tions, Springer-Verlag, Heidelberg.
Lindman, B., Zeppezauer, M., & Åkeson, Å. (1972) *Biochim. Biophys. Acta 257*, 173–177.
Norne, J. E., Bull, T. E., Einarsson, R., Lindman, B., & Zeppezauer, M. (1973) *Chem. Scr. 3*, 142–144.
Norne, J. E., Hjalmarsson, S. G., Lindman, B., & Zeppezauer, M. (1975a) *Biochemistry 14*, 3401–3408.
Norne, J. E., Lilja, H., Lindman, B., Einarsson, R., & Zeppezauer, M. (1975b) *Eur. J. Biochem. 59*, 463–473.
Reynolds, C. H., & McKinley-McKee, J. S. (1975) *Arch. Biochem. Biophys. 168*, 145–162.
Reynolds, C. H., Morris, D. L., & McKinley-McKee, J. S. (1970) *Eur. J. Biochem. 14*, 14–26.
Rossman, M. G., Liljas, A., Brändén, C.-I., & Banaszak, L. J. (1975) *Enzymes, 3rd Ed. 11*, 61–102.
Söderberg, B.-O., Zeppezauer, E., Boiwe, T., Nordström, B., & Branden, C.-I. (1970) *Acta Chem. Scand. 24*, 3567–3574.
Stephens, R. S., & Bryant, R. G. (1976) *Mol. Cell. Biochem. 13*, 101–112.
Theorell, H., & Yonetani, T. (1963) *Biochem. Z. 338*, 537–553.
Ward, R. L., & Happe, J. A. (1971) *Biochem. Biophys. Res. Commun. 45*, 1444–1450.
Ward, R. L., & Cull, M. D. (1974) *Biochim. Biophys. Acta 365*, 281–284.
Wyeth, P., & Prince, R. H. (1977) *Inorg. Perspect. Biol. Med. 1*, 37–71.
Yonetani, T. (1963) *Biochem. Z. 338*, 300–316.
Zeppezauer, M., Lindman, B., Forsén, S., & Lindqvist, I. (1969) *Biochem. Biophys. Res. Commun. 37*, 137–142.
Zeppezauer, E., Jörnwall, H., & Ohlsson, I. (1975) *Eur. J. Biochem. 58*, 95–104.

Comparative Study on the Structure and Stability of Bovine Seminal Ribonuclease, Its Monomeric Bis(S-carboxymethylated-31,32) Derivative, and Bovine Pancreatic Ribonuclease†

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ABSTRACT: The secondary and tertiary structure of dimeric seminal ribonuclease, its monomeric bis(S-carboxymethylated-31,32) derivative (MCM-sRNase), and bovine pancreatic ribonuclease (RNase A) were compared by circular dichroism, difference absorption, and fluorescence techniques. The far-ultraviolet circular dichroism spectrum of MCM-sRNase is quite similar to that of RNase A and shows a minimum at 209 nm and a shoulder near 220 nm. Conversely, the spectrum of native seminal RNase is clearly different and shows a single, negative, broad trough centered at about 215 nm, reminiscent of β-structure content. The most relevant feature in the near-ultraviolet circular dichroism spectrum of seminal RNase is the absence of a positive peak near 242 nm, which instead is present in MCM-sRNase and in pancreatic RNase A. Since this positive ellipticity has been previously assigned to a contribution of exposed tyrosine residue(s) in RNase A, it is proposed that dissociation of dimeric seminal RNase into its monomeric MCM-sRNase leads to exposure of tyrosine residue(s). The deep burial of these residues in seminal RNase and their partial exposure in MCM-sRNase have been evidenced also by difference absorption, fluorescence emission, and fluorescence quenching measurements. The efficiency of quenching by added solutes in seminal RNase is much less than that observed with MCM-sRNase or pancreatic RNase A. MCM-sRNase was more sensitive than native seminal RNase and RNase A to thermal denaturation, as evidenced by monitoring the thermal unfolding of the secondary structure by circular dichroism measurements at 220 nm and of the tertiary structure by tyrosine fluorescence emission measurements. Analogously, the denaturing action of urea was more pronounced with MCM-sRNase than with native seminal RNase or pancreatic RNase A. These results indicate that the dimeric structure of seminal RNase exerts a significant protective effect toward protein denaturing agents. This effect may be physiologically significant in maintaining an effective concentration of ribonuclease in seminal fluids.

Bovine seminal ribonuclease is a dimer containing two identical peptide chains of 124 amino acid residues linked through two intermolecular disulfide bridges (D'Alessio et al., 1972a,b, 1975; Di Donato & D'Alessio, 1973). Sequence analysis of the peptide chain of seminal RNase revealed 23 amino acid substitutions compared with pancreatic RNase A,[1] but the 8 residues of half-cystine present in RNase A are observed at identical positions in the subunit chain of seminal RNase (Suzuki et al., 1976). This extensive sequence homology raises the possibility that the subunits of seminal RNase and pancreatic RNase A have similar three-dimensional structure and analogous mechanism of action. In fact, not only do the two enzymes have similar catalytic properties (Floridi et al., 1972), but in addition selective reduction of the intermolecular disulfide bridges at cystine residues in positions 31 and 32 of the polypeptide chain, followed by S-alkylation, affords a monomeric derivative of seminal RNase which is catalytically active (D'Alessio et al., 1975). This stable derivative, selectively S-alkylated seminal RNase (bis(S-

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Abbreviations used: CD, circular dichroism; MCM-sRNase, monomeric bis(*S*-carboxymethyl)cysteine-31,32-ribonuclease (seminal); RNase A, the major component of bovine pancreatic RNase; $AcNH_2$, acrylamide; ATA, *N*-acetyl-L-tyrosinamide.

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carboxymethylated-31,32)ribonuclease, seminal, MCM-sRNase) may be regarded as a component of a system of two homologous monomers (MCM-sRNase and RNase A) and two homologous dimers (seminal RNase and artificially dimerized pancreatic RNase A; Crestfield et al., 1962; Wang et al., 1976). In view of the extensive knowledge of the molecular and catalytic properties of pancreatic RNase A, this set of protein molecules could represent a particularly useful and interesting system for structure–function studies in enzyme molecules.

In this paper, various aspects of the secondary and tertiary structure of seminal RNase and MCM-sRNase have been evaluated from circular dichroism, difference absorption, and fluorescence emission measurements. The results of these spectroscopic studies have been compared with the structural features of pancreatic RNase A. The stability to thermal, pH, and urea denaturation of the three RNase molecules was also investigated. The present study on the solution structural properties of seminal RNase and its monomeric derivative would complement the ongoing X-ray crystallographic analysis on the native dimeric enzyme (L. Mazzarella, private communication).

Experimental Section

Materials. Seminal RNase was prepared from bull semen and its homogeneity checked as described by D'Alessio et al. (1972a). Its monomeric derivative MCM-sRNase was prepared by limited reduction with dithiothreitol of the native dimeric protein followed by S-alkylation with iodoacetic acid (D'Alessio et al., 1975). RNase A (type XII-A) was obtained from Sigma Chemical Co. (St. Louis, MO). The protein concentration was calculated from $A_{278nm}^{0.1\%} = 0.46$ for seminal RNase (D'Alessio et al., 1972a), $A_{278nm}^{0.1\%} = 0.48$ for MCM-sRNase (Parente et al., 1977), and $A_{278nm}^{0.1\%} = 0.71$ for RNase A (Puett, 1972). Acrylamide (Serva Chemical Co., Heidelberg, West Germany), cesium chloride (C. Erba, Milan, Italy), potassium iodide (C. Erba), and dithiothreitol (Pierce Chemical Co., Rockford, IL) were all used without further purification. Urea (C. Erba) was recrystallized from 95% ethanol and only fresh solutions were used. *N*-Acetyl-L-tyrosinamide was a Sigma product and was shown to be homogeneous by thin-layer chromatography. All other materials were obtained from commercial sources and were used without further purification.

Circular Dichroism. The CD measurements were made at room temperature on a Cary Model 61 dichrograph with constant nitrogen flushing. Cylindrical quartz cells of 0.1 cm path length and a protein concentration of about 0.1 mg/mL were used in the far-ultraviolet region of 200–250 nm. In the near-ultraviolet region of 250–300 nm, protein concentration of 0.9–1.0 mg/mL and a path length of 1 cm were used. The reduced mean residue ellipticities, $[\theta]$, are expressed in deg cm^2 dmol^{-1} and were calculated from the relationship $[\theta] = \theta M/10lc$, where θ is the observed ellipticity in deg, M is the mean residue molecular weight (taken as 117 (D'Alessio et al., 1975) for seminal RNase and MCM-sRNase, and 110.5 (Puett, 1972) for pancreatic RNase A), l is the cell path length in cm, and c is the protein concentration in g/mL. The ellipticity melt experiments (melting profile measurements; Fujita & Imahori, 1975) were carried out at 220 nm in a water-jacketed cell compartment connected to a Haake thermoregulator. The protein solution temperature was increased in a stepwise manner (2 deg/min) from 20 to 80 °C. The temperature inside the cell was measured continuously by means of a thermistor probe inserted directly into the solution in the neck of the cell.

Fluorescence. Fluorescence measurements were made with a Perkin-Elmer Model MPF-2A spectrofluorometer, equipped with a thermostated cell holder and connected to a Hitachi QPD_{33} recorder. Samples were excited in 1-cm square quartz cells at 280 nm. The absorbance of samples examined never exceeded 0.1 in order to minimize errors due to light absorption by the samples. The contribution to the intensity of fluorescence by the solvent buffer or contaminants was routinely measured by a fluorescence scan from 290 to 400 nm upon excitation at 280 nm. Temperature effects were measured by continuously recording the intensity of fluorescence emission at 305 nm while the temperature inside the cell was increased at a constant rate (2 deg/min) by circulating water through the cuvette holder with a Haake thermostat. The temperature inside the cell was directly measured by a thermistor probe immersed into the solution through the cap of the cell.

Acrylamide, CsCl, and KI quenching of fluorescence emission was performed at 25 °C as described by Lehrer (1971). The decrease in intensity of emission, upon excitation at 280 nm, was followed as a function of the quencher concentration at 305 nm for the protein samples, as well as for the model compound *N*-acetyltyrosinamide. The buffer used in each case was 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl.

Difference Spectroscopy. Ultraviolet difference spectra were obtained by using a Cary 15 double beam recording spectrophotometer equipped with a 0–0.1 absorbance scale. Matched 1-cm, 1-mL quartz cells were used. In different compartments were placed the protein samples in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl (absorbance at 280 nm was 0.4) and 2 mM dithiothreitol in the same buffer. The base line was recorded from 320 to 240 nm and, after inversion of the sample cell, the resultant spectrum was recorded twice. The temperature was 22 °C. A spectrum was also recorded after heating the sample cell at 70 °C for 5 min.

Other Methods. Absorbance values at single wavelengths were measured with a Hitachi Perkin-Elmer Model 139 spectrophotometer, while continuous spectra were obtained with a Cary 15 double-beam spectrophotometer. Measurements of pH were made with a Metrohm Model E510 pH meter equipped with a combined glass electrode.

Results

Structural Studies. Far-Ultraviolet CD. The far-ultraviolet CD spectra of seminal RNase, MCM-sRNase, and pancreatic RNase A are shown in Figure 1. A broad negative peak centered at about 215 nm is observed with seminal RNase with negative ellipticity at all wavelengths being less than the corresponding values for MCM-sRNase and RNase A. The shape of the spectrum is more reminiscent of β structure since no evidence of peaks or shoulders is seen near 208 and 222 nm which are characteristics of α-helix content (Greenfield & Fasman, 1969). Conversely, MCM-sRNase and pancreatic RNase A show CD spectra of similar shape, including the extremum at 209 nm and a shoulder near 220 nm. Some differences, however, in extent of negative ellipticity are seen with these two proteins. These results would indicate that dissociation of seminal RNase by cleavage of interchain disulfide bonds leads to the refolding of some residues into a helical structure.

Near-Ultraviolet CD. MCM-sRNase shows negative ellipticity values higher than those of native seminal RNase, but somewhat lower than those of pancreatic RNase A (Figure 1). Our near-ultraviolet CD spectrum of pancreatic RNase

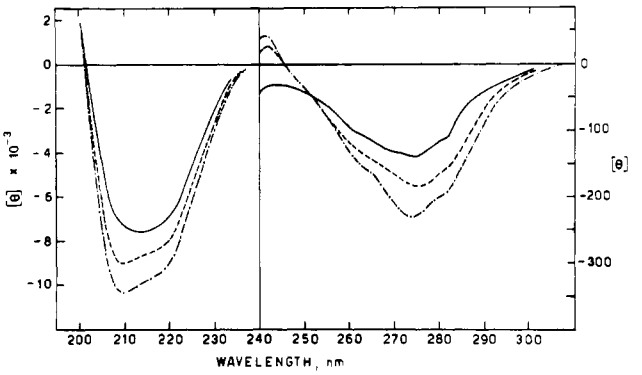

FIGURE 1: Circular dichroic spectra of seminal RNase (—), MCM-sRNase (---), and pancreatic RNase A (—·—) in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. Spectra were recorded at room temperature. The protein concentrations were 0.09–0.1 mg/mL in the far-ultraviolet region (200–250 nm) and 0.9–1.0 mg/mL in the near-ultraviolet region (250–310 nm).

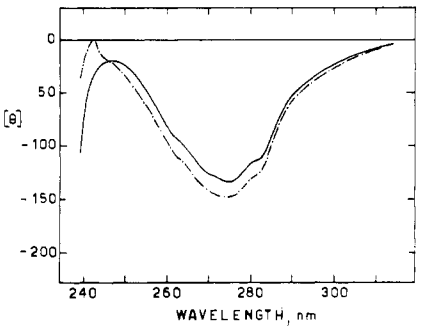

FIGURE 2: Near-ultraviolet circular dichroic spectra of seminal RNase reduced with 1 mM dithiothreitol in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. The protein concentration was 1.0 mg/mL. The spectra were recorded before (—) the addition of the reducing agent and after 10 min (—·—) of reaction. The temperature was 22 °C.

A agrees well with the data of others (Simons et al., 1969; Pflumm & Beychok, 1969; Puett, 1972). The CD extremum near 242 nm, which has been attributed in RNase A to the contribution of exposed tyrosyl groups (Simons & Blout, 1968; Strickland, 1974, and references cited therein), is not observed with seminal RNase, which instead shows negative ellipticity in this region. Since, on the other hand, the extremum at 242 nm is seen with MCM-sRNase, it is conceivable to relate it to the intermolecular covalent association of the two subunits in dimeric seminal RNase. In addition, on the basis of previous band assignments, it would imply exposure of tyrosyl residues upon dissociation of seminal RNase.

Figure 2 shows the changes of near-ultraviolet CD spectra upon reduction of native seminal RNase in the presence of dithiothreitol, i.e., under conditions where selective reduction of intersubunit disulfide bonds and partial dissociation (~30%) of the subunits occur (D'Alessio et al., 1975). The increase in negative ellipticity at 250–290 nm and in particular the decrease near 242 nm observed upon reduction of the native enzyme are in agreement with the results shown in Figure 1 and obtained with the isolated monomeric species MCM-sRNase.

Ultraviolet Difference Spectra. In order to substantiate the results described above, it was of interest to follow the selective reduction and partial dissociation of seminal RNase by measuring difference absorption spectra (Herskovits & Laskowski, 1962; Herskovits, 1967) (Figure 3). Under controlled conditions of reduction both tyrosine and phenylalanine chromophores underwent an alteration in their environment. The fine structure of the difference spectrum below

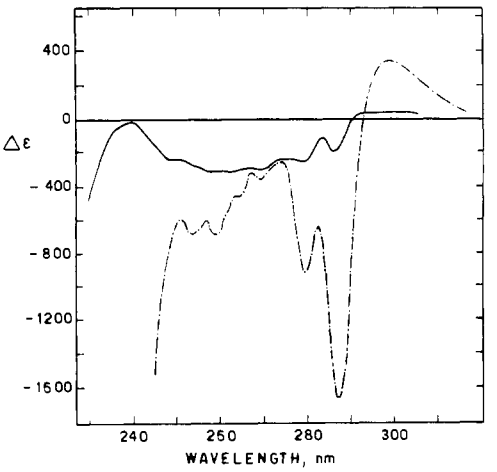

FIGURE 3: Difference spectra produced by reduction of seminal RNase at a protein concentration of 0.86 mg/mL in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl and 1 mM dithiothreitol. The difference spectra were recorded after 10 min at 22 °C (—) and after heating the sample cuvette at 70 °C for 5 min (—·—). The data are expressed as difference in molar absorption, Δε.

270 nm indicates changes in the environment of phenylalanine residues. The appearance of a double negative trough near 287 and 278 nm is characteristic of a blue shift of the tyrosine absorption band, which is usually interpreted as an increased exposure of tyrosine residues to the solvent (Donovan, 1969, 1973). Heating the seminal RNase solution in the presence of dithiothreitol at 70 °C for 5 min induces a large difference spectrum, indicative of a drastic alteration of the tyrosine environment.

Fluorescence and Fluorescence Quenching. The fluorescence emission of the protein molecules under study is due entirely to tyrosine residues since they lack tryptophan residues. Upon excitation at 280 nm, the three RNase molecules showed emission maxima near 303 nm which are typical of tyrosine residues (Brand & Witholt, 1967). The quantum yields of seminal RNase and MCM-sRNase in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl have been found to be very low, approximately one-third of that observed with pancreatic RNase A. Low quantum yields can be ascribed to buried tyrosine residues, which are usually thought to be nonfluorescent (Cowgill, 1968; see also Discussion).

Ionic quenchers such as iodide and cesium ions (Lehrer, 1971) as well as the neutral quencher acrylamide (Eftink & Ghiron, 1976, 1977) were used to measure the exposure of tyrosyl residues in the RNase molecules under study. A convenient method for quantitating the efficiency with which an added compound can quench the fluorescence of an excited species is through the relationship derived by Stern & Volmer (1919), $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the fluorescence intensities of the species in the absence and in the presence of the quencher, respectively, [Q] is the quencher concentration and K_{sv} is the Stern–Volmer constant. The results of the quenching of tyrosine fluorescence of seminal RNase, MCM-sRNase, pancreatic RNase A, and the model compound *N*-acetyltyrosinamide are shown in Figure 4. The most striking feature of the results is the high steric shielding of the tyrosine residues observed in native seminal RNase since both iodide as well as cesium ions do not have a quenching effect to any measurable degree. The efficiency of quenching of these ions with MCM-sRNase and pancreatic RNase A falls in between the extreme cases of native seminal RNase and of the model compound *N*-acetyltyrosinamide, reflecting the intermediate topographical positioning of their tyrosyl

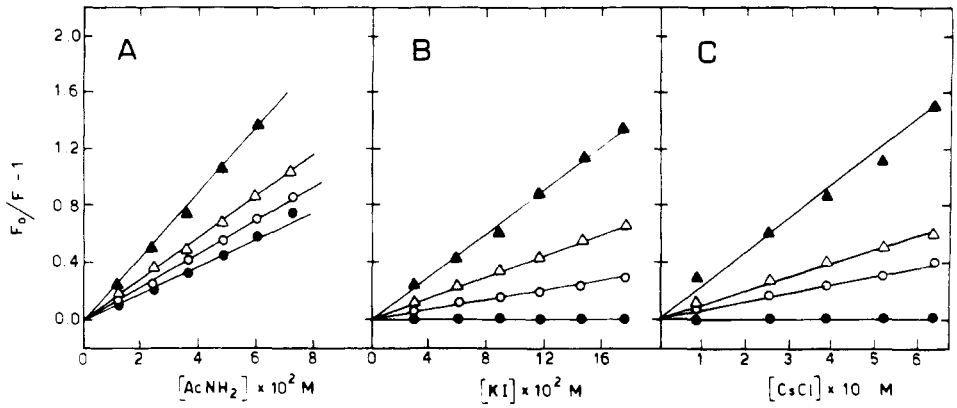

FIGURE 4: Stern–Volmer plots for acrylamide (A), potassium iodide (B), and cesium chloride (C) quenching of tyrosine fluorescence of seminal RNase (●—●), MCM-sRNase (○—○), pancreatic RNase A (△—△), and the model compound *N*-acetyltyrosinamide (▲—▲). The wavelength of excitation was 280 nm and emission was measured at 305 nm. Spectra were recorded at 25 °C. The proteins were dissolved at a concentration of about 0.1 mg/mL in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl.

residues. MCM-sRNase appears to possess tyrosine residues which are not completely protected from collision with the ionic probes, as it is in the case for native seminal RNase, but more shielded than those in pancreatic RNase A. Acrylamide quenching of tyrosine fluorescence occurs also with seminal RNase and the effects observed parallel those obtained with ionic quenchers, since the same order of quenching for the three proteins is observed.

Stability Studies. Heat. The stability of the proteins under study to thermal denaturation was assessed by measuring the dichroic signal at 220 nm, which is a sensitive parameter of the polypeptide backbone conformation (Greenfield & Fasman, 1969). As shown in Figure 5, with seminal RNase and pancreatic RNase A, there is a small, linear decrease in negative ellipticity below 50 °C, followed by a larger decrease between 50 and 70 °C, indicative of a major conformational change. The transition midpoints calculated from the sigmoidal curves are the denaturation or melting temperatures (T_m) which occur at 61 and 60 °C for seminal RNase and pancreatic RNase A, respectively. Conversely, MCM-sRNase unfolds at a lower temperature, showing a transition curve with a T_m of 51 °C. The thermal unfolding processes were found largely reversible and the extent of reversibility was related to the maximum temperatures reached during the melting experiments. However, no attempts were made to find out conditions governing full reversibility of the process of thermal unfolding.

The 42–47% reduction of negative ellipticity at 220 nm observed at 70–80 °C (Figure 5) with the three proteins is indicative of extensive unfolding. At high temperatures the protein molecules appear to retain some secondary structure, since the far-ultraviolet CD spectra at 70–80 °C of the proteins under study differ from those expected for random coil polypeptides, which show positive ellipticity in the region 210–230 nm (Greenfield & Fasman, 1969). However, it should be observed that there is no general agreement on the CD spectrum of a polypeptide in an aperiodic structure (Holladay & Puett, 1976).

The thermal stability of the tertiary structure of the three proteins was investigated by tyrosine fluorescence measurements. Whereas the curve of intensity of fluorescence against temperature for a model compound, e.g., *N*-acetyltyrosinamide, decreases monotonically with increasing temperature, with proteins deviations from a monotonic decrease are often observed (Steiner & Edelhoch, 1962; Gally & Edelman, 1962; Brand & Witholt, 1967). This is usually taken as indicative of a modification in the environments of the fluorophores and

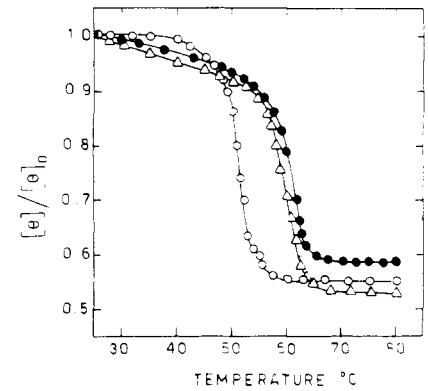

FIGURE 5: Effect of temperature on the mean residue ellipticity, $[\theta]$, at 220 nm of seminal RNase (●—●), MCM-sRNase (○—○), and pancreatic RNase A (△—△), all dissolved in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl at a protein concentration of 0.08–0.1 mg/mL. The results are reported as the ratio of $[\theta]/[\theta]_0$ as a function of temperature, where $[\theta]_0$ is the ellipticity value at 25 °C.

consequently of a transition in tertiary structure of the protein molecule (Cowgill, 1968). The fluorescence intensities of the tyrosyl residues of native seminal RNase and pancreatic RNase A fall monotonically with increasing temperature until about 50 °C (Figure 6). Above this temperature, the intensities increase, particularly sharply with seminal RNase, and finally fall again at much higher temperatures. The profile of fluorescence intensity vs. temperature obtained with RNase A is in agreement with that previously reported (Gally & Edelman, 1962). In the case of MCM-sRNase, the sigmoidal curve of intensity against temperature is shifted to lower temperature. The temperature dependence of fluorescence emission intensity reveals transitions in the same range as it is observed by using far-ultraviolet CD measurements. The T_m values calculated from the curves shown in Figure 6 are the same, within experimental error, as those obtained from CD data (Figure 5).

Urea. Unfolding profiles obtained with protein denaturants (urea, guanidine hydrochloride) offer a useful method for examining the stability, or lack of stability, of protein molecules (Tanford, 1968, 1970; Pace, 1975). Figure 7 shows the changes of negative ellipticity values measured at 220 nm as a function of urea concentration for seminal RNase, MCM-sRNase, and pancretic RNase A. The overall unfolding profiles are very similar for seminal RNase and pancreatic RNase A, which appear rather stable to urea

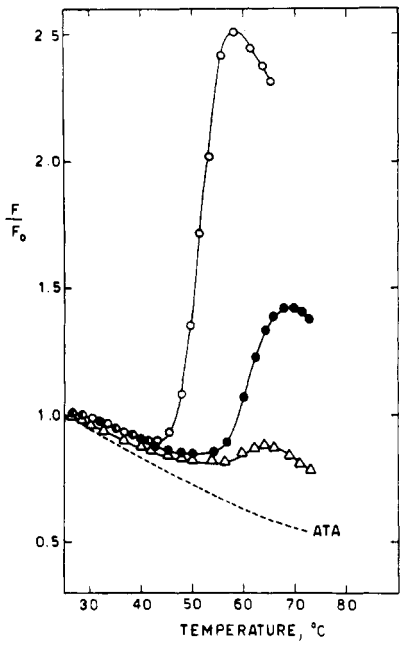

FIGURE 6: Temperature dependence of relative fluorescence intensity of seminal RNase (●—●), MCM-sRNase (○—○), and pancreatic RNase A (△—△) and of the model compound *N*-acetyltyrosinamide (---). Excitation was carried out at 280 nm and relative emission intensity measured at 305 nm. The results are reported as the ratio of F/F_0 as a function of temperature, where F_0 is the relative fluorescence emission intensity at 25 °C.

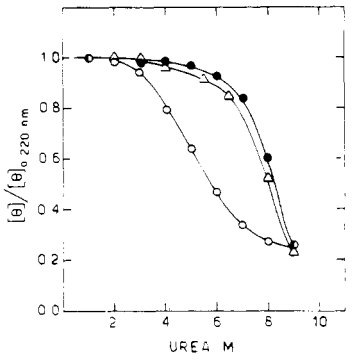

FIGURE 7: The variation of the mean residue ellipticity, $[\theta]$, at 220 nm for seminal RNase (●—●), MCM-sRNase (○—○), and pancreatic RNase A (△—△) with urea concentration. Measurements were performed after 20-h incubation at room temperature at about 0.1 mg/mL protein concentration in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl.

denaturation (Salahuddin & Tanford, 1970). MCM-sRNase, on the other hand, is unfolded at a lower urea concentration, with a transition midpoint occurring near 5 M urea.

pH. The effect of pH on the fluorescence intensity of RNases is depicted in Figure 8. In the pH range 3–9 the protein molecules appear to be stable, since no structural transition is revealed by changes in the environment of the fluorescent tyrosines. The fluorescence intensity is quenched in alkali since tyrosyl residues ionize in the proteins and tyrosinate is nonfluorescent (Brand & Witholt, 1967). In acid solution, below pH 2.5, a dramatic increase in fluorescence intensity is seen with seminal RNase and MCM-sRNase. The acid transition observed with MCM-sRNase occurs at a somewhat higher pH than for the native seminal RNase. The enhancement of tyrosine fluorescence intensity should be related to changes in conformation since far-ultraviolet CD spectra (not shown) of all three RNases at pH 2 indicated significant unfolding.

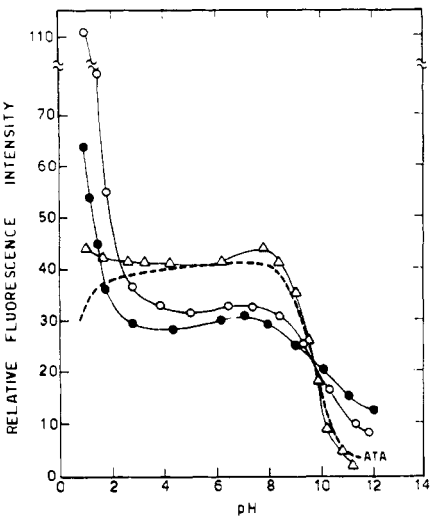

FIGURE 8: The pH dependence of the intensity of fluorescence measured at 305 nm, upon excitation at 280 nm, for seminal RNase (●—●), MCM-sRNase (○—○), and pancreatic RNase A (△—△) and for the model compound *N*-acetyltyrosinamide (---). Measurements were performed after adjusting the pH of a protein solution at pH 7.0, 0.1 mg/mL, in a 3-mL cuvette with 3 N NaOH or 3 N HCl.

Discussion

Secondary Structure. When two homologous protein molecules contain sufficient similarities in their primary structures, they usually exhibit similarities also in the folding of the polypeptide backbone. The data presented in this paper, unlike other studies on the characterization of functionally identical proteins from different sources, allow the comparison between proteins from the same species. The structure of bovine pancreatic RNase A is compared with those of bovine seminal RNase and of its monomeric, catalytically active derivative MCM-sRNase. Extensive similarities were found between pancreatic RNase A and monomeric MCM-sRNase. In fact, the far-ultraviolet CD spectrum of MCM-sRNase was found to be very similar to that of pancreatic RNase A (Figure 1). At pH 7.0, both protein molecules show CD spectra of similar shape with a negative extremum at 209 nm and a shoulder near 220 nm, which are usually taken as indication of an α-helix content (Greenfield & Fasman, 1969). The observed negative ellipticity values for MCM-sRNase are somewhat lower than those observed with pancreatic RNase A. This could indicate some differences in secondary structure between the two protein molecules, but it should be born in mind some experimental limitations in CD measurements. These include instrument calibration (Cassim & Yang, 1969) and protein concentration measurements, so that the calculated $[\theta]$ could be off by a few percent.

The far-ultraviolet CD spectrum of native seminal RNase is clearly different from the spectrum observed with both MCM-sRNase and pancreatic RNase A. This would imply that quaternary structure affects the secondary structure of native seminal RNase. A single negative broad trough centered at about 215 nm is observed and the spectrum is more reminiscent of a β-structure content (Greenfield & Fasman, 1969). Use of the numerical data proposed by Greenfield & Fasman (1969) for the quantitation of the CD spectra would indicate that dissociation of seminal RNase by cleavage of interchain disulfide bonds leads to the refolding of some 7% of the polypeptide chain into a helical structure. This figure, however, should be regarded as only approximate. It is becoming more and more clear that CD determinations of secondary structures in proteins have several limitations, since

the methods of calculations are empirical and by necessity involve many assumptions (Chen et al., 1974; Garnier et al., 1976; White, 1976). On the other hand, the structures found for a number of proteins, including pancreatic RNase A, are in fairly good agreement with those known to exist from X-ray diffraction studies. Whether our estimates of a higher α-helix content in MCM-sRNase by comparison with native seminal RNase are accurate remains to be seen when the X-ray structure of this protein becomes available (L. Mazzarella, work in progress).

The State of Tyrosine Residues. Investigations which use both crystals and solutions indicate that, in pancreatic RNase A, the hydroxyl groups of Tyr-73, -76, and -115 are free to interact with the solvent, whereas Tyr-25, -92, and -97 are located inside the protein molecule and hydrogen bonded to other groups belonging to the protein (Wyckoff et al., 1970; Richards & Wyckoff, 1971; Strickland, 1974). In the polypeptide chain of seminal RNase, 4 tyrosine residues occur in positions 25, 73, 92, and 97 (Suzuki et al., 1976). Therefore, the exposed tyrosine residues at positions 76 and 115 of pancreatic RNase A are missing in seminal RNase.

In the near-ultraviolet region, the CD spectrum of seminal RNase shows a broad negative envelope centered at about 275 nm and the intensity of negative ellipticity in the 250–300-nm region is less than that observed in MCM-sRNase and pancreatic RNase A. This lower intensity is in agreement with the proposal that the near-ultraviolet CD spectrum of pancreatic RNase A arises mainly from the phenolic rings which have their hydroxyl groups exposed to the solvent and in particular from the interactions of the exposed residues Tyr-73 and Tyr-115 (Horwitz & Strickland, 1971; Strickland, 1974, and references cited therein). Since Tyr-115 is absent in seminal RNase and substituted with a serine residue, these interactions cannot occur. Similar observations and explanations have been previously made with rat ribonuclease, which is homologous to the pancreatic bovine enzyme and has only 4 tyrosine residues in positions 25, 92, 97, and 115, with Tyr-73 being replaced by a histidine residue (Klee & Streaty, 1970).

Upon dissociation of native seminal RNase, an increase in negative ellipticity in the 250–300-nm region is observed (Figures 1 and 2). On the basis of the considerations reported above, it can be inferred that the dimer–monomer conversion leads to an increased exposure of tyrosine residue(s). However, it should be born in mind that disulfide bonds may represent significant contributions to the optical activity in the region of side-chain chromophores (Sears & Beychok, 1973; Strickland, 1974). Accordingly, it could be argued that the spectral differences observed in the near-ultraviolet CD spectrum between seminal RNase and MCM-sRNase could arise in part from the loss, through chemical modification, of the intrinsic optical activity of the intersubunit disulfide bonds and in part from changes in the optical activities of other chromophores (Bewley, 1977).

A distinct feature of the near-ultraviolet CD spectrum of seminal RNase involves the negative ellipticity shown near 242 nm, where both the other two protein molecules show a positive extremum. The positive peak at 242 nm observed with bovine pancreatic RNase A has been attributed to a contribution of exposed tyrosine residues (Pflumm & Beychok, 1969; Simons & Blout, 1968; Simons et al., 1969; Puett, 1972; Strickland, 1972). Rat pancreatic ribonuclease, where the tyrosine residues located at positions 73 and 76 (both exposed) in bovine pancreatic RNase A are missing, does not have a positive ellipticity near 242 nm (Klee & Streaty, 1970). It was thus proposed that one or both of these two tyrosine residues contribute to the positive dichroic signal near 242 nm (see Strickland, 1974, for a review). If this interpretation is correct, considering that Tyr-76 is missing in seminal RNase, we could infer that the positive ellipticity in MCM-sRNase could result from Tyr-73, which could be exposed to the solvent medium upon dissociation of native seminal RNase. Alternatively, it may be argued that near Tyr-25 conformational changes could take place more readily than anywhere else, considering that this residue is in close proximity to the intermolecular disulfide bonds at positions 31 and 32 of the polypeptide chain. This would be in line with the proposal of Simons & Blout (1968) that Tyr-25 contributes significantly to the positive peak at 242 nm of RNase A.

The burial of most tyrosine residues in seminal RNase and their partial exposure upon dissociation of the native dimeric protein have been indicated by additional spectroscopic techniques, such as differential absorption, tyrosine fluorescence emission, and fluorescence quenching. Selective reduction of intermolecular disulfide bonds of seminal RNase under controlled conditions produced a negative difference spectrum with characteristics related to the tyrosine chromophore (Figure 3). The small molar absorbance change of $-210\ M^{-1}\ cm^{-1}$ at 287 nm indicates that only a small change in the environment of tyrosine residues has occurred, since a transfer of a single residue from the hydrophobic interior of a protein to essentially unhindered contact with solvent would imply a molar absorption difference of $-750\ M^{-1}\ cm^{-1}$ (Donovan, 1973). However, it should be recalled that, under the experimental conditions used for selective reduction of the native enzyme, only partial dissociation ($\sim$30%) occurs (D'Alessio et al., 1975). A more significant change in tyrosine environment occurs after heating the seminal RNase solution in the presence of dithiothreitol since the observed molar absorbance change at 287 nm of $-1700\ M^{-1}\ cm^{-1}$ corresponds to the exposure of more than two tyrosine residues.

The quantum yields of tyrosine fluorescence in seminal RNase and MCM-sRNase are extremely low, only about one-third of that of pancreatic RNase A. This can be ascribed to buried positioning of tyrosine residues in the seminal RNase molecule. Buried residues are usually thought to be nonfluorescent, owing largely to hydrogen bonding to peptide and other polar groups in the interior of the protein molecule (Cowgill, 1968). Upon denaturation by high temperature or acid, the unfolding of seminal RNase and MCM-sRNase leads to the exposure of tyrosyl residues with concomitant dramatic effects on the intensity of tyrosine fluorescence (Figures 6 and 7).

An alternative explanation can also be given since the low fluorescence of pancreatic RNase A has been explained in terms of internal quenching by sulfur-containing amino acid residues and in particular by disulfide groups (Cowgill, 1970). In fact, it has been shown that sulfur-containing molecules quench the fluorescence of tyrosine and tryptophan (Arian et al., 1970; Bent & Hayon, 1975; Longworth, 1968). More recently (Morgan et al., 1978), it has been proposed that fluorescence quenching in pancreatic RNase A is due to the existence of regions in which the side chains of sulfur-containing amino acids (cystines and methionines) alternate in space with side chains of tyrosine, leading to an S–π interaction which causes quenching of tyrosine fluorescence. Considering that seminal RNase and MCM-sRNase have a higher number of sulfur-containing amino acid residues (disulfides and thioethers) than pancreatic RNase A, an even lower fluorescence is expected and in fact found.

An excellent way to experimentally determine the degree of exposure of aromatic residues (tryptophan and tyrosine) in proteins in solution is by fluorescence quenching (Lehrer, 1971). This technique was used to complement the results discussed above and to discriminate between burial of tyrosine residues or quenching of fluorescence by sulfur amino acids (see above). Ionic quenchers such as KI and CsCl (Lehrer, 1971) have been used in this study, but also the neutral quencher acrylamide (Eftink & Ghiron, 1976, 1977) has been used since the quenching effect of polarizable ionic quenchers can be influenced by electrostatic effects due to the polyelectrolyte nature of a protein molecule (Lehrer, 1971). Acrylamide, which does not show these shortcomings, was proposed as an extremely efficient collisional quencher, capable of permeating the protein matrix and reporting on the "depth of the burial" of a fluorophore (Eftink & Ghiron, 1976, 1977). The results obtained with ionic quenchers indicate that in seminal RNase the tyrosine residues are completely protected from collision with these ionic probes, whereas acrylamide does show a measurable quenching of tyrosine fluorescence. The observed order of quenching with all three quenchers is *N*-acetyltyrosinamide > pancreatic RNase > MCM-sRNase > seminal RNase and is consistent with the topographical positioning of tyrosyl residues inferred from the above discussed spectroscopic techniques.

Irie & Suito (1975) found that only 1–2 out of 8 tyrosine residues per molecule of seminal RNase titrate normally with a pK_a of about 9.8 and are accessible to chemical reagents. Interestingly, some tyrosine residues were not titrated, even in 8 M urea or 6 M guanidine hydrochloride, thus greatly differing from the homologous tyrosines of RNase A (Richards & Wyckoff, 1971). These results, indicating deep burial of some tyrosine residues in seminal RNase, are in general agreement with the data reported in this study.

Stability. Native dimeric seminal RNase showed a heat stability similar to that of pancreatic RNase A. This was evident from melting profiles obtained by monitoring the loss in secondary structure by CD measurements at 220 nm, as well as by monitoring changes in the tyrosine fluorescence emission which can be related to changes in tertiary structure. By using these two thermal unfolding probes, which are sensitive to different regions of the protein molecules, the same transition temperature, T_m, was obtained, i.e., 61 °C for seminal RNase and 60 °C for pancreatic RNase A. This is usually taken as an indication of a two-state unfolding process (Lumry et al., 1966). Conversely, monomeric MCM-sRNase is less thermostable and unfolds near 51 °C. The results on urea or acid denaturation of the three RNase molecules parallel those obtained with thermal denaturation, indicating that MCM-sRNase is less stable than both seminal and pancreatic RNase. In particular, the acid transition observed with MCM-sRNase occurring at somewhat higher pH than for native seminal RNase (Figure 8) can be interpreted in terms of a loose structure of monomeric MCM-sRNase with respect to that of native seminal RNase.

It is tempting to speculate on the molecular mechanism of the stabilization observed in dimeric seminal RNase with respect to monomeric MCM-sRNase. Many extracellular proteins, which require greater stability to fluctuating environmental conditions, contain intrachain disulfide cross-links, which have the presumed function to stabilize the native conformation. Usually, stabilization of protein molecules by disulfide bonds is explained in terms of destabilization of a cross-linked polypeptide chain in the unfolded state due to a loss of entropy (see Johnson et al., 1978, for a recent discussion). A similar explanation can also be given for the stabilizing effect of interchain disulfide bonds in seminal RNase.

Noncovalent intersubunit interactions in seminal RNase should also enhance stability. With polypeptides or relatively small proteins in aqueous solution, aggregation by intermolecular contacts due to hydrophobic interactions is often observed, leading to the burial of nonpolar residues in the hydrophobic core of the molecule. The burial of hydrophobic tyrosine residue(s) upon dimerization of the subunits in seminal RNase, as observed by several spectroscopic techniques, could indicate the acquisition of hydrophobic interactions enhancing the stability of the dimeric molecule. On the other hand, on the basis of far-ultraviolet CD spectra, dimerization appears to be accompanied also by changes in secondary structure, which could also influence stability of the protein molecule.

There are a few other examples of protein association resulting in structural changes and stability. A situation similar to the case of seminal RNase may be found with apoA-II, an apoprotein of the human high density lipoprotein complex. ApoA-II is composed of two identical chains linked by a single disulfide bond. In other species, the protein lacks this bond and the molecule has half the molecular weight of human apoA-II. It appears that the chain of 77 amino acid residues has only marginal stability because of its small size, but acquires additional structure and stability when it dimerizes (Gwynne et al., 1975). Another example is given by the S peptide of pancreatic RNase A. The peptide is largely unfolded in aqueous solution, while in the binding process to S protein it undergoes a coil-to-helix transition (Scatturin et al., 1967). In addition, whereas S protein unfolds at 34 °C (Simons et al., 1969), RNase S′, obtained by mixing equimolar quantities of S peptide and S protein, unfolds near 45 °C (Sherwood & Potts, 1965; Takahashi et al., 1969).

Finally, it seems relevant to recall previous results obtained from the exposure of native seminal RNase, MCM-sRNase, and pancreatic RNase A to the proteolytic action of trypsin (Parente et al., 1976). Seminal RNase and RNase A have been found completely resistant to digestion, whereas MCM-sRNase was a good substrate for trypsin and was rapidly cleaved. The data presented in this paper are well in line with these results. They all lead to the conclusion that the dimeric structure of seminal RNase exerts a significant protective effect on the protein molecule not only toward protein denaturing agents such as heat and urea but also toward proteolytic enzymes. The advantage in stability gained by association of the two monomeric globular entities could influence to a significant degree the steady-state concentration of active seminal RNase. In conclusion, the dimeric structure of seminal RNase may be a physiologically significant device for maintaining an effective concentration of ribonuclease even in an adverse environment. In this respect, these considerations parallel those recently reported by Wang & Hirs (1977) in a study on the influence of heterosaccharides in porcine pancreatic ribonuclease on the conformation and stability of the protein.

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References

Arian, S., Benjamini, M., Feitelson, J., & Stein, G. (1970) *Photochem. Photobiol. 12*, 481–487.

Bent, D. V., & Hayon, E. J. (1975) *J. Am. Chem. Soc. 97*, 2612–2619.

Bewley, T. A. (1977) *Biochemistry 16*, 4408–4414.
Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11*, 776–856.
Cassim, J. Y., & Yang, J. T. (1969) *Biochemistry 8*, 1947–1951.
Chen, Y. H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.
Cowgill, R. W. (1968) *Biochim. Biophys. Acta 168*, 417–430.
Cowgill, R. W. (1970) *Biochim. Biophys. Acta 207*, 556–559.
Crestfield, A. M., Stein, W. H., & Moore, S. (1962) *Arch. Biochem. Biophys., Suppl. 1*, 217–222.
D'Alessio, G., Floridi, A., De Prisco, R., Pignero, A., & Leone, E. (1972a) *Eur. J. Biochem. 26*, 153–161.
D'Alessio, G., Parente, A., Guida, C., & Leone, E. (1972b) *FEBS Lett. 27*, 285–288.
D'Alessio, G., Malorni, M. C., & Parente, A. (1975) *Biochemistry 14*, 1116–1121.
Di Donato, A., & D'Alessio, G. (1973) *Biochem. Biophys. Res. Commun. 55*, 919–928.
Donovan, I. W. (1973) *Methods Enzymol. 27D*, 497–520.
Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry*, Part A (Leach, S. J., Ed.) pp 158–180, Academic Press, New York.
Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.
Eftink, M. R., & Ghiron, C. A. (1977) *Biochemistry 16*, 5546–5551.
Floridi, A., D'Alessio, G., & Leone, E. (1972) *Eur. J. Biochem. 26*, 162–167.
Fujita, S. C., & Imahori, K. (1975) *Pept. Polypeptides Proteins, Proc. Rehovot Symp., 2nd*, 217–229.
Gally, J. A., & Edelman, H. H. (1962) *Biochim. Biophys. Acta 60*, 499–509.
Garnier, J., Salesse, R., Rerat, B., Rerat, C., & Blake, C. (1976) *J. Chim. Phys. Phys.-Chim. Biol. 73*, 1018–1023.
Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.
Gwynne, J., Palumbo, G., Osborne, J. C., Jr., Brewer, H. B., Jr., & Edelhoch, H. (1975) *Arch. Biochem. Biophys. 170*, 204–212.
Herskovits, T. T. (1967) *Methods Enzymol. 11*, 748–775.
Herskovits, T. T., & Laskowski, M. (1962) *J. Biol. Chem. 237*, 2481–2492.
Holladay, L. A., & Puett, D. (1976) *Biopolymers 15*, 43–59.
Horwitz, J., & Strickland, E. H. (1971) *J. Biol. Chem. 246*, 3749–3752.
Irie, M., & Suito, F. (1975) *J. Biochem. (Tokyo) 77*, 1075–1084.
Johnson, R. E., Adams, P., & Rupley, J. A. (1978) *Biochemistry 17*, 1479–1484.
Klee, W. A., & Streaty, R. A. (1970) *J. Biol. Chem. 245*, 1227–1232.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
Longworth, J. W. (1968) *Photochem. Photobiol. 7*, 587–596.
Lumry, R., Biltonen, R., & Brandts, J. F. (1966) *Biopolymers 4*, 917–944.
Morgan, R. S., Tatsch, C. E., Gushard, R. H., McAdon, J. M., & Warme, P. K. (1978) *Int. J. Pept. Protein Res. 11*, 209–217.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Parente, A., Branno, M., Malorni, M. C., Welling, C. W., Libonati, M., & D'Alessio, G. (1976) *Biochim. Biophys. Acta 445*, 377–385.
Parente, A., Albanesi, D., Garzillo, A. M., & D'Alessio, G. (1977) *Ital. J. Biochem. 26*, 451–466.
Pflumm, M. N., & Beychok, S. (1969) *J. Biol. Chem. 244*, 3973–3981.
Puett, D. (1972) *Biochemistry 11*, 1980–1990.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647–806.
Salahuddin, A., & Tanford, C. (1970) *Biochemistry 9*, 1342–1347.
Scatturin, A., Tamburro, A. M., Rocchi, R., & Scoffone, E. (1967) *J. Chem. Soc., Chem. Commun.*, 1273–1274.
Sears, D. W., & Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry*, Part C (Leach, S. J., Ed.) pp 445–593, Academic Press, New York.
Sherwood, L. M., & Potts, J. T., Jr. (1965) *J. Biol. Chem. 240*, 3799–3805.
Simons, E. R., & Blout, E. R. (1968) *J. Biol. Chem. 243*, 218–221.
Simons, E. R., Schneider, E. G., & Blout, E. R. (1969) *J. Biol. Chem. 244*, 4023–4026.
Steiner, R. F., & Edelhoch, H. (1962) *Nature (London) 193*, 375–376.
Stern, O., & Volmer, M. (1919) *Phys. Z. 20*, 183–188.
Strickland, E. H. (1972) *Biochemistry 11*, 3465–3474.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113–175.
Suzuki, H., Greco, L., Parente, A., Farina, B., La Montagna, R., & Leone, E. (1976) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.) Vol. 5, Suppl. 2, p 93, N.B.R. Foundation, Washington, D.C.
Takahashi, T., Irie, M., & Ukita, T. (1969) *J. Biochem. (Tokyo) 65*, 55–62.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–282.
Tanford, C. (1970) *Adv. Protein Chem. 24*, 1–95.
Wang, D., Wilson, G., & Moore, S. (1976) *Biochemistry 15*, 660–665.
Wang, F.-F. C., & Hirs, C. H. W. (1977) *J. Biol. Chem. 252*, 8358–8364.
White, H. A. (1976) *Biochemistry 15*, 2906–2912.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305–328.