengths the RNA structure can be so rigid that there is very slow hydrolysis by either dimeric or monomeric ribonucleases.

The precipitation assays measure the low molecular weight oligonucleotides; if the dimeric enzyme gave fragments of a different size from those yielded by the monomer this would affect the observed ratio of activities. The precipitation assay used by Bartholeyns (1973) and Bartholeyns and Moore (1974) differed from earlier procedures in that the precipitation was carried out in a more dilute nucleotide solution; the absorbance was measured on the supernatant perchloric acid solution without dilution, whereas Anfinsen et al. (1954) performed the precipitation in more concentrated solution and diluted the supernatant for spectrophotometric measurement. In our hands the results of the former procedure are very sensitive to the mode of addition of the precipitant; the procedure closer to that of Anfinsen et al. (1954) used in the present experiments has given reproducible results from day to day and operator to operator.

The spectrophotometric measurement with double-stranded RNAs also has the limitation that it is not a measurement of the number of phosphate ester bonds hydrolyzed in the substrate. It is the most convenient assay for obtaining the results in Table I, which are useful in providing a guide to conditions for the preparation of a cross-linked dimer with optimum activity. The aim of the present study has been the preparation of a cross-linked derivative of bovine pancreatic ribonuclease for comparison with the bovine seminal ribonuclease (D'Alessio et al., 1975) in terms of enzymic and physiological properties; the latter properties can be expected to be a function of molecular size as well as the increased ability to act on double-stranded substrates which the two dimeric enzymes share.

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