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The Abnormal Carboxyl Groups of Ribonuclease. I. Preparation and Properties of Methylated Ribonuclease*

Clarence A. Broomfield,† John P. Riehm, and Harold A. Scheraga

ABSTRACT: Previous work has indicated that ribonuclease A contains at least two carboxyl groups which do not titrate normally, and it has been postulated that these abnormal carboxyl groups are associated with the abnormal tyrosine residues known to be present in the molecule. The present work is an attempt to locate in the amino acid sequence those carboxyl groups which are abnormal, by esterifying the "exposed" carboxyl groups under conditions where the "buried" residues do not react. It has been found that a product could be obtained which contained three free carboxyl groups. This product possessed essentially no enzymatic activity; the activity was largely regained by saponification under mild conditions. At least two of the abnormal tyrosine residues were not normalized in the methylated derivative, as indicated by the spectrophotometric titration curve, although there is an apparent loosening of the structure.

In the acid range, the methylated protein underwent low-temperature transitions and thermally induced transitions which were similar to those of ribonuclease. That the folded structure of the derivative is similar to that of the native protein is shown by the difference spectrum of the methylated protein versus ribonuclease at neutral *p*H and low temperature. These results, together with the location in the sequence of the three free carboxyl groups (the subject of the following paper), provide information about the role of specific side-chain interactions in maintaining the folded structure of ribonuclease.

In order to fit the experimental titration curve of ribonuclease A to a theoretical curve, it is necessary to assume that the intrinsic ionization constants, $pK°$, of some of the carboxyl groups are abnormally low. Tanford and Hauenstein (1956) assumed that five of the ten side-chain carboxyl groups have a $pK°$ of 4.0, while the remaining five groups have a more normal $pK°$ of 4.70. To explain ultraviolet difference spectral data and conformational changes at low *p*H, Hermans and Scheraga (1961) have assumed that one carboxyl group has a $pK°$ of 2.5, another a $pK°$ of 3.65, and the remainder a $pK°$ of 4.6. The latter authors have also postulated the existence of interactions involving carboxyl groups and "buried" tyrosyl side chains (Shugar, 1952, Tanford *et al.*, 1955) in a nonpolar region.

Since the sequence of amino acids in ribonuclease A is known, the identification of the abnormal carboxyl groups as well as the buried tyrosine residues would provide valuable information about the conformation

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of the protein in solution. Cha and Scheraga (1963) and Donovan (1963) have reported the identification of two and one, respectively, of the abnormally titrating tyrosine residues, and work is currently in progress in this laboratory to identify the third. The present work was carried out in order to determine the location in the amino acid sequence of the abnormal carboxyl groups.

It might be expected that carboxyl groups which are involved in intramolecular interactions, such as hydrogen bonding in the postulated nonpolar regions, would be less reactive than similar groups not so involved. Therefore, it was decided to attempt to identify the abnormal groups by preferentially reacting those which are most accessible, and then determining which carboxyl residues remain free. It would be preferred, of course, to carry out the desired modification in an aqueous medium since the purpose of this work is to obtain information about the structure of the molecule in water. However, there are a number of difficulties inherent in such a procedure, the most important of which is the competition of the water with the protein for the specific reagent. Attempts were made to react the carboxyl groups of ribonuclease A in an aqueous solution, and the results are reported in an accompanying paper (Riehm and Scheraga, 1965).

A more practical approach to the problem was esterification of the carboxyl groups in methanol, using HCl as a catalyst. It was possible to find conditions under which a product which consistently contained three free carboxyl groups was obtained. This product was shown to possess a folded structure and contained at least two abnormal tyrosyl groups.

It is important to note that, despite the fact that the conformation of ribonuclease in HCl is different from that at neutral *p*H, a partially methylated derivative is obtained which, like native ribonuclease, has abnormal tyrosyl residues which are near carboxyl groups. Hence, it is reasonable to assume that the partially methylated derivative, prepared at low *p*H, refolds to the native conformation in water at neutral *p*H.[1]

The esterified material was essentially inactive enzymatically but could be reactivated to a large extent by mild alkaline hydrolysis of the ester groups. This paper describes the preparation and some physicochemical properties of the derivative, and the next paper (Riehm *et al.*, 1965) describes the identification of the free carboxyl groups in the amino acid sequence.

Experimental

Materials. Ribonuclease (either the 5-times-recrystallized or the type II chromatographed material) was obtained from the Sigma Chemical Co. The ribonuclease A fraction was prepared by chromatography of the commercial product on a 7.5 × 60-cm column of unsieved Amberlite IRC-50, XE-64 resin according to the procedure of Hirs *et al.* (1953), and then deionized by passage through a 5.0 × 40-cm column of MB-1 mixed-bed resin (Rohm and Haas Co.). The material was then lyophilized and stored at 2° until needed.

Ribonucleic acid (RNA) was purchased from Nutritional Biochemicals Corp. 2,3-Diaminopropionic acid and 2,4-diaminobutyric acid were obtained from Mann Research Laboratories, Inc. Ninhydrin and hydrindantin were Dougherty Chemical Co. products. All other reagents were either reagent grade or the best grade available.

Methods

Ribonuclease Methyl Ester. The ester was prepared according to a modification of the procedure described by Fraenkel-Conrat and Olcott (1945), a procedure which has been shown to selectively esterify carboxyl groups (Saroff *et al.*, 1953). The conditions for obtaining this methylated derivative were empirically determined. Approximately 250 mg of ribonuclease A was suspended in 25 ml of cold absolute methanol (2°) and the suspension was stirred in a 35-ml capacity polyethylene container by means of a Teflon-coated magnetic stirring bar. To this suspension was slowly added 150 μl of concentrated HCl. The protein immediately dissolved, but then partially reprecipitated as a very fine colloidal suspension. The stirring bar was removed, and the container was tightly covered and then fixed to a slowly revolving motor which stirred the suspension by inverting the container twice per second. The reaction was allowed to proceed for 168 hours at 2°. At the end of this time, the reaction mixture was poured into 25 ml of cold deionized water and the small amount of product adhering to the sides of the reaction vessel was dissolved by pouring the solution back and forth between the containers. The solution was dialyzed in the cold against three changes of 0.001 M HCl and then lyophilized. The product was stored in a desiccator at 2° until needed.

Potentiometric Titrations. These measurements were made in 0.15 M KCl, using a Radiometer Model PHM 4 meter equipped with a G222B glass electrode and a Type K100 calomel reference electrode. The meter was standardized against phthalate, borate, tetroxalate, and mixed phosphate buffers. Each of these buffers was prepared according to the recommendations of Bates (1954). Titrations were performed with approximately 1 M HCl and carbonate-free 1 M KOH. Carbon dioxide was excluded by continuously purging the titration vessel with water-saturated, carbon dioxide–free nitrogen. These experiments were generally not carried out above *p*H 9.0, since high *p*H favors saponification of the ester groups; measurements above *p*H 7.0 were made as rapidly as possible.

Saponification Studies. These studies were made using the equipment described for the titration experiments. The *p*H of a ribonuclease–methyl ester solution (approximately 50 mg in 5 ml of water) was adjusted to *p*H 10.44 by the addition of 1 M KOH. The solution

[1] Of course, the possibility always exists that, in the refolded partially methylated protein, the abnormal tyrosyl residues could be near different carboxyl groups than in the native molecule.

was then maintained at this *p*H until the rate of base uptake had become very slow. Again, the solution was purged with water-saturated, carbon dioxide-free nitrogen.

Spectrophotometric Titrations and Transition Studies. These experiments were carried out in a Cary Model 14 spectrophotometer. The instrument was equipped with cell blocks in a manner such that the temperatures of the sample and reference cells could be independently and accurately controlled by circulating water from external baths.

Ribonuclease Activity Measurements. Enzymatic assays were carried out in a Beckman Model DU spectrophotometer according to the procedure of Kunitz (1946). The spectrophotometer was equipped with thermospacers so that a constant temperature (25°) could be maintained during the reaction. RNA in 0.1 M acetate buffer at *p*H 5.0 was the substrate.

Amino Acid Analyses. These were performed with the Technicon amino acid analyzer. All hydrolyses were carried out in 6 N HCl in sealed, evacuated ampules for 22 hours at 116°. Calculations of the amino acid compositions of ribonuclease and ribonuclease methyl ester were made by assuming that the hydrolysate contained the theoretical number (12) of alanine residues.

The Lossen Rearrangement. The Lossen rearrangement, a reaction in which the esters of glutamic acid and aspartic acid are converted to their respective diamino acids, was carried out on the methylated derivative by a procedure which was similar to that described by Gallop *et al.* (1960). These workers performed this reaction on methylated gelatin. Approximately 20 mg of protein was treated for 1 hour at 40° and at *p*H 7.0 with 5 ml of 1 M hydroxylamine. After this time, the protein was precipitated with two volumes of cold acetone, washed twice with methanol and once with ether, and then dried *in vacuo*. This hydroxamic acid derivative was dissolved in 5 ml of water and the *p*H was adjusted to 8.0. An equivalent volume of 1% dinitrofluorobenzene (v/v) in ethanol was added and the reaction was allowed to proceed at *p*H 8.0 for 15 minutes at room temperature. After it was extracted twice with ether and once with petroleum ether, the aqueous phase was made 0.1 N with respect to NaOH, heated to 100° for 2 minutes, and then brought to dryness at 35° in a rotary evaporator. The residue was taken up in 3 ml of 6 N HCl and prepared for hydrolysis prior to amino acid analysis. A control (ribonuclease A) was also carried through this procedure.

Results

Rates of Methylation. In the process of determining optimal conditions for the methylation reaction, it was found that the nature of the product depended on the temperature and on the HCl concentration. High temperatures (room temperature and above) yielded a protein which was quite insoluble in water. The same result was obtained when high concentrations of HCl were employed. On the other hand, the conditions finally selected (and described in the experimental section) produced a soluble protein.

Table I illustrates the rate of carboxyl group disappearance as a function of reaction time. The number of groups titrated up to *p*H 7.6 appears to level off at 7.5 groups. If this value is compared to approximately 15.5 groups obtained for ribonuclease (Tanford and Hauenstein, 1956), it appears that eight of the eleven carboxyl groups have been removed. The other 4.5 groups titrated are the four histidines and about half of the α-amino group. This observation is consistent with subsequent findings which show the presence of three free carboxyl groups in the methylated protein. Table I also includes the enzymatic activity of the protein as a function of reaction time. The activity approaches a limiting value of less than 1% of the original activity. Therefore, it is concluded that the methylated protein has either undergone significant conformational changes about the active site or else that one or more carboxyl groups which are necessary for catalytic activity have undergone methylation. Vithayathil and Richards (1961) prepared a fully methylated ribonuclease which was enzymatically inactive. However, they were able to regenerate activity by adding the methylated protein to the S-protein.

TABLE I: Number of Groups Titrated[a] and the Per Cent Activity Found, Following Methylation.

Reaction Time (hours)	Number of Groups Titrated (between *p*H 2.0 and 7.6)	Per Cent Activity
0.1	14.7	100
1	13.5	97
2	13.5	
4.5	13.2	
8.3	12.8	81
24	10.8	43
45	10.0	17
120	7.4	<1
168	7.7	<1
336	7.6	

[a] Between *p*H 2.0 and 7.6, eleven carboxyl groups, four histidine groups, and a fraction (*ca.* 0.5) of the single α-amino group of ribonuclease are titrated.

Potentiometric Titrations. Potentiometric titration curves (performed at 25 and 10°) of the methylated protein are shown in Figure 1. In each case, the experimental points were fitted to a calculated curve by assuming that the protein contained three carboxyl groups. The experimental points obtained from the 25° runs were best fitted to a theoretical curve in which all the carboxyl groups had a $pK°$ equal to 4.6. Since a $pK°$ of 4.6 is indicative of normally ionizable carboxyl

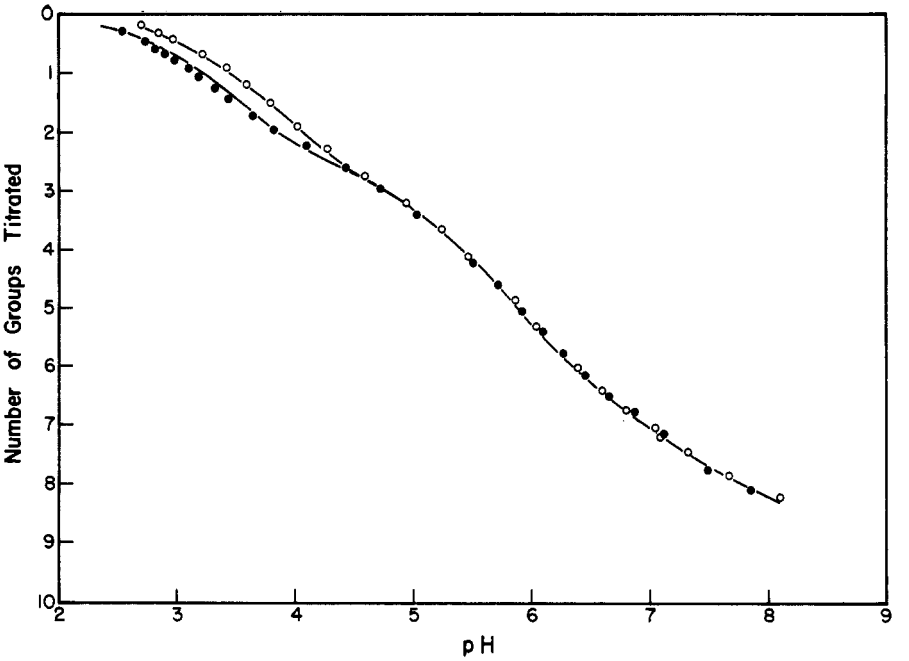

FIGURE 1: Potentiometric titration curves of ribonuclease methyl ester in 0.15 M KCl. The open circles are data at 25°, the solid circles are data at 10°, and the curves are theoretical, assuming the following parameters: at 25°, three carboxyl groups, all having a $pK°$ of 4.6, four imidazole groups with a $pK°$ of 6.6, and one α-amino group with a $pK°$ of 7.8. At 10°, two carboxyl groups with a $pK°$ of 4.3, one with a $pK°$ of 4.6, and the remainder of the groups the same as at 25°. At both temperatures, w was taken as 0.056. The positions of the curves relative to each other and to the zero on the ordinate are arbitrary.

groups, it is concluded that the carboxyls in the methylated protein titrated normally at this temperature. On the other hand, to obtain the best fit for the 10° data, it was necessary to assume that two of the carboxyls possessed a slightly lower $pK°$ (4.3). These observations may be understood if one refers to the transition temperature curves shown in Figure 2. At 25°, a significant proportion of the molecules has undergone a transition (unfolding) at pH values near 5.5. In addition, as the titration at this temperature is carried through the acid range, the transition temperature of the methylated protein is lowered. Therefore, as the titration of the carboxyl groups is carried out, a larger proportion of the molecules unfolds. As a result of this unfolding, the carboxyl groups appear to titrate normally.

The unfolding of the protein at 10° is insignificant, even at pH values approaching 4.0. Therefore, the tendency for two of the carboxyl groups to titrate at an abnormally low pH was detected. Nevertheless, even at this temperature, a transition did occur as the pH was lowered (see Figure 2). Therefore, it is believed that the observed $pK°$ values of these carboxyls at 10° could be even lower if the protein remained in its native conformation.

It should be noted that a proper fit of the experimental points (at either temperature) to a theoretical curve was accomplished by assuming a value for w, the electrostatic factor, of 0.056. This is the same value found by Cha and Scheraga (1960) for the titration of ribonuclease A in a guanidine hydrochloride–urea solvent. This indicates that the structure of the methylated protein is a less tightly folded one than is that of ribonuclease A.

Demethylation Studies. The ester groups may be removed from the methylated derivative by hydrolysis at basic pH. If the pH is held below the point at which ribonuclease A is irreversibly denatured, a large proportion of the enzymatic activity may be regained. The results of a typical hydrolysis experiment are shown in Figure 3. In this case, the pH was held at 10.44 by periodic and accurately measured additions of NaOH until the rate of base uptake had become very slow. At the end of this time (26 hours at 25°), it was calculated that 7.65 groups per molecule had been hydrolyzed. The enzymatic activity of the resulting product was found to be 81% of the activity found for ribonuclease A. After standing in the cold for an additional 48 hours, the activity of this product rose slightly to 87% of that found for ribonuclease A. Nevertheless, owing to the probability of carbon dioxide uptake, no measure of base consumption was made to determine whether or not additional ester groups had been hydrolyzed.

The number of groups removed per molecule (7.65) tends to corroborate other evidence that there were eight ester groups per molecule. However, because of the length of time required for the reaction to go to

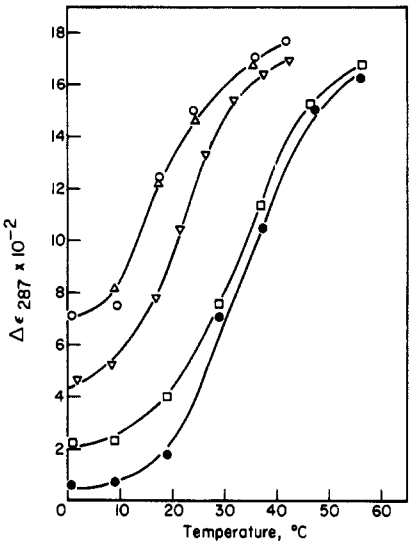

FIGURE 2: Difference extinction coefficient at 287 mμ of ribonuclease methyl ester, as a function of temperature. These are typical curves which were selected to show the change in shape and magnitude as the *p*H of the solution is increased. ○, *p*H 1.11; △, *p*H 1.38; ▽, *p*H 2.45; □, *p*H 4.00; ●, *p*H 5.44. In each case, the sample solution (maintained at 0°) was identical to the reference solution.

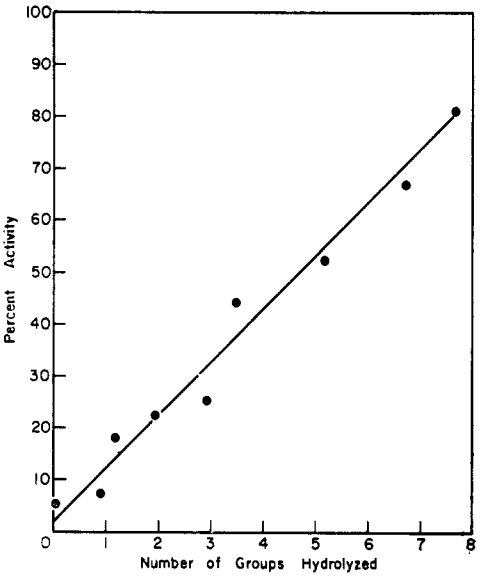

FIGURE 3: The recovery of enzymatic activity by ribonuclease methyl ester upon hydrolysis of the ester groups at *p*H 10.44 and 25°. The final point on this curve represents a reaction time of 26 hours. Activity increased to a slightly higher value upon standing in the refrigerator for an additional 2 days.

TABLE II: Amino Acid Content of Ribonuclease A and of Ribonuclease Methyl Ester Following the Lossen Rearrangement (moles of amino acid/mole of protein).

Amino acid	Theory	Ribonuclease A Control	Methylated Ribonuclease
Aspartic acid	15[a]	14.66[a]	12.81[a]
Glutamic acid	12[a]	11.79[a]	7.52[a]
Valine	9	8.88	7.81
2,3-Diaminopropionic acid			1.91
2,4-Diaminobutyric acid			2.86

[a] These numbers correspond to the sum of the free acid and amide forms.

completion and the limited stability of the *p*H-meter, it was not possible to determine this value more precisely.

The Lossen Rearrangement. To obtain an independent check on the number of carboxyl groups in the methylated derivative, and at the same time to gain some knowledge of the identity of the amino acids which were resistant to methylation, the esterified protein was subjected to the Lossen rearrangement. This reaction converts the esters of amino acids to their respective diamino acids (Gallop *et al.*, 1960). Therefore, the decrease in the aspartic acid, glutamic acid, and valine content (the carboxyl-terminal residue in ribonuclease is valine) upon treating the methylated derivative according to the procedure of Gallop *et al.* (1960) should be a measure of the number of groups esterified. Table II reports the aspartic acid, glutamic acid, and valine content of the methylated derivative following the Lossen rearrangement. The results of the control experiment, in which ribonuclease A was treated in a manner identical to that of the methylated protein, are included in Table II. The values shown for ribonuclease A, carried through this procedure, closely approximate the theoretical values. In contrast, the methylated derivative shows a decrease of approximately two aspartic acid residues, one valine residue, and either four or five glutamic acid residues per mole of protein. The observed decrease in glutamic acid content was such that a clear choice could not be made between four and five residues. Nevertheless, it should be pointed out that the conversion of the amino acid esters to their corresponding hydroxamic acid derivatives has been reported by Gallop *et al.* (1960) to be only 90% effective. Therefore, if one considers that the decrease in the glutamic acid content represents only a 90% conversion of the ester groups present, then the actual number of esterified glutamic acid residues would approximate five.

Table II also includes the yields of the two amino

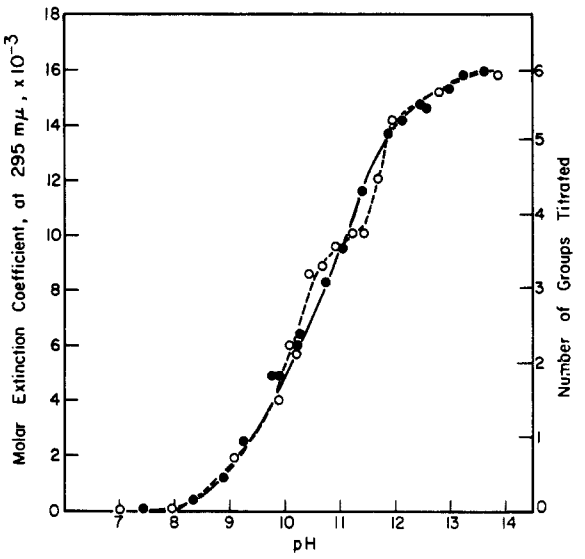

FIGURE 4: Spectrophotometric titrations of ribonuclease methyl ester at 25° (solid circles) and at 1° (open circles). The scale for the number of groups titrated was calculated by assuming an extinction coefficient of 2700 per residue.

acids resulting from the rearrangement reaction: 2,3-diaminopropionic acid and 2,4-diaminobutyric acid. These amino acids were found only in those experiments in which the methylated derivative was used. The concentrations of these derivatives were calculated using the color equivalents reported by Gallop *et al.* (1960). The acid arising in this reaction from the esters of aspartic acid residues, 2,3-diaminopropionic acid, was eluted from the amino acid column approximately 30 ml prior to the ammonia peak. The yields of this acid accounted for the decrease in the aspartic acid content of the methylated protein. 2,4-Diaminobutyric acid, which arises in this rearrangement from the esters of glutamic acid residues, was eluted immediately after and not totally separated from 2,3-diaminopropionic acid. In this case, it may be seen that the decrease in glutamic acid content was not completely accounted for by the yield of 2,4-diaminobutyric acid. A plausible explanation for the low yield of 2,4-diaminobutyric acid is the possibility of α-γ-carboxyl interchange during hydroxamic acid formation. This possibility has been discussed by Gallop *et al.* (1960). Nevertheless, the decrease in two aspartic acid residues, one valine residue, and approximately five glutamic acid residues strongly suggests that of the eleven carboxyl groups in ribonuclease A (five aspartic acids, five glutamic acids, and the single carboxyl-terminal valine), the methylated derivative contains three free aspartic acid residues. The valine derivative (isobutyraldehyde) does not produce a positive ninhydrin color test.

It should be pointed out that the experimental procedure, as outlined in the previous section, caused noticeable decreases in the content of those amino acids (lysine, histidine, methionine, and cystine) which could react with dinitrofluorobenzene or could undergo chemical alterations during the basic treatment (Bohak, 1964). These observations were also noted in the control experiment. The yields of the other amino acids (except aspartic acid, glutamic acid, and valine in the hydrolysate of the methylated derivative) were comparable to their concentrations expected in a ribonuclease A hydrolysate.

Spectrophotometric Titrations. One characteristic of native ribonuclease is the abnormal titration of three of its six tyrosyl residues (Tanford *et al.*, 1955). Since the objective of the present investigations is the identification of those carboxyl groups which are believed to be hydrogen bonded to the "buried" tyrosine groups in a nonpolar environment (Hermans and Scheraga, 1961), it is of interest to demonstrate that the methylated protein also possesses abnormal tyrosyl residues.

Spectrophotometric titration curves, run at 25° and at 1°, are shown in Figure 4. While the abnormal groups are not obvious in the 25° titration data, it is clear from the data at 1° that the methylated derivative contains tyrosyl groups which titrate with a higher pK than do the normal groups. These observations suggest that the methylated derivative is unfolded (with respect to the tyrosyl groups) at 25°, while at 1° the molecule does possess a folded conformation. It is noteworthy that the pK of these residues (as is seen from the 1° data) is somewhat lower than in ribonuclease A. This is probably indicative of an overall loosening of the structure (even at 1°) relative to the ribonuclease A molecule. A similar loosening of structure has been noted by Ooi and Scheraga (1964) in tryptic modifications of ribonuclease A.

Although it was possible to demonstrate that the derivative did contain abnormal tyrosyl residues, it is difficult to ascertain the exact number of such groups. As is seen in Figure 4, the break in the titration curve occurs between three and four groups. This observation indicates that at least two and perhaps three tyrosyls titrated at a higher pH.

Transition Studies. It is known that ribonuclease undergoes conformational changes when the pH of a neutral solution is brought through the acid range (Hermans and Scheraga, 1961). The extent of unfolding at any given pH depends on the temperature of the solution. These conformational alterations can be studied by observing the optical density decrease at 287 mμ, and this phenomenon has been attributed to a change in the environment of tyrosyl residues. In addition, since these structural changes occur over the range of the carboxyl titration, it has been inferred that some carboxyl groups are involved with tyrosyl residues in a nonpolar region in maintaining the native conformation of the protein.

Hermans and Scheraga (1961) have noted that a cooled solution of ribonuclease at low pH has a negative difference spectrum with respect to a solution at the same temperature and at neutral pH. It was postulated by these workers that this negative difference spectrum arose from the protonation of one or more

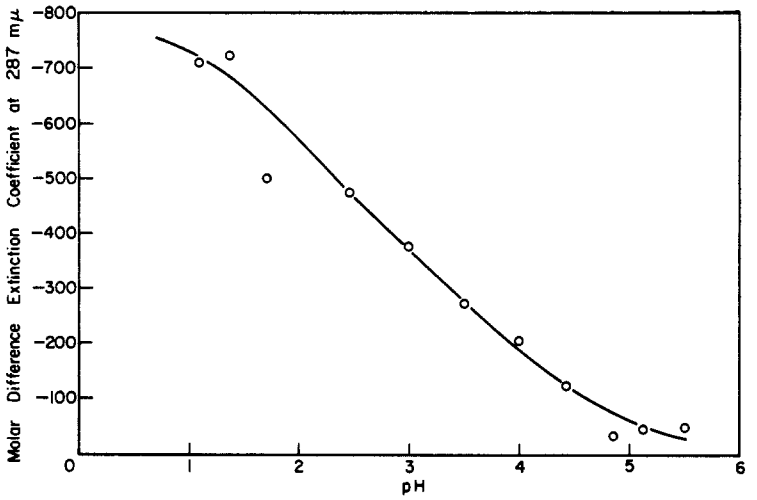

FIGURE 5: Difference spectrophotometric titration of ribonuclease methyl ester at 2°. The points are the extinction coefficients of a solution at the indicated *p*H. The reference was a solution of the same material at the same concentration and at *p*H 6.5.

carboxyl groups which were involved in carboxylate-tyrosine interactions. Protonation is accompanied by a small perturbation of the tyrosyl residues. It was further proposed that these alterations do not grossly affect the nonpolar region about the tyrosyl residues.

Figure 5 illustrates a similar study performed on the methylated derivative. This curve differs from that of ribonuclease (Hermans and Scheraga, 1961) in that the point of inflection (pK_{obsd}) occurs at a higher *p*H; i.e., near *p*H 3.5 instead of *p*H 1.5 for ribonuclease. This result again indicates a looser structure for the methylated protein.

Hermans and Scheraga (1961) also noted that solutions of ribonuclease underwent a much larger optical density decrease at acid *p*H values when the temperature of these solutions was raised. Such transitions could be reversed by lowering the temperature. It was postulated that these optical density changes were caused by gross unfolding of the molecule such that the tyrosyl residues were brought from their hydrophobic environment into an aqueous one.

Typical transition curves (of the methylated derivative) at constant *p*H are shown in Figure 2. These transitions were at least 80% reversible and in most instances were found to be completely reversible. The small amount of irreversibility noted in a few cases was probably due to the extended periods of time during which these samples were allowed to stand at high temperature.

As can be seen from Figure 2, even at low temperature, where the transition curves are fairly flat, the limiting $\Delta\epsilon_{287}$ value differed. If an isotherm at 2° were to be drawn through these curves and the $\Delta\epsilon_{287}$ values plotted as a function of *p*H, a titration curve would be obtained which would be similar to the curve shown in Figure 5.

The midpoint of the transition curve has been defined as the transition temperature, T_{tr}. This parameter

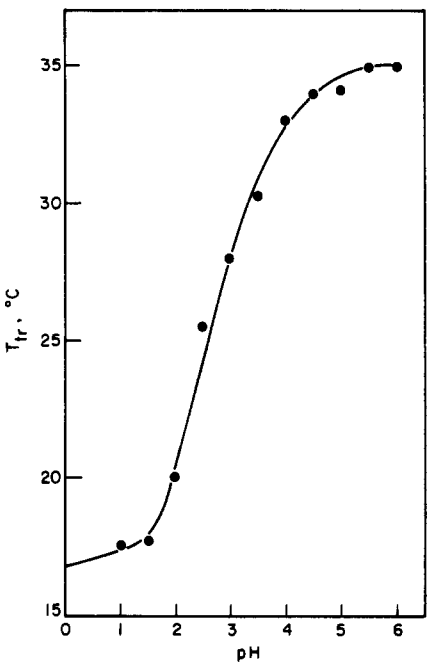

FIGURE 6: Transition temperatures of ribonuclease methyl ester as a function of *p*H. The transition temperature was taken as the temperature at which one-half of the total change in the difference extinction coefficient had occurred, between that at 0° and the maximum.

can be plotted as a function of *p*H, as is shown in Figure 6. The resulting curve is similar to that of ribonuclease, except that the entire curve is shifted to lower temperatures, and the difference in transition temperature between the low and high *p*H regions is about 18°, as compared with a 35° difference for ribonuclease. These

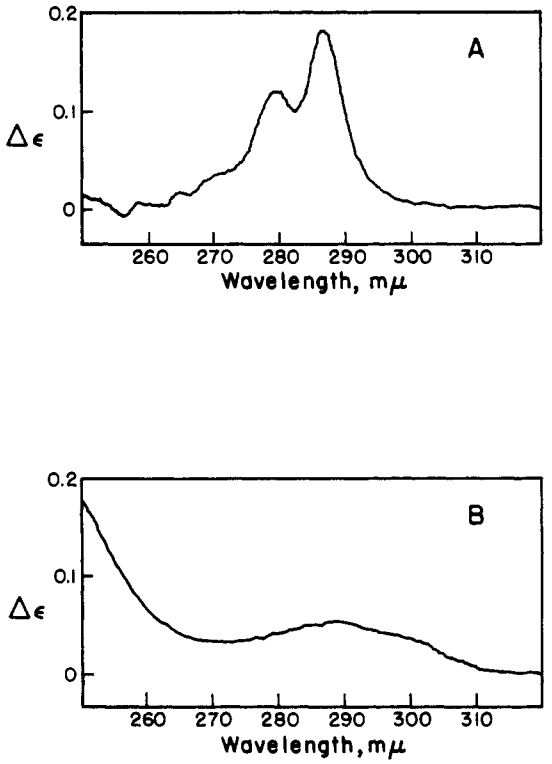

FIGURE 7: Typical difference spectra of ribonuclease methyl ester. (A) Methyl ester at *p*H 1.5, 0.9°, as the reference solution versus methyl ester at *p*H 7.0, 5.1°, as the sample solution. (B) Methyl ester at *p*H 7.8, 0.9°, as the reference solution versus ribonuclease A at *p*H 7.0, 5.1°, as the sample solution. The concentrations of these solutions were identical.

observations are consistent with the looser structure previously proposed for the methylated protein.

Additional evidence that the structure of the derivative is essentially the same as that of ribonuclease A is the difference spectrum of the methyl ester at *p*H 7.8 versus ribonuclease A at *p*H 7.0. Such data are shown in Figure 7B. Although there is a spectrum, it is not the typical tyrosine perturbation spectrum usually obtained when a protein is observed to be unfolding, an example of which is shown in Figure 7A. The latter spectrum is typical of that obtained with the methylated derivative at low *p*H, or high temperature, and closely resembles that of denatured ribonuclease. It is important to note that a *fully* esterified ribonuclease derivative, prepared under different experimental conditions (Sela *et al.*, 1957), was spectrophotometrically identical to denatured (or unfolded) ribonuclease.

Discussion

In spite of the fact that the methylated protein was prepared under conditions where the conformation of the protein was quite different from that in aqueous solution, there apparently is formed, upon solution in water, a derivative which reverts to a conformation very similar to that of ribonuclease. The ultraviolet absorption spectrum of our partially methylated protein, at neutral *p*H, is remarkably similar to that of ribonuclease, a fact which is not true for a *fully* methylated derivative (Sela *et al.*, 1957). In addition, the methylated protein undergoes, in the acid range, low-temperature transitions and thermally induced transitions which are similar to those of ribonuclease. The methylated protein also contains abnormally titratable tyrosyl and carboxyl groups.

Although the physicochemical properties of the methylated protein are similar to those of ribonuclease, there are definite indications that the methylated protein possesses a looser structure than does ribonuclease. As was noted previously, the low-temperature transitions of the methylated protein occur at a somewhat higher *p*H (Figure 5) than in the native molecule, and the transition temperatures of the methylated protein, at a given *p*H (Figure 6), are lower than they are in ribonuclease. In addition, it was necessary to carry out experiments at low temperatures in order to observe the abnormal behavior of the carboxyl and tyrosyl groups in the methylated protein. These observations should not be overly surprising in view of the fact that the derivative possesses a totally different electrostatic charge than does ribonuclease.

All experimental observations indicated that the derivative contained three free carboxyl groups. Titration, saponification, and the Lossen rearrangement techniques consistently implied that eight of the eleven carboxyl groups (known to be present in ribonuclease) were methylated. In addition, the Lossen rearrangement technique indicated that the three free carboxyl groups were aspartic acid residues.

The exact number of tyrosine-carboxylate interactions in the methylated protein is uncertain. Spectral studies indicated that at least two and perhaps three tyrosines titrated abnormally. In addition, potentiometric titrations (at 10°) implied that two carboxyl groups titrated at an abnormally low *p*H. These observations suggest that at least two of the free carboxyl groups in the methylated protein could be involved in tyrosine-carboxylate interactions. Physicochemical evidence is lacking to indicate that the third carboxyl is a "buried" group. Nevertheless, it should be pointed out that the only evidence for the number of abnormal carboxyl groups is the titration of the protein at 10°. Although the best fit to the experimental points was obtained by assuming that the protein contained one carboxyl group with a $pK°$ equal to 4.6, and two carboxyl groups with $pK°$ equal to 4.3, a reasonable fit was also obtained by assuming one carboxyl group with $pK°$ equal to 4.2 and two carboxyl groups with $pK°$ equal to 4.4. Therefore, the possibility that the third carboxyl group is also a "buried" group should be considered.

It appears that this derivative possesses many of the characteristics of the native molecule and that at least two of the free carboxyls in this derivative could

well be the "buried" carboxyls of ribonuclease A. Therefore, a study was carried out (Riehm *et al.*, 1965) to determine which carboxyl groups remained free. A discussion of the nature of the particular interactions in which these groups may be involved will be reserved for that communication.

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